ANALYSIS OF VALPROIC ACID INDUCED SIDE EFFECTS: POLYCYSTIC OVARY SYNDROME AND WEIGHT GAIN

by

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This thesis is dedicated to my family and those who suffer from a mental illness

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Abstract

Objective: Valproic acid (VPA) is a medication for the treatment of bipolar disorder, epilepsy, or migraine headache. VPA treatment is associated with weight gain and in women can induce symptoms of polycystic ovary syndrome (PCOS). This thesis attempted to develop an animal model for VPA induced side effects *in vivo* and determined *in vitro* the effect of VPA on the development of adipocytes (adipogenesis) and secretion of the hormone leptin.

Results: In rats, VPA treatment was not associated with alterations in estrous cycling, weight gain, aromatase activity, or serum levels for estradiol, testosterone, or leptin. However, cystic follicles were present in 50% of the drug treatment animals compared to 25% of saline treated animals. In mouse and human preadipocytes, VPA inhibited adipogenesis and reduced the growth rate of mouse preadipocytes. VPA treatment during adipogenesis reduced mRNA levels for the transcription factors, peroxisome proliferator-activated receptor gamma (PPARγ) and steroid regulatory binding protein (SREBP)1a, as well as the protein levels for PPARγ, SREBP1a, and CCAAT/enhancer binding protein alpha (C/EBPα). VPA and other histone deacetylase (HDAC) inhibitors blocked adipogenesis, whereas valpromide, which does not inhibit HDACs, did not prevent adipogenesis. In mature adipocytes, acute VPA treatment reduced leptin mRNA levels and protein secretion, and VPA catabolites were incorporated into lipids. The reduction of leptin occurred independent of changes in glucose uptake, PPARγ, SREBP1a, or C/EBPα protein levels, degradation of leptin mRNA, or alterations in the rate of lipogenesis or amount of intracellular free fatty acids.

Conclusions: *In vivo,* VPA treatment in female rats had limited usefulness for modeling VPA-induced PCOS symptoms, with the exception of the development of cystic follicles. *In vitro* VPA inhibited adipogenesis highlighting a requirement for HDAC activity in adipogenesis. VPA also reduced preadipocyte growth, decreased leptin production and secretion, and did not alter lipid metabolism in mature adipocytes. Paradoxically, in humans VPA treatment induces weight gain and increases serum leptin levels, suggesting that VPA may be affecting multiple systems that regulate energy balance

List of Abbreviations and Symbols

α-MSH	α-melanocyte stimulation hormone
4PB	4 phenylbutyric acid
5-HT	serotonin
ACS	acyl-CoA synthetase
ActD	actinomycin D
ADD-1	adipocyte determination and differentiation factor-1
AgRP	agouti-related peptide
ALLN	N-acetyl-leucine-leucine-norleucinal
AML	acute myeloid leukemia
AMPA	amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPK	AMP-dependent protein kinase
ANOVA	analysis of variance
ASP	acylation-stimulating protein
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BAT	brown adipose tissue
BBB	blood-brain barrier
bcl-2	B-cell lymphoma/leukemia-2
BD-Gal4	GAL4 DNA binding domain plasmid
BDNF	brain derived neurotrophic factor
BHLHLZ	basic helix-loop-helix leucine zipper
BMI	body mass index

BSA	bovine serum albumin
C	cleaved
C/EBP	CCAAT/enhancer binding protein
C/EBPα	CCAAT/enhancer binding protein alpha
C/EBPβ	CCAAT/enhancer binding protein beta
C/EBPδ	CCAAT/enhancer binding protein delta
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine-monophosphate
CART	cocaine- and amphetamine-regulated transcript
CF	cerebrospinal fluid
CHO	Chinese hamster ovary
CHOP	C/EBP homologous protein
CHX	cyclohexamide
CL	corpus luteum
CLA	conjugated linoleic acid
cpm	counts per minute
CMV	cytomegalovirus
CNS	central nervous system
CP	choroid plexus
CRE	cyclic AMP response element
CREB	cAMP response element binding protein
CS	calf serum
DAG	diacylglycerol

DBD	DNA binding domain
DEX	dexamethasone
DHA	dicosahexaenoic acid
DHA	docosahexaenoic acid
DHAP	dihydroxyacetone phosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
dpm	disintegrations per minute
DTT	dithiothreitol
DVS	Divalproex Sodium
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ETOH	ethanol
$FADH_2$	reduced flavin adenine dinucleotide
FBS	fetal bovine serum
FC	follicular cysts
FDA	Federal Drug Agency
FFA	free fatty acids
F-MDI	Fajas et al. differentiation inducing medium
FR-luc	luciferase reporter plasmid
FSH	follicle-stimulating hormone

GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUT4	glucose transporter 4
GnRH	gonadotropin-releasing hormone
GRP41	orphan G protein coupled receptor 41
GSK-3	glycogen synthase kinase 3
GSK-3β	glycogen synthase kinase 3beta
h	hours
HCL	hydrochloric acid
HDAC	histone deacetylase
HDL	high density lipoproteins
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRE	hypoxia response element
HSL	hormone-sensitive lipase
IBMX	isobuthylmethylxanthine
IGF	insulin-like-growth factor
IL-6	interleukin 6
IMPase	inositol monophosphatase
IP_3	inositol 1,4,5-triphosphate
IRS	insulin receptor substrate
Jak	janus kinase
JNK	c-jun N-terminal kinase
KCL	potassium chloride

kg	kilogram
KOH	potassium, hydroxide
KRH	Krebs-Ringer-HEPES
L	liter
LBD	ligand binding domain
LDL	low density lipoproteins
LH	luteinizing hormone
M	molar
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MC4R	melanocortin 4 receptor
MCH	melanin-concentrating hormone
MDI	medium to induce differentiation
$MgS0_4$	magnesium sulfate
MI	differentiation medium
min	minutes
MPOA	medial preoptic area
mRNA	messenger RNA
MRS	magnetic resonance spectroscopy
mTOR	mammalian target of rapamycin
Na^+	sodium
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide

NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NEFA	non-esterified free fatty acids
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NPY	neuropeptide Y
ob	obese
Ob-R	leptin receptor, long isoform
P	precursor
PAI-1	plasminogen activator inhibitor 1
PAT	perilipin, adipose differentiation-related protein, and TIP47
PBS	phosphate buffered saline
PCOS	polycystic ovary syndrome
PFA	paraformaldehyde
PI	phosphoinositol
PI3-K	phosphatidylinositol 3-kinase
pKa	proton acid dissociation constant
PKA	protein kinase A
FAS	fatty acid synthase
PL	phospholipids
PMSF	phenyl methyl sulfonyl fluoride
POMC	proopiomelanocortin
PPAR	peroxisome proliferator-activated receptor

PPARα	peroxisome proliferator-activated receptor alpha
PPARβ	peroxisome proliferator-activated receptor beta
PPARγ	peroxisome proliferator-activated receptor gamma
PPAR-Gal4	GAL4 DNA binding domain / PPARγ ligand binding domain
PTP1B	protein tyrosine phosphatase IB
PDE3B	phosphodiesterase 3B
PUFA	polyunsaturated fatty acids
ROS	rosiglitazone
RSK	ribosomal protein S6 kinase-1
GAP43	growth cone associated protein 43
RT	room temperature
RXR	retinoid X receptor
SCAP	SREBP cleavage-activating protein
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulfate
SE	standard error of the mean
SHBG	sex-hormone binding globulin
SRE	sterol regulator element
SREBP	steroid regulatory binding protein
SRF	serum response factors
SSC	saline sodium citrate
ssc	side-chain cleavage
Stat	signal transducers and activators of transcription

t10c12 CLA	t10c12 conjugated linoleic acid
TAG	triacylglycerol
TCA	tricarboxylic acid
TGFβ	transforming growth factor beta
TGZ	troglitazone
TK	thymidine kinase
TLC	thin layer chromatography
SDS	sodium dodecyl sulfate
TNFα	tumor necrosis factor alpha
TSA	trichostatin A
VLDL	very low density lipoproteins
VPA	valproic acid
VPM	valpromide
WAT	white adipose tissue
Wnt	wingless
Zen-DIM	Zen-Bio differentiation inducing medium

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Many different people shape one's life and I feel so fortunate during my studies at Dalhousie to have been enriched by the friendship of so many wonderful people. My supervisor, Dr. Mark Nachtigal made this opportunity possible for me and I will always have fond memories of times in animal care, in the lab, socializing outside the lab, and where I think we spent the most time - sitting in front of his computer talking about science. I am grateful for this experience and his mentorship. Within the lab, I have had the opportunity to interact with three fabulous post-doctoral research fellows –Drs. Yang-Xin Fu, Gabriel Bertolesi and my "newly found cousin", Trevor Shepherd. These guys have been so helpful to me and exemplify the type of "fellow" I hope to become. I have also had the advantage of working in an excellent environment created by the many faculty members, students, support staff, and technicians within Mark's lab, the Department of Pharmacology, and the Animal Care Facility. I especially want to note the contributions of Lesley Dunfield my across the bench partner, and Michelle Mujoomadar, our pseudo lab member. Moreover, I have enjoyed my experiences in my other home in the Department of Psychiatry where I have gain many friendships and been given the honor to be involved in clinical research. I am especially in debt to my mentor Dr. Stan Kutcher, who has been the fuel that lighted my interest in the study of mental illness.

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Chapter 1

INTRODUCTION

Valproic acid (VPA) is a commonly prescribed medication for the treatment of epilepsy, bipolar disorder, or migraine headaches. This well-tolerated medication has a low rate of inducing serious side effects and is a highly effective therapeutic agent. However, VPA treatment is often associated with significant weight gain and in female patients can be associated with the occurrence of polycystic ovary syndrome (PCOS). The mechanism by which VPA produces both its therapeutic effects and side effects remain to be elucidated. The work presented in this thesis explores the mechanism by which VPA induces weight gain and symptoms of PCOS in women. Specifically the work assesses an animal model for VPA induced side effects *in vivo* and determines the effect of VPA on the development and biology of adipocytes *in vitro*.

VALPROIC ACID

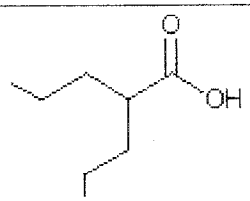
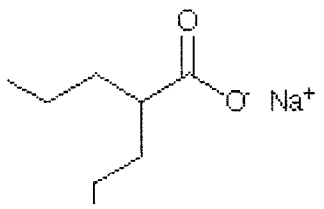
VPA was first produced in 1882 and used as a solvent for organic compounds (1). The clear, colorless, eight-carbon branched chain fatty acid derived the generic name of VPA from the chemical name of 2-propylvaleric acid. In 1962, 80 years after the synthesis of VPA, the therapeutic potential of VPA was fortuitously discovered when VPA was used as a solvent for experimental anticonvulsants and the investigators in the laboratory of Dr. G. Carraz recognized that VPA itself had anticonvulsant activity (2). The first clinical trial testing the safety of VPA in the treatment of epilepsy was reported the following year (3), and the drug was approved in France in 1967 for the treatment of epilepsy, and subsequently in the United States by the Federal Drug Agency (FDA) in 1978 (reviewed in 4).

As a therapeutic agent, VPA is often prescribed in a formulation that contains the sodium salt form of VPA, sodium valproate, a white powder that is highly soluble in polar substances (water, ethanol) (Table 1). Because both VPA and sodium valproate are difficult to formulate into a solid oral dosage form (VPA is an oily liquid and sodium valproate is very hygroscopic and liquefies rapidly), the combination of VPA and its sodium salt (1:1 molar ratio) was patented in the 1980s and marketed under the name of Divalproex Sodium (DVS) by Abbott Laboratories (reviewed in 4, 5). Statistics from Abbott Laboratories show that in the United States DVS produced sales of $927 million in 2003, making DVS one of Abbott's Laboratories biggest selling products (6). VPA is available throughout the world in numerous formulations and salts, and the term VPA in this thesis will refer to any of these VPA-based compounds.

Pharmacokinetic Properties of VPA

The fatty acid structure of VPA explains a number of its characteristics, in terms of its distribution, sites of action, and routes of elimination. In humans, the bioavailability of VPA is between 96-100% which is consistent with VPA crossing the intestinal mucosal membranes and not being subject to first-pass metabolism (reviewed in 7). The rate of absorption of VPA varies between the formulations with peak serum concentrations occurring after approximately 1-2 hours (h) for conventional pills, 3-6 h for enteric coated tablets, and 10-12 h for sustained-released tablets (reviewed in 8). The half-life of VPA in humans is around 12 h with some differences occurring between the different formulations (reviewed in 9). VPA has a pKa of 4.56 and thus at a physiological pH of 7.4 in serum, VPA is highly ionized into a carboxylate moiety, valproate (10).

Table 1 The molecular names and structure of VPA and sodium valproate

Compound	Chemical Name(s)	Molecular Formular/Weight	Molecular Structure
Valproic Acid	2-Propylpentanoic Acid 2-Propylvaleric Acid Di-n-dipropylacetic Acid	$C_8H_{16}O_2$ 144.2	
Sodium Valproate	Sodium 2-propylvalerate Sodium 2-propylpentanoate	$C_8H_{15}NaO_8$ 166.2	

This results in a relatively small amount of VPA being distributed by passive diffusion into tissues, since only the non-ionized, lipid soluble portion of VPA can undergo diffusion (reviewed in 11). In serum, a high proportion (70-94%) of VPA is also bound to albumin, with lower percentages bound in elderly patients and pregnant women, and in the presence of increasing amounts of free fatty acids within the serum (reviewed in 12). Due to VPA being mainly ionized at physiological pH and the high proportion of VPA that is bound to serum proteins, the volume of distribution of VPA is around 0.14 L/kg in humans, indicating that most of VPA is confined to the circulation and extracellular fluids (reviewed in 8).

Uptake and Efflux of VPA

Despite these physiochemical properties VPA is able to rapidly enter the brain as demonstrated by it's ability to terminate status epilepsy in less than 20 minutes (min) following a loading intravenous dose of VPA (reviewed in 13). Tissue distribution studies in rodents and dogs have also demonstrated that peak concentrations of VPA in the cerebrospinal fluid (CF) occurs within minutes following treatment (reviewed in 7). Uptake and exit of VPA within the central nervous system (CNS) can occur at either the choroid plexus (CP, blood-cerebrospinal fluid barrier) and/or the blood-brain barrier (BBB, brain capillary endothelium). The uptake of VPA at both the CP and BBB is suggested to occur mainly (approximately 2/3rd of entry) by a saturable carrier-mediated process and to a lesser degree through passive diffusion (14, 15). Within the BBB, the uptake of VPA is hypothesized to occur through a medium-chain fatty acid transporter, since VPA uptake is inhibited by medium- and long-chain fatty acids but not by short-chain monocarboxylic acids (14).

In addition to the carrier-mediated uptake of VPA into the CNS, pharmacokinetic studies report that a carrier-mediated system is also responsible for the efflux of VPA out of the CNS. Efflux occurs mainly at the BBB and unlike the transport for uptake, is sensitive to probenecid, an organic anion transport inhibitor (16). A variety of studies involving either animals or humans have demonstrated that the rate of efflux exceeds the rate of uptake for VPA in the CNS (reviewed in 7). The avid efflux of VPA results in a low amount of VPA in the CNS, with a brain-to-unbound plasma concentration ratio of <0.5 at steady state. This low amount of VPA within the CNS is the most likely explanation for why patients receiving VPA require a relatively large amount of VPA to achieve therapeutic efficacy (reviewed in 7).

Metabolism of VPA

VPA is almost completely metabolized in patients, with a very small amount of VPA being found in urine. The metabolic fate of VPA is highly complex and results in the production of at least 14 different metabolites (reviewed in 9). Conjugation reactions account for around 50% of VPA metabolism and another 40% can be accounted for through metabolites formed by β-oxidation.

Conjugation to D-glucuronic acid is one of the major routes of VPA biotransformation that results in the production of VPA-glucuronide (reviewed in 17). Glucuronidation occurs in microsomes and is a common method for the metabolism of a variety of carboxylic acids. The polar hydroxyl groups of glucuronide make VPA more water-soluble and thus facilitate its excretion. In addition to conjugation with glucuronide, VPA also undergoes conjugation with carnitine, glycine, and coenzyme A (reviewed in 17). The conjugation of VPA with carnitine forms valproylcarnitine.

Carnitine is an amino acid derivative localized in high energy demanding tissues (skeletal muscle, liver, etc) and is essential for the intermediary metabolism of fatty acids (reviewed in 18). The conjugation of VPA with carnitine has received clinical attention due to reports of carnitine deficiencies in patients treated with VPA (19). The urinary excretion of valproylcarnitine represents less than 10% of all acylcarnitines and less than 1% of VPA metabolites in the urine (19). Thus the formation of valproylcarnitine appears to be insufficient to account for tissue carnitine deficiencies in patients. Moreover, it is more likely that these deficiencies are due to insufficient uptake of carnitine, since recent clinical data supported that carnitine deficits in VPA treated patients occur mainly in patients who are heterozygous for carnitine transporter defects (20, 21).

Similar to all fatty acids that are metabolized, VPA is activated by undergoing conjugation with coenzyme A, by acyl-CoA synthetase (ACS), to form VPA-CoA (22, 23). The formation of VPA-CoA allows VPA to react with additional enzymes and subsequently undergo ß-oxidation (22, 23). The metabolism of VPA through ß-oxidation occurs primarily in the mitochondria of liver cells (22, 23). Since VPA is a branched chain fatty acid the enzymes responsible for the catabolism of VPA differ from those involved in the breakdown of endogenous straight-chain fatty acids (reviewed in 17). The main metabolites formed from ß-oxidation of VPA include $\Delta^{2(E)}$–VPA, 3-hydroxyl-VPA, and 3-oxo-VPA (17, 22). Several of the metabolites formed by ß-oxidation have been demonstrated to exhibit anticonvulsant activity in animals models; however the brain concentrations of these metabolites are likely too low to produce therapeutic effects, with the exception of $\Delta^{2(E)}$–VPA. The $\Delta^{2(E)}$–VPA metabolite is suggested to have

therapeutic effects in humans, since this metabolite is a more potent anticonvulsant in animals models when compared to VPA, and remains in the brain longer than VPA (reviewed in 17). Due to these properties, clinical trials have been initiated with this compound and there is considerable interest in the development of similar second-generation VPA therapeutics (reviewed in 11, 24).

In addition to conjugation and ß-oxidation, VPA undergoes a variety of oxidation reactions within the endoplasmic reticulum (ER) to produce a variety of metabolites (reviewed in 9). These metabolites include 1) hydroxylation products arising from cytochrome P450 metabolism (3-OH-VPA, 4-OH-VPA, 5-OH-VPA), 2) ketones arising from the oxidation of 3-OH-VPA or 4-OH-VPA (3-oxo-VPA, 4-oxo-VPA), and 3) dicarboxylic acids arising from oxidation of 4-OH-VPA and 5-OH-VPA (propylglutaric acid, propylsuccinic acid). A small proportion of VPA (0.3%) also undergoes cytochrome P-450 desaturation in liver microsomes to produce more $\Delta^{4(E)}$–VPA. Overall VPA metabolism occurs through both mitochondrial and microsomal processes. Studies examining the relative contribution of these two processes have demonstrated that as the dosage of VPA increases, the amount of metabolism occurring in the mitochondria is reduced with an accompanying increase in metabolism occurring in the microsomes by glucuronidation (17). Thus, depending on the dosage of VPA, the proportions of the different VPA metabolites will vary, which may be clinically important if the metabolites are determined to be active in contributing to the therapeutic or side effect profile of VPA.

Species Differences in the Pharmacokinetics of VPA

The pharmacokinetic properties of VPA vary between different species. The pathway for VPA biotransformation is similar in all species, yet there are dramatic differences in VPA elimination and half-life (reviewed in 11). The half-life of VPA is estimated to be 0.6 h in rhesus monkeys, 0.8 h in mice, 2.5 h in rats, 9 h in cats, and 9-18 h in humans. Since half-life is proportional to the volume of distribution and inversely related to clearance, the differences in half-life of VPA between species is partly due to differences in clearance, since the volume of distribution for VPA in all species is similar to the species extracellular volume. A difference in the rate of clearance for VPA between species is partly due to differences in the amount of VPA that is bound to plasma proteins. For example, 80-95% of VPA is bound to plasma proteins in humans compared to only 12% in mice. However, differences in clearance between species cannot be fully explained by plasma binding, since in the case of rhesus monkeys, 80% of VPA is bound to plasma proteins yet the half-life of VPA is 0.6 h. These species differences make it difficult to sustain therapeutic levels of VPA when modeling VPA effects in rodents and non-human primates.

Pharmacodynamic Properties of VPA

Clinical Indications for VPA

The FDA has approved the use of VPA for epilepsy, bipolar disorder, and migraine headaches. VPA has demonstrated efficacy in animals models of epilepsy and in the treatment of both human partial (local onset) and generalized (bilateral onset) seizures (reviewed in 4, 8). A review of clinical trials evaluating VPA treatment in 808 adults and 585 children with epilepsy, reported a greater than 75% reduction in seizure

frequency in 53% and 78% of patients with partial or generalized seizures, respectively (8). In this review, the mean oral VPA dose ranged from 20-40 mg/kg/day and mean plasma concentrations ranged from 50-100 *u*g/ml (8). Moreover, in a summary of trials that used VPA treatment in monotherapy, 62-86% of patients were reported to be in remission from seizures for at least 1 year, which was noted to be similar to the remission rates of patients on other antiepileptic drugs (8).

In addition to the frequent use of VPA for the treatment of epilepsy, VPA is a leading adjunctive and alternative monotherapy to lithium for the treatment of acute mania, cyclothymia, mixed state, and rapid cycling bipolar disorder (reviewed in 25). A Cochrane Review assessing VPA treatment in acute mania reported VPA had a similar efficacy to lithium and carbamazepine, yet was more efficacious when compared to placebo (26). The dosage of VPA used for the treatment of bipolar disorder is similar to the dosage used to treat epilepsy, with patients receiving between 750-1500 mg/day and efficacious responses being observed with serum levels ranging from 45-120 *u*g/ml (27).

In 1996, VPA also received FDA-approval for the treatment of migraine headaches. VPA is as effective as propranolol for migraine treatment and is useful in treating acute migraine attacks when given as an intravenous therapy (reviewed in 28). Randomized trials investigating VPA in the treatment of migraines reported approximately 50% of patients receiving VPA have a 50% reduction in migraine attack frequency, when VPA was used at dosages ranging from 600-1000 mg/day and serum levels ranging from 75-120 *u*g/ml.

In addition to these FDA approved indications, VPA is being tested as a chemotherapeutic agent due to the recently exciting preclinical evidence suggesting that

VPA can influence tumor growth, invasion, and differentiation (reviewed in 29). Most recently, VPA has been shown to induce apoptosis in cells expressing a multi-drug resistance phenotype from patients with acute myeloid leukemia (AML) (30). In addition to this study, the antineoplastic properties of VPA have been demonstrated in many tumor models, with the exception of estrogen-dependent tumor models, where VPA can enhance estrogen receptor expression and increase proliferation in the presence of estrogens (31). These preclinical studies have led to ongoing Phase II trials investigating VPA use in cancer therapy. For example, current clinical trials being funded by the United States National Cancer Institute are evaluating VPA treatment of myelodysplastic syndromes, myeloid or chronic lymphocytic leukemia, and in patients with Kaposi's Sarcoma (32). Others have also reported that VPA therapy may be useful in the treatment of schizophrenia, neuropathic pain, panic disorder, and agitation in dementia, however these reports are mainly anecdotal and await empirical validation (reviewed in 25, 33).

In summary, similar doses of VPA can produce anti-epileptic, mood stabilizing, or prophylactic effects against migraine headaches. VPA may also prove to be useful in cancer therapy. The ability of VPA to be efficacious in the treatment of this variety of human diseases is remarkable, considering the mechanism(s) by which VPA produces these diverse number of effects remains largely unknown.

VPA Effects on GABA Signaling

The oldest and most highly investigated hypothesis regarding VPA's mechanism of action has focused on VPA enhancing the main inhibitory neurotransmitter, γ-aminobutyric acid (GABA) (reviewed in 11, 33, 34-36). *In vitro* VPA increases GABA

synthesis through inhibiting 3 enzymes that reduce the production of GABA (GABA transaminase, succinic semialdehyde dehydrogenase, α-ketoglutarate dehydrogenase) through the GABA-shunt of the tricarboxylic acid (TCA) cycle. These effects were suggested to mediate VPA's ability to increase GABA levels by 15-45% in rodent brains following acute VPA administration and account for the increases in GABA in plasma and cerebral spinal fluid of patients and volunteers treated with VPA. However, the concentration of VPA that is required to increase GABA levels *in vitro* may not be present *in vivo* and the broad spectrum of VPA effects in different types of seizures does not support the idea that VPA acts predominantly as a GABA agonist. Moreover, *in vivo* nuclear magnetic resonance (NMR) spectroscopy studies do not support the notion that VPA increases GABA. For example, chronic VPA treatment in rats decreased the amount of GABA in the CNS (37) and patients with refractory seizures being treated with VPA had no significant alterations in brain GABA levels (38). Thus, although enhanced GABA transmission may contribute to VPA's anti-epileptic activity in rodents, preliminary *in vivo* imaging studies do not support the idea that VPA can enhance GABA levels within the CNS of humans.

VPA Effects on Sodium Channels and the Glutamatergic System

VPA has been well documented to alter neuronal excitability, which has led to investigations examining the effect of VPA on ion channels other than the GABA receptor/chloride channel. VPA can inhibit voltage-gated sodium (Na^+) channels, through reducing the maximal Na^+ conductance, as well as delaying the recovery of voltage-dependent Na^+ channels (reviewed in 11, 33). This work has primarily been completed in neuronal culture models. However work in hippocampal slice models did not reproduce

these results, suggesting that VPA effects may not be due to the effects on Na^+ channels in tissue. VPA was also been demonstrated to modulate excitatory neurotransmission by regulating the glutamatergic system, as recently reviewed by Zarate *et al.* (39). Acute VPA treatment can stimulate glutamate release in cerebral cortical slices and potentiate glutamate-evoked intracellular calcium activity. An increase in the release of glutamate by VPA is paradoxical since VPA is used in the treatment of mania and epilepsy, with are generally associated with increased excitatory neurotransmission. However, the acute effects of VPA may be counterbalanced with chronic VPA treatment, which can increase glutamate transporter expression and increase the uptake and removal of glutamate. Moreover chronic VPA treatment may alter the expression or function of the glutamate receptors including the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. Specifically, VPA has been demonstrated to suppress NMDA-evoked depolarization, dampen seizure activity induced by AMPA receptor agonists in rodents, as well as decrease the expression of the AMPA GluR1 subunit in rodents. Similar to the effect of VPA on GABA transmission, spectroscopy studies measuring the concentration of glutamate in whole brain or from regional areas have documented conflicting findings in rodents, and the small study done in patients with refractory epilepsy found increased glutamate concentrations in 2/5 patients receiving VPA (38). In order to help identify if alterations in GABA or glutamate mediate the effects of VPA, future prospective studies will need to measure alterations in these amino acids before and after VPA therapy using NMR spectroscopy.

VPA Inhibits Histone Deacetylases

VPA is being tested as a chemotherapeutic agent due to VPA's ability to inhibit histone deacetylases (HDACs), which was discovered in 2001 by two independent laboratories (40, 41). As outlined in detail in Chapter 3 of this thesis, deacetylation of core histones by HDACs is correlated with repression of target gene expression, thus inhibition of HDACs by VPA can de-repress transcription. VPA and specific VPA analogs can inhibit a variety of HDACs with an *in vitro* IC_{50} within therapeutic dosages and induces hyperacetylation of histone H3 and H4 in cells with a order of potency that parallels their *in vitro* potencies (e.g. VPA>2-ethylhexanoic acid>4-pentenoic acid) (42). The molecular mechanism for the anti-tumor effects of HDAC inhibition by VPA treatment remains unknown, but is associated with alterations in cell cycle regulators (such as cyclin D, p21, p27), induction of pro-apoptotic factors (such as caspase-3, tissue inhibitor of matrix metalloproteinase-3 and insulin-like growth factor binding protein-3), and inhibition of angiogenesis (42-47).

In addition to mediating the anti-tumor effects of VPA, the ability of VPA and VPA analogs to inhibit HDAC activity correlates with the teratogenic activity of VPA and its analogs, supporting the notion that the teratogenic property of VPA is mediated by HDAC inhibition (41). In contrast, the ability of VPA and its analogs to inhibit HDACs does not correlate with the antiepileptic or mood stabilizing properties of these agents, implying that these actions are most likely not mediated by HDAC inhibition. For example, *in vivo* valpromide (VPM) is an anticonvulsant and mood stabilizing agent, but is not teratogenic and is not an inhibitor of HDACs (41, 48). Therefore the HDAC

inhibitory properties of VPA likely mediate the chemotherapeutic and teratogenic properties of VPA.

VPA Effects on Intracellular Signaling Pathways

Research examining the mechanism for the mood stabilizing properties of VPA has identified that mood stabilizers, such as lithium and VPA, have common effects on neuronal cell signaling pathways and gene expression, which may contribute to their therapeutic effects (reviewed in 49, 50-54). Similar to many therapeutic agents, it is likely that there are multiple mechanisms of action for VPA. Criteria have been suggested to aid in identifying if alterations in cell signaling pathways or alterations in gene expression may be therapeutically relevant for the treatment of bipolar disorder (49, 55). For example, it has been proposed that alterations in cell signaling pathways should occur by dissimilar mood stabilizers and manipulations of the gene or signaling pathway should produce behavioral changes in experimental animal models. Currently no alterations in cell signaling pathways or direct gene targets have been proven to meet all of the suggested criteria. It is difficult to test whether alterations in a gene produce similar behavioral changes or mimic VPA's affects, since there is a lack of validated models for bipolar disorder (reviewed in 56). Despite these obstacles, VPA and lithium (two dissimilar mood stabilizers) have been identified to share common cellular targets that are under investigation as possible mediators for VPA effects. Included in these common effects are alterations in the phosphoinositol (PI), mitogen-activated protein kinase (MAPK), and wingless (Wnt) / β-catenin signaling pathways.

VPA and the Phosphoinositol Cycle

Inositol metabolism and alterations in the PI cycle have been implicated in the etiology and treatment of bipolar disorder since lithium was first hypothesized to deplete inositol levels (reviewed in 57). Cells need to maintain sufficient levels of inositol in order to synthesize phosphoinositides, and more importantly regulate signaling through the PI cycle. Lithium has been demonstrated to deplete intracellular levels of inositol due to inhibition of inositol monophosphatase (IMPase), which regulates recycling of inositol and controls the *de novo* synthesis of inositol (reviewed in 57). Unlike lithium, VPA does not inhibit IMPase activity, but produces a variety of similar effects as lithium on the PI cycle as demonstrated in yeast. VPA treatment in yeast decreases intracellular myo-inositol mass, reduces IMPase gene expression, and increases expression for two yeast enzymes (ino1, ino2) that are involved in inositol biosynthesis (58, 59). Most recently, VPA has also been shown in transformed yeast to inhibit a human enzyme called 1D-myo-inositol 3-phosphate synthase, which catalyzes the rate-limiting step in *de novo* inositol synthesis (60).

It remains to be discovered whether the effects of VPA in yeast will correspond to similar effects in mammalian cells or neurons. Work with sensory neurons has suggested that VPA can modulate inositol metabolism. VPA decreases growth cone collapse and increases growth cone formation in neurons, which can be reversed by co-treatment with inositol or inhibition of prolyl oligopeptidase, an enzyme thought to play a role in increasing the inositol precursor, inositol 1,4,5-triphosphate (IP_3) (61). Since similar effects on growth cone morphology were observed with lithium or VPA in sensory neurons, the mood stabilizing effects of these drugs was hypothesized to be modulated by

a reduction in inositol and IP_3 activity; however it is unknown whether VPA directly affected inositol or IP_3 activity in these sensory neurons (reviewed in 49).

Experimental studies with brain slices from mice, rats, and monkeys demonstrate that VPA can increase IP_3 activity, yet *in vivo* studies report only slight or no increases in brain inositol or IP_3 activity in animals (reviewed in 49). Clinical magnetic resonance spectroscopy (MRS) studies have reported similar myo-inositol levels in VPA-treated euthymic patients, when compared to euthymic patients receiving lithium or controls that have no psychiatric history (62). These authors suggest that their data supports the hypothesis that VPA induces normalization of PI cycle, since it has been previously reported that bipolar patients that are symptomatic and not receiving medications have abnormally high levels of myo-inositol (62). However, in the case of lithium, it has been demonstrated that the reduction in brain myo-inositol levels occur prior to a therapeutic response, suggesting that modulation of inositol levels may be a trigger for alterations in other signaling pathways that may bring about lithium's therapeutic effects (reviewed in 50). Therefore, more prospective *in vivo* investigations are required in order to assess whether VPA modulates the PI cycle in patients.

VPA and the Mitogen-Activated Protein Kinase Pathway

VPA treatment can induce the MAPK signaling pathway, as assessed by VPA inducing the phosphorylation of extracellular signal-regulated kinase (ERK), ERK1 and ERK2 in neuronal cells, as well as in rat hippocampus and frontal cortex (55, 63). VPA can also activate a reporter gene assay that utilizes a response element for MAPK target gene expression, which can be blocked by co-treatment with MAPK inhibitors or expression of a dominant negative Ras or Raf, which are upstream components of the

MAPK pathway (63). Independent of effects on two other MAPKs (c-jun N-terminal kinase (JNK) and p38), VPA can also trigger the phosphorylation of downstream components of the MAPK pathway including c-jun, cAMP response element binding protein (CREB), and ribosomal protein S6 kinase-1 (RSK) (55).

Activation of the MAPK pathway by VPA has also been reported to induce the expression of MAPK target genes including growth cone associated protein 43 (GAP43), brain derived neurotrophic factor (BDNF), and B-cell lymphoma/leukemia-2 (bcl-2) (55, 63). Recently, the induction of bcl-2 by VPA has been demonstrated to be cell-type specific. VPA increases bcl-2 expression in rodent and human neuronal cells (64), does not alter expression in human or rat glia cells (63-65), and decreases expression in endometrial cancer cell lines (44). Since numerous pathways including the MAPK pathway can modulate bcl-2 expression (66), it will be important for future work to identify how VPA and other mood stabilizers modulate expression of these genes in different cell types.

The physiological consequences of activating the MAPK pathway by VPA has been suggested to promote the neurotrophic effects of mood stabilizers within the CNS (reviewed in 53). Whether VPA has neurotrophic effects *in vivo* and whether these effects rely on the MAPK pathway remains to be established. The possibility that the ERK pathway may be important in the anti-manic properties of VPA, was further highlighted by the variety of changes in affective-like behaviors observed in rodents with genetic manipulations of the BDNF-ERK kinase pathway (reviewed in 67).

VPA and the Wnt/ β-catenin Pathway

Similar to the discovery of the effects of mood stabilizing agents on the PI cycle, VPA was discovered to have effects on the Wnt/ β-catenin signaling pathway (reviewed in 49, 52, 53, 68). Within this pathway an intermediary called disheveled, inhibits glycogen synthase kinase 3β (GSK-3β), a constitutively active enzyme that was first identified as one of several kinases that phosphorylates and inhibits glycogen synthase (reviewed in 69). In addition to its effects on glycogen synthase, GSK-3β can bind in a complex with other proteins to facilitate the phosphorylation of cytoplasmic β-catenin leading to β-catenin degradation. Activation of the Wnt pathway inhibits GSK-3β phosphorylation allowing the accumulation and nuclear translocation of β-catenin resulting in expression of numerous target genes, including those involved with apoptosis and embryonic development.

VPA treatment can increase the steady state levels of β-catenin in mouse and human neuronal cells (41, 70) and increase the cytoplasmic pool of β-catenin in rat cortex. Since the accumulation of β-catenin occurs after a minimum of 10 h of VPA treatment in cell culture, VPA is hypothesized to directly increase transcription of β-catenin rather than stabilize existing β-catenin pools, which is in agreement with the experimental data demonstrating VPA increased β-catenin messenger RNA (mRNA) levels (41). It is controversial whether VPA also contributes to β-catenin accumulation by the inhibition of GSK-3β phosphorylation, as has been proven for lithium (reviewed in 49). *In vitro* assays have found that VPA does not alter GSK-3β mediated phosphorylation of a glycogen synthase peptide (41) or microtubule-associated protein (MAP)-1B peptide (71), or phosphorylation of an endogenous MAP protein (tau) in

mouse neuronal cells (41). Moreover, *in vivo* administration of VPA does not alter GSK-3β protein or activity in rat cortex (72). In contrast to these findings, VPA can inhibit GSK-3β mediated phosphorylation of a CREB peptide and increases the incorporation of radiolabeled phosphates into two putative GSK-3β substrates *in vitro* (70). These conflicting results suggest that VPA may selectively inhibit GSK3-β substrates.

The ability of VPA to alter GSK3-β phosphorylation is still undetermined, however VPA treatment was recently shown to induce a modest increase in phosphorlyated GSK-3β on serine-9 in human neuronal cells (73), yet did not alter GSK-3β serine-9 phosphorylation in mouse cerebellar granule cells (71). When examining the downstream effects of GSK3-β phosphorylation, VPA induced Wnt-dependent reporters in transient reporter gene assays was associated with inhibition of HDACs (41). Inhibition of HDAC activity by VPA has also been recently demonstrated to be associated with the increased phosphorylation of serine-9 GSK3-β by VPA in human neuronal cells, thus supporting the hypothesis that VPA action on GSK3-β is a result of HDAC inhibition.

Overall the HDAC inhibitory properties of VPA may account for the induction of β-catenin expression and altered GSK-3β activity. Despite VPA and lithium modulating the Wnt/β-catenin pathway by different mechanisms, the common effect of these two drugs on induction of β-catenin raises the intriguing hypothesis that mood stabilizers may therapeutically regulate neuroprotection and modulate circadian rhythms in bipolar disorder by this mechanism (reviewed in 68).

TOLERABILITY OF VPA

One of the benefits of VPA therapy is that VPA has a broad therapeutic margin of safety and is associated with only rare idiosyncratic reactions and a variety of common general side effects (reviewed in 8, 33, 74). The most common general side effect of VPA therapy is gastrointestinal effects, which occur in approximately 10-45% of patients and include nausea, vomiting, and heartburn. These side effects typically occur during the initiation of VPA therapy and occur less frequently with use of sodium valproate, enteric-coated, and control release formulations. In less than 10% of patients receiving VPA, dose-related tremor, dermatological effects (rash or alopecia), and neurological effects, such as drowsiness, irritability, and ataxia can occur, with an increased occurrence in patients on polytherapy. Weight gain is another common side effect that is observed in patients receiving VPA, and as discussed in detail in Chapter 3, does not appear to be associated with VPA formulation or dosage and is sustained with continual treatment. Although weight gain is well documented to be a side effect of VPA treatment in CNS disorders, weight gain may be a potential benefit of VPA therapy in trials that are investigating the efficacy of VPA in patients with cancer.

Female patients on VPA can also experience many of the symptoms associated with PCOS, which as discussed in detail in Chapter 2, increase patient health risks and can lead to infertility. Males treated with VPA may also experience a reduction in reproductive function, with recent clinical studies identifying reduced testicular volumes in men treated with VPA (75, 76) and animal studies reporting a reduction in epididymal weight with no accompanying changes in fertility in male rats treated with VPA (77). Additionally, in women of childbearing age, the use of VPA is cautioned due to the

teratogenic effects of VPA, which have been noted previously to be a consequence of HDAC inhibition by VPA (41). Spina bifida occurs in 1-2.5% of offspring from mothers treated with VPA during the first trimester of pregnancy. Since the onset of seizures or acute manic episodes are also considered harmful to developing embryos, often VPA treatment during pregnancy must be sustained in combination with a reduction in dose, switching to a sustained release formulation, and intensive prenatal monitoring with folic acid supplementation.

Metabolic disturbances including hypocarnitinemia and hyperammonemia can also be observed in patients receiving VPA. These conditions occur mainly in patients that have existing metabolic aberrations that are likely unmasked by the added stress of VPA metabolism. Reversible thrombocytopenia with associated inhibition of platelet aggregation is a more common side effect of VPA therapy yet is often associated often classified as mild with a platelet count around 101-150 x $10^3/mm^3$. The rare and idiosyncratic, non-dosage related VPA-induced side effects include reversible idiopathic hepatitis and hemorrhagic pancreatitis. These events are extremely rare and fatal, for example 29 fatalities occurred due to hepatotoxicity in over one million patients receiving VPA between 1987 to 1993 in the United States of America (78). Despite the rare occurrence of these fatal side effects, development of more efficacious derivatives of VPA that are not associated with as many side effects are in development since side effects, such as weight gain, may not be fatal but can lead to noncompliance and increased health risk associated with obesity. Aside from HDAC inhibition being responsible for the teratogenic effects of VPA, it is largely unknown how VPA induces this side effect profile.

SUMMARY

VPA is a short chain fatty acid that is used in the treatment of epilepsy, bipolar disorder, migraine headaches, and is being investigated as a therapeutic agent in treatment of certain forms of cancer. Therapy with VPA is relatively safe with common side effects being non-fatal yet associated with an overall decrease in health. The mechanism by which VPA produces both its therapeutic and side effect profile is unknown, but likely involves multiple actions including alterations of neurotransmitters, inhibition of HDACs, and effects on intracellular signaling pathways.

In the second chapter of this thesis, the study described addresses the question of whether VPA could induce symptoms of PCOS in rodents. This study was undertaken in an attempt to create a rodent model for VPA-induced PCOS. In the third and fourth chapter, studies examining the effect of VPA on adipocyte development and mature adipocyte biology are described. These studies were completed to determine if VPA can have effects on adipocytes, with a larger goal of trying to understand more about the potential mechanisms for VPA induced weight gain.

CHAPTER 2

VALPROIC ACID FAILS TO INDUCE POLYCYSTIC OVARY SYNDROME IN FEMALE RATS

Portions of this chapter appeared in the following publication:

Lagace DC, Nachtigal MW. (2003) *Valproic acid fails to induce polycystic ovary syndrome in female rats.* Progress in Neuro-Psychopharmacology and Biological Psychiatry 5824:1-8. (79)

ABSTRACT

Purpose: Valproic acid (VPA) treatment in female patients is associated with the occurrence of a variety of endocrine side effects that include many characteristic symptoms of polycystic ovary syndrome (PCOS). The aim of our study was to prospectively measure whether VPA treatment was associated with the presentation of PCOS symptoms in rats. **Methods:** Normal estrous cycling female rats (n=22) were treated perorally three times daily with VPA (330 mg/kg/day), divalproex sodium (DVS; 300 mg/kg/day) or phosphate-buffered saline for a minimum of 30 days. PCOS-associated symptoms were assessed including determining changes in estrous cycle, weight, estradiol and testosterone levels, aromatase activity, and ovarian morphology. **Results:** There was no significant difference in the mean number of days animals were in proestrus-estrus or metestrus-diestrus between the three groups. All animals gained weight during the study and there were no appreciable differences in mean weight gain or serum leptin levels between groups. Total serum estradiol or testosterone levels and ovarian aromatase activity were not significantly different between the groups. The number of corpora lutea was not significantly different between the groups, however cystic follicles were present in 50% of the drug treatment animals compared to 25% of saline treated animals. **Conclusions:** VPA and DVS treatment was associated with a higher proportion of animals developing cystic follicles but did not mimic the VPA–induced PCOS that is observed in women. Thus, it appears that the rat has limited usefulness for modeling VPA-induced symptoms associated with PCOS.

Keywords: Polycystic Ovary Syndrome (Pcos), Rat, Female, Valproic Acid

INTRODUCTION

One concern with the use of VPA therapeutically is the association between VPA treatment and symptoms characteristic of polycystic ovary syndrome (PCOS). This introduction will summarize the main characteristics of PCOS, review the clinical literature on the association between VPA treatment and PCOS, and describe cell culture and animal studies that have tested VPA effects on reproductive-endocrine systems.

Polycystic Ovary Syndrome (PCOS)

PCOS is one of the most common endocrine disorders occurring in 4-15% of women of reproductive age in the general population (80, 81). There is large variability in the reported incidence rates for PCOS due to the lack of consensus on the definition of PCOS. First defined by Stein and Leventhal in 1935 (82), PCOS was described as a disorder that had symptoms of polycystic ovaries, amenorrhea (no menstrual bleeding for >6 months), hirsutism (excessive bodily hair growth), and obesity. In 1990, at the National Institute of Health Conference on PCOS, the diagnostic criteria for PCOS did not include the occurrence of polycystic ovaries and only required the presence of chronic menstrual irregularity, and hyperandrogenism (83). Hyperandrogenism can be classified biochemically by elevated androgen (testosterone) concentrations, or clinically by the occurrence of hirsutism, acne, or male-pattern hair loss (alopecia). Polycystic ovaries was removed from the definition of PCOS because approximately 20% of the general population that had polycystic ovaries, had no clinical manifestations of PCOS (84). However, since 1990, there has been debate about the inclusion/exclusion of polycystic ovaries from the definition of PCOS and at the 2003 Rotterdam PCOS Consensus, PCOS was re-defined to include a broader spectrum of symptoms with the

cardinal features of hyperandrogenism and polycystic ovary morphology (85). In both the 1990 and 2003 consensus it was also recognized that other endocrine abnormalities including elevated serum luteinizing hormone (LH) and insulin resistance are common features of PCOS (85). The increase in LH release is not accompanied by alterations in the other main gonadotropin hormone, follicle-stimulating hormone (FSH), and therefore results in a high LH:FSH ratio (greater than 2) (86). Insulin resistance leading to hyperinsulinemia is also often observed in patients with PCOS and highly associated with obesity, which is present in around 50% of PCOS patients (87). Although the definition of PCOS continues to evolve over time, PCOS has remained to be called a syndrome, and as such, requires a combination of symptoms resulting in pathology. It is necessary for patients with PCOS to be treated since PCOS is a risk factor for many health problems, including infertility, non-insulin dependent diabetes mellitus, ischemic heart disease, dyslipidemia, and endometrial carcinoma (80, 81). Furthermore, the symptoms associated with PCOS are recognized to cause psychological distress and decreased quality of life (88).

Based on the pathology and efficacy of certain therapeutic interventions for PCOS, there are three main theories postulated for the pathogenesis of PCOS, namely the hypothalamic-pituitary, ovarian morphology, and insulin hypothesis (83, 87, 89). The release of gonadotropins (LH and FSH) from the pituitary is regulated by the pulse amplitude and frequency of the gonadotropin-releasing hormone (GnRH) from the hypothalamus. LH and FSH regulate reproductive steroidogenesis, which occurs mainly within the ovary. As shown in Figure 2.1, LH stimulates androgen production from the stroma (thecal) cells by increasing P450ssc (P450 side-chain cleavage or CYP11A1)

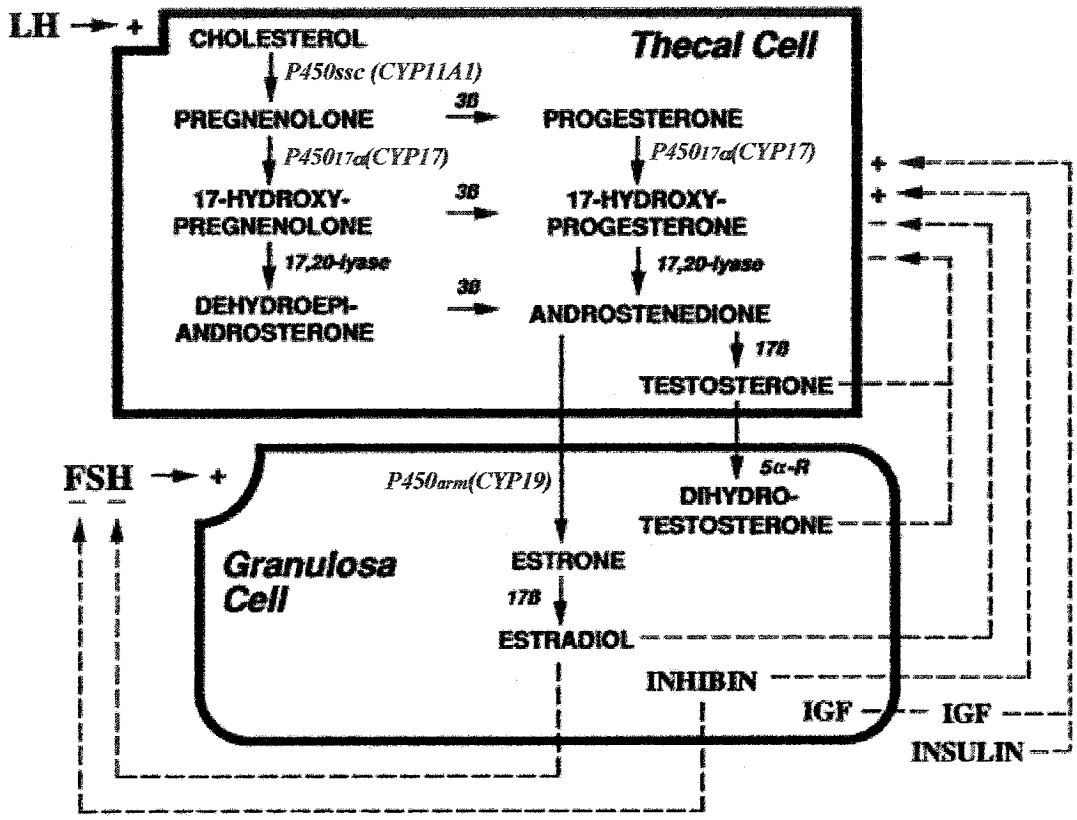


Figure 2.1 The two cell hypothesis of ovarian steroidogenesis
Testosterone and estradiol are mainly produced in the thecal and granulosa cells respectively within the ovary. IGF:insulin-like growth factor; *3B:* 3*B*–hydroxysteroid dehydrogenase; *17B:* 17 *B*-hydroxysteroid dehydrogenase; *5α-R:* 5α reductase; *arm:* aromatase; *17α:* 17α hydroxylase; *17–20-lyase:* C17,C20 lyase; *ssc:* side-chain cleavage. Modified from R.J. Norman 2002 (90).

production and activity leading to increased androgen levels. FSH regulates oestradiol biosynthesis from androgens within the granuolsa cells through increasing P450 aromatase (CYP19) enzyme levels, which converts androstenedione to estrone (86, 90).

The hypothalamic-pituitary hypothesis suggests that the primary defect in PCOS is an increase in LH release (87). In support of this hypothesis, suppressing LH release through treatment with GnRH analogues alleviates some symptoms of hyperandrogenism and PCOS (91). The mechanism underlying the increased LH release is unidentified but may be due to abnormalities in GnRH pulsatility, sensitization of the GnRH receptors, or imbalance of the normal inhibitory (e.g. dopaminergic) and stimulatory (e.g. opioidergic) regulation of GnRH release (87, 92).

The second hypothesis about the pathogenesis of PCOS suggests there is dysfunction within the ovary that leads to overproduction of androgens (87). In support of this hypothesis, ovarian wedge resection or destroying androgen tissue within the ovary reduces the levels of androgens and restores follicular maturation and ovulation (89, 93). Overproduction of androgens is suggested to be due to dysreguation of P450-17α-hydroxylase (CYP17) (94, 95), reduction in P450 aromatase activity (96), and/or inhibition of inhibin B, which suppresses FSH and androgen production in the thecal cells (Figure 2.1) (87, 97). Deregulation of androgen synthesis has also been reported to be an intrinsic property of PCOS thecal cells based on experiments with human thecal cells isolated from follicles of patients with PCOS compared to controls (95).

Insulin resistance and the resulting hyperinsulinemia is a common feature of PCOS and is the third hypothesis suggested to account for the pathology of PCOS (87, 89). Hyperinsulinemia is hypothesized to contribute to hyperandrogenism by influencing

androgen synthesis and increasing the circulating pools of bioavailable androgens. Evidence that insulin and insulin-like growth factor (IGF) enhance androgen synthesis and augments the thecal cell response to LH has been demonstrated *in vitro*, but has yet to be replicated by *in vivo* modeling (98, 99). Insulin and IGF can also increase the free (or bioavailable) androgens, by suppressing hepatic sex-hormone binding globulin (SHBG) production (89). The importance of SHBG in determining the amount of available androgens has been documented in women that carry a rare SHBG gene variant which impairs hepatic SHBG secretion, resulting in hyperandrogenism and PCOS (100). Furthermore, insulin sensitizing agents, such as metformin and thiazolidinediones (101, 102), which are used as therapeutic agents for PCOS, are able to reduce insulin levels, decrease the levels of free androgens, and reduce the symptoms associated with PCOS. In addition alterations in lifestyle that promote weight loss (improved diet, increase exercise) are associated with increased insulin sensitivity and can be effective in treating PCOS, especially in obese women (87).

Unfortunately, none of the three hypotheses are able to explain all cases of PCOS. High serum LH levels, hyperandrogenism, or hyperinsulinemia are not present in all patients with PCOS (86, 87). Therefore the three hypothesized mechanisms should not be viewed as mutually exclusive, which seems appropriate considering the variability in presentation of PCOS in patients.

Polycystic Ovary Syndrome and VPA

The literature examining the rates of PCOS following VPA treatment comprises studies involving patient populations with epilepsy or bipolar disorder (reviewed in 103, 104). A high incidence rate of PCOS symptoms following VPA therapy was first

reported in a Finnish study involving women with epilepsy (105). Further publications by this group reported that VPA-induced endocrine side-effects were 1) associated with weight gain and hyperinsulinemia that were postulated to contribute to the development of hyperandrogenism and polycystic ovaries (106), 2) reversed by replacing VPA treatment with another anticonvulsant, lamotrigine (107), and 3) observed as early as one month following initiation of VPA treatment (108). More recently this group has published a multicenter study that describes the frequency of polycystic ovaries or hyperandrogenism, or both, occurred in 70% (26/37) of patients receiving VPA, compared to 20% of patients receiving the mood stabilizer carbamazepine, and 19% (10/52) of healthy female controls (109). The occurrence of these characteristic PCOS symptoms were also more prevalent in obese patients on VPA (9/14, 79%), when compared to lean patients on VPA (9/23, 39%) (109).

Since the initial findings in 1993, there has been an intense debate about whether VPA induces PCOS in patients with epilepsy (reviewed in 103, 110, 111-113). This debate is fueled by conflicting results from clinical studies completed by a variety of groups examining the rates of PCOS, polycystic ovaries, and/or hyperandrogenism in women treated with VPA for epilepsy. In agreement with earlier studies, Murialdo *et al.* (114) confirmed that ovulatory dysfunction and cystic ovaries were more prevalent in patients with epilepsy treated with VPA (40%), when compared to patients using other anticonvulsant therapies (13%). The rate of PCOS symptoms have also been reported to be increased in women with epilepsy on VPA who are not receiving oral contraception (13%, n=33), when compared to women on other antiepileptics who are not receiving oral contraception (2%, n=5), or women on VPA who are taking oral contraceptives

(3%, n=20) (115). Moreover, hyperandrogenism and weight gain has been observed in younger girls treated with VPA prior to development of puberty (116, 117). In contrast to these studies, four cross sectional studies including 105, 93, 65 and 43 patients have reported no association between treatment with VPA and increased occurrence of PCOS symptoms (118-121). In three of these studies, VPA treatment was not associated with increased occurrence of PCOS, hirsutism or cystic ovaries compared to treatment with other anticonvulsants, but was associated with significant alterations in biochemical parameters associated with PCOS including increased testosterone (118, 122), a high LH:FSH ratio (118), as well as increased postprandial insulin levels (120, 121).

Recent studies examined the occurrence of PCOS in patients with bipolar disorder treated with VPA have also reported contradictory results (reviewed in 123). In a preliminary report on 10 patients receiving either VPA or lithium, no significant differences in the rate of PCOS symptoms were found between the two groups and no patients on VPA had PCOS (124). In a study of 32 women with bipolar disorder, O'Donovan *et al. (125)* reported 41% of women with bipolar disorder receiving VPA had polycystic ovary syndrome. Similarly, in a study of 38 women with bipolar disorder, McIntyre *et al.* (126) reported that VPA treatment was associated with a significant increase in menstrual abnormalities and hyperandrogenism, when compared to treatment with lithium. Due to the small sample sizes of these studies it is difficult to extrapolate these results that currently suggest VPA treatment in women with bipolar disorder may be associated with PCOS symptomology.

When examining the literature on VPA and PCOS, VPA treatment appears to be associated with a higher incidence rate for PCOS symptoms in patients treated for

epilepsy and bipolar disorder. Contradictions and variability have likely arisen between the studies due to the absence of a uniform definition of PCOS, variable incidence rates for PCOS symptoms, lack of large sample sizes necessary to show significant differences with adequate power, and the high number of patients receiving multiple medications and/or having a secondary axis disorder. It is likely that these methodological issues may be overcome in the ongoing large international prospective studies that are assessing the association between PCOS and antiepileptic drug use in drug naive patients (104).

Modeling VPA-Induced Endocrine Effects

Studies have assessed whether VPA affects hormone biosynthesis by examining VPA effects in cell culture systems utilizing human ovarian thecal cells, porcine (pig) ovarian follicular cells, and human pancreatic islet cells. VPA effects on steroidogenesis were first reported in cultured follicles from pig ovaries (127). In this model, VPA increased the testosterone:estradiol ratio by decreasing P450 aromatase activity and preventing androgen conversion to estrogens (127-129). More recently, VPA has also been demonstrated to increase androgen biosynthesis in a human ovarian thecal cell model (130). In this study, VPA increased the secretion of dehydroepiandrosterone, Δ4-androstenedione, and 17α-hydroxyprogesterone, under both a basal non-stimulated condition and a stimulated condition with saturating concentrations of the adenylate cyclase activator, forskolin. VPA treatment also increased P450 17α-hydroxylase (CYP17), and P450 cholesterol side-chain cleavage (CYP11A) promoter activity, as well as protein and mRNA levels for these enzymes in the thecal cells. These effects produced a similar phenotype in thecal cells from patients with PCOS, suggesting that VPA can induce a PCOS like phenotype in thecal cells. However, VPA treatment further

augmented androgen release in the thecal cells isolated from PCOS patients suggesting VPA can also alter androgen production in patients with a genetic predisposition for PCOS. In addition to affecting human thecal cells, others have demonstrated that VPA treatment in human pancreatic islet cells elicits a time and dose-dependent increase in insulin secretion (131). These results are in agreement with the suggested hypothesis that VPA treatment in women increases serum insulin levels by directly modulating pancreatic insulin secretion (120, 121). Together these *in vitro* studies support the notion that VPA has effects on peripheral endocrine cells and modulates cellular activity in a fashion consistent with PCOS characteristics.

In addition to cell culture models, four studies have examined the effect of VPA on female reproductive-endocrine function *in vivo*, using rats or rhesus monkeys. Bruguerolle *et al.* (132) first reported that female rats treated with sodium valproate treatment (10-200 mg/kg) for 21 days did not significantly modify the estrous cycle. Tauboll *et al.* (133) showed that female rats treated with VPA (200 mg/kg) for 90 days had significantly reduced serum total testosterone and an increased incidence of ovarian follicular cysts compared to control animals. Roste *et al.* (134) also showed that ovaries from rats treated with VPA had a cystic morphology that was not present in rats treated with another anticonvulsant drug, lamotrigine. Although these studies demonstrated symptoms of PCOS following VPA treatment in rats, none of these studies prospectively measured all of the symptoms associated with PCOS, which include changes in weight gain, steroid production, estrous cycling, and ovarian morphology in the same animals. In a study of rhesus monkeys, VPA was given to 7 normally cycling primates for 12-15 months and outcome measures were collected both prior to and concurrent with treatment

(135). In the monkeys, VPA therapy did not induce cyclic hormonal changes (high LH, high LH:FSH), morphological abnormalities (cystic follicles) or hormonal characteristics (hyperandrogenism, hyperinsulemia) of PCOS, but did induce a significant increase in weight. This study suggested that VPA treatment may not induce PCOS, however these results must be interpreted in light of the pharmacokinetic profile of VPA in monkeys, since the dose of VPA given to the monkeys produced close to therapeutic levels of VPA, yet the therapeutic levels may not have been sustained with the twice/day dosing regiment (135).

The purpose of the study described in this chapter was to measure whether VPA treatment was associated with the induction of pathological conditions associated with PCOS in female rats. This study began in September 2000, and at the time only the two earliest studies examining VPA effects in females rats had been published (132, 133). In order to advance our understanding of whether VPA had effects in female rats the design of this study excluded non-estrous cycling rats at baseline, as well as prospectively evaluated a variety of characteristic PCOS symptoms before and after treatment. If VPA treatment induced symptoms of PCOS in rats, it could provide a useful model to evaluate the mechanism for VPA-induced PCOS.

MATERIAL AND METHODS

Animals

Thirty-six adult female Sprague-Dawley albino rats (50-70 days old, weight between 200-225 grams) were purchased from Charles River Canada. Rats were housed in pairs in the animal care facilities of Dalhousie University and maintained on a 12:12 hour light/dark cycle (7 a.m.-7 p.m.). They were provided a diet of regular rodent chow and tap water *ad libitum* during the study. The Animal Care Committee of Dalhousie University approved all experimental procedures.

Drugs

The sodium salt form of valproic acid (2-Propyl Pentanoic Acid Sodium Salt, Sigma, P4543) was dissolved in sterile phosphate buffered saline (PBS; pH 7.4, stock solution: 260 mg/ml). Divalproex sodium (DVS; Epival™, Abbott, stock solution 200 mg/ml) tablets were purchased from the Queen Elizabeth II Health Sciences Center, Pharmacy Department, crushed and suspended in PBS to make a uniform suspension.

Handling and Study Inclusion Criteria

Following 10 days of acclimation to the laboratory facilities, animals were weighed and vaginal smears were completed daily between 7:30-9:30 a.m. in order to habituate the animals to handling and to monitor estrous cycling. For inclusion into the study, rats had to exhibit two or more successive 4-day estrous cycles. Fourteen rats were excluded from the study since they did not meet this criterion following 40 days of handling. The remaining 22 animals completed the study. An outline of the study protocol is provided in Figure 2.2.

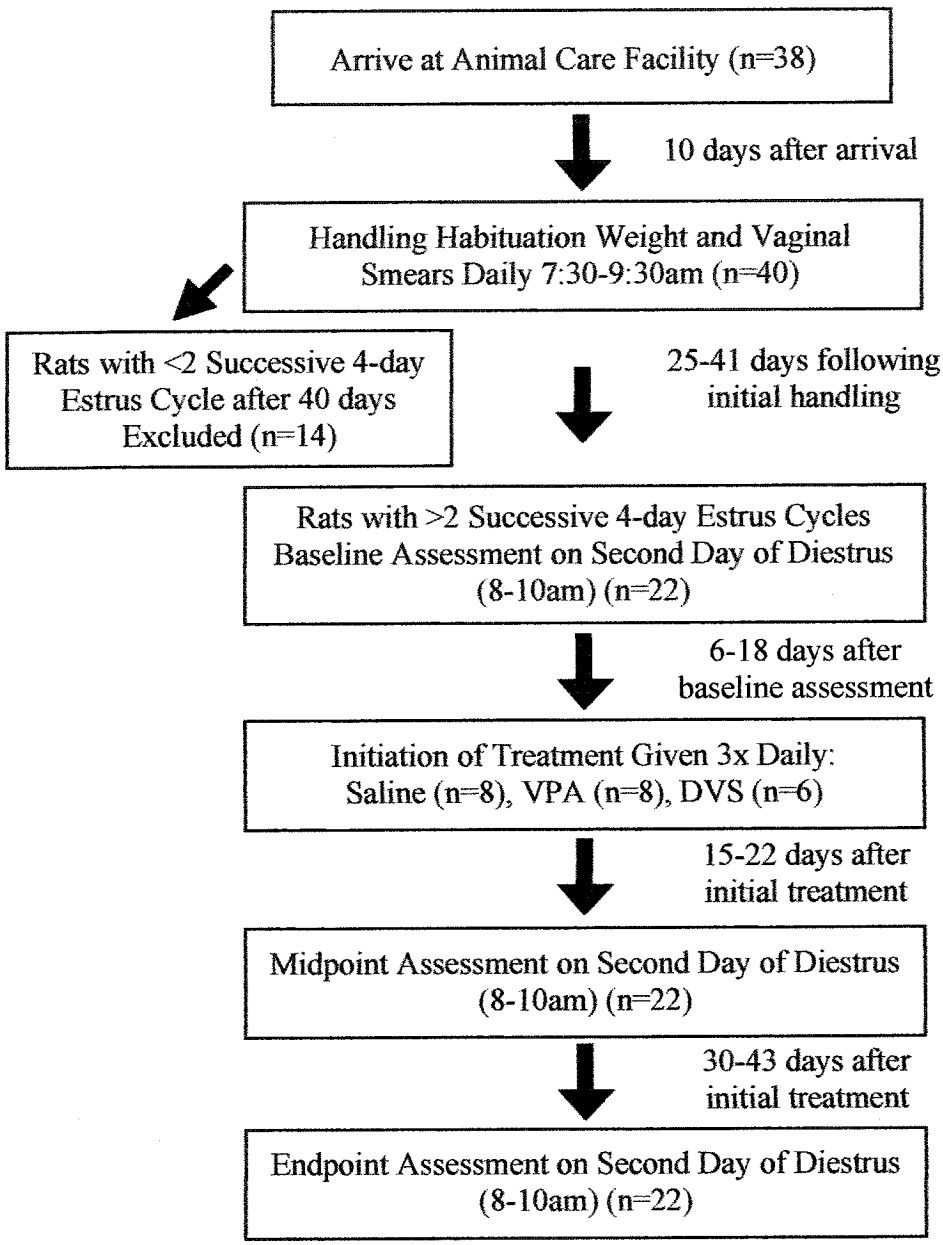


Figure 2.2 Animal study protocol
There is a range of days indicated for baseline, midpoint and endpoint assessments, as well as for initiation of treatment with valproic acid (VPA) or divalproex sodium (DVS), because these procedures were always completed on the second day of diestrus during the menstrual cycle.

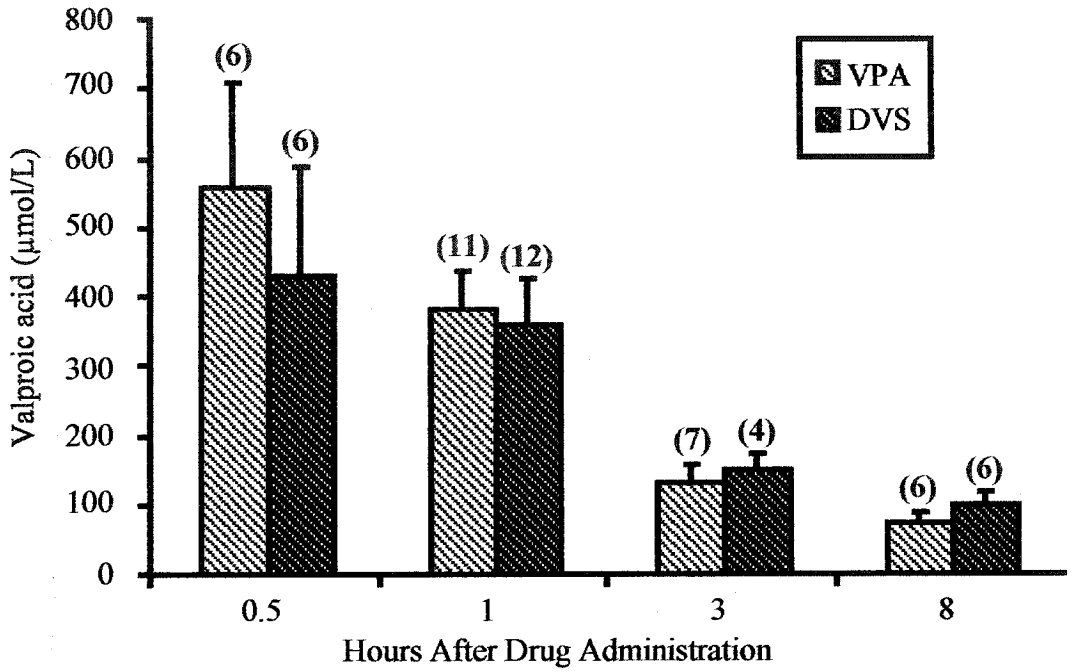
Treatment Protocol

After meeting entry criteria, blood was drawn for baseline assessment, which was done between 8-10 a.m. on the second day of diestrus. After the rats had exhibited one normal 4-day estrous cycle following baseline assessment, the rats were randomly assigned and treated with either: *Phosphate-buffered saline* (Saline, n=8), *divalproex sodium* (DVS, n=6), or *valproic acid* (VPA, n=8).

In a pilot study blood samples were collected from twenty rats treated with VPA or DVS for at least 2 weeks. The samples were collected at a maximum of once per week at either 0.5, 1, 3, or 8 h following treatment. These rats were not included in our experimental population and demonstrated that VPA plasma levels on average were less than 150 μmol/L 8 h following treatment (Figure 2.3). In order to maintain elevated plasma VPA levels animals were treated three times daily (7:30 - 9:00 a.m.; 4:00 - 5:30 p.m.; 10:00 - 11:30 p.m.).

Drugs were administered to the animals perorally by direct pipetting into their mouth. This protocol was chosen based on pilot work in the laboratory demonstrating intraperitoneal injection of VPA was associated with stress (vocalizations and no weight placed on hind leg of injection side; DCL and MWN, unpublished observation) and published reports of peroral feeding with gastric tube being associated with esophageal rupture (133). DVS and VPA were administered at a dose of 300 mg/kg/day and 330 mg/kg/day, respectively, for a minimum of 30 days. These doses were selected because this was the maximal amount of drug that could be dissolved in a concentrated stock solution and administered in a volume of less than 200 μl.

A



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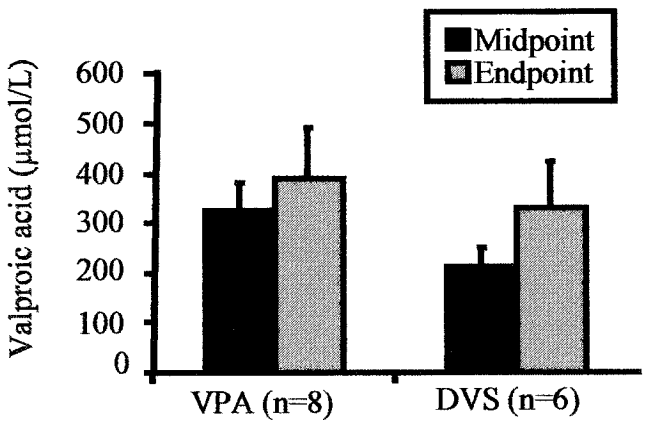


Figure 2.3 Mean valproic acid (VPA) serum levels
A) Pilot study demonstrating time course of mean serum levels (µmol/L) of VPA in rats following oral administration of VPA or divalproex sodium (DVS). Numbers in parentheses indicate the total number of animals per treatment group. **B)** Mean VPA serum levels at midpoint and endpoint assessment 1 h following treatment with either VPA or DVS. Bars represent standard error of the mean.

A midpoint assessment occurred between 8-10 a.m. on the second day of diestrus between 15-22 days following the initiation of treatment. After at least 30 days of treatment, and when animals were in their second day of diestrus, animals were sacrificed 1 hr following their morning treatment and the endpoint blood sample was collected. Any animal that was in standing estrus for greater than 7 consecutive days after 30 days of treatment was sacrificed during estrus.

Vaginal Smears

Vaginal smears were obtained daily between 07:30-08:30 h. A blunt ended eye dropper containing saline was used to obtain cellular material. The cells that desquamate from the epithelium were smeared on a slide; slides were then dried and stained with Dip Quick Stain (CDMV Inc.). Staging the estrous cycle was categorized according to Montes and Luque (136).

Blood Collection

Blood samples were obtained between 8:30-11:00 a.m. on the second day of diestrus. Immediately before blood collection, rats were warmed under a heat lamp for 5 minutes, followed by placing the tail in a warm (40°C) water bath for 2 min. The rat was placed into a plastic holding device and blood (0.5-2 ml) was drawn from the lateral tail vein. The blood sample was allowed to clot at room temperature and then centrifuged for 10 minutes at 3200xg. Serum was collected and store at –20°C until analyzed.

Serum Hormone and Valproic Acid Levels

Serum total 17-beta-estradiol was analyzed using an Immulite Kit (Diagnostic Products Corp.) with an automated chemiluminescence machine at the Atlantic Veterinary College, University of Prince Edward Island. Sensitivity for estradiol is 15

pg/ml and the antibody used is highly specific with ≤2% cross reactivity with other steroids.

Serum total testosterone and leptin were measured by radioimmunoassay (Immunotech Testosterone Assay Kit, Medicorp; Rat Leptin Radioimmunoassay Kit, Linco). Sensitivity for testosterone is 0.1 ng/ml and the antibody used is highly specific with ≤2% cross reactivity with other hormones, with the exception of 5α-dihydrotestosterone (10%) and 19-nor testosterone (5%). Sensitivity for leptin is 0.5 ng/ml with100% specificity for mouse or rat leptin.

Serum VPA concentrations were determined using a fluorescence polarization immunoassay (AxSYM Valproic Acid Assay, Abbott), at the Department of Clinical Chemistry at the Queen Elizabeth II Health Sciences Center, Halifax, Canada.

Aromatase Activity

In vitro aromatase activity was measured by an adaptation of the radiometric assay described previously (137-139). Briefly, one ovary from each animal was placed in 0.5 ml of 50 mM phosphate buffer (pH 7.4) and then subjected to a 1 min burst in a Polytron homogenizer. The homogenate was then centrifuged at 3,500xg for 20 min at 4°C. The resulting supernatant was decanted and protein concentration determined by Bradford Assay using Bio-Rad Protein Assay Reagent (BioRad). The reaction mixture (volume 0.5 ml) contained phosphate buffer, 0.24 mM of nicotinamide adenine dinucleotide phosphate (NADPH), 380 nM [1β-^{3}H] androstenedione (NEN Life Science Products, Specific Activity 25.9 Ci/mm) and 100 ug of protein extract. Control incubations were performed without any tissue sample (background value). Reaction tubes were capped, vortexed, and incubated with constant shaking for 30 min at 25°C.

After 30 min, reactions were terminated by the addition of 0.5 ml ice-cold water. Organic compounds were extracted by the addition of 1.5 ml of ice-cold chloroform and continuous vortexing for 10 min. The mixture was centrifuged (3,200g 10 min) and 0.8 ml of the aqueous phase was extracted and incubated for 10 min with 0.8 ml of 5% dextran-coated charcoal suspension. The sample was centrifuged (3,200g 10 min) and 1 ml of supernatant was extracted and added to 1.5 ml of ice-cold chloroform, vortexed for 5 min and then centrifuged (3,200g 10 min). The activity of 0.8 ml of the highly purified aqueous solution was counted in 7 ml of Ready Safe Liquid Scintillation Cocktail Fluid (Beckman) using an automated counter. Background values were subtracted and results were expressed as aromatase activity in picomoles/h/mg protein.

Histology

After excision, the ovaries and uterus were cleaned of fat. Uterine wet weight was determined gravimetrically following cleaning. The right and left ovaries were randomly assigned for histology or for assessment of aromatase activity. For histology, ovaries were fixed in 4% paraformaldehyde for 48 h, snap frozen on dry ice and sectioned at 40μm on a freezing microtome. Ovarian material was viewed by examining consecutive serial sections and matched sections were used for staining with hematoxylin and eosin. A treatment-blinded observer counted the number of cysts and corpora lutea in matched serial sections, using a minimum of three consecutive sections to verify. Cystic follicles were defined as those follicles that did not have an oocyte and displayed a large antral cavity with an attenuated granulosa cell layer (140, 141),

Statistical Analysis

Values are expressed as mean ± standard error of the mean (SE). For outcome measurements that were assessed at one time point during the study, a one-way analysis of variance (ANOVA) was completed to examine any differences between the groups. For outcome measures that were assessed at two or more time points during the study, a repeated measure ANOVA was used to determine differences that occurred over time and between the treatment groups. For subsequent group comparisons, *post hoc* analysis was completed using the Bonferroni test. Categorical outcome measures were examined using a Fisher's chi-square. Person correlation coefficients were determined for serum leptin and weight. All tests were considered significant at $p<0.05$. Data analysis was done using SPSS Base 8.0 for Windows.

RESULTS

Serum Levels of VPA

Our goal was to determine whether rats treated with VPA or DVS would exhibit the same PCOS-like symptoms reported in female patients, which are typified by weight gain, hyperandrogenism, amenorrhea, and polycystic ovaries (110, 142). In humans, therapeutic doses of VPA result in plasma levels ranging from 50-150 μg/ml (349-1039 μmol/L) (25). It is difficult to obtain steady state therapeutic serum levels of VPA in rats because VPA has a low solubility and a markedly reduced half-life (2-5h) compared to humans (9-18 hr) (reviewed in 11). Previous studies in rats have administered VPA either once or twice daily at doses ranging from 10-600 mg/kg/day (132-134), whereas we administer the drug three times daily in order to achieve peak drug levels more frequently. Analysis of VPA serum levels after oral administration of either VPA (330 mg/kg/day) or DVS (300 mg/kg/day) in rats produced similar results (Figure 2.3A). Average VPA serum levels 1 h after treatment at the midpoint and endpoint assessment were lower in animals treated with DVS, however this was not statistically different from animals treated with VPA (Figure 2.3B). The animals were noted to tolerate the treatment well and did not demonstrate any signs of drug toxicity.

Animal Weight

Animals in all three groups gained weight as they aged from approximately 8.5 weeks to 20 weeks old during the study (Figure 2.4). Statistical analysis using a repeated measure ANOVA demonstrated an overall significant change in weight over time with no significant differences between the treatment groups. Post hoc testing identified significant increases in weight when comparing baseline and midpoint weight in animals

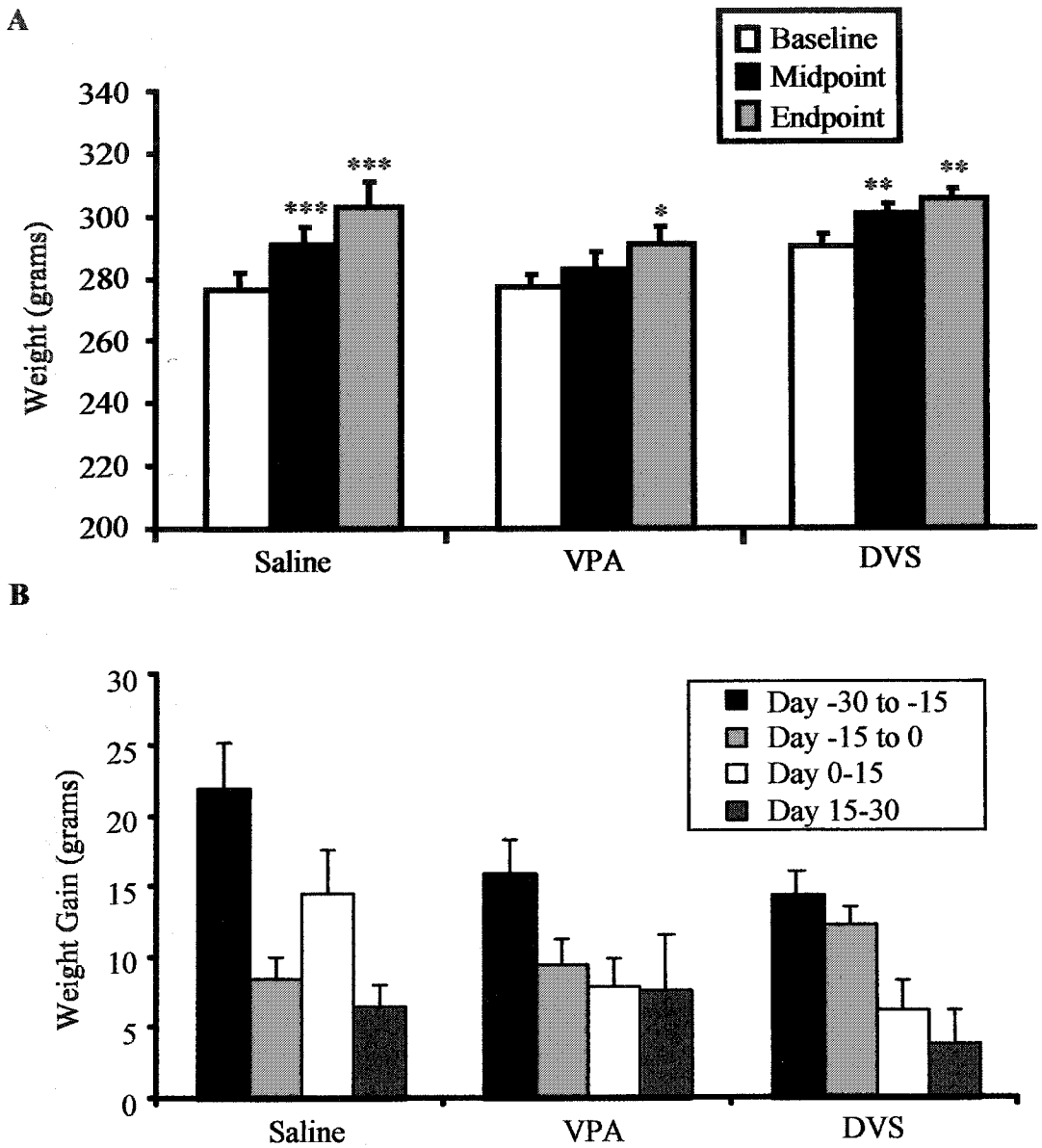


Figure 2.4 Changes in weight following treatment with VPA or DVS
A) Mean weight at baseline, midpoint, and endpoint for animals treated with saline (n=8), VPA (n=8) or DVS (n=6). No significant differences between groups, significant increase over time when compared to baseline, Post-hoc comparisons compared to baseline* $p<0.05$, **$p<0.01$, *** $p<0.005$ **B)** Mean weight gain for animals treated with saline, VPA, DVS before and after initiation of treatment (day 0).

treated with either DVS and saline, as well as in all three groups of animals when comparing baseline and endpoint weight (Figure 2.4A). Although non-significant, animals in the DVS group weighed more at baseline (average 290.8 ± 2.7 g) when compared to animals in the saline (average 276.5 ± 3.5 g) or VPA (average 273.9 ± 2.9 g) group. This finding was not expected since the animals were randomly assigned to groups. Since there was this difference in weight at baseline, we also evaluated weight gain throughout the study by determining mean weight gain during two 15-day periods before and after treatment initiation (day 0) (Figure 2.4B). Although there were no statistically significant differences in weight gain between the groups, there is a noticeable trend for all animals to have more weight gain 30-15 days prior to the start of treatment, when most animals were undergoing handling habituation and vaginal smear. When examining the weight gain that occurred 15 days after the beginning of treatment (day 0-15), there was a non-significant trend for differences between the groups ($p=0.06$), with the saline group having more weight gain when compared to the drug-treated rats. These results suggest that VPA does not induce weight gain in rats.

Serum Leptin Levels

As discussed in detail in Chapter 5, leptin is an adipocyte-derived hormone that is critical for controlling appetite and energy balance in rodents (143). Leptin levels are often significantly elevated in obese patients treated with VPA (126, 144). In the rats, serum leptin levels appeared to stay relatively constant throughout the study period, ranging from 3 to 6 ng/ml (Figure 2.5). These results are similar to published leptin levels for female rats on the second day of diestrus (3.2 ng/ml) (145). Statistically, there was an overall significant change in serum leptin levels over time, but no significant

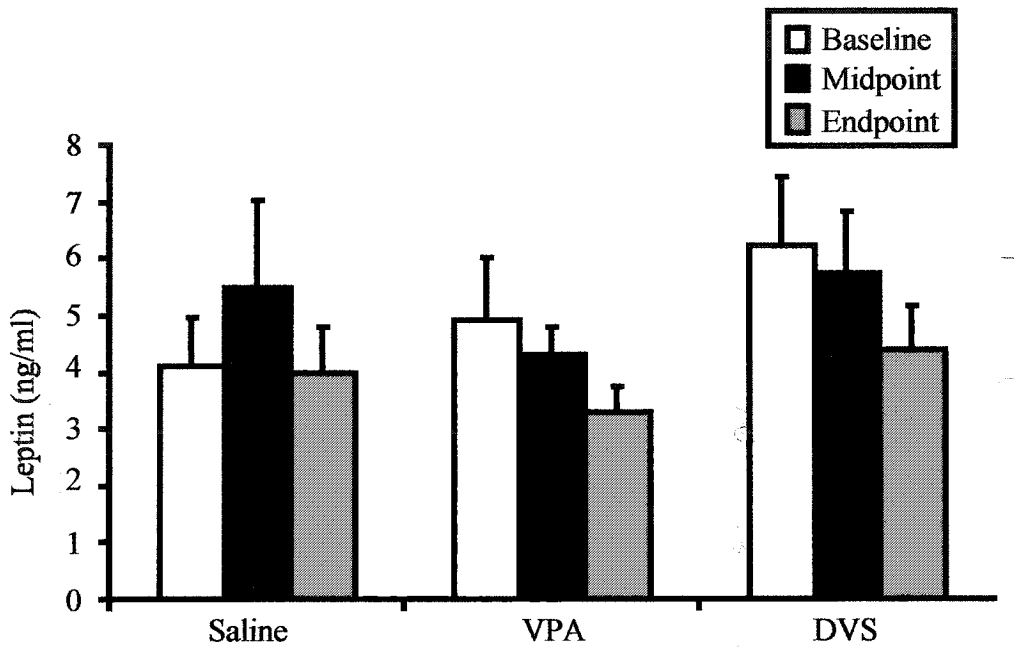


Figure 2.5 Serum leptin levels during treatment with VPA or DVS
Mean serum leptin levels at baseline, mid-point, and end-point for animals treated with saline (n=8), VPA (n=8) or DVS (n=6). No significant differences between groups, ANOVA.

differences between treatment groups. Post-hoc analysis demonstrated that for each group of animals, there were no significant differences in serum leptin levels between time points.

Correlations between Weight and Serum Leptin Levels

In rats, weight and serum leptin levels are correlated (reviewed in 146). When examining the correlation between weight and serum leptin level at baseline, midpoint and endpoint for all animals in the study (n=22), there was a significant positive correlation with higher serum leptin levels occurring in animals that weighed more (Figure 2.6A, 2.6B).

When examining the same correlation for animals treated with either saline, VPA, or DVS, none of the groups had a significant correlation between leptin and weight at baseline (Figure 2.6A, 2.6B). Only the animals treated with PBS had a significant positive correlation ($p<0.05$) between leptin and weight at midpoint and endpoint. The square of the correlation coefficients for saline treated animals demonstrates that at midpoint and endpoint, linear regression explains 68% and 59% of the variations in leptin values over the rat's weight range. For DVS and VPA treated animals, leptin levels and weight are not significantly correlated at midpoint or endpoint. For the VPA treated animals, a linear regression model at both midpoint and endpoint accounted for 0.9% and 4.9% of the variation in leptin levels over the rat's weight range. This data must interpreted in light of the lack of significant correlation at baseline, yet suggests that VPA treatment may be associated with loss of correlation between serum leptin levels and weight.

A Pearson Correlation Coefficients for Serum Leptin Levels and Weight

	Baseline	Midpoint	Endpoint
All Groups (n=22)	0.52 (p=0.01)	0.54 (p=0.01)	0.63 (p=0.02)
Saline (n=8)	0.45	0.82 (p=0.02)	0.77 (p=0.02)
VPA (n=8)	0.49	0.03	0.07
DVS (n=6)	0.75	0.81	0.44

B

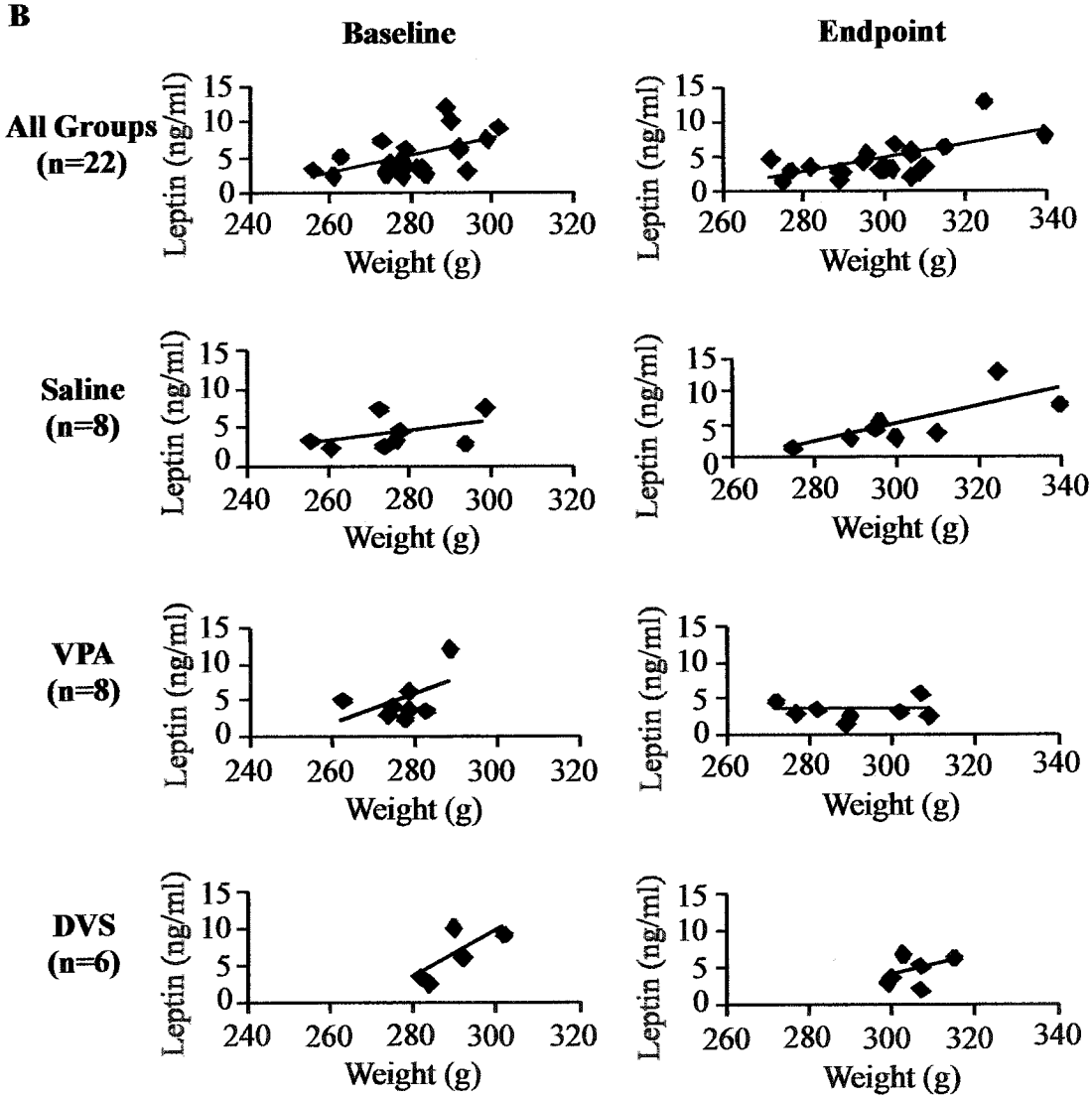


Figure 2.6 Correlation between serum leptin levels and weight
A) Pearson correlations coefficients between weight and serum leptin levels for all the rats in study, and independently for saline, valproic acid (VPA) and divalproex sodium (DVS) treated rats. Significant p values are indicated in brackets. **B)** Scatterplots of weight and serum levels at baseline (left graphs) and endpoint (right graphs) for all rats in the study and for saline, VPA, and DVS treated rats. Best fit linear line are shown.

Estrous Cycles

To determine whether estrous cycles were altered with VPA treatment, we categorized the stage of the estrous cycle daily using vaginal smears. A typical estrous cycle consists of 12 hours in proestrus, 30 hours in estrus, 6 hours in metestrus, and 48 hours in diestrus, with the whole cycle occurring in 4-5 days (136). The stage of the cycle is determined by the composition of the cells obtained by vaginal smear as shown in Figure 2.7A.

Thirty days prior to the initiation of treatment, animals in each of the three groups presented a typical 4-5 day estrous cycle, with an average total number of 14/30 days in proestrus-estrus and between 15-16/30 days in diestrus (Figure 2.7B). Similarly, 30 days after the initiation of treatment, there were no significant differences in the mean number of days animals were in proestrus-estrus or diestrus between the groups. Although there were no statistically significant differences in these mean values before and after treatment, there was a large difference in the variance for the number of days in proestrus-estrus and diestrus after treatment initiation (Figure 2.7B). This large variation in mean number of days occurred in all treatment groups and demonstrates that the rat estrous cycle may have been sensitive to experimental manipulations associated with treatment (increased handling, pipetting in mouth).

By the end of the study, 29% of drug-treated rats (2/8 VPA, 2/6 DVA) had been in constant estrus for at least 14 days, and 12.5% of the saline treated rats (1/8) had been in constant estrus for 10 days. These results indicate that a small proportion of rats in all of the groups developed anovulation.

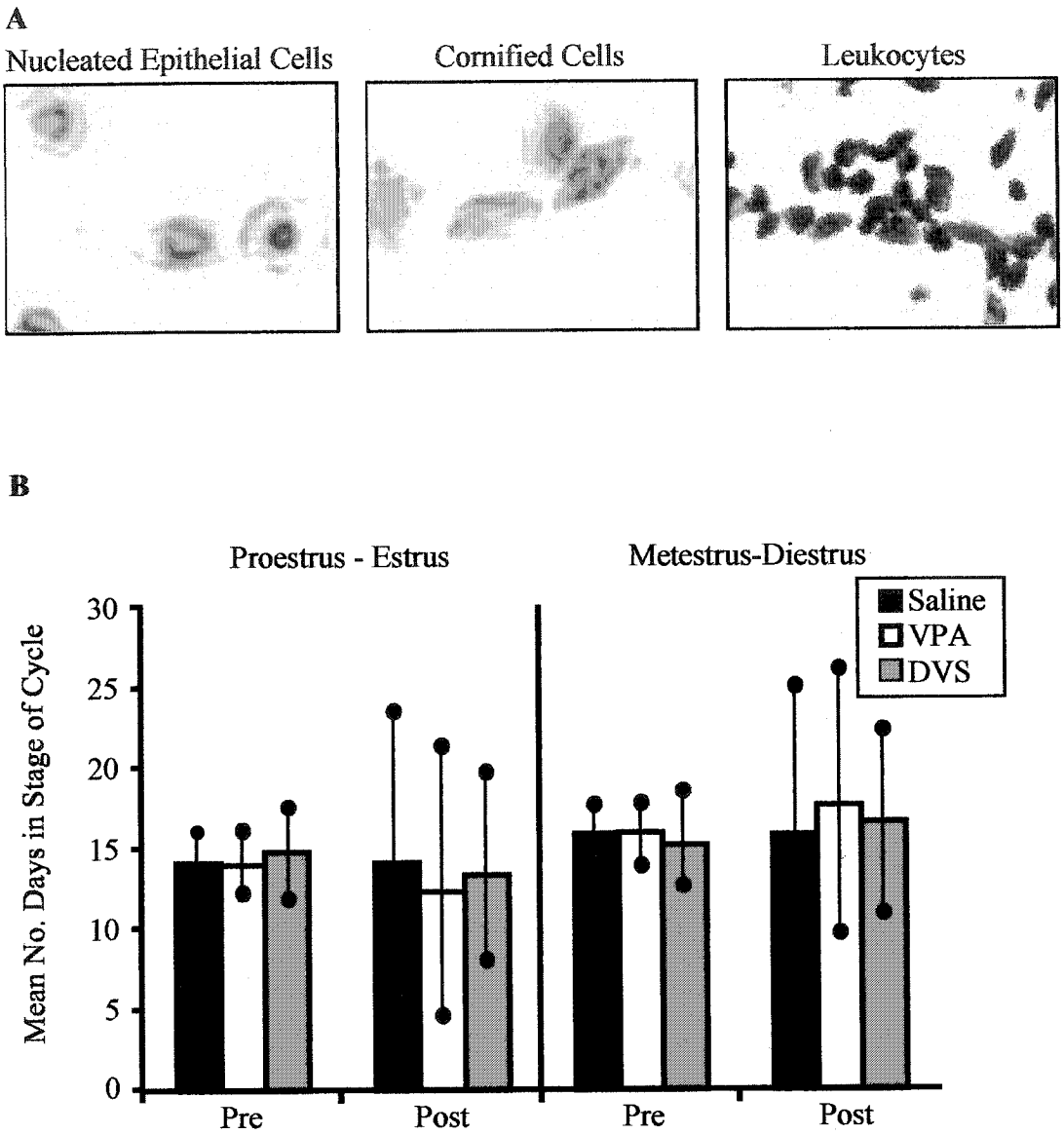


Figure 2.7 Estrus cycle following treatment with VPA or DVS
A) Representative pictures of stained vaginal smears demonstrating nucleated epithelium cells, cornified cells, and leukocytes. Estrus cycle stage is identified by smears comprising of mainly: nucleated epithelium cells in proestrus, cornified cells in estrus, cornified cells and leukocytes in metestrus, and only leukocytes in diestrus. **B)** Comparisons between the mean number of days the rats were in proestrus-estrus and metestrus-diestrus as assessed before (pre) and after (post) 30 days of treatment with saline (n=8), VPA (n=8) or DVS (n=6). Vertical lines indicate the range of days for animals within each group.

Ovarian Morphology

Female patients and rats receiving VPA have been observed to develop ovarian cysts (113, 133, 134). We analyzed ovarian morphology at the end of the study period and found some ovaries had cystic morphology as demonstrated in Figure 2.8. Follicular cysts (FC) are characterized by a highly attenuated granulosa cell layer and a hypertrophied thecal cell layer comprised of larger polygonal lipid filled cells (147). Polycystic morphology is the product of complete or arrested atresia of development of secondary follicles in the absence of ovulation (147). The corpus luteum (CL) forms following ovulation and is primarily responsible for the synthesis and release of progesterone, a hormone essential for the establishment and maintenance of pregnancy in mammals. In the absence of pregnancy, the CL will decrease in mass in a process, termed luteolysis, which occurs between proestrus and estrus (148). Thus the presence of CL is a marker for the number of follicles that have undergone ovulation.

The number of animals that had FC, as well as the total number of CL and cysts per ovarian section were determined by a counter blinded to treatment assignment (Figure 2.9). There was no statistically significant difference in the mean number of CL or FC between the three treatment groups (Figure 2.9).

There was a significant difference in the number of animals that had FC between the groups with 25% (n=2/8) of control animals and 50% (n=3/6 and 4/8) of drug treated animals having at least one cystic follicle/ovarian section. In addition, animals that remained in constant estrus (4 in drug treated and 1 in the saline treated group) had an abnormally large number of FC (range: 5-9) and relatively fewer CL (range 0-4).

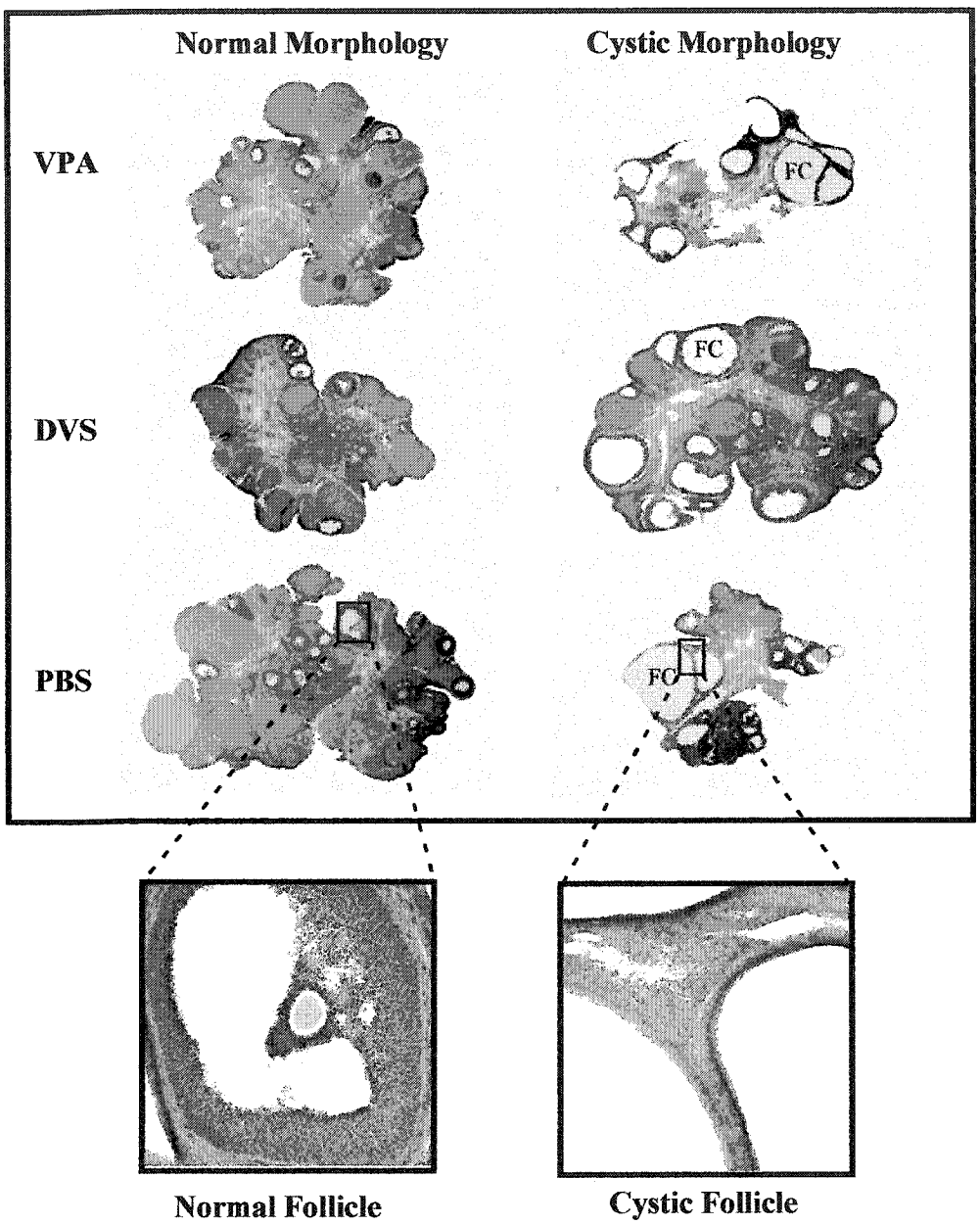
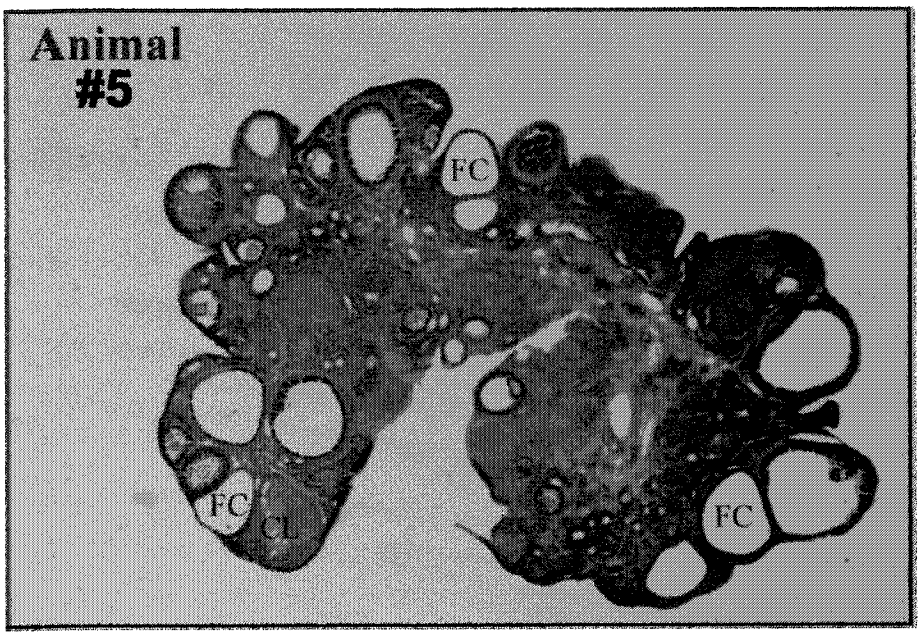


Figure 2.8 Representative ovarian morphology
Sections of ovaries harvested at endpoint. Normal, healthy follicles, at all stages of development (primordial, primary, secondary, antral, plus numerous corpora lutea), typify normal ovarian morphology. Follicular cysts (FC) were found in ovaries from animals in all treatment groups.

A



B

	Corpora lutea (CL)		Follicular Cysts (FC)	
	Mean (SE)	**Range**	**Mean (SE)**	**Range**
Saline	7.1 (1.6)	0-16	1.1 (0.7)	0-5
VPA	6.8 (1.7)	0-15	1.3 (0.6)	0-5
DVS	6.5 (1.5)	1-10	0.5 (0.2)	0-1

Figure 2.9 Number of corpora lutea and cystic follicles following treatment with VPA or DVS

A) Representative section of an ovary demonstrating corpora lutea (CL) and follicular cysts (FC) **B.** Mean number and range of corpora lutea and cystic follicles in animals treated with saline (n=8), VPA (n=8), or DVS (n=6). Numbers in parentheses indicate standard error from the mean.

Uterine Wet Weight

As a further determinant of endocrine function we measured the wet weight of the uterus. Uterine wet weight is used in rat screening tests for the detection of estrogen-disrupting chemicals, with higher uterine weight being associated with chemicals that have higher estrogenic potency (149). The uterine wet weight was not significant between the animals in the three groups. The mean (±SE) for uterine wet-weight was 162.9± 4.7, 159.1±6.2, 152.5±5.9 (mg/kg body weight) for saline, DVS, and VPA treated rats, respectively.

Serum Estradiol Levels

From serum samples that were collected at baseline, midpoint and endpoint, total estradiol and total testosterone serum levels were measured. Throughout the study period, total estradiol levels rose in all treatment groups (Figure 2.10A). This was confirmed statistically with an overall significant change in total estradiol over time in the absence of any differences between treatment groups. When comparing the differences in serum estradiol levels between time points within each group, post-hoc analysis demonstrated a significant difference between baseline and midpoint levels for saline and VPA treated rats, as well as significant difference between baseline and endpoint levels for all three groups.

Serum Testosterone Levels

Since hyperandrogenism is associated with VPA treatment in patients (113), we measured total serum testosterone levels. There was no significant difference in total serum testosterone levels between the groups or between time points (Figure 2.10B).

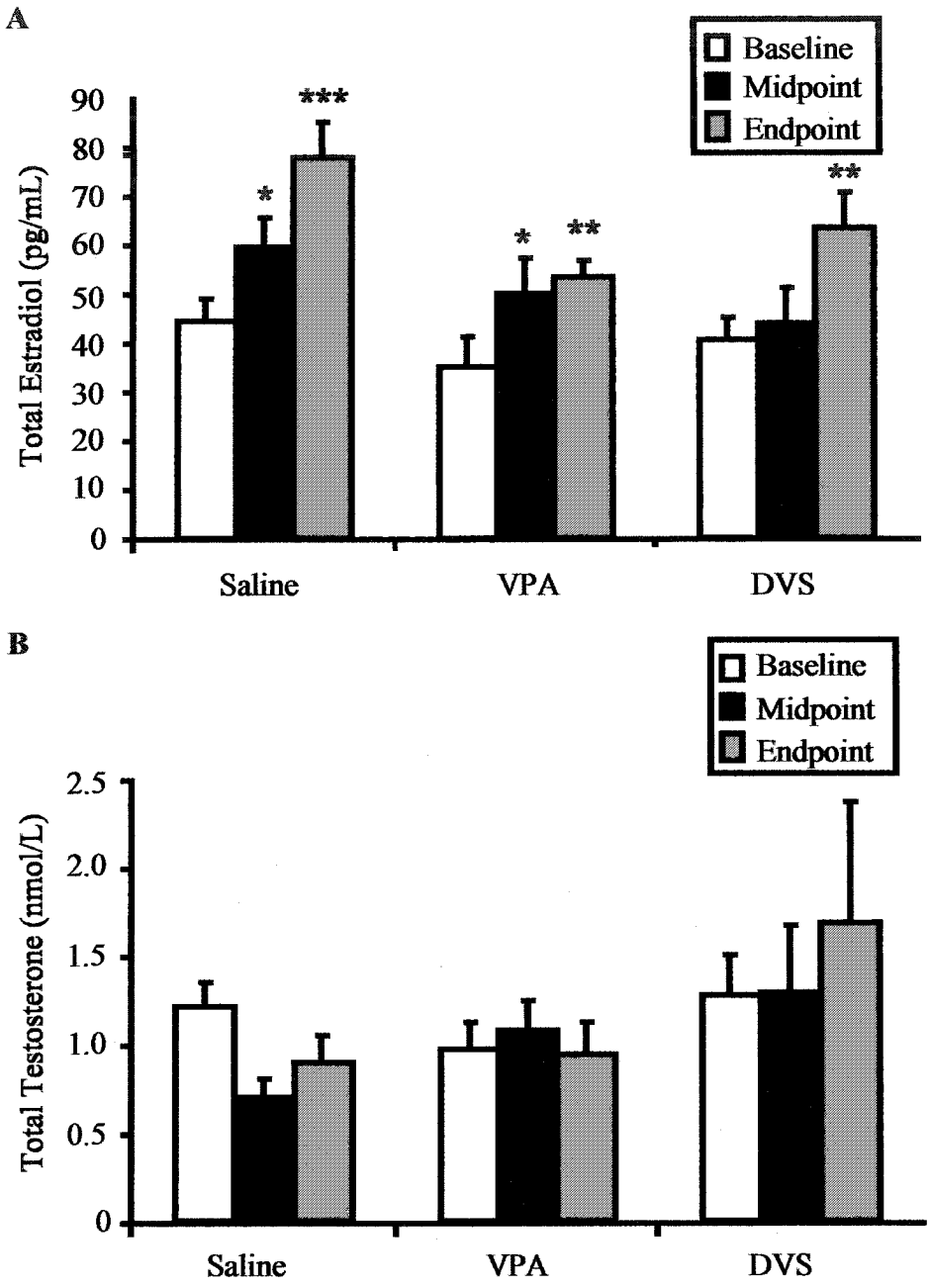


Figure 2.10 Estradiol and testosterone levels during treatment with VPA or DVS

Serum total estradiol **(A)** and total testosterone **(B)** levels measurements at basline, midpoint, and endpoint for rats treated with saline (n=8), VPA (n=8) or DVS (n=6). No significant differences between groups, significant increase over time when compared to baseline, Post-hoc analysis as compared to baseline, * $p<0.05$, ** $p<0.01$, *** $p<0.005$. Bars represent standard error of the mean.

Throughout the study these values ranged from 0.7-1.6 nmol/L, which is consistent for total serum testosterone levels in female rats during the second day of diestrus (133, 150).

Ovarian Aromatase Activity

VPA treatment of follicles *in vitro* increases the testosterone:estradiol ratio due to decreasing aromatase activity (128, 129). In the animals within this study there was no significant differences in mean aromatase activity between the groups, with mean (±SE) aromatase activity being 1.25±0.26, 1.02±0.19, and 1.18±0.12 (pmol/ hr /mg protein) for the saline, VPA, and DVS group, respectively. This activity is within the normal range for untreated cycling female rats (0.5-1.6 pmol/ hr /mg protein) (138, 151).

DISCUSSION

Women treated with VPA can experience symptoms that are characteristic of PCOS including enlarged ovaries with multiple follicular cysts, menstrual irregularities, high androgen production, and obesity (110-112, 125). The aim of this study was to prospectively determine whether rats treated with VPA displayed symptoms of PCOS to determine whether this paradigm provided a useful model to evaluate VPA-induced PCOS. Our results show that during treatment with VPA animals demonstrated differences in ovarian morphology, but did not differ in estrous cycle, serum estradiol or testosterone levels, or weight gain when compared to control animals.

VPA Induction of Cystic Follicles

Polycystic ovaries in the normal population have a prevalence of approximately 20%, whereas rates as high as 43% have been reported in women with epilepsy receiving VPA therapy (81, 105). In our study, we found that 25% (n=2/8) of control animals and 50% (n=3/6 and 4/8) of drug treated animals had FC. There was no significant difference in the mean number of FC between the animals, most likely due to the large range and high variability between animals. Two previous studies examining VPA effects on ovarian morphology did not report the percentage of animals with FC following VPA treatment, but did report that rats treated with 200 and 600 mg/kg VPA for 90 days had a significantly increased mean number of ovarian FC compared to saline-treated animals (133, 134). Similar to our studies, there was a large standard error associated with the mean number of FC. Our results were not significant, yet their results were significant most likely due to the larger sample sizes (15-19 animals per group). The combination of

our data and these previous studies support the hypothesis that VPA induces the formation of FC in rats.

VPA Effects on Ovulation

Infertility is often one of the presenting features of PCOS, most likely due to anovulation (83). Treatment with VPA in women is associated with significantly higher rates of irregular menstrual cycling including anovulation (no menstruation) and oligomenorrhea (cycle length longer than 35 days) (105, 114, 125, 126). In rats, ovulation can be indirectly assessed through the occurrence of regular estrous cycles and the presence of corpora lutea within the ovary. Bruguerolle *et al.* (132) previously reported normal estrous cycling in female rats following treatment with a relatively low dose of sodium valproate (10 to 200 mg/kg). In this study, there were no significant differences in the number of animals that developed a stage of constant estrus (29% vs. 12.5%, drug vs. control), or mean number of days in each stage of the cycle between drug-treated and saline-treated rats. Irrespective of group, the rats had more irregular cycling following treatment onset as indicated by the large variance in number of days in each of the stages of the cycle, which may be attributable to the handling and stress of experimental manipulation. In addition, treatment with VPA or DVS was not associated with differences in the average number of CL; however the rats that had developed constant estrus had relatively few CL, suggesting that 29% of drug-treated rats and 12.5% of rats were anovulatory. Previously studies found that rats treated with 600 mg/kg VPA had a significant decrease in the number of CL, which was not present in rats treated with 50-400 mg/kg VPA (133, 134). Furthermore, rhesus monkeys treated for 12-15 months with VPA have regular menstrual cycles and histological evidence of ovulation in their

ovaries (135). Overall these findings suggest that VPA treatment is not associated with a high incidence rate of anovulation in rats or monkeys.

VPA Effects on Serum Testosterone and Estradiol Levels

Patients with PCOS have increased androgen levels in the absence of significant alterations in serum estradiol levels (83, 87). Similarly, women treated with VPA have increased androgen levels yet no alterations in serum estradiol levels (105, 113, 117, 118). Like human serum estradiol levels, rat serum estradiol levels are dynamically regulated during the estrous cycle. Serum estradiol levels surge on the second day of diestrus, plateau at the initiation of proestrus, fall dramatically with the preovulatory LH surge, and then remain low until the end of estrus (152). In this study, estradiol levels were measured on day 2 of diestrus and for an unidentified reason increased in both drug and saline treated groups throughout the study period. No significant differences in estradiol levels have been reported in rats treated for 90 days with 50 or 200 mg/kg/day VPA and in rhesus monkey treated for 12-15 months with 960 mg/kg/day VPA (133-135). Overall, treatment with VPA in monkeys and rats is not associated with alterations in serum estradiol levels.

Hyperandrogenism occurs in 17 to 63% of patients treated with VPA compounds (105, 109, 118). VPA has also been demonstrated to increase androgen biosynthesis in a human ovarian thecal cell model (130) and increase testosterone and estradiol secretion in porcine ovarian follicular cells (127-129). In this study, despite rising levels of estradiol, total testosterone levels remained constant throughout the study and there were no differences between drug- and saline-treated rats. Touboll *et al.* (133) reported previously that after 90 days of VPA treatment female rats had lower total testosterone levels

[control = 1.44 ± 0.44, VPA treated (200 mg/kg/day) = 0.98 ± 0.56, (p=0.01)]. In rhesus monkeys, VPA treatment did not alter androgen levels, nor the LH response to a GnRH agonist challenge (135). Therefore, unlike in humans, VPA treatment in rats and monkeys does not induce a state of hyperandrogenism.

Previous work examining the effect of VPA on steroid production in cultured porcine follicles showed that VPA inhibited the conversion of testosterone to estradiol by inhibiting P450 aromatase (CYP19) (127). In this chapter, no significant difference in ovarian aromatase activity was found between VPA, DVS, or saline-treated animals. Irrespective of group assignment, rats had no alterations in serum testosterone levels yet had increased serum estradiol. It is unknown why this difference occurred. The measurement of aromatase activity in this study would be unable to detect changes in aromatase activity during the study, since activity was measured in the harvested ovarian tissue at the end of the study.

VPA Induced Weight Gain

Both obesity and PCOS commonly occur concurrently in women of reproductive age (87). Several reports examining weight gain in patients with epilepsy suggest that between 40 – 60% of adult patients will gain between 17 to 30 lbs (7- 14 kg) while taking VPA (106, 107, 118, 144, 153). Unlike the proportion of humans that gain weight following VPA treatment, drug treated rats did not show increased weight gain when compared to controls. In the only prospective study to examine weight change following VPA treatment, after an initial lag in weight gain in 12 month old male rats fed a high dose of VPA (600 mg/kg), VPA treated rats had similar weight compared to control animals by the end of the study period (154). In two other studies examining VPA

therapy for 90 days in female rats, no alterations in weight were detected at endpoint assessment (133, 134). In contrast to the effect of VPA in rats, VPA therapy in rhesus monkeys induced a significant increase in weight gain after 9 months of treatment (135). These results suggest that VPA's ability to induce weight gain may be a characteristic of primate physiology.

The mechanism(s) leading to weight gain with VPA therapy is unknown. Weight is the outcome of energy balance and is controlled through a variety of mechanisms (reviewed in 155). Patients treated with VPA who gain weight have increased serum leptin levels, increasing by 3.5 fold on average one-year post treatment initiation (144). The increase in leptin following VPA treatment has been hypothesized to simply reflect an increase in body mass, since patients that do not gain weight on VPA have no significant changes in serum leptin levels (144). Serum leptin levels also do not differ between women with PCOS and weight-matched controls (87). In this study, leptin levels did not significantly differ between rats treated with VPA, DVS, or saline. However, at the endpoint of the study leptin levels and body weight were not correlated in VPA-treated animals, but they were correlated in saline-treated rats. These data suggest that treatment with VPA may alter "leptin homeostasis" despite having no direct affect on body mass in rats. Further work examining the direct effects of VPA on leptin secretion and mRNA levels is discussed in Chapter 5.

Limitations of the Model

The results from this study must be interpreted within its limitations, including the small sample size. We initially planned to have 12 animals per group, however, 14 of the 36 animals were excluded due to abnormal estrus cyclicity prior to treatment. The

incidence of abnormally cycling rats and the frequency of cystic follicle development in the normal rat population must be considered in the experimental design. The problem with including rats that have abnormal cycling at the onset of the study, which most likely occurred in previous studies (132-134), is that it is difficult to determine drug specific effects. By including animals that have normal estrous cycles this study demonstrates that VPA could induce cystic follicles in rats that had no prior endocrine abnormalities. However, by excluding animals that have abnormal estrous cycles this study is excluding the 1/3 of the population that were "at risk" for endocrine abnormalities. VPA treatment is prescribed to women independent of their current endocrine status and women with bipolar disorder or epilepsy may have an increased risk for irregular cycling (113). Thus, it is possible by excluding the "at risk" animals and studying only the normal cycling rats, significant differences in a larger, unselected population of rats were missed. In fact, it may be interesting for future work to study both normal cycling and abnormal cycling rats to determine if VPA produces different effects between these two populations.

Laboratory rats were chosen for this study due to logistical reasons (availability of a rodent facility), as well as the large amount of literature examining the distinct ovarian cyclic pattern of estrus, and similar biochemical processes and anatomic features between normal female rats and women. Furthermore, the pharmacokinetics of VPA in rats has been well studied and although it is difficult to achieve stable therapeutic drug levels in rodents, we were able to somewhat overcome this problem by treating the animals three times a day. Some of the disadvantages of using rats include the relatively limited amount of blood that can be withdrawn at one time point, the stress associated with

handling the animals, and the inability to assess ovarian morphology in live animals. The limited amount of serum that could be obtained also prohibited the assessment of many other biochemical parameters that are associated with PCOS, including LH, FSH, and insulin levels. In the study examining VPA effects in rhesus monkeys, sufficiently large blood samples were obtained to allow for measurements of LH, FSH, insulin, as well as responses to a GnRH agonist and a glucose tolerance test (135). However, none of these parameters were significantly different in the monkeys before and after VPA treatment. Thus, despite this study being unable to measure a wider set of hormone parameters, VPA did not induce hyperandrogenism, high LH:FSH ratio, or hyperinsulinemia in rats or monkeys, which are highly prevalent in human PCOS.

Future Questions

Laboratory rats have been used in a variety of experiment models for PCOS which include: injection of estradiol valerate, neonatal androgenization, chronic administration of dehydroepiandrosterone or an aromatase inhibitor (letrozole), and maintenance of animals in constant light (156, 157). These models have led to a better understanding of PCOS pathogenesis and helped to discover improved treatments for women with PCOS, yet none of these models are able to produce all the pathological symptoms that are observed in PCOS in humans. Although there is currently no known genetic cause for PCOS, it may be that PCOS develops as a consequence of abnormal genetic factors that interact with environment factors (such as treatment with VPA) leading to clinical pathology. If PCOS has a genetic etiology, rats may not possess this genetic abnormality and thus we may not be able to model PCOS. The notion that PCOS arises from altered genetics has several lines of support including familial clustering of

PCOS within first-degree relatives and linkage analysis suggesting putative candidate gene(s) may be located on chromosome 19 (reviewed in 89). In addition, cultured PCOS thecal cells when compared to control thecal cells can produce altered levels of androgens and have increased mRNA levels for several P450 enzymes, including CYP11A1 and CYP17 (89). It is possible that VPA may only induce PCOS in women that have this unidentified genetic abnormality. In support of this idea, VPA can augment androgen release in the thecal cells isolated from PCOS patients compared to thecal cells from controls, suggesting that VPA effects are altered in cells that have a genetic predisposition for PCOS (130). Thus investigations into the possible pathogenesis of PCOS may help to elucidate why a proportion of females treated with VPA develop PCOS.

Several proposed mechanisms have been suggested to explain why VPA treatment is associated with PCOS (113, 123). As described above, VPA may have direct effects on androgen production in ovaries, as supported by data from cell culture models, but not from rodent or primate models. VPA may also affect the neurotransmitter systems that influence hypothalamic-pituitary regulation. It has been hypothesized that VPA may directly effect GnRH neurons which are mainly located in the medial preoptic area (mPOA) of the hypothalamus (158). During sexual maturation, morphological changes occur within the GnRH cell population, with bipolar cells transforming into unipolar cells with irregular spine-like surfaces (159). VPA administration in pubertal mice can alter the density of the inhibitory inputs to GnRH neurons within the mPOA, as well as reduce the maturation of GnRH neurons within the mPOA in juvenile, seizure-prone, inbred mice (160, 161). Although these findings may have relevance to how VPA

effects endocrine function in children, the effects on GnRH maturation were not observed in adult mice and may not be relevant to VPA effects in adult women. However, maturation of GnRH neurons is only one part of the complex development of the GnRH pulse generator and it remains to be determined if VPA has other unidentified actions on the hypothalamic-pituitary axis.

In addition to VPA's action on the ovary and hypothalamic-pituitary axis, it has been hypothesized that VPA may induce the development of PCOS due to the central nervous system pathology associated with epilepsy and bipolar disorder. Indeed, untreated women with epilepsy or bipolar disorder appear to have higher rates of menstrual and endocrine abnormalities, however in many studies VPA treatment was associated with a significant increase in these abnormalities (113, 123). Thus, the theory has been raised that epilepsy or bipolar disorder may predispose individuals to be susceptible for VPA-induced PCOS.

Animal models of epilepsy, using amygdala kindling, have a significant increase in FC formation (162). This model could be used to test whether VPA treatment would reveal a significant increase in PCOS symptoms in kindled rats. In order to determine whether side effects associated with VPA therapy are due to drug treatment, are part of the underlying central pathology of epilepsy/bipolar disorder, or a combination of drug treatment and central pathology, future prospective multi-centered clinical studies including women treated with VPA that have different diseases (migraines, cancer) should be undertaken.

CONCLUSION

With the exception of cystic ovaries, rats fail to exhibit VPA-induced endocrine side effects that are characteristic of PCOS. This conclusion is based on the strengths (homogeneous population, prospective nature, frequent dosing) and limitations (sample size) of this study. In women treated with VPA, there is mounting evidence that a proportion of women will develop PCOS. This is a concern due to the increased health risk and lack of identification of this side-effect in VPA prescribing information. In rats and monkeys VPA treatment is not associated with the development of PCOS, yet this finding does not discount that VPA treatment is associated with the induction of PCOS in humans.

CHAPTER 3

INHIBITION OF HISTONE DEACETYLASE ACTIVITY BY VALPROIC ACID ATTENUATES ADIPOGENESIS

Portions of this chapter appeared in the following publication:

Lagace DC, Nachtigal MW. (2004) *Inhibition of Histone Deacetylase Activity by Valproic Acid Blocks Adipogenesis.* The Journal of Biological Chemistry 279:18851-60. (163)

ABSTRACT

Objective: Like many psychotropic medications valproic acid (VPA) induces weight gain through an unidentified mechanism. VPA is a histone deacetylase (HDAC) inhibitor and histone deacetylation can modulate the process of differentiation in some cell types. It is unknown whether VPA can directly affect adipocyte development and thus this study examined whether VPA modulated adipogenesis. **Results:** VPA treatment inhibited mouse 3T3-L1 and human preadipocyte differentiation, as well as reduced the growth rate of 3T3-L1 preadipocytes. Reduced rates of adipogenesis occurred if VPA treatment was added either once at the onset of treatment in differentiation medium or added daily following the removal of the differentiation medium. Moreover, induction of differentiation by peroxisome proliferator-activated receptor gamma (PPARγ) agonists was blocked by VPA in both mouse and human preadipocytes. In contrast to previous work, VPA was not a ligand for PPARγ in reporter gene assays, suggesting that inhibition of differentiation by VPA was not due to direct effects of VPA on PPARγ-induced transcription. VPA, like other HDAC inhibitors (including trichostatin A (TSA)) inhibited adipogenesis and increased histone H3 acetylation. In contrast, the VPA derivative valpromide, which does not possess HDAC inhibitory effects, did not prevent adipogenesis. VPA treatment did not affect early clonal expansion nor expression of CCAAT/enhancer binding protein alpha (C/EBPα) mRNA, which are required for preadipocyte differentiation. VPA or TSA treatment, however, reduced mRNA levels for the transcription factors, PPARγ and steroid regulatory binding protein (SREBP1a), as well as the protein levels for PPARγ, SREBP1a, and C/EBPα. **Conclusions:** VPA treatment reduced both the number of preadipocytes as well as the development of

adipocytes and the expression of highly regulated adipocyte transcription factors. VPA effects *in vitro* attenuated adipogenesis, yet *in vivo* VPA induced weight gain, suggesting that VPA may be affecting multiple systems that regulate energy balance *in vivo*. The similar effects of VPA and HDAC inhibitors on the processes of differentiation highlight a requirement for HDAC activity in adipogenesis.

Keywords: Adipogenesis/Histone Deacetylase/Valproic Acid

INTRODUCTION

VPA treatment can induce weight gain and contribute to the development of obesity. Numerous hypotheses have been postulated to explain why VPA induces weight gain, and VPA effects on multiple systems involved in energy balance most likely underlie this side effect. In this chapter VPA treatment is demonstrated to attenuate the process of adipogenesis, which occurs when preadipocytes differentiate into adipocytes.

VPA-INDUCED WEIGHT GAIN

The propensity of VPA and other psychotropics to induce weight gain has been the subject of many recent reviews (164-167). In general the reported incidence rate for weight gain associated with VPA treatment varies widely from 4-71% between trials (reviewed in 166), due to differences in patient populations and varieties of definitions for weight gain. In a double-blind trial (n=480) for the treatment of epilepsy, a larger proportion of VPA-treated patients (20%) had significantly more weight gain (>5.5kg) when compared to carbamazepine-treated patients (6%) (168). Similarly, in a multi-centered 3-year comparative trial assessing VPA and carbamazepine for the treatment of epilepsy, weight gain was the most frequent adverse event associated with VPA therapy in both children and adults (169, 170). For the treatment of bipolar disorder, a randomized controlled 1-year follow-up study (n=187) reported that weight gain, defined as a greater than 5% increase over baseline weight, was more common in patients receiving VPA (27%) than placebo (7%) (171). In another 47-week randomized study of 123 patients with bipolar disorder, VPA induced weight gain was similar to the weight gain observed in patients receiving olanzapine, an antipsychotic drug that has also been well documented to increase weight (172). These large long-term randomized trials

provide the most robust evidence that chronic treatment with VPA results in weight gain in a significant number of patients.

The mechanism underlying VPA-induced weight gain has not been elucidated. Data from several different trials show that age, gender, medical condition, dose and serum concentrations of VPA, as well as family history of bodyweight problems, are not significantly correlated with the gain in weight associated with VPA treatment (173-176). There are conflicting reports investigating whether the weight of patients (measured by body mass index (BMI)) before treatment initiation can predict VPA-induced weight gain. In adults, lower baseline BMI scores were predictive of weight gain with VPA (175), in younger adults baseline BMI scores were not predictive of weight gain (177), and in children higher baseline BMI scores were predictive of weight gain with VPA (173). Therefore combining information on age and BMI score prior to treatment initiation may help identify whether individuals are susceptible to gain weight on VPA, however these conclusions must be viewed with caution due to the limited number of trials that have addressed this question.

A number of studies have also noted that patients that gain weight on VPA report appetite stimulation and increased thirst and quenching with calorie-rich beverages (174, 176, 178). When measured quantitatively in one study, VPA treatment was not associated with an increase in total intake of calories, monosaccharides or disaccharides (179), yet it is well known that it is difficult to get an accurate measurement of food intake from humans (reviewed in 155). The increase in appetite reported for patients receiving VPA treatment has been suggested to result from enhanced GABA transmission within the hypothalamic axis of the CNS. Yet, as recently reviewed by Van

den Pol (180) the role of GABA and glutamate in regulating energy balance has yet to be determined. It is unlikely that VPA's ability to induce weight gain is solely due to increased GABA transmission, since other anticonvulsants that increase GABA, including tiagabine, are not reported to induce weight gain (181).

Another group of hypotheses have suggested that the weight gain associated with VPA therapy is due to alterations in serum carnitine, leptin, and insulin levels, which are all known to play a role in energy homeostasis (reviewed in 165, 166). VPA treatment can be associated with carnitine deficiency (20, 182-184) and as discussed in the introduction on VPA (Chapter 1), carnitine is involved in the breakdown of fatty acids by β-oxidation. However, a prospective study has recently demonstrated that carnitine levels in patients receiving VPA therapy do not correlate with VPA-induced weight gain, suggesting that carnitine does not mediate VPA induced weight gain (178). In contrast, significant increases in both serum leptin and insulin levels have been reported in patients that gained weight following 1 year of therapy with VPA, and were suggested to be part of the pathogenesis of VPA-induced weight gain (144). The increase in leptin following VPA treatment has been hypothesized to simply reflect an increase in body mass (adipocyte number or size), since patients that do not gain weight on VPA have no significant changes in serum leptin levels (185). Higher serum insulin levels have been associated with VPA-induced weight gain in a variety of trials (106, 121, 144, 178). Unlike the increase in leptin that is associated with increased body mass, the significant increase in insulin occurs in VPA treated patients compared against weight matched controls (185). Recent work has demonstrated that VPA can increase insulin secretion from human pancreatic β-cells (131). These authors suggest that the modulation of

insulin by VPA occurs by either 1) VPA directly stimulating insulin secretion, which has been demonstrated for other fatty acids (reviewed in 186) or by 2) VPA competing for binding with serum albumin (187) resulting in an increase in serum free fatty acids which then stimulates insulin secretion (131). The ability of VPA to modulate insulin secretion raises the hypothesis that VPA has direct effects on peripheral tissues, which may contribute to VPA effects in energy homeostasis. This chapter will explore whether VPA effects adipose cell development.

Alterations in Adipose Tissue During Weight Gain

A significant increase in the incidence of obesity in the Western world has led to intense research examining the factors that contribute to weight gain and obesity (reviewed in 188). The development of weight gain and obesity occurs when energy intake (feeding) exceeds total body energy expenditure (physical activity, basal metabolism, adaptive thermogenesis) for an extended period of time (reviewed in 155). The physiological system that controls energy balance, includes long and short term afferent signals mainly produced from adipose tissue, liver, pancreas, and gut, which are integrated within the arcuate nucleus of the hypothalamus and result in efferent outputs that regulate appetite, energy expenditure, hormonal milieu, energy partitioning, reproduction, and growth (reviewed in 189). During weight gain and the onset of obesity, excess energy is stored in the form of triacylglycerol (TAG) within adipocytes.

Adipocytes are found within adipose tissue, which is present in numerous anatomical localizations and depots within the body. Functionally, adipose tissue is divided into brown adipose tissue (BAT) that is responsible for energy dissipation and white adipose tissue (WAT) that is responsible for energy storage (reviewed in 190).

BAT is present only in mammals and functions to generate heat for the maintenance of body temperature and arousal from hibernation. The brown appearance of BAT is due to the high content of respiratory chain pigments (cytochromes) that are involved in the production of heat by the uncoupling of the mitochondrial respiratory chain by the uncoupling protein 1. In rodents BAT is critical for thermogenesis and has an important role in total energy homeostasis and body weight regulation. For example, certain dietary components in rodents can increase energy intake yet not affect body fat because of increased energy expenditure by BAT (191). In humans there is inter-individual variations in the amount of BAT in adults and the function of BAT is less clear. Similar to all larger mammals, BAT in humans is highly expressed within the fetus and then decreases in infancy. In adult humans BAT has been identified in various anatomical sites, yet it is estimated on average that 1 in 200 of the total number of adipocytes in humans are within BAT (reviewed in 190). The relatively lower amount of BAT compared to WAT is hypothesized to reflect the lower requirement for thermogenesis by BAT in humans.

In contrast to BAT, WAT accumulates in obesity and primarily functions to store energy. WAT is divided into subcutaneous and visceral adipose tissue, with the visceral stores being associated with a higher risk for diseases that are associated with obesity such as Type II Diabetes (reviewed in 190). The WAT is comprised of approximately $1/3^{rd}$ mature adipocytes and a combination of blood vessels, preadipocytes, fibroblasts and macrophages (192). WAT mass increases during weight gain by an increase in adipocyte size (hypertrophy) and from an increase in the number of adipocytes (hyperplasia) (193). Hypertrophy occurs through the balance of the synthesis

(lipogenesis) and breakdown (lipolysis) of TAG in mature adipocytes, whereas hyperplasia results from the interaction between preadipocyte proliferation, differentiation, and cell death. The hypothesis that hyperplasia occurs in adipose tissue is a more recent and controversial issue. Adipocyte hyperplasia has been observed in many rodent models of obesity, including high fat-fed rats (194), transgenic mice over-expressing the insulin-responsive glucose transporter 4 (GLUT4) in adipose tissue (195), and estrogen-receptor-α knockout mice (196, 197). It has also been observed that preadipocytes in human WAT can differentiate *in vitro* into mature adipocytes (198, 199). More recently, human WAT obtained by liposuction has been demonstrated to contain multipotent stem cells that can differentiate *in vitro* into mesenchymal lineages (adipogenic, chondrogenic, myogenic, or osteogenic cells) in the presence of lineage-specific induction factors (200-202). Thus, it is generally becoming more accepted that preadipocytes exist within WAT and that differentiation can occur in humans.

ADIPOGENESIS

The process of differentiation from preadipocyte into mature adipocyte is characterized by a number of highly controlled temporal events. Cells are "committed" to the adipocyte lineage once a multipotent stem cell has become a preadipocyte and lost the ability to differentiate into other mesenchymal lineages. In the early 1970s clonal preadipocyte immortal fibroblast cell lines, designated as 3T3-L1 and 3T3-F442A, were established from Swiss 3T3 cells that were derived from disaggregated 17- to 19-day mouse embryos (203). Subsequent preadipocyte cell lines have been established, however the earlier models remain highly utilized in the study of adipogenesis and have led to a wealth of information about adipocyte differentiation (204). The main processes

that have been identified to occur during differentiation are described below and depicted in Figure 3.1. These include growth arrest of preadipocytes, induction of differentiation, subsequent alterations in cell morphology, clonal expansion, and alterations in gene expression and lipid metabolism.

Committed preadipocytes are required to withdraw from the cell cycle before undergoing adipocyte conversion. When preadipocyte cell lines and primary cells are grown to confluence, a state of growth arrest occurs (reviewed in 193, 205). Contact inhibition elicits growth arrest, although contact inhibition may not be a necessary component of adipogenesis. For example, differentiation can occur when growth arrest is induced in growing, non-confluent, NIH-3T3 cells that express activated PPARγ (206). In this model, activation of PPARγ is demonstrated to modulate the activity of transcription factors that regulate the cell cycle. Specifically a progressive decease in the DNA-binding activity of E2F, which is involved in initiating the S phase of the cell cycle, occurs following activation of PPARγ (206). However, since these studies have been completed in transformed cells, it remains to be established whether growth arrest can occur independent of contact inhibition in other models of adipogenesis.

Growth arrested preadipocytes undergo differentiation when treated with inducing agents. At the time of the addition of inducing medium, cells are usually defined to be at day 0 of the differentiation process (Figure 3.1). Preadipocyte cell lines and primary cell culture models vary in their responsiveness to inducing agents and thus various combinations of agents are used to induce differentiation (204).

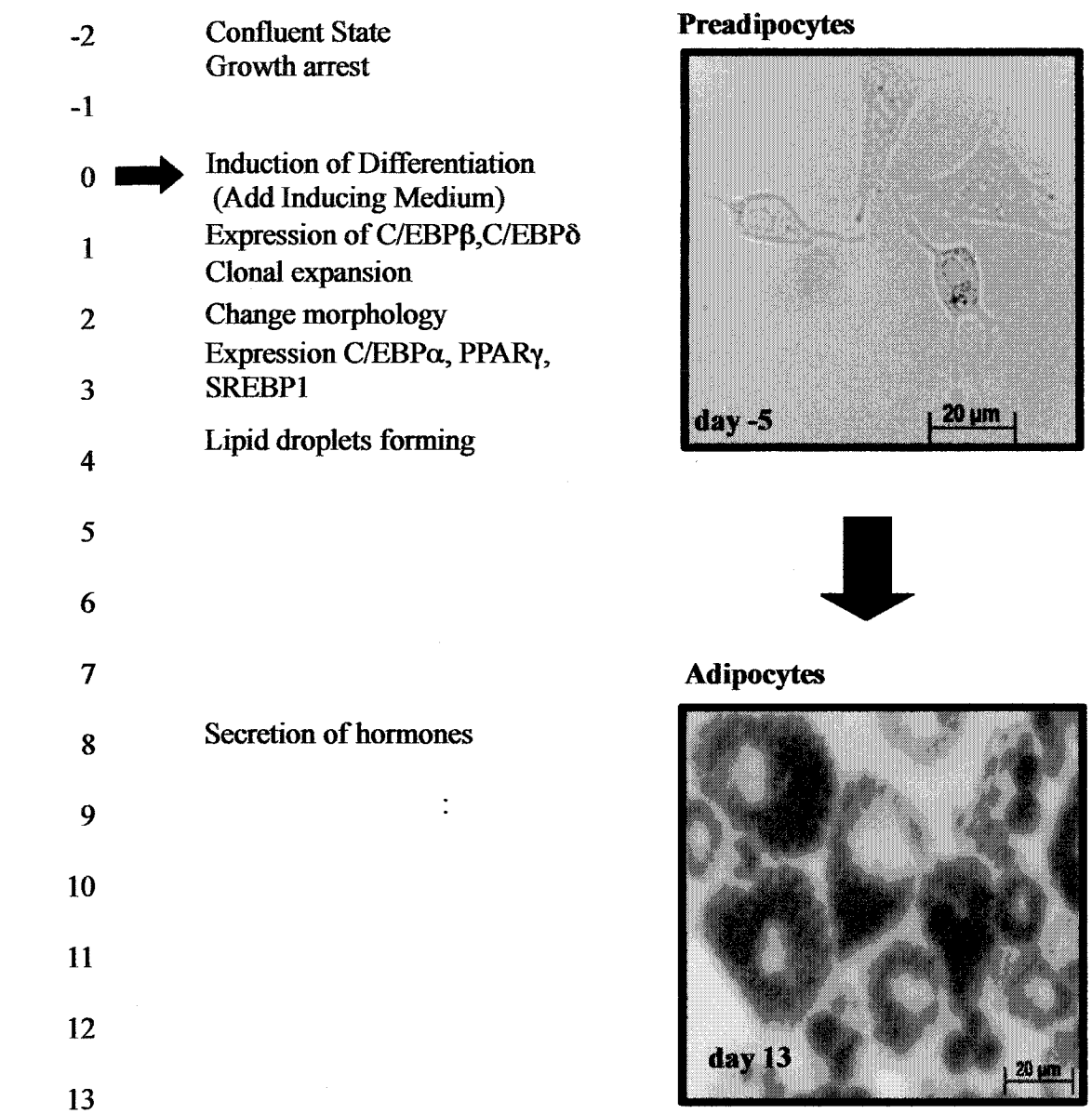


Figure 3.1 Main processes occurring during adipogenesis
General progress of events that occur in the differentiation of preadipocytes into adipocytes during days -2 to 13 in culture. The events between day 0 and day 3 generally occur within the order presented. Pictures on right demonstrate typical morphology of 3T3-L1 preadipocytes prior to confluence at day -5, and lipid droplets in mature adipocytes stained with Oil Red O at day 13.

In preadipocyte cell lines, the standard adipogenic inducing medium, contains serum, insulin, dexamethasone (DEX) and isobuthylmethylxanthine (IBMX). Activation of insulin/insulin-like-growth factor (IGF)-1 signaling pathway by insulin is a necessary part of adipogenesis and occurs through the activation of the insulin receptor substrate 1 and 2 (IRS-1, IRS-2) leading to the activation of phosphatidylinositol 3-kinase (PI3-K) signaling and its downstream target, protein kinase B (207, 208). DEX is one of many synthetic glucocorticoids that are potent inducers of adipogenesis. In preadipocytes, glucocorticoids activate the glucocorticoid receptors and are involved in promoting the expression of CCAAT/enhancer binding protein delta (C/EBPδ) and PPARγ (209). IBMX is also a common component of the differentiation medium and has multiple actions including being an inhibitor of phosphodiesterases, a competitive antagonist of adenosine-1 receptors and an activator of the cyclic adenosine-monophosphate (cAMP) pathway (204). Since other cAMP activators, such as dibutyryl cAMP or forskolin, have also been demonstrated to promote differentiation in preadipocyte cell lines, IBMX is accepted to induce differentiation by activation of cAMP (210-212). The necessity of insulin, DEX and/or IBMX to trigger or accelerate the rate of differentiation varies among the established cell lines, yet generally these components highlight the role for the insulin/ IGF-1, glucocorticoid, and cAMP signaling pathways in preadipocyte differentiation.

Following the addition of the inducing medium, preadipocytes synchronously reenter the cell cycle and undergo one-two rounds of mitosis during a process called mitotic clonal expansion (reviewed in 205). Mitosis occurs in 3T3-L1 preadipocytes, at 24-36 h and again at 48-60 h after induction of differentiation (213). The requirement for

clonal expansion during adipogenesis is controversial (214), however the most recent work by Tang *et al.* (213) strongly supports that clonal expansion is a requirement for differentiation of preadipocyte cell lines. This study demonstrated that treatment with different cell-cycle inhibitors produced varying amounts of inhibition of cycle-cell marker expression, which correlated with relative decreases in mitosis and adipogenesis (213). In comparison to preadipocyte cell lines, primary preadipocyte cell cultures do not require mitosis in order to undergo differentiation (215). Primary cultures have an enhanced amount of differentiation when they are cultured in the absence of serum and do not undergo cell division (215). These results have led to the suggestion that primary preadipocyte cells may undergo cell division *in vivo* and may be in a latter stage of development when obtained from primary adipose tissue.

Change in cell shape is one of the first hallmarks of adipogenesis. Preadipocytes change from a fibroblastic to spherical shape due to alterations in production of cytoskeletal components and the amount and type of extracellular matrix (ECM). These changes are not associated with the accumulation of lipids since these changes precede the activation of transcription factors associated with an adipocyte phenotype and lipid metabolism (216). During initiation of differentiation there is a significant decrease in cytoskeleton components such as actin and tubulin expression (216) and degradation of the fibronectin-rich ECM produced by the preadipocytes (217). Many of the specific proteins that are involved in these alterations have recently been identified. For example, ENC-1, a Kelch-related actin-binding protein is expressed following the induction of preadipocyte differentiation and is required for adipogenesis and cytoskeleton reorganization (218). Similarly, proteolytic degradation of the ECM is a necessary

component of adipogenesis and occurs through the actions of a kallikrein-dependent plasminogen cascade, which allows for the cleavage and removal of fibronectin from the microenvironment of preadipocytes (217).

The molecular changes that occur during adipogenesis include alterations in a daunting number of genes and proteins. Proteomic analysis has described alterations in the profile of over 2000 protein species during adipogenesis (219). Microarray analysis found 109 genes and 447 uncharacterized expressed sequence tags have over a 10-fold difference in expression following preadipocyte differentiation (220). Within this complexity, several prominent transcription factors, including PPARγ, SREBP, and members of the C/EBP family have been already been well characterized to be regulators of adipocyte differentiation.

The C/EBPs are a family of leucine zipper transcription factors that include 6 family members (α, β, γ, δ, ε, and C/EBP homologous protein (CHOP)) and function as homo- or heterodimeric complexes (reviewed in 221). As cells undergo differentiation, maximal expression of C/EBPβ and C/EBPδ occurs rapidly within 4 h after addition of the differentiation inducing medium. While C/EBPδ expression is maintained for 48 h, C/EBPβ expression gradually declines up to day 8 (222, 223). These proteins are necessary for adipogenesis, with overexpression of either isoform in preadipocytes enhancing adipogenesis and mice lacking either or both isoforms showing reduced adipocyte tissue mass (224, 225). Both C/EBPβ and C/EBPδ are transcriptional activators of the C/EBPα gene, yet a lag of 30 hours occurs between the expression of C/EBPβ and C/EBPδ and subsequent expression of C/EBPα (223). The delay in activation of C/EBPα is hypothesized to be necessary since C/EBPα is anti-mitotic and

thus would inhibit the early process of mitotic clonal expression (223, 226). The expression of C/EBPα is delayed by at least two known repressive mechanisms. Firstly, although C/EBPβ and C/EBPδ are expressed they do not have DNA-binding activity due to formation of an inactive heterodimer between C/EBPβ-CHOP-10, which dissociates following the down-regulated of CHOP-10 after clonal expansion (221, 226, 227). Moreover, within the C/EBPα promoter there is an Sp regulator element (GT-box, binding Sp1, Sp3) that overlaps with a CUP/AP-2α regulator element and is inactivated by CUP-AP2α, which is also down-regulated after clonal expansion (226). Once turned on, C/EBPα expression is maintained throughout the adipocyte lifespan with maximum expression occurring around day 5 of differentiation (228). There is compelling evidence that C/EBPα expression is critical for preadipocyte differentiation with 1) expression of C/EBPα being sufficient to induce differentiation in the absence of inducing medium (229), 2) expression of C/EBPα antisense blocking differentiation (230), and 3) C/EBPα knockout mice having an absence of WAT in many depots (231). C/EBPα activates the transcription of many genes in adipocytes but is most notable for its role to promote the expression of the insulin receptor, thus conferring insulin sensitivity in adipocytes (232).

The PPARs are a subgroup of the large nuclear hormone receptor superfamily, which are ligand activated transcription factors (reviewed in 233, 234). There are three known protein isoforms of PPAR, including PPARα, PPARβ (also called PPARδ), and PPARγ, which individually act as obligate heterodimers with the retinoid X receptor (RXR). Of these isoforms, PPARγ is highly expressed in adipose tissue and plays a central role in adipogenesis (reviewed in 235, 236).

Recent knockout mice studies have also highlighted the role for PPARγ in mature adipocyte biology and the differentiation of adipocytes *in vivo*. When a floxed PPARγ gene was ablated in adipocytes by the Cre recombinase expressed under the control of the adipocyte specific aP2 promoter, mice had normal weight gain during the first 6 months of life yet 50% of their white adipocytes were hypertrophic and over 80% of the adipocytes died (237). These results support that PPARγ is essential for post differentiation survival of adipocytes with its inactivity resulting in fat cell loss and compensatory hypertrophy. Furthermore, when PPARγ was ablated in adipocytes using the tamoxifen-dependent Cre-estrogen receptor recombination system, within a few days adult mice had a reduction in body fat due to the death of mature PPARγ-null adipocytes, demonstrating PPARγ is essential for the *in vivo* survival of mature adipocytes (238). However, the reduction in body fat in these PPARγ-null mice was not maintained after a few weeks due to the replacement of the PPARγ-null adipocytes with PPARγ-expressed adipocytes, mostly likely derived from the preadipocytes. Thus, together these findings highlight that PPARγ is essential for the survival of mature adipocytes, in addition to its role in inducing differentiation.

There are two isoforms of PPARγ, $PPAR\gamma_1$ and $PPAR\gamma_2$. $PPAR\gamma_2$ has an extra 30 amino acids in its amino terminus due to alternative promoter usage (239). $PPAR\gamma_1$ is more widely expressed in a variety of tissues (large intestine, kidney, adipose tissue) compared to $PPAR\gamma_2$, which is predominately expressed in adipose tissue and has minimal expression in liver and muscle (239). The activation of PPARγ is strongly up-regulated during adipogenesis and ectopic expression and/or ligand activation of PPARγ in preadipocytes or nonadipogenic fibroblastic cells (NIH-3T3 cells) promotes

differentiation and up-regulation of a number of adipogenic genes (233, 235). The functional difference between the two PPARγ isoforms in adipogenesis is being debated yet recent work has provided evidence that PPARγ$_2$ may have an enhanced ability to induce preadipocyte differentiation (240, 241). Using artificial transcriptional suppressors of the endogenous PPARγ genes and ectopic expression of individual PPARγ isoforms, the level of expression of PPARγ$_2$, and not PPARγ$_1$ correlated with the degree of adipogenesis (240). These results supported PPARγ$_2$ as the transcription factor critical for adipogenesis. In contrast, retroviral expression of PPARγ$_2$ and PPARγ$_1$ in NIH-3T3 cells demonstrates both of these isoforms have similar intrinsic ability to induce differentiation, with PPARγ$_2$ only being different from PPARγ$_1$ in its ability to induce differentiation in response to low PPARγ ligand concentrations (241). When combined, these findings suggest that the 30 extra amino acids in PPARγ$_2$ increase the ability of PPARγ$_2$ to activate transcription and ultimately increase adipogenesis. In agreement with this hypothesis, PPARγ$_2$ compared to PPARγ$_1$ has enhanced ability to bind some components of coactivator complexes that are required for adipocyte differentiation (241).

Due to the interplay between PPARγ and C/EBPα, it has been difficult to ascertain whether these two factors work independently or together in one pathway to induce adipogenesis (242). In mice, homozygous knockout of C/EBPα is lethal days after birth, whereas PPARγ knockout is lethal early in gestation (embryonic day 10) secondary to a deficit in placenta development (231, 243). The formation of PPARγ and C/EBPα occurs simultaneously in differentiation and there are many reports of how these transcription factors regulate each other's expression. Heterozygous PPARγ knockout

mice express reduced levels of C/EBPα (244), and homogeneous C/EBPα knockout mice have reduced levels of PPARγ (232). Overexpression of PPARγ or C/EBPα in NIH-3T3 cells can also induce differentiation, suggesting expression of either one of these factors is sufficient for adipogenesis to occur. However, in C/EBPα null fibroblast cells differentiation can proceed when PPARγ$_2$ is overexpressed (232), but PPARγ null fibroblast cells remain defective in differentiation when C/EBPα is overexpressed (242), supporting the idea that PPARγ is the proximal effector in adipogenesis (242). Overall, it is generally accepted that both PPARγ and C/EBPα are the two key transcription factors necessary for adipogenesis, with PPARγ having some adipogenic effects that are independent of C/EBPα (214, 245).

The SREBPs were first identified and given two names in 1993 by two separate groups working independently on cholesterol metabolism (246) and preadipocyte differentiation (247). The name SREBP was coined by the Goldstein and Brown research group based on the ability of these proteins to bind to a sterol regulator element (SRE) in DNA for the regulation of cholesterol metabolism (246). The other name for SREBP is adipocyte determination and differentiation factor-1 (ADD-1) which was coined by the Spiegelman research group working on adipocyte transcription (247). There are three SREBP isoforms, with the SREBP-1 gene producing SREBP1a and SREBP1c, and the SREPB-2 gene producing SREBP2 (reviewed in 248). ADD-1 has subsequently been recognized to be homologous to the human SREBP1c isoform (249).

The SREBPs are members of the basic helix-loop-helix leucine zipper (bHLHLZ) family of transcription factors, yet they are unique in that they are synthesized as precursor proteins and have a unique dual DNA binding specificity (248). In liver cells,

it has been well characterized that SREBPs are synthesized as precursors which embed into the ER and undergo proteolytic cleavage allowing the amino-terminus of the protein to be targeted to the nucleus where it can activate SREBP target genes (reviewed in 250). SREBPs have a dual binding specificity and can bind as a monomers to DNA directly at repeat SREs and also form dimers which bind at inverted repeat E-boxes (251). During adipogenesis of preadipocyte cell lines, the expression of SREBP-1 dramatically increases, similar to the profile of PPARγ (252). Since the initial characterization of SREBP in adipocyte differentiation was done with the SREBP1c/ADD1 isoform, most work on the role of SREBP in differentiation has been completed using this isoform.

Unlike PPARγ or C/EBPα, the expression of SREBP1c is unable to induce an adipocyte phenotype in fibroblasts, yet expression of a dominant negative SREBP1c is able to abolish adipogenesis (252). Thus, it appears that *in vitro*, SREBP1c activity is required for adipogenesis, but may not be adipogenic in the absence of PPARγ or C/EBPα. Through interactions with PPARγ, several lines of evidence support that SREBP can promote differentiation. Preadipocyte differentiation can be blocked with expression of dominant negative SREBP1c yet can be reversed upon expression of a ligand for PPARγ (253). This work suggested that SREBP1c expression produces factor(s) which activate PPARγ, a theory that was supported by experimental evidence demonstrating that medium from fibroblasts transfected with SREBP could activated a GAL4-PPARγ ligand binding domain fusion protein (253). More recently, it has been suggested that SREBP1 and SREBP2 can both act as a transcription factors and regulate the promoter activity of PPARγ (254). This work has led to the suggestion that SREBP can induce the expression of PPARγ and enhance adipogenesis.

In adipose tissue the expression of SREBP1c mRNA is 3 times greater than the expression of SREBP1a. In cultured preadipocyte cell lines there is contradictory evidence whether SREBP1a and/or SREBP1c is increased during differentiation (249, 253-256). The results from overexpression experiments with SREBP1 in mice, has raised interest into the differences in the contribution of SREBP1a and SREBP1c to adipogenesis. Overexpression of a dominant active form of SREBP1c in adipocytes in mice produces a decrease in WAT tissue and reduction in expression of adipocyte differentiation markers, resulting in a phenotype that resembles lipodystrophy (257). In striking contrast, overexpression of SREBP1a in adipocytes resulted in adipocyte hypertrophy and increase in fatty acid secretion in mice (258). The differences in the phenotypes of the SREBP1a and SREBP1c mice suggest that these isoforms, which have identical DNA binding domains and differ only in their transactivation domain, must have differentiating properties that dramatically effect adipocyte development.

As mentioned previously, in addition to PPARγ, C/EBPα and SREBP1, there are numerous other genes that are temporally and/or functionally linked to the process of adipogenesis (reviewed in 193, 259, 260). In this report we examined the effect of VPA treatment on the process of adipogenesis and measured the protein and mRNA levels for three transcription factors, PPARγ, C/EBPα and SREBP1a.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation

Mouse 3T3-L1 were obtained from the American Type Culture Collection (ATCC) and subcultured in 5% CO_2 at 37^{0}C. Cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen), with 10% heat inactivated calf serum (CS) (Invitrogen), and 100 U/ml penicillin G with 100 μg/ml streptomycin (Invitrogen). Medium was changed every two days and preadipocytes were maintained at <50% confluence.

For 3T3-L1 differentiation experiments, 2 days after preadipocytes reached confluence they were treated with medium to induce differentiation [MDI: DMEM, 10% fetal bovine serum (FBS; CanSera), 250 nM dexamethasone (DEX; Sigma), 500 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma), and 100 nM (0.58 μg/ml) human insulin (Roche Molecular Biochemicals)]. After 2 days in MDI, preadipocytes were cultured in DMEM containing 10% FBS, and 5 μg/ml insulin. Subsequent medium changes occurred every second day.

Valproic acid (VPA, Sigma) and 4 phenylbutyric acid (4PB, Sigma) were dissolved in phosphate buffered saline (PBS), whereas valpromide (VPM, Katwijk Chemie B.V.) and trichostatin A (TSA, Sigma) were dissolved in dimethyl sulfoxide (DMSO), and applied as indicated in figure legends. DMSO levels were kept under 0.1%.

For PPARγ agonist induced differentiation, preadipocytes (2 days post confluent) were treated with DMEM, 10% FBS, 100 nM insulin, and a PPARγ agonist. The PPARγ agonist used included troglitazone (TGZ, Biomol Research Laboratories) and rosiglitazone (ROS; BRL49653, kind gift of Dr. C. Sinal, Dalhousie University). TGZ is

a PPARγ ligand (*in vitro* 3T3-L1 IC_{50}=2 μM) and was used at a dose of 10 μM, which has previously been demonstrated to induce differentiation in 3T3-L1 cells (5, 261). ROS is a more potent, higher affinity PPARγ ligand (*in vitro* 3T3-L1 = 4nM) and was used at a dosage of 1 μM, which has also been previously demonstrated to induce adipogenesis in 3T3-L1 cells (261-263). The medium was changed to DMEM, 10% FBS, 5 μg/ml insulin, and the PPARγ agonist, with subsequent media changes every other day.

During the differentiation process, cells were photographed using a camera (Nikon) on an inverted microscope, and the resultant photos were cropped and adjusted for brightness and contrast using Adobe Photoshop. For images in Figure 3.2, cells were visualized on a phase-contrast Axiovert 200 microscopy at 10x or 40x magnification (Carl Zeiss, Gottingen, Germany), and images were captured using an Axiocam and Axiovision 3.0 software. Images were subsequently cropped using Adobe Photoshop.

Human adipocyte experiments were conducted by Zen-Bio Inc. (Contract #: DAL040403). Primary human preadipocytes were obtained from patients undergoing liposuction surgery. Two lots of cells were used including those from an individual [L091901, male, 48 years old, BMI: 25.07] and a mixed lot SL0023 (6 female individuals, average age 48, average BMI: 26.07).

Details for primary preadipocyte differentiation can be found at www.zen-bio.com. Briefly, preadipocytes were maintained in preadipocyte media which included 1:1 (v/v) DMEM/Ham's F12 nutrient broth, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 10% FBS, and antibiotics. Two-day post confluent preadipocytes were exposed to the initiation of differentiation medium which

included DMEM/Ham's F12 nutrient broth, 15 mM HEPES buffer, 3% FBS, 33 *u*M Biotin, 17 *u*M Pantothenate, 100 nM human insulin, 1 *u*M DEX, 0.25 mM IBMX, and antibiotics. Following 3 days of treatment in differentiation medium, adipocytes were maintained in adipocyte medium, which included DMEM/Ham's F12 nutrient broth, 15 mM HEPES buffer, 3% FBS, 33 *u*M Biotin, 17 *u*M Pantothenate, 100 nM human insulin, 1 *u*M DEX, and antibiotics.

In some experiments the primary cells were differentiated in either 1 *u*M ROS (BRL49653) or 10 *u*M Zen-Bio proprietary non-TZD PPARγ agonist during the time cells were maintained in the differentiation and adipocyte media, as indicated in figure legends. For each experiment, a triplicate set of cells were treated with 5 ng/ml tumor necrosis factor alpha (TNFα) as a control, since TNFα has been well documented to block preadipocyte differentiation. The addition of ROS, Zen-Bio PPARγ agonist, or TNFα was included with each medium change. Cells were photographed at day 13 of differentiation by Zen-Bio Inc.

Oil Red O Staining

Oil Red O is a lysochrome, which means it is a fat-soluble dye and is therefore soluble in all lipids, especially TAG (264). Cells were stained with Oil Red O (C.I. 26125, Sigma) and quantified as previously described by Kasturi and Joshi (265). Briefly, cells were washed with PBS, fixed in 4% paraformaldehyde (PFA) for 10 min, rinsed with water, followed by two washes in 70% ethanol (ETOH). Lipid droplets were stained for 15 min at room temperature with Oil Red O using a concentration of 6 parts saturated Oil Red O dye (Sigma) in isopropanol and 4 parts water. The stained cells were washed four times for 5 min with 70% ETOH.

Following staining, the 12-well plate containing the stained cells was scanned into the computer and the resultant photos were cropped and adjusted for brightness using Adobe Photoshop. Following scanning, stain dissolved within the lipids droplets was extracted with 100 µl isopropanol and the absorbance of the dye was measured at 520 nm using a microplate scanning spectrophotometer (PowerWave$_X$, Bio-Tek Instruments). If necessary a dilution of the sample was made with isopropanol to ensure absorbance readings were <2 as per specifications of spectrophotometer.

[^{3}H] Thymidine Incorporation

Cells were pulsed labeled with [^{3}H] thymidine (2 μCi/ml, Amersham) at 37^{0}C for 1 hour prior to harvesting (1 h, 10 h, 20 h, or 30 h after the induction of differentiation), similar to the method of Tang and Lane (266). Briefly, cells were placed on ice and rinsed twice with ice cold PBS. To precipitate DNA, cells were incubated with 10% trichloroacetic acid for 1 h at 4^{0}C. Cells were then washed with absolute alcohol at room temperature (RT) and allowed to air dry at RT for 2 h. DNA was extracted using 0.1 M sodium hydroxide (NaOH) for at least 1 h at RT and radioactivity was counted in acidified scintillation fluid.

Growth Curve Assay

3T3-L1 preadipocytes were plated on day 0 at 4 x 10^4 cells/well in growth medium in six-well plates. On day 1 and day 3, cells were fed new medium. Cells were treated daily with 1 mM VPA. Cells from duplicate wells were counted using a hemacytometer over a 4 day period. Cells at the end of the experiment were photographed using a camera (Nikon) on an inverted microscope, and the resultant photos were cropped and adjusted for brightness and contrast using Adobe Photoshop.

Analysis of TAG Synthesis

Cells were pulse labeled with [^{14}C] acetic acid (250 nCi/ml, Amersham) for 2 h at 37^{o}C. The [^{14}C] acetic acid will be metabolized and incorporated into acetyl CoA, which is subsequently incorporated into newly synthesized triacylglycerol. Cells were rinsed twice with cold PBS and scraped into 1ml methanol:water (5:4, v/v) and sonicated. The organic and aqueous phases were extracted using chloroform:methanol (1:2, v/v) with 0.58% sodium chloride (NaCl). The organic phase was washed three times with ideal upper phase buffer (0.57% methanol:NaCl:choloroform, 45:47:3, v/v/v), evaporated and then resuspended in chloroform. Radiolabeled lipids were resolved by thin layer chromatography (TLC) in petroleum ether:diethyl ether:acetic acid (60:40:1, v/v/v) on Whatman Silica Gel 60 TLC Plates (Fisher) . Standards (Sigma, 99F7817) were run in separate lanes on same plates as samples and identified using iodine staining. TLC plates were exposed to film and the radiolabeled lipids identified by co-migration with authentic standards were scraped into vials and quantified by scintillation counting.

Northern Blot Analysis

RNA was isolated using Trizol reagent (Invitrogen). Twenty-thirty micrograms of total RNA was separated on a 1.5% agarose/0.67% formaldehyde gel and transferred to BrightStar Plus membrane (Ambion Inc.). The mouse PPARγ2 (gift from Dr. B. Spiegelman, Dana-Farber Cancer Institute, Boston), mouse C/EBPα (gift from Dr. O. MacDougald, University of Michigan Medical School, Ann Arbor), and mouse SREBP1a (gift from Dr. J. Goldstein, University of Texas Southwestern Medical Center, Dallas) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were labeled with [α-^{32}P] dATP (2 μCi/ml) using the Strip-EZ DNA Probe Synthesis and Removal Kit

(Ambion Inc.). The blots were prehybed for 1 h and incubated with $1x10^6$ cpm/ml cDNA probe overnight at 42°C in ULTRAhyb buffer (Ambion Inc.). Blots were washed at 42°C in low (2 x saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS)) and high (0.1 x SSC, 0.1% SDS) stringency wash solution. Each blot was stripped using Strip-EZ DNA Kit and re-probed to detect target mRNA. Signals were visualized by autoradiography and quantified using a Molecular Dynamics Storm Phosphoimager.

Western Blot Analysis

Cells were washed 2 times in ice cold PBS, extracts isolated using high-salt lysis buffer [10 mM Tris-Cl pH7.4, 500 mM NaCl, 2 mM ethylenediaminetetraacetic (EDTA), 1 mM dithiothreitol (DTT), 1% Triton X 100, 1x complete protease inhibitors (Roche), 1 mM phenyl methyl sulfonyl fluoride (PMSF), sonicated, clarified by centrifugation (10 min at 15 000 x g), and quantified by Bradford analysis (BioRad Laboratories). For SREBP1a analysis, cells were treated for 4 h before harvesting and again during lysis with 25 μg/ml of the proteasome inhibitor, N-acetyl-leucine-leucine-norleucinal (ALLN, Sigma). Twenty-forty micrograms of protein extract per lane were separated by SDS-PAGE in the presence of 1% ß-mercaptoethanol using a 7% or 12% gel as indicated in figure legends and transferred to nitrocellulose.

Blots were blocked in 5% milk for 1 h following by incubation with a primary antibody overnight. Primary antibodies from Santa Cruz Biotechnology Inc. included anti-PPARγ (monoclonal, E-8), anti-SREBP1a (monoclonal, 2A4), and anti-C/EBPα (polyclonal, 14AA). Other primary antibodies included anti-acetyl-histone H3 (polyclonal, 06-599, Upstate Cell Signaling Solutions), anti-HDAC1 (polyclonal, PC544 Oncogene Research), and anti-actin (polyclonal A2066, Sigma). After washing in Tris-

buffered saline, blots were incubated with sheep anti-rabbit or sheep anti-mouse IgG-horseradish peroxidase conjugated secondary antibody (Chemicon International). Protein expression was visualized with enhanced chemiluminescence (Perkin Elmer Corp) and signal quantified using NIH Image software. Western blots were stripped in stripping solution (62.5mM Tris pH 6.8, 2% SDS, 100mM ß-mercaptoethanol) for 15 min at 50°C, washed, blocked and re-probed.

Transient Transfection and PPARγ Two-Hybrid Reporter Gene Assay

Reporter gene assays were conducted using HepG2 cells maintained in DMEM with 10% FBS. The HepG2 cells were a kind gift of Dr. C. Sinal (Dalhousie University, Halifax). Cells were plated in 24-well plates at $7.5x10^4$ cells/well the day prior to transfection. Cells were transfected with the mammalian two-hybrid transfection mixture of 200 ng reporter plasmid (FR-luc) and 12.5 ng of either the GAL4 DNA binding domain (DBD) expression plasmid (BD-Gal4) or the expression plasmid for GAL4 DBD fused with the human PPARγ ligand binding domain (PPAR-Gal4). The FR-luc, BD-Gal4, or PPAR-Gal4 plasmids were kind gifts from Dr. C. Sinal (Dalhousie University, Halifax). All wells also had 100 ng of internal reference plasmid pCMV β-galactosidase and the total DNA was increased up to 450 ng using pBluescript II KS plasmid (Stratagene). Cells were transfected in triplicate in normal growth medium overnight with FuGENE6 transfection reagent (Roche Molecular Biochemicals). The subsequent day, cells were treated overnight with compounds. Twenty hours after addition of drugs cells were harvested and luciferase activity determined using the Enhanced Luciferase Assay kit (BD Pharmingen). Results were normalized using β-galactosidase activity and represent the mean data from three independent experiments.

Statistical Analysis

Values are expressed as mean ± standard error of the mean. For outcome measurements that were assessed at one time point during the study, a one-way ANOVA was used for multiple group comparisons, and paired t-test for comparisons between 2 groups. For outcome measures that were assessed at two or more time points during the study, a repeated measure ANOVA was used to determine differences that occurred over time and between the treatment groups. For subsequent group comparisons, *post hoc* analysis was completed using the Bonferroni test. All tests were considered significant at $p<0.05$. Data analysis was done using SPSS Base 11.0 for Windows.

RESULTS

3T3-L1 Preadipocyte Cell Model

Adipocyte differentiation was modeled using the mouse 3T3-L1 cell line. The 3T3-L1 preadipocytes had a characteristic flat fibroblastic morphology and were propagated at less than 50% confluence (Figure 3.2A). Cells used for experiments were allowed to grow into a confluent state and changed shape to resemble a cobblestone morphology (Figure 3.2B). The preadipocytes were then induced to differentiate in medium (MDI) consisting of DMEM containing 10% FBS, DEX, IBMX, and insulin for 2 days. Following this treatment, cells were maintained in culture using growth medium containing 10% FBS and insulin. Approximately 2 days after the addition of MDI, cells displayed a spherical morphology and began to accumulate noticeable small lipid droplets by day 3-4. At 13 days following the addition of MDI, cells contained numerous lipid droplets as stained with the Oil Red O dye (Figure 3.2C, D).

VPA Reduces 3T3-L1 Preadipocyte Cell Number

Increased adipocyte tissue mass can result both from an increase in fat-cell number (hyperplasia) and increase in fat-cell size (hypertrophy). Fatty acids, including docosahexaenoic acid (DHA, 22:6) (267) can decrease the growth rate of 3T3-L1 preadipocytes. Preadipocytes were seeded at equal density and cell number was counted daily for four days in the absence or presence of VPA. The effects of 1 mM (143 μg/ml) VPA were evaluated because this dosage is clinically relevant, with therapeutic serum levels being > 45-120 μg/ml (trough serum levels) and toxicology studies demonstrating serum concentrations ≤ 450 μg/ml produce limited toxicity (268).

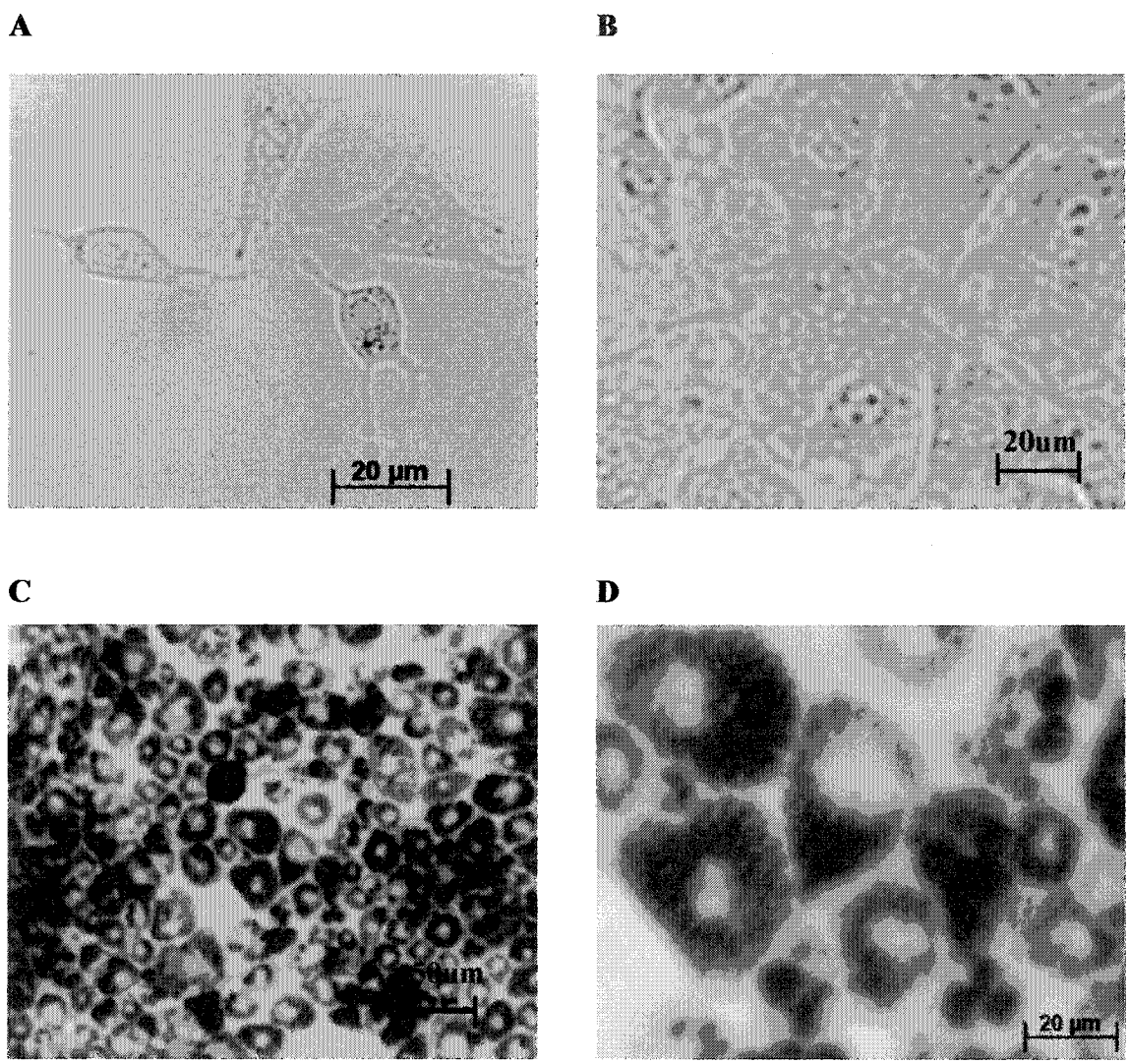


Figure 3.2 3T3-L1 cell model of preadipocyte differentiation
A) Propagated 3T3-L1 preadipocytes have characterisitic fibroblastic morphology.
B) Confluent 3T3-L1 preadipocytes form a cobblestone morphology.
C & D) Following 13 days after the addition of medium to induce differentiation (MDI), adipocytes form and have a spherical shape with numerous lipid droplets as demonstrated by Oil Red O staining.

Over time there was a significant increase in the number of cells and a significant interaction between treatment with VPA and time (Figure 3.3A). There was a significant reduction in the number of preadipocytes after 3 and 4 days of VPA treatment. At day 4 the untreated and VPA treated cells had achieved a state of confluence. The decrease in the cell number after 3-4 days may have been due to the size of the cells. VPA treated cells appeared to have a more irregular shape when compared to the cobblestone pattern of the untreated cells (Figure 3.3B). These results suggest that VPA can reduce the number of preadipocytes, but does not prevent the cells from obtaining a state of confluence over time.

VPA Does Not Induce Differentiation of 3T3-L1 Preadipocytes

A previous study suggested that VPA may act as a PPARγ ligand that is capable of inducing adipocyte differentiation in the absence of MDI (269). To determine whether VPA could induce adipocyte differentiation, two days after mouse 3T3-L1 preadipocytes had grown to a state of confluence, the cells were cultured in DMEM medium containing 10% FBS, insulin and VPA (1 or 5 mM) for two days. Cells were subsequently maintained in culture in DMEM medium containing 10% FBS and insulin, and treated daily with VPA.

After 13 days in culture in the absence or presence of VPA (1 mM), very few (approximately less than 1%) cells stained red with Oil Red O (Figure 3.4A). In contrast almost 100% of cells differentiated in presence of MDI and no VPA stained red (Figure 3.4A). These data show that chronic VPA treatment was unable to induce differentiation of 3T3-L1 cells into adipocytes in the absence of MDI.

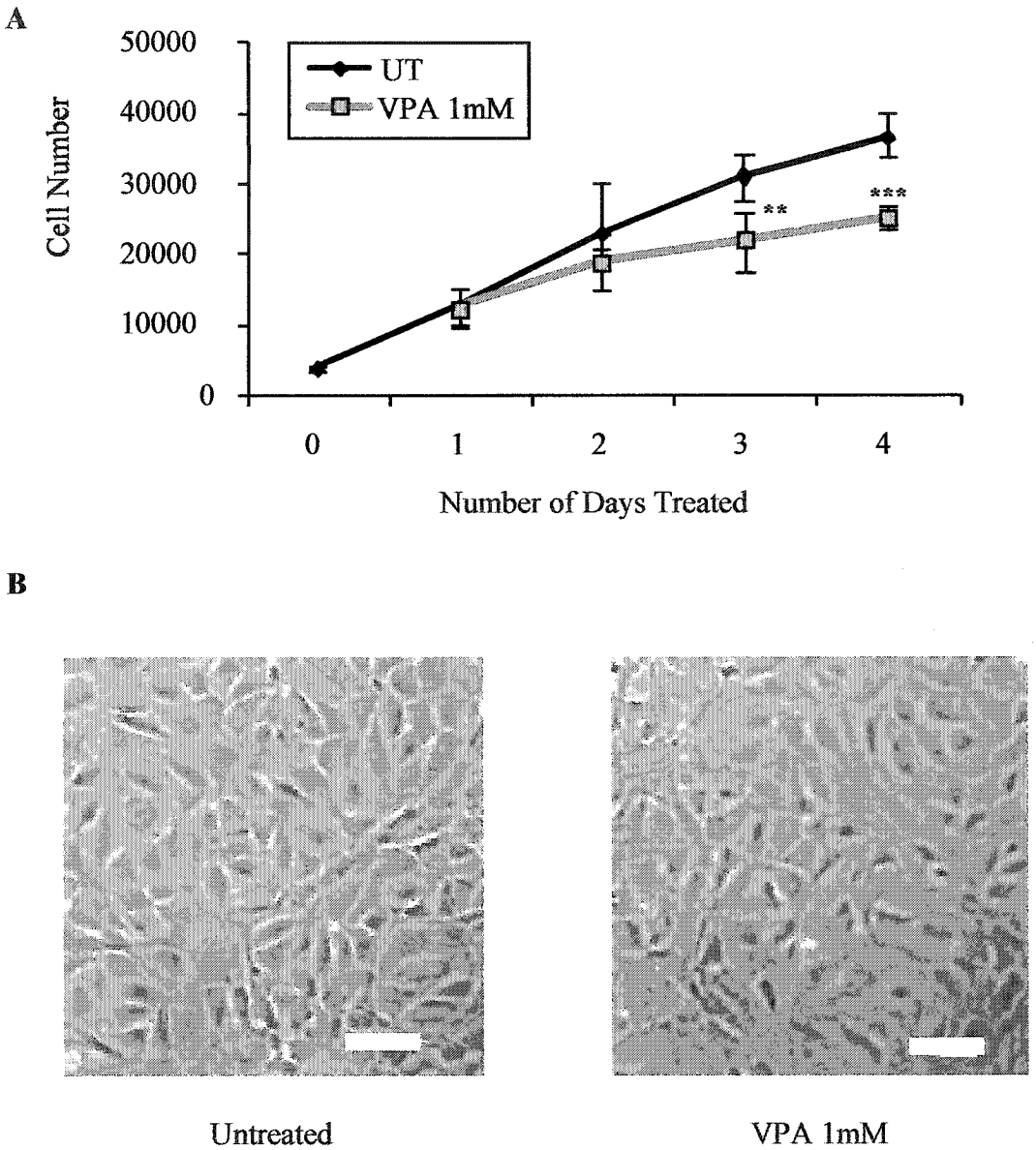


Figure 3.3 VPA effects on 3T3-L1 preadipocytes cell number
A) Preadipocytes were grown in the absence (UT) or presence of 1mM VPA for 1-4 days. Over time there was a significant increase in the number of cells. VPA treatment reduced the number of cells at day 3 and 4. Post-hoc analysis, compared to UT, **$p<0.01$, *** $p<0.005$. The data are the mean error of three independent experiments performed in triplicate. **B)** Untreated and VPA treated cells at day 4 of growth curve are confluent. White scale bar=50μm.

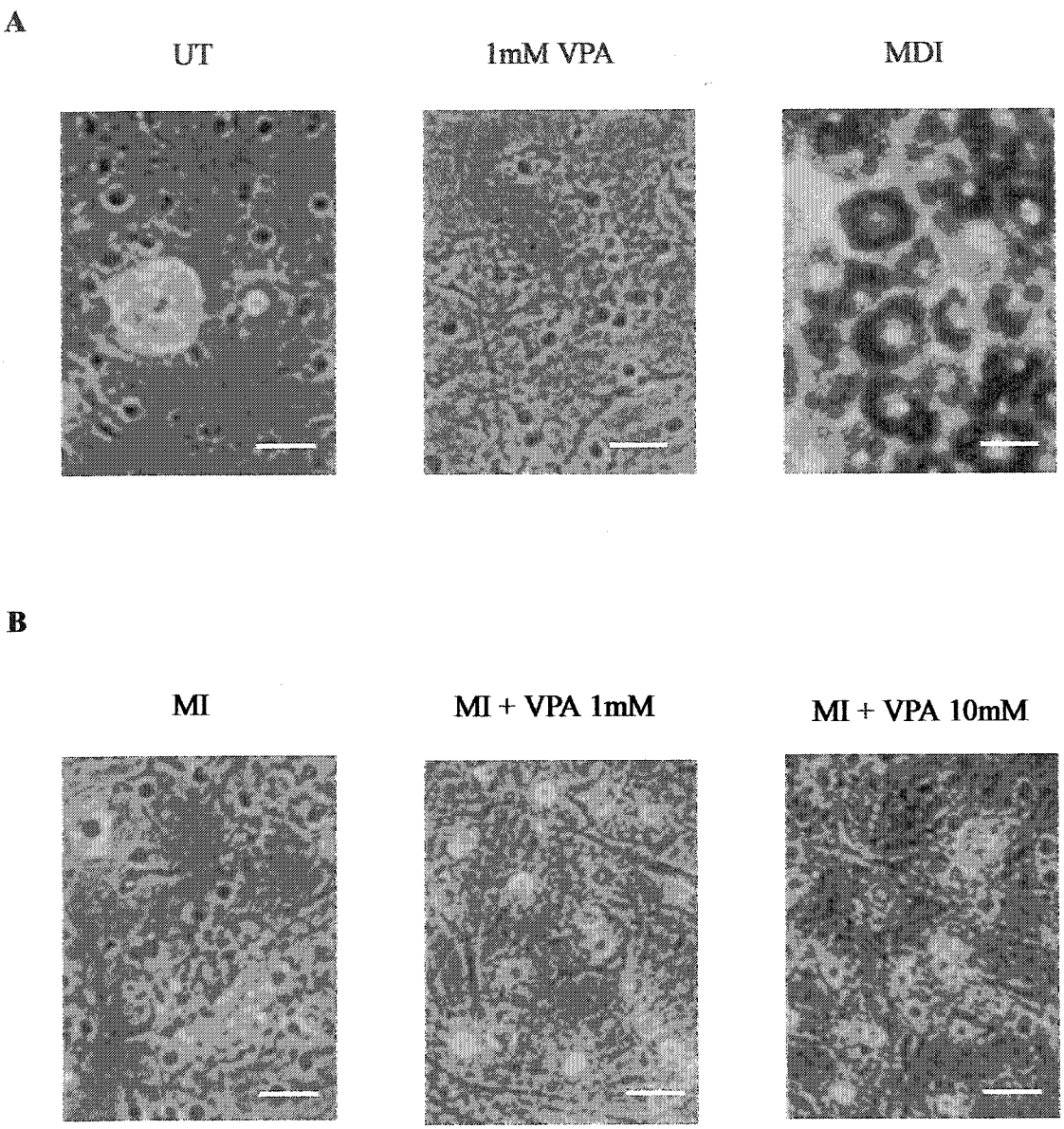


Figure 3.4 VPA does not induce differentiation of 3T3-L1 preadipocytes
A) Preadipocytes were induced to differentiate in the presence of DMEM, 10% FBS, and insulin, in MDI or in the absence (UT) or presence of 1 mM VPA. **B)** Preadipocytes were induced to differentiate in the presence of DMEM, 10% FBS, insulin and IBMX (MI) in the presence of 1 mM or 10 mM VPA. Cells in A and B were stained with Oil Red O and white scale bar=50μm.

Wiper-Bergeron et al. (270) have published that treatment with 10 mM VPA induced the differentiation of 3T3-L1 cells when cells were differentiated in the presence of differentiation medium (MI). MI consists of DMEM, 10% CS, 500 μM IBMX and 50 nM insulin, and is different from the MDI (described in methods) in that it does not contain DEX, has 50 nM versus 100 nM insulin, and contains CS instead of FBS. Experiments conducted to replicate these findings showed that very few cells differentiated in the presence of MI when compared to the amount of cells differentiating in the presence of MDI (Figure 3.4B). Contrary to previous findings, treatment with either 1 mM or 10 mM VPA in the presence of MI did not increase the number of differentiated adipocytes (Figure 3.4B).

VPA Inhibits MDI-Induced Differentiation

Since VPA did not induce differentiation, the effect of VPA treatment on MDI-induced preadipocyte differentiation was examined. Daily treatment with VPA significantly inhibited MDI-induced adipocyte differentiation in a dose-dependent manner; shown by the decrease in Oil Red O staining measured 13 days after the initiation of differentiation (Figure 3.5A). Under higher magnification, almost 100% of the MDI-treated cells displayed lipid droplet formation, whereas in the presence of MDI and VPA there was a reduction in the number of cells that had lipid droplets (Figure 3.5B). The amount of Oil Red O stain can be quantified following extraction from the cells. In the presence of MDI and 1 mM VPA there was on average a 56% (±10%) reduction in Oil Red O staining when compared to cells induced to differentiate in MDI (Figure 3.5C).

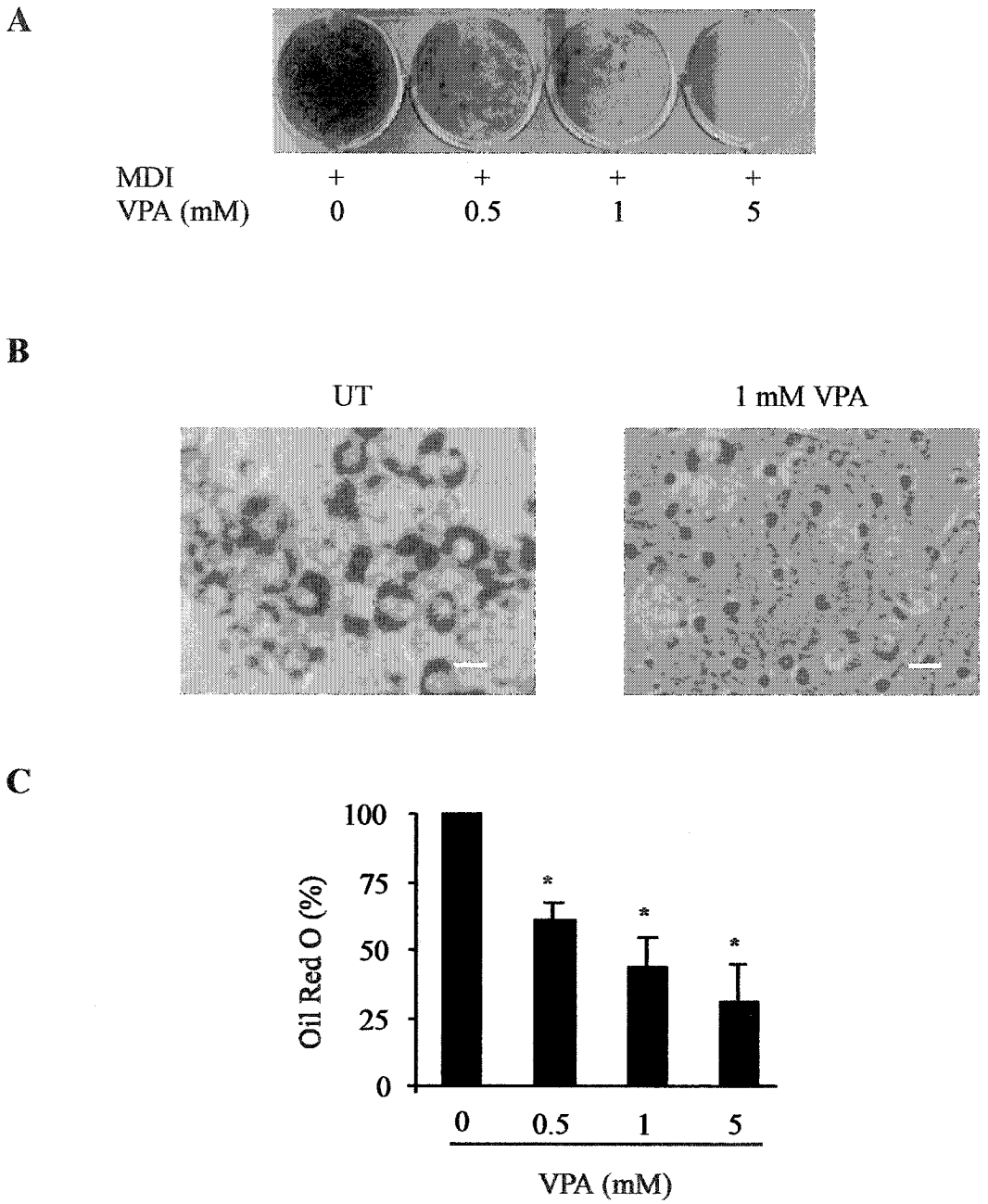


Figure 3.5 VPA inhibits differentiation of mouse 3T3-L1 preadipocytes
A) Cells were grown in the presence of MDI medium (DMEM, 10% FBS, IBMX, DEX, and insulin) for the first 2 days, and treated daily with VPA (0-5mM). VPA dose-dependently reduced adipocyte differentiation. **B)** High magnification (200x) picture of cells cultured using the same conditions as (A) demonstrating MDI induced adipocyte formation, which was attenuated by VPA treatment. White scale bar=50μm. **C)** Quantification of Oil Red O staining of cells cultured in MDI confirmed that VPA treatment significantly reduced formation of adipocytes. Data represents mean percent levels compared to UT (set at 100% differentiation) from three independent experiments, * $p<0.001$.

In addition, to quantify the decrease in TAG levels following daily treatment with VPA, cells were metabolically labeled with [^{14}C]-acetic acid at day 13 post differentiation. Chronic VPA treatment significantly decreased TAG synthesis by 51% (untreated 21,723±4,782 dpm/mg protein vs. 1 mM VPA 10,698±2,855 dpm/mg protein, $p<0.001$, mean from 3 independent experiments). Therefore both a reduction in Oil Red O staining and TAG synthesis demonstrated that VPA can inhibit adipogenesis.

VPA Inhibits Differentiation of Human Preadipocytes

Zen-Bio Inc. was contracted to conduct a double-blind study to examine the effects of VPA on differentiation of primary human subcutaneous preadipocyte cultures. These studies were conducted on two cell lots, one from a single individual (L091901) and the other from a pooled sample containing adipocytes from 6 individuals (SL0023). The effect of VPA on adipocyte differentiation was measured in the absence and presence of Zen-Bio differentiation inducing medium (Zen-DIM). In both lots of cells, VPA did not induce differentiation of human preadipocytes (Appendix A, bottom panels). Lot L091901 exhibited some differentiation in the absence of VPA, which appeared to be inhibited by VPA treatment (Appendix A, right panels). Due to the relatively few cells that differentiated under these conditions, it was not possible to quantify TAG levels in these cultures because it was below the range of detection. When human preadipocytes were induced to differentiate in the presence of Zen-DIM and VPA, VPA inhibited differentiation (Appendix B). Quantification of TAG levels revealed that VPA significantly inhibited differentiation, to a similar degree as TNFα, a known inhibitor of preadipocyte differentiation (Appendix B).

VPA Treatment at Initiation of Differentiation Inhibits Adipogenesis

To examine whether the inhibitory effects of VPA on 3T3-L1 differentiation occurred following a single (acute) treatment with VPA, 1 mM VPA was added once concurrent with MDI at the onset of differentiation. After the addition of VPA there was a significant reduction in adipocyte formation measured at days 6-13 (Figure 3.6A, B). This finding suggested that VPA treatment might affect critical steps that occur at the onset of differentiation.

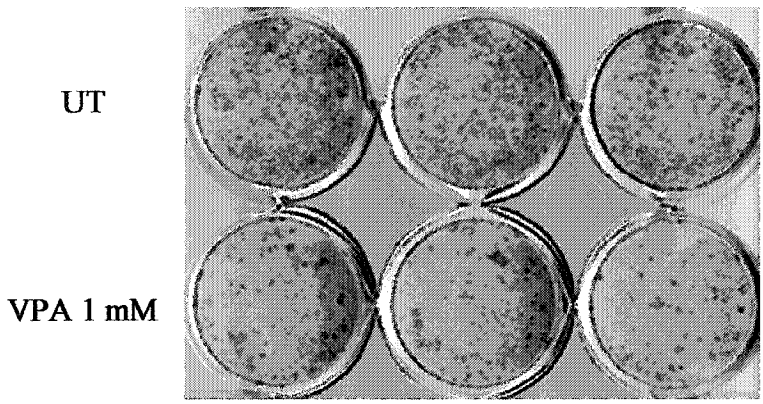
Clonal Expansion is Not Affected by VPA Treatment

After reaching confluence preadipocytes become quiescent, however in response to differentiation medium they undergo mitotic clonal expansion. Since mitotic clonal expansion is necessary for differentiation, VPA effects on reentry into the cell cycle were assessed by measuring [^{3}H]-thymidine incorporation into cellular DNA. Differentiating preadipocytes exhibited a well-characterized entry into, and exit from the S phase of the cell cycle at ~12-16 hours following addition of the MDI (Figure 3.7). In the presence of VPA, this curve remained unchanged, indicating that VPA did not affect mitotic clonal expansion.

Temporal Effects of VPA Treatment on Adipocyte Differentiation

To examine the time period of differentiation during which VPA was acting to inhibit adipogenesis, experiments were conducted to test whether daily VPA treatment at different time periods during the differentiation process could inhibit adipogenesis. Treatment of confluent preadipocytes one day prior to the initiation of differentiation (day –1) with MDI did not prevent adipocyte formation as quantified by Oil Red O staining (Figure 3.8B).

A



B

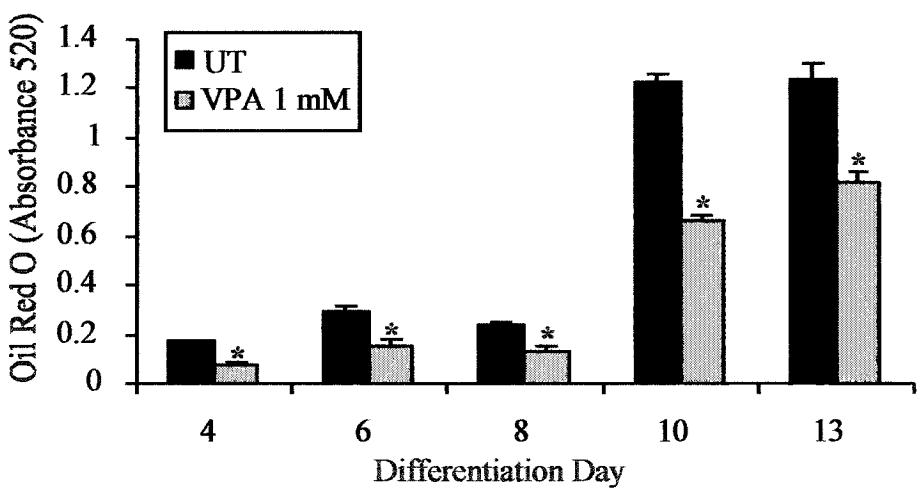


Figure 3.6 Differentiation is inhibited following a single treatment with VPA **A)** 3T3-L1 cells induced to differentiate in MDI and treated once at day 0 with 1 mM VPA. At day 10, cells have noticably reduced amounts of Oil Red O staining compared to untreated (UT) cells. **B)** Quantification of mean reduction in Oil Red O staining as measured from days 4-13. Data represent mean from one experiment performed in triplicate; similar findings were obtained in three independent experiments, compared to untreated (set at 100% differentiated), ANOVA, post-hoc compared to UT on each day, *=$p<0.05$.

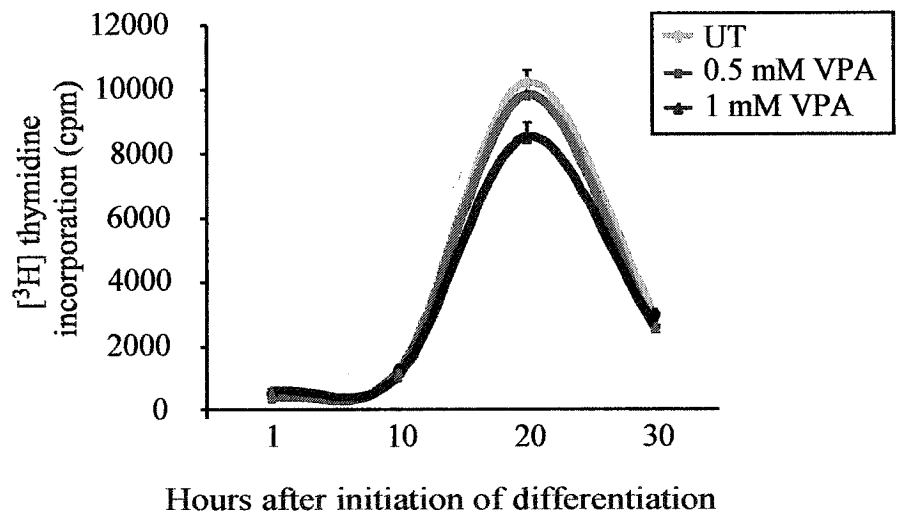


Figure 3.7 Clonal expansion is not affected by VPA treatment
[^{3}H] thymidine incorporation into cellular DNA measured at 1, 10, 20, and 30 hours following the addition of MDI in to 3T3-L1 cells demonstrating no significant difference in clonal expansion in the absence (UT) or presence of VPA. Representative data from one experiment performed in triplicate; similar data was obtained in two separate experiments.

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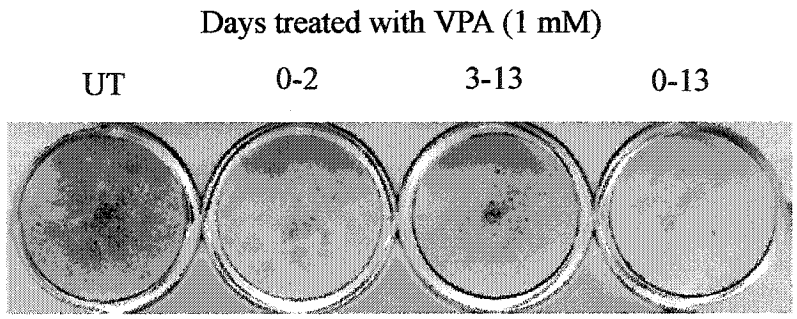

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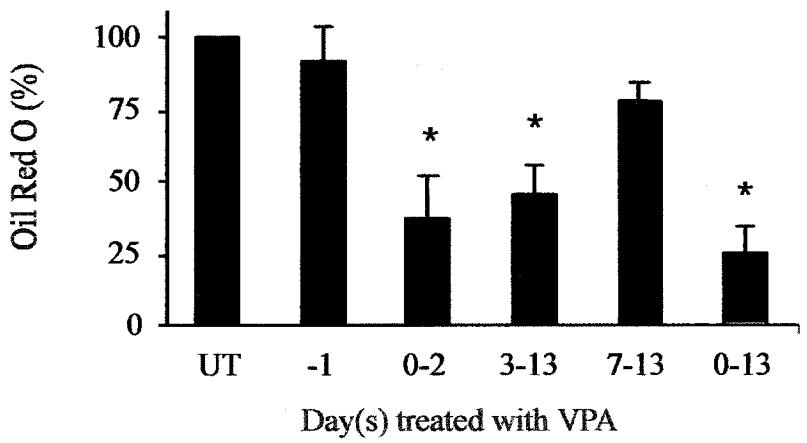

Figure 3.8 Temporal effects of VPA treatment on adipocyte differentiation
A) 3T3-L1 cells induced to differentiate in MDI (day 0-2) and treated daily with 1 mM VPA (days 0-2, 3-13, or 0-13) had noticeably reduced amounts of Oil Red O staining compared to untreated cells (UT). **B)** Quantification of Oil Red O staining in cells differentiated with MDI (days 0-2) and either untreated (UT), pre-treated with VPA 1 day prior to MDI (-1) or treated daily with VPA during differentiation (days 0-2, 3-13, 7-13, or 0-13). Data represents mean percent levels compared to UT (set at 100% differentiation) from three indepedent experiments * $p<0.001$.

VPA treatment concurrent with MDI (day 0-2) caused inhibition of differentiation to the same extent as daily VPA treatment beginning after the removal of the MDI (days 3-13) (Figure 3.8A, B). Daily VPA treatment beginning 7 days after the initiation of differentiation (day 7-13) also reduced Oil Red O levels; however this reduction was not significant compared to untreated cells (Figure 3.8B). These results suggest that VPA is affecting critical events that occur both at the onset of differentiation, as well as events required during the initial maturation of the adipocytes.

Inositol Supplementation Does Not Reverse Inhibition of Differentiation by VPA

VPA can disrupt inositol metabolism and this effect has been implicated in the ability of VPA to increase growth cone size *in vitro* since these effects can be reversed by supplementation with inositol (61). Inositol levels increase during differentiation of adipocytes (271), raising the possibility that VPA's ability to inhibit adipocyte differentiation may be due to inositol depletion.

To test this hypothesis, the medium was supplemented with 1 mM inositol daily in the presence or absence of VPA throughout the differentiation process. Inositol supplementation did not cause any significant difference in adipogenesis, although there was a non-significant reduction in Oil Red O staining in the presence of inositol (Figure 3.9A, B). In the presence of both inositol and VPA, there was a significant reduction in adipogenesis, as assessed by Oil Red O staining (Figure 3.9A, B). Thus, inhibition of differentiation by VPA does not appear to result from inositol depletion.

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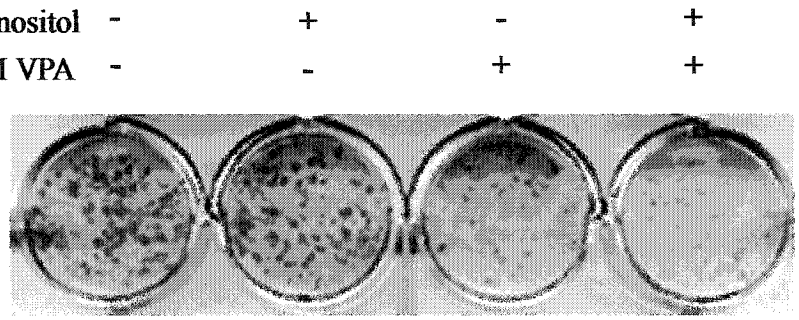

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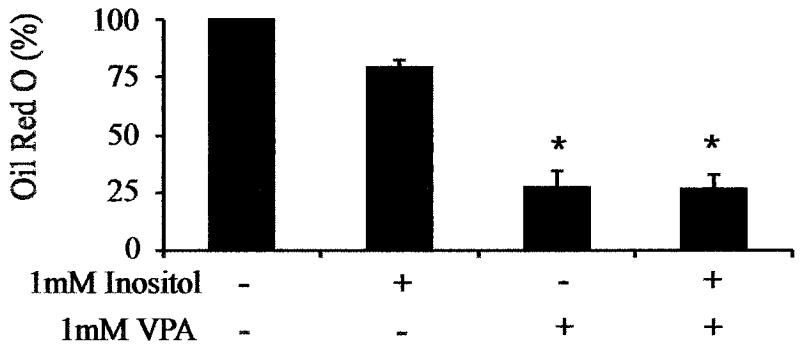

Figure 3.9 Inositol does not reverse VPA-induced inhibition of differentiation **A)** 3T3-L1 cells induced to differentiate in MDI (day 0-2) and in the absence (-) or presence of daily treatment with either 1mM inositol or VPA. Daily treatment with 1 mM VPA reduced amounts of Oil Red O staining. **B)** Quantification of Oil Red O staining at day 13, in cells cultured as defined in A. Data represents mean percent levels compared to UT (set at 100% differentiation) from three independent experiments, * $p<0.01$.

VPA Inhibits PPARγ-induced Differentiation in 3T3-L1 and Human Preadipocytes

PPARγ agonists induce differentiation of adipocytes, whereas PPARγ antagonists inhibit differentiation (233). The PPARγ ligands troglitazone (TGZ) and the more potent rosiglitazone (ROS) were used to induce differentiation of 3T3-L1 preadipocytes. When differentiation was induced with either ROS or TGZ, the amount of differentiation occurring by day 13 following induction was reduced relative to cells differentiated with MDI (Figure 3.10A). However, when co-treated with VPA and either TGZ or ROS, differentiation of 3T3-L1 cells was inhibited by 60% and 43% respectively (Figure 3.10A, B), suggesting that VPA can inhibit differentiation mediated by PPARγ.

Zen-Bio was contracted to examine the effects of VPA on ROS-induced differentiation of human preadipocytes. Similar to the effects of Zen-DMI, ROS induced differentiation in appropriately 50% of the cells in both lots, with a few more cells in the single lot (L091901) appearing to differentiate when compared to cells in the mixed lot (SL0023) (Appendix C, top panel). Daily treatment with VPA (1 mM) inhibited differentiation of human preadipocytes in both lots of cells (Appendix C, bottom panel). In agreement with the Oil Red O staining, VPA (0.5-5 mM) treatment of both lots of cells significantly reduced TAG levels (Appendix C). Compared to treatment of cells with TNFα, VPA induced a greater reduction in TAG levels, suggesting that VPA can inhibit differentiation of preadipocytes more than TNFα. These data show that VPA inhibits PPARγ-induced preadipocyte differentiation.

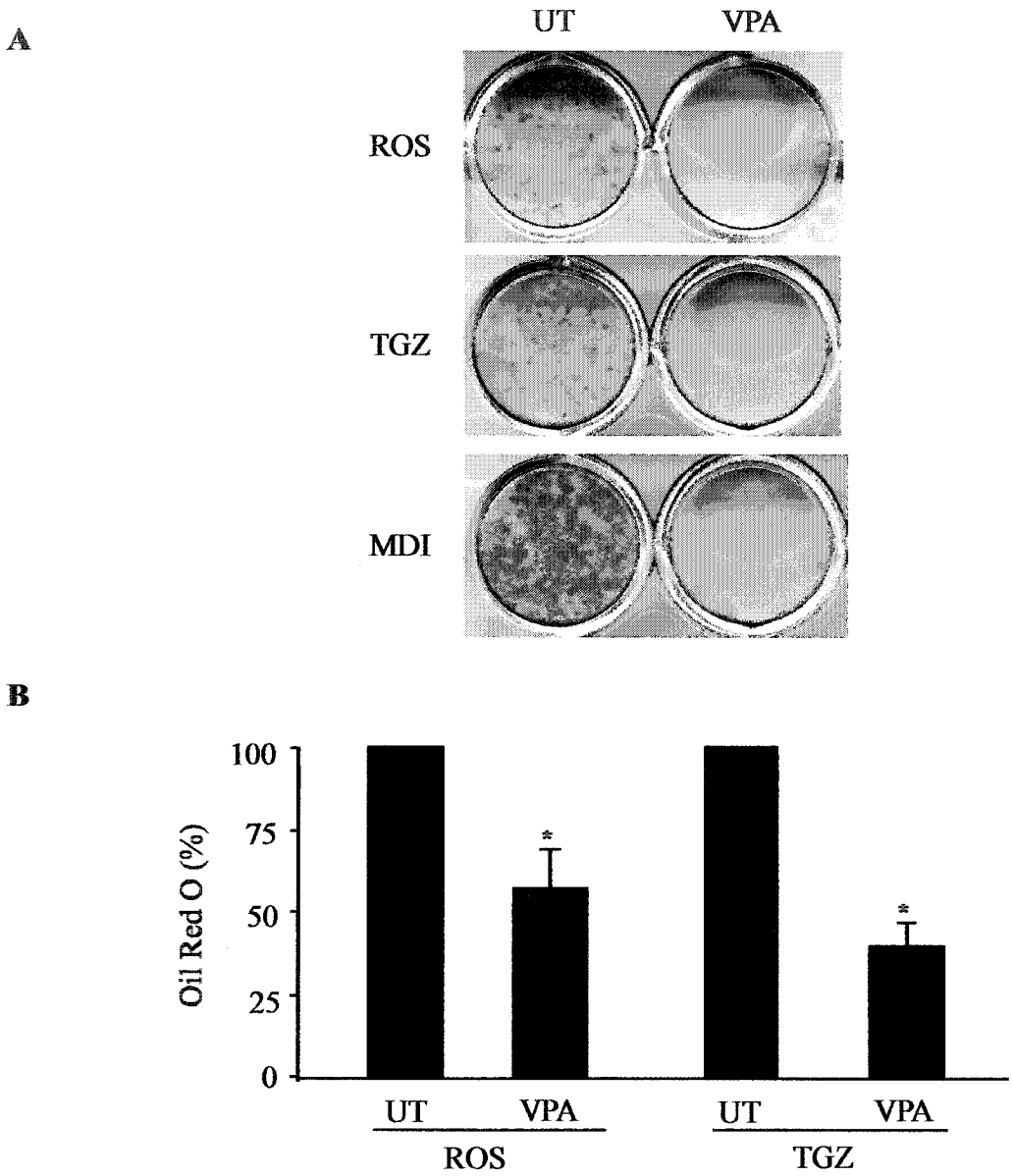

Figure 3.10 VPA reduces PPARγ-induced differentiation of 3T3-L1 preadipocytes **A)** Daily VPA treatment reduces Oil Red O staining in 3T3-L1 cells induced to differentiate (day 0-2) with either 1 μM rosiglitazone (ROS) or 10 μM troglitazone (TGZ). Cells differentiated with MDI (day 0-2) in the same experiment demonstrate relatively more differentiation. **B)** Quantification of Oil Red O staining of 3T3-L1 cells differentiated as described in A. Data represents mean percent levels compared to UT (set at 100% differentiation) from three indepentent experiments. * $p<0.001$.

VPA is not a PPARγ Ligand

Due to VPA's ability to block PPARγ-induced differentiation, reporter assays were conducted to assess whether VPA interacted with the ligand binding domain (LBD) of PPARγ. Since TGZ and ROS had equivalent activities, TGZ was used in these experiments as the positive control for activation of the PPARγ LBD. Due to the low transfection efficiency obtained in 3T3-L1 cells (data not shown), these experiments were completed in human hepatoblastoma HepG2 cells. HepG2 cells are a good alternative model since they endogenously express PPARγ mRNA and protein (272). Cells were transfected with the GAL4 DNA binding domain (DBD) expression plasmid (BD-Gal4) or the expression plasmid for GAL4 DBD fused with the PPARγ ligand binding domain (LBD) (PPAR-Gal4). Activation of the PPAR-Gal4 allows it to bind to the Gal4 response element in the FR-luc reporter plasmid, which subsequently activates transcription of luciferase.

VPA induced a weak activation of PPAR-Gal4, which was similar to the activation of the control plasmid BD-Gal4 lacking the PPARγ-LBD (Figure 3.11). In contrast, TGZ induced a significant increase in PPAR-Gal4 activity (40 fold increase) when compared to the activity of the control plasmid, BD-Gal4. These results suggest that VPA non-specifically increased the activation of PPAR-Gal4. VPA can increase the activity of many reporter genes in transient transfection experiments, most likely due to the ability of VPA to inhibit HDAC (40). The HDAC inhibitor, TSA, induced an increase in BD-Gal4 and PPAR-Gal4 reporter activity that was similar to that observed with VPA (Figure 3.11). These data suggest that non-specific activation of reporter genes may be an indirect effect of HDAC inhibition.

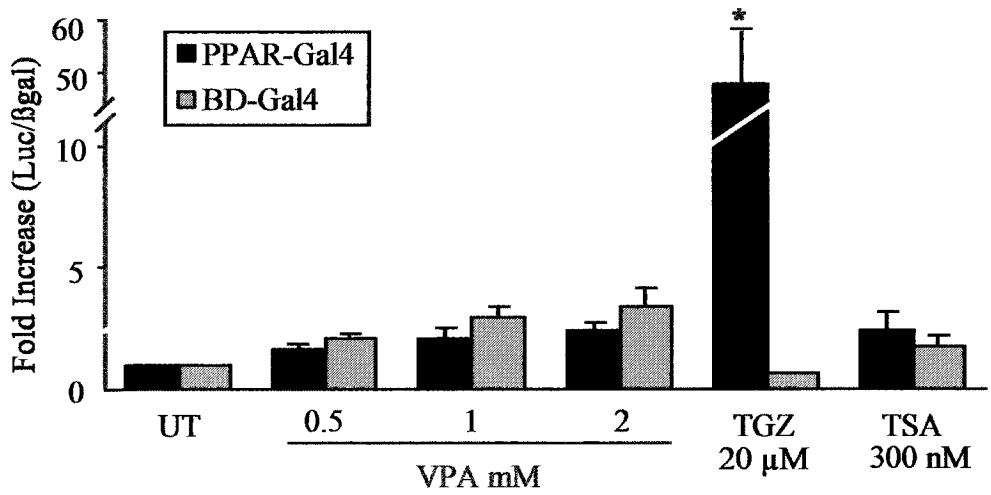

Figure 3.11 Activation of PPAR-Gal4 transcriptional activity by VPA
Fold induction in luciferase activity in the presence the reporter plasmid FR-luc and the Gal4 expression plasmid (BD-Gal4) or the Gal4 expression plasmid with the PPARγ ligand binding domain (PPAR-Gal4) following treatment with either VPA (0.5-2 mM), TGZ (20 µM) or TSA (300 nM) in HepG2 cells. Data are normalized with ß-gal activity; similar findings were obtained in three independent experiments. TGZ induces a significant increase in activity in the presence of PPAR-Gal4, compared to BD-Gal4, t-test, * $p<0.001$.

PPARγ partial agonists or antagonists can inhibit 3T3-L1 differentiation (273, 274). Since VPA inhibits 3T3-L1 differentiation the effect of VPA on the dose response curve of TGZ-induced PPARγ activity was determined to assess whether VPA may be acting as a PPARγ partial agonist or antagonist. TGZ dose-dependently induced PPARγ activity with a mean fold induction of luciferase activity ranging from 23±2.5 to 35±5.2 (Figure 3.12A). TGZ did not alter BD-Gal4 reporter activity (Figure 3.12B). If VPA was acting as a partial agonist or antagonist it should inhibit TGZ activity. VPA induced a mean 2.5±0.23 fold increase (Figure 3.12C) in TGZ-induced PPARγ activity [range: 53±6.3 to 72±16.0 (Figure 3.12A); however, this fold increase by VPA was similar to the increase in BD-Gal4 reporter activity (Figure 3.12B, C). These data imply that VPA (0.5-2 mM) does not act as a PPARγ partial agonist or antagonist, and thus it is hypothesized that VPA effects are meditated by the HDAC inhibitory activity of VPA.

In agreement with VPA producing its effects on the PPARγ reporter assay through acting as a HDAC inhibitor, both TSA and 4-phenylbuturate (4PB) induced similar fold activation in the presence of either PPAR-Gal4 or BD-Gal4 plasmids (Figure 3.13). Treatment of the cells with TGZ (20 μM) alone or co-treatment with a HDAC inhibitor (TSA or 4PB) induced a significantly higher amount of activation with PPAR-Gal4 compared to BD-Gal4. Compared to treatment with TGZ, or TGZ and 4PB, the combination treatment of TGZ and TSA induced a significant increase in PPAR-Gal4 activity. In contrast, co-treatment of either TSA, 4PB, or VPA did not induce a significant increase in PPAR-Gal4 activity, suggesting that the three drugs may work through a similar mechanism to non-specifically activate reporter gene activity and thus cannot produce a synergistic effect.

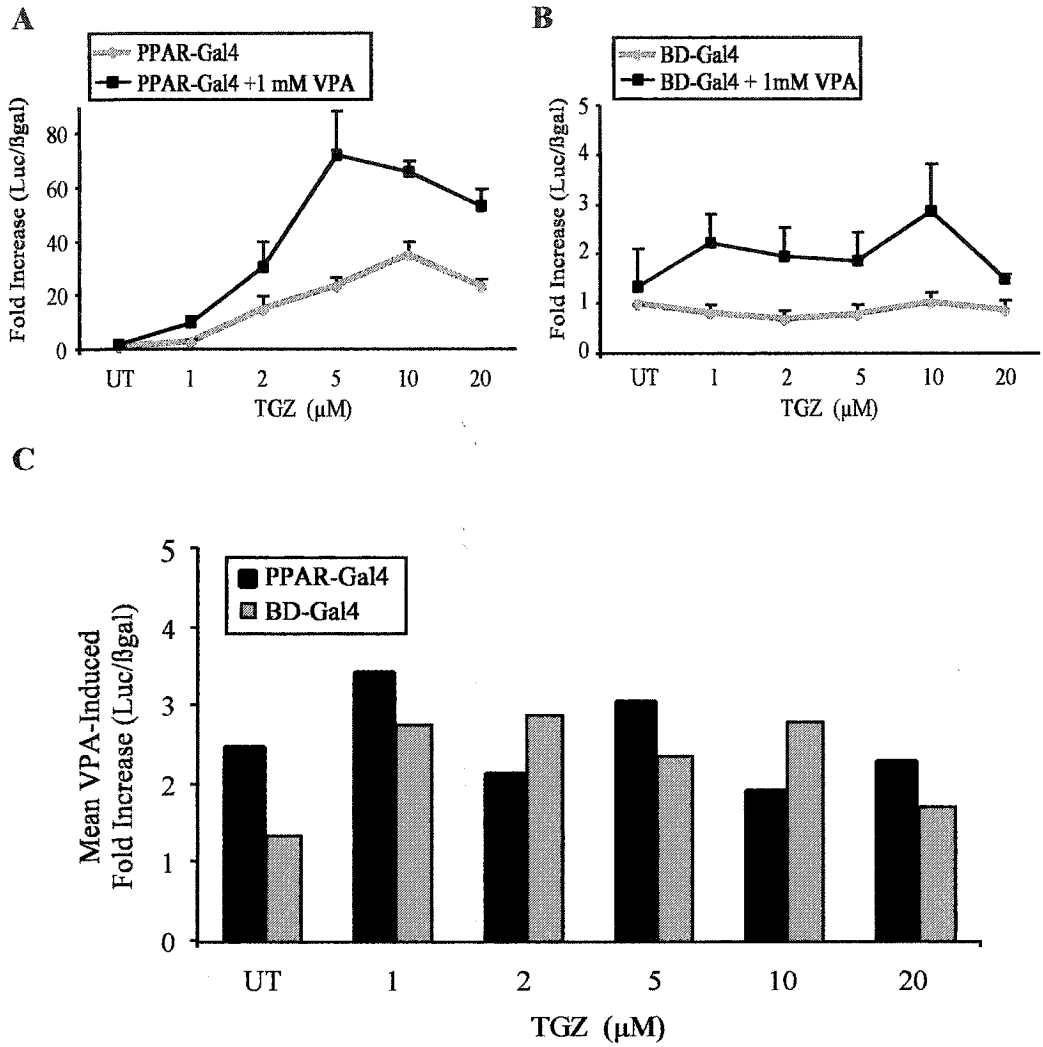

Figure 3.12 VPA activation of PPAR-Gal4 is not specific to VPA interactions with the PPARγ LBD

A) Dose-response curve (1-20 µM) for TGZ-induced increase of PPAR-Gal4 activity in the absence or presence of 1 mM VPA. **B)** Dose-response (1-20 µM) for TGZ-induced increase of BD-Gal4 activity in the absence or presence of 1 mM VPA. **C)** Summary of VPA-induced fold increase from experiments described in A and B demonstrating that the differences in activation of BD-Gal4 versus PPAR-Gal4 induced by VPA are not different. Data are normalized with ß-gal activity; similar findings were obtained in three independent experiments.

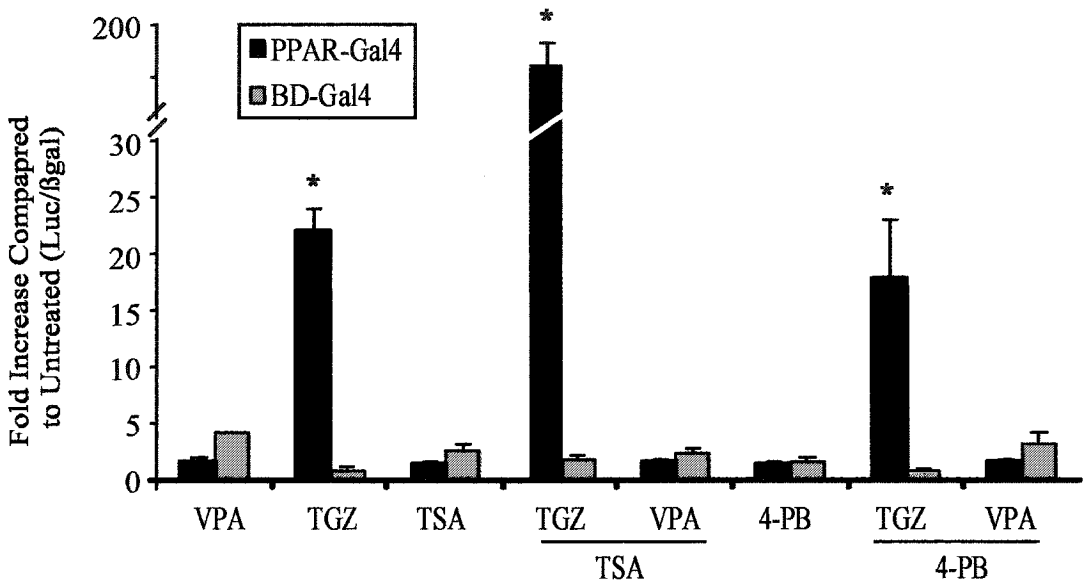

Figure 3.13 Activation of PPAR-Gal4 transcriptional activity by HDAC inhibitors

Fold induction in luciferase activity in the presence of the Gal4 expression plasmid (BD-Gal4) or the Gal4 expression plasmid with the PPARγ ligand binding domain (PPAR-Gal4) following treatment with either VPA (1 mM), TGZ (20 μM), TSA (300 nM), or 4-PB (1.5 mM) in HepG2 cells. In the presence of TGZ, there was always a significant increase in activity in PPAR-Gal4, compared to BD-Gal4, t-test, *$p<0.01$. Data are normalized with ß-gal activity; fold induction relative to untreated cells, set at 1; similar findings were obtained in three independent experiments.

VPA Treatment Inhibits PPARγ and SREBP1a mRNA Levels

The onset of differentiation in adipocytes involves activation of gene expression including C/EBPα/ß/δ, PPARγ, and SREBP1a. Northern analysis was conducted to determine whether the reduction in differentiation by VPA was accompanied by changes in gene expression. Prior to the onset of differentiation preadipocytes have undetectable levels of C/EBPα, PPARγ, and SREBP1a mRNA (Figure 3.14A). Twenty-four hours following the initiation of differentiation a weak signal for the three transcripts was detectable (Figure 3.14A). At day 1, 2 and 5 following the initiation of differentiation, VPA-treated cells had reduced levels of PPARγ and SREBP1a mRNA, and no change in C/EBPα mRNA levels compared to control cells (Figure 3.14A & B). Since VPA treatment did not alter C/EBPα mRNA levels, the expression of C/EBPß and C/EBPδ mRNA levels following VPA treatment were not explored, because expression of C/EBPα is highly dependent on expression of C/EBPß and C/EBPδ (223, 226). We found that VPA reduced GAPDH mRNA levels at day 2 and day 5 by 33±6% and 29±8%, respectively, and therefore could not use GAPDH mRNA levels to normalize for equal loading. Since there was no change in C/EBPα levels at day 2 and 5 following treatment, it is unlikely that the reduction in PPARγ and SREBP1a gene expression is due to unequal loading; confirmed by 18S rRNA loading (Figure 3.14A).

VPA Treatment Reduces PPARγ, SREBP1a and C/EBPα Protein Levels

In order to determine if VPA-induced a concomitant reduction in mRNA and protein levels for PPARγ or SREBP-1a, total cell protein lysates were obtained from cells treated with VPA. Although VPA treatment during adipogenesis was not associated with alterations in C/EBPα mRNA levels, C/EBPα protein levels were also assessed.

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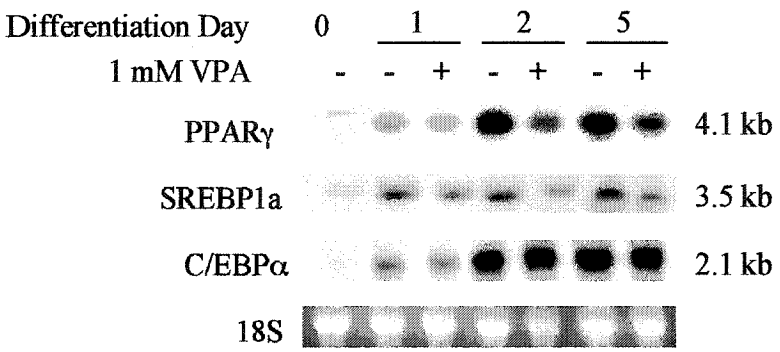

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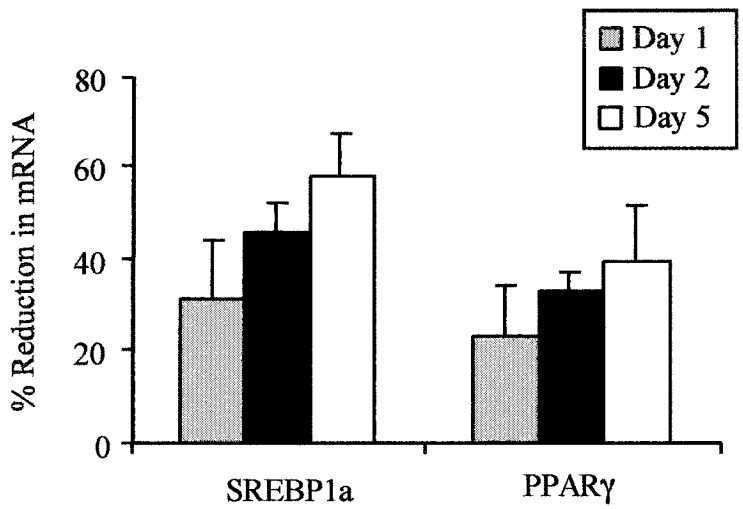

Figure 3.14 VPA reduces SREBP1a and PPARγ, but not C/EBPα, mRNA
A) Northern analysis of mRNA from 3T3-L1 cells prior to addition of MDI medium (day 0) and days 1, 2, and 5 after addition of MDI in the absence or presence of 1 mM VPA. **B)** Mean percent reduction in mRNA levels for SREBP1a and PPARγ comparing 1 mM VPA treated to UT cells from two independent experiments, performed in duplicate at each time point.

PPARγ has two isoforms, PPARγ$_1$ and PPARγ$_2$ that are undetectable in preadipocytes (day 0), are expressed at low levels one day following initiation of differentiation (day 1), and are clearly detectable at all time points tested after day 3 (Figure 3.15A). VPA treatment caused a similar reduction in PPARγ$_1$ and PPARγ$_2$, with a maximum reduction of greater than 50% on day 7 (Figure 3.15C). After detection of PPARγ, blots were stripped and incubated with an antibody against C/EBPα, which detects both the 42 kDa and 30 kDa alternative translation products. While C/EBPα proteins were undetectable in preadipocytes, a weak signal was detected at day 1 of differentiation, and relatively high levels of expression were detected at days 3-5 (Figure 3.15A). Compared to untreated cells, daily VPA treatment reduced the amount of both p42 and p30 C/EBPα protein (Figure 3.15C), which was surprising since no change in C/EBPα mRNA levels were detected in the presence of VPA.

The precursor (P; 125 kDa) and the cleaved (C; 68 kDa) active form of SREBP1a were also detected in differentiating adipocytes (Figure 3.15B). In preadipocytes expression of both the P and C form of SREBP1a was detected by western blot analysis. In the absence of VPA treatment, the expression of the SREBP1a (P) was increased at day 1 after inducing differentiation, whereas SREBP1a (C) was expressed at higher on day 4 and 7. Daily VPA treatment did not have an effect on the amount of SREBP1a (P), but consistently decreased the SREBP1a (C) at day 4 and later time points (Figures 3.15B, C).

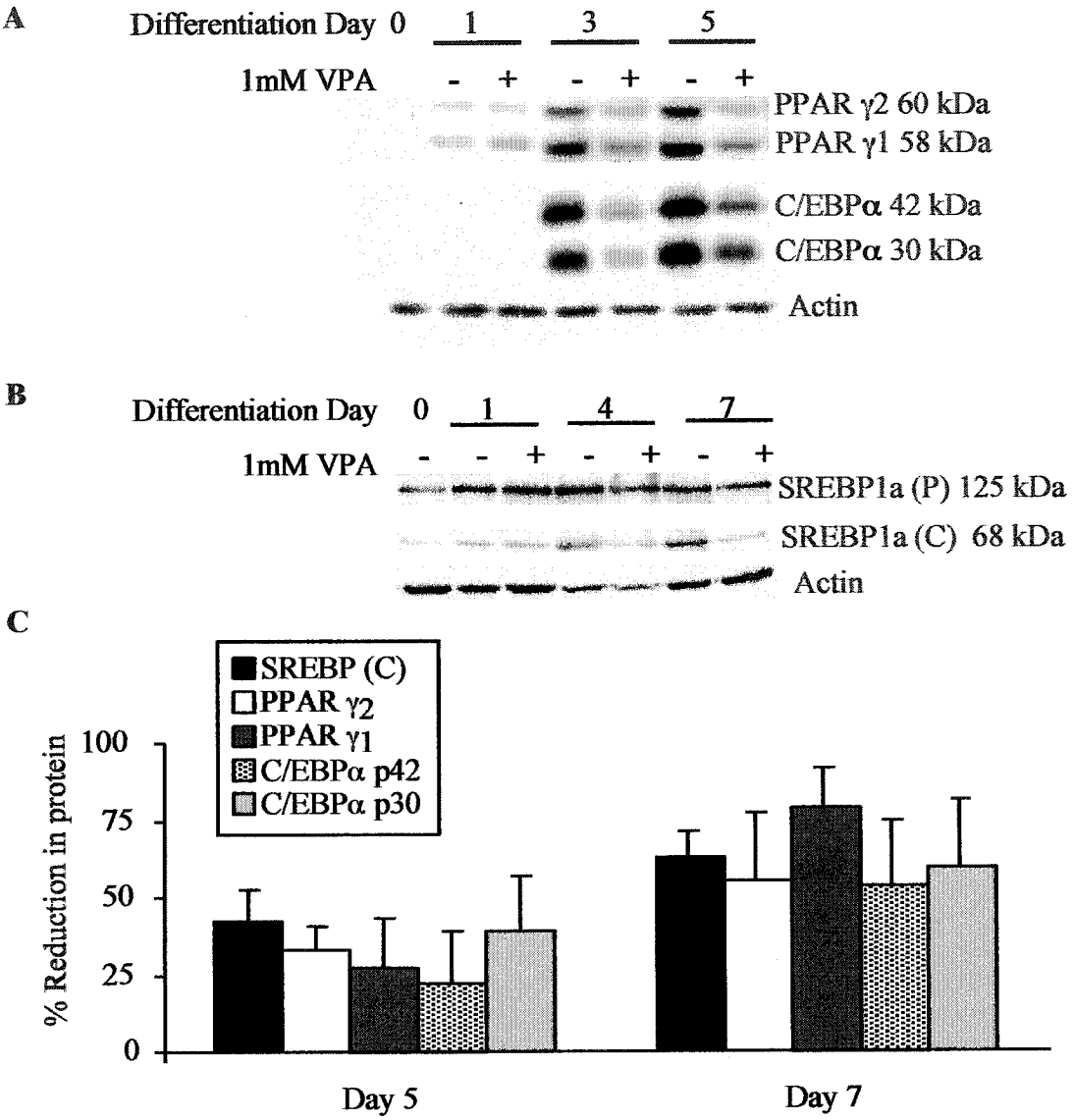

Figure 3.15 VPA treatment reduces PPARγ1 and PPARγ2, p30 and p41 C/EBPα, and SREBP1a (C) protein levels

A) Western analysis of whole cell protein extracts from 3T3-L1 cells obtained prior to addition of MDI medium (day 0) and days 1, 3, and 5 after addition of MDI in the absence or presence of 1 mM VPA. **B)** Western analysis of whole cell protein extracts from 3T3-L1 cells prior to (day 0) or after the addition of MDI medium (days 1, 4, and 7). Daily VPA (1 mM) treatment reduces protein levels for mature SREBP1a (C), but does not affect precursor SREBP1a (P) protein. **C)** Mean percent reduction in mature SREBP1a, PPARγ and C/EBPα protein levels at day 5 and 7 after initiation of differentiation, comparing VPA (1 mM) treated to untreated cells. Data is representative of two independent experiments performed in triplicate at each time point.

HDAC Inhibition and Adipogenesis

HDAC activity is involved in a variety of cellular differentiation processes such as myogenesis (275), osteoclastogenesis (276), and differentiation of the intestinal epithelium during organogenesis (277). HDAC activity was recently also suggested to modulate adipogenesis and affect the expression of PPARγ and C/EBPα (269, 270). VPA inhibits adipogenesis and HDACs with an *in vitro* IC_{50} in the mM range (41), therefore the next set of experiments investigated whether other HDAC inhibitors had effects similar to VPA on adipogenesis.

TSA is used as a reference substance in research for the development of new inhibitors of HDACs because it is the most potent HDAC inhibitor with an *in vitro* IC_{50} within the nM range (reviewed in 278). Daily treatment with TSA during preadipocyte differentiation caused a dose-dependent inhibition in adipogenesis, with doses of greater than 3 nM producing a greater than 50% reduction in adipocyte formation when assessed by Oil Red O quantification (Figure 3.16A). Since 1mM VPA treatment also inhibited differentiation on average by 56% (Figure 3.16C), all future experiments used a 3 nM dose of TSA. Similar to the effect of VPA treatment on adipogenesis, TSA treatment once at day 0 or throughout differentiation caused a reduction in Oil Red O staining (Figure 3.16B). Similar to VPA and TSA, 4-phenylbutyrate (4PB; 1.5 mM) also inhibited 3T3-L1 differentiation, within its known HDAC IC_{50} range (Figures 3.17) (reviewed in 278, 279). In contrast, daily treatment with VPM (1 mM), an amide analog of VPA that does not inhibit HDAC activity (41), did not significantly affect 3T3-L1 differentiation (Figure 3.17). These results support the hypothesis that inhibition of adipogenesis is associated with inhibition of HDAC activity.

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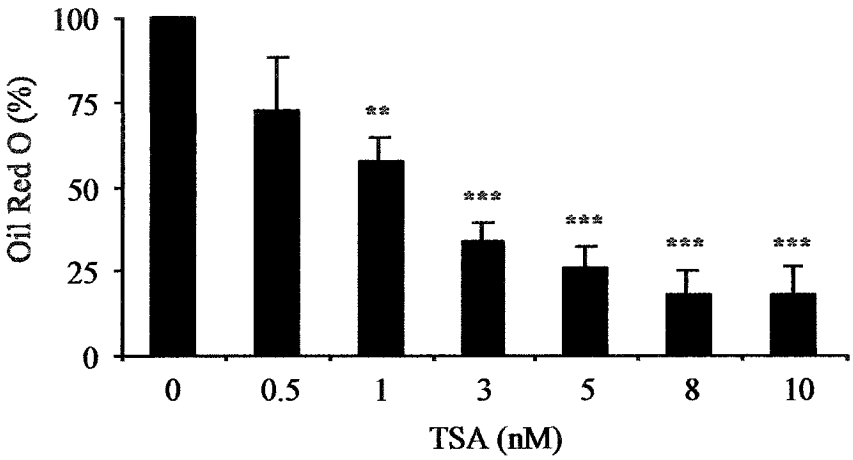

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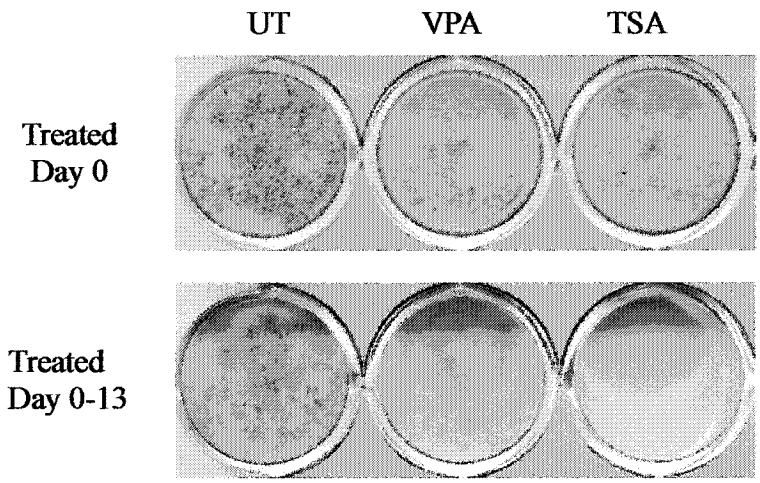

Figure 3.16 TSA inhibits differentiation of 3T3-L1 preadipocytes
A) Quantification of Oil Red O staining of cells cultured in MDI demonstrating daily (day 0-13) treatment with TSA significantly reduced formation of adipocytes dose-dependently. Data represents mean percent levels compared to UT (set at 100% differentiation) from three independent experiments, **$p<0.01$,*** $p<0.001$. **B)** Cells were grown in the presence of MDI medium (IBMX, DEX, and insulin) for the first 2 days, and treated at day 0 or daily from 0-13 with TSA (3 nM) or VPA (1 mM). Similar to VPA, TSA reduces adipocyte differentiation.

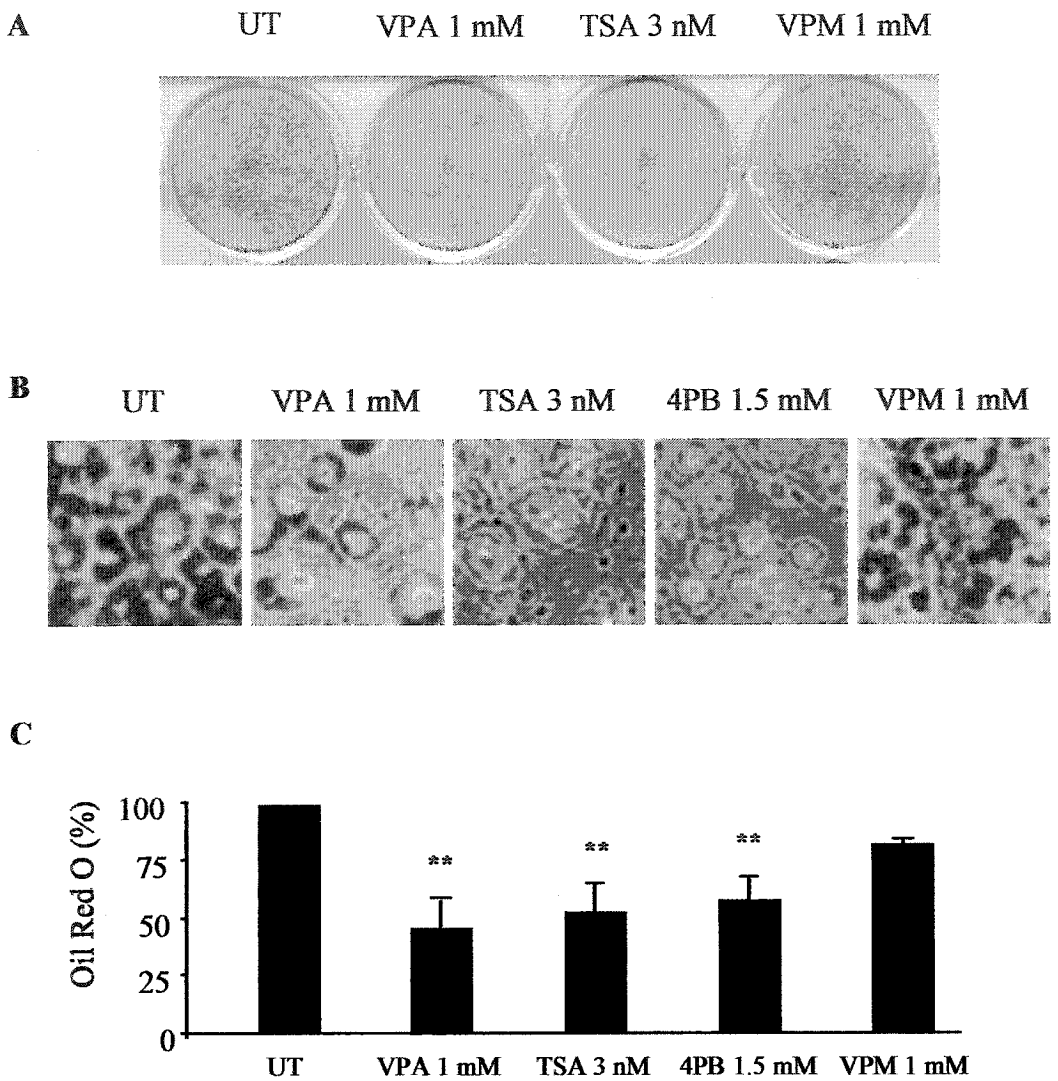

Figure 3.17 Inhibition of differentiation by HDAC inhibitors
A) Cells stained with Oil Red O 13 days after initiation of differentiation in MDI. In comparison to untreated (UT) cells, daily treatment with the HDAC inhibitors VPA (1 mM) and TSA (3 nM) inhibited differentiation, whereas valpromide (VPM 1 mM), which does not inhibit HDACs, does not affect adipocyte differentiation. **B)** Higher magnification of cells cultured as described in A. Cells treated with the HDAC inhibitor 4PB are also shown to have a reduced number of differentiated adipocytes. **C)** Quantification of Oil Red O staining of cells cultured using the same conditions as A and B. Data represents mean percent level compared to UT (set at 100% differentiation) from three independent experiments. ** $p<0.01$.

Concurrent with my studies investigating HDAC inhibition and adipogenesis, published work by Fajas *et al.* (269) suggested that treatment with HDAC inhibitors, including VPA and 4PB, promoted the differentiation of 3T3-L1 preadipocytes. When compared to the MDI medium used in our experiments, the differentiation inducing medium used by Fajas *et al.* (269) (F-MDI) contained 4 times the amount of DEX (1 μM versus 0.25 μM) and 17 times the amount of insulin (10 μg/ml, versus 0.58 μg/ml). To determine if the differences in our results were due to the differences in the composition of the differentiation inducing medium, experiments were conducted using F-MDI. When differentiation was induced with either F-MDI or MDI, almost all cells had the characteristic morphology of adipocytes and contained lipid droplets at day 13 after initiation of differentiation (Figure 3.18A). Treatment with either VPA or 4PB daily throughout differentiation significantly inhibited F-MDI- or MDI-induced differentiation (Figure 3.18B). These results suggest that the differences in the differentiation medium used in this study and the work of Fajas *et al.* (269) were not responsible for the contrasting results obtained.

TSA, but not VPM Reduces PPARγ, SREBP1a and C/EBPα mRNA Levels

We determined whether the reduction in adipocyte differentiation by TSA affected C/EBPα, PPARγ, and SREBP1a mRNA expression. Similar to VPA treated cells, TSA treatment caused reduced levels of PPARγ and SREBP1a mRNA levels (Figure 3.19A, B). Unlike VPA treatment, which had no effect on C/EBPα mRNA levels (Figure 3.14, 3.19A), TSA treatment reduced C/EBPα mRNA levels (Figure 3.19A, B). while VPM treatment which was not associated with changes in adipogenesis, also had no effect on PPARγ, SREBP1a or C/EBPα mRNA levels (Figure 3.19A).

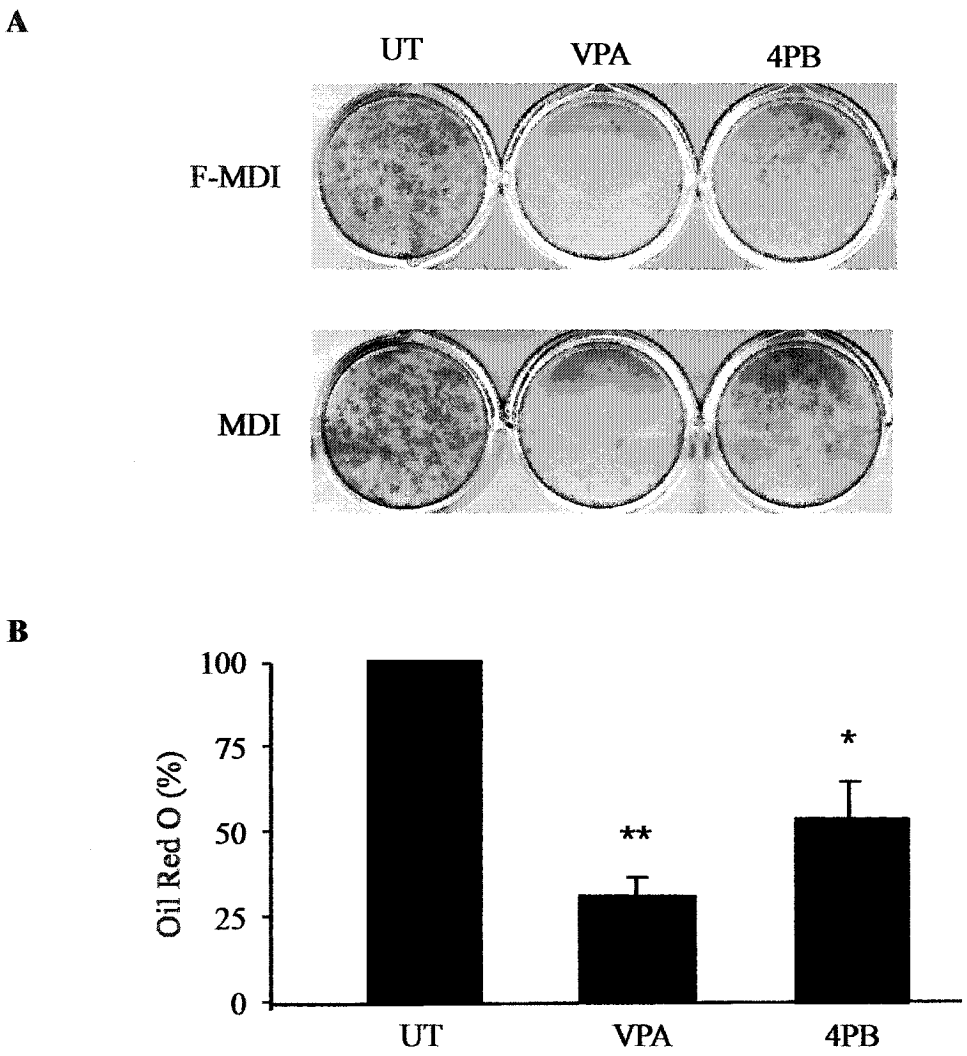

Figure 3.18 VPA and 4PB inhibit differentiation induced by F-MDI or MDI. **A)** Cells were grown in the presence of F-MDI or regular MDI medium from day 0-2, and treated daily from 0-13 with 4PB (1.5 mM) or VPA (1mM). In both F-MDI and MDI, VPA and 4PB reduced adipocyte differentiation. **B)** Quantification of Oil Red O staining of cells cultured in F-MDI confirmed that daily (day 0-13) VPA or 4PB treatment significantly reduced formation of adipocytes. Data represents mean percent levels compared to UT (set at 100% differentiation) from three independent experiments, * $p<0.01$, *, $p<0.001$.

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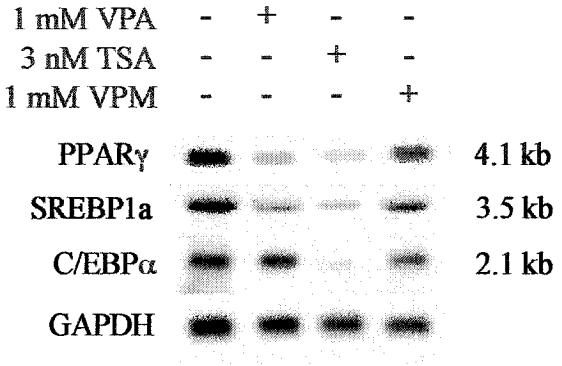

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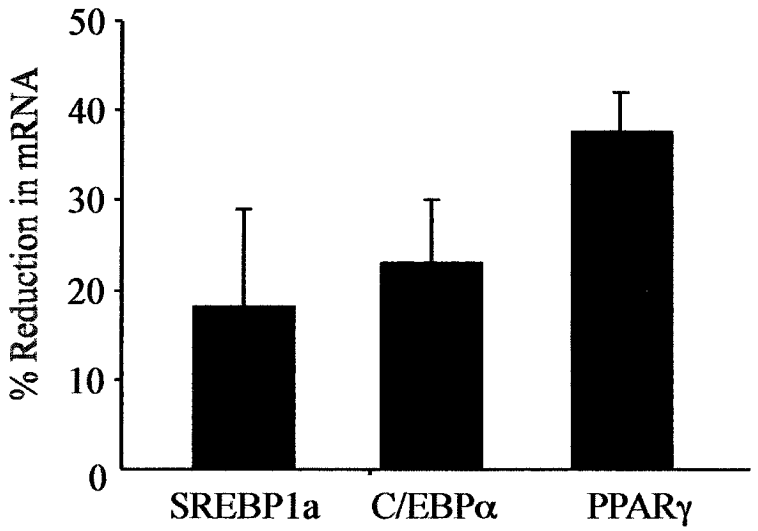


Figure 3.19 TSA reduces SREBP1a and PPARγ, and C/EBPα mRNA levels **A)** Northern analysis of mRNA from 3T3-L1 cells obtained 5 days after addition of MDI. Cells were treated daily with VPA, TSA, or VPM. VPA and TSA, but not VPM, reduced SREBP1a, PPARγ, and C/EBPα. **B)** Mean percent reduction in mRNA levels for SREBP1, C/EBPα, and PPARγ comparing TSA treated to UT cells from two independent experiments, performed in triplicate at each time point.

TSA, but not VPM Reduces PPARγ, SREBP1a and C/EBPα Protein Levels

Similar to the effect of VPA treatment, TSA treatment reduced the protein levels for PPARγ_1, PPARγ_2, p42 and p30 C/EBPα, and the cleaved form of SREBP1a (C) but not SREBP1a (P) at day 7 of differentiation (Figure 3.20A-C). In contrast to the effects of VPA or TSA, VPM treatment, which does not inhibit differentiation, did not affect protein expression (Figure 3.20A).

HDAC Inhibitors Induce Higher Levels of Acetylated Histone H3 and Do Not Alter HDAC1 Protein Levels

To examine the HDAC inhibitory activity of VPA, TSA and VPM, 3T3-L1 cells were treated with VPA (1 mM), TSA (3 nM), or VPM (1 mM) daily and histone acetylation was assessed at day 3 and 7 following initiation of differentiation. Compared to untreated cells, daily VPA and TSA treatment during preadipocyte differentiation induced histone H3 acetylation as assessed at day 3 and 7 during differentiation (Figure 3.21A). Some histone H3 acetylation was observed in VPM treated cells, but the levels were consistently less than that produced by VPA or TSA and often appeared unchanged compared to untreated cells (Figure 3.21A).

Wiper-Bergeron *et al.* (270) demonstrated that during 3T3-L1 cell differentiation, HDAC1 protein levels are decreased. In contrast to these findings, HDAC1 protein levels in 3T3-L1 cells were not significantly different between preadipocytes (day 0) and developing adipocytes (days 1, 3, 5) in the absence or presence of VPA, TSA, or VPM (Figure 3.21B, day 5 data not shown). Thus in this model, HDAC1 protein levels are not dynamically reduced during differentiation, nor altered by HDAC inhibition.

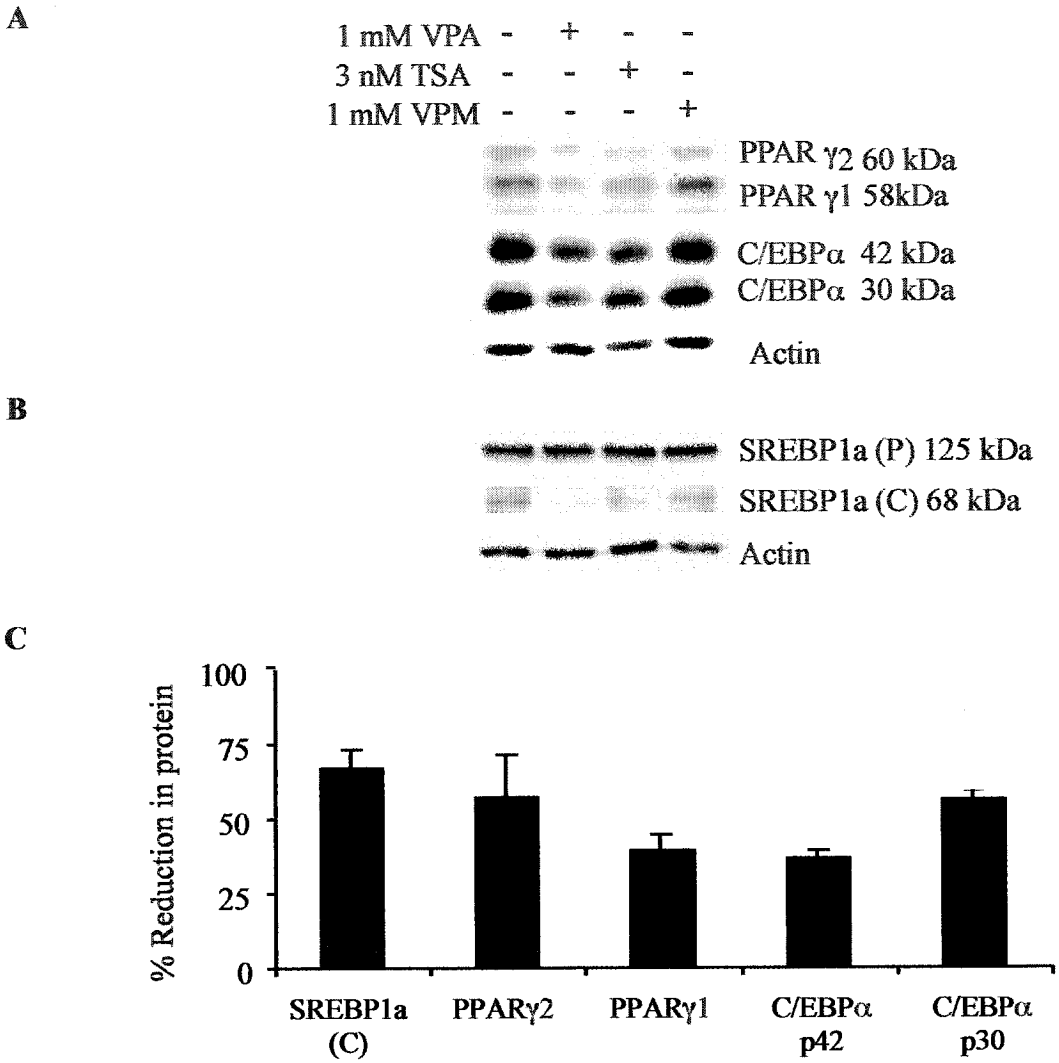
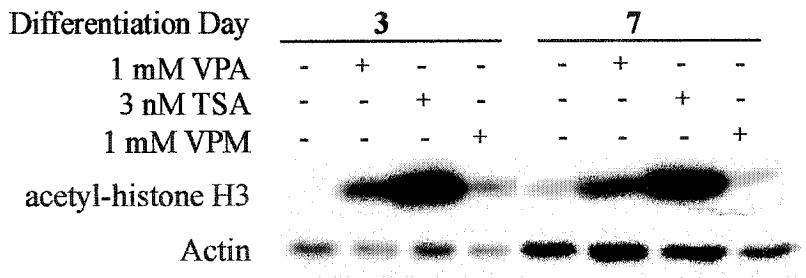


Figure 3.20 TSA, but not VPM reduces PPARγ, SREBP1a, and C/EBPα protein levels

A) Western analysis of 3T3-L1 cells obtained 7 days after the addition of MDI. Daily TSA or VPA treatment reduced PPARγ1, PPARγ2, p30 and p42 C/EBPα protein levels, when compared to untreated cells. **B)** Western analysis of whole cells protein extracts obtained at day 7 after the addition of MDI, demonstrating daily TSA or VPA treatment reduced SREBP (C), but does not affect SREBP (P) protein levels. **C)** Mean percent reduction in protein levels comparing TSA (3 nM) treated to untreated cells from two independent experiments, each performed in duplicate.

A



B

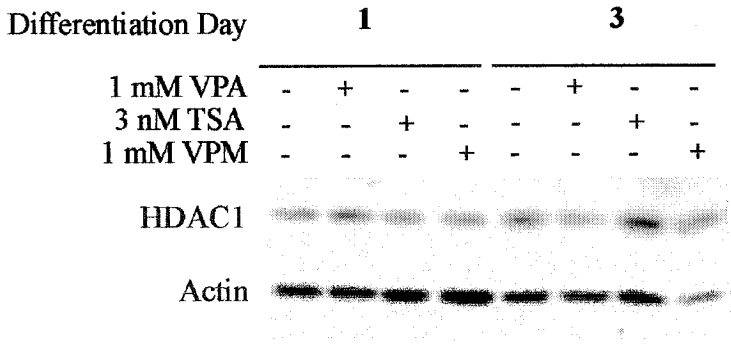


Figure 3.21 Treatment with VPA and TSA induces acetylated histone H3, but does not alter HDAC1 protein levels

A) Western analysis of 3T3-L1 cells obtained at day 3 and day 7 after the addition of MDI. Daily VPA and TSA treatment induces high levels of acetylated histone H3, compared to UT and VPM-treated cells. **B)** Western analysis of 3T3-L1 cells obtained at day 1 and day 3 after the addition of MDI. There was no difference in expression levels for HDAC1 between cells that were untreated (UT), or treated daily with VPA, TSA, or VPM.

DISCUSSION

The results in this chapter demonstrate that VPA treatment prevents mouse and human adipocyte differentiation *in vitro*. Inhibition of HDAC activity with VPA, TSA and 4PB inhibited adipogenesis, whereas VPM, a VPA analogue that does not possess HDAC inhibitory properties did not prevent adipogenesis. The reduction in differentiation by inhibitors of HDAC was accompanied by a reduction in the synthesis of TAG and decrease in protein levels for PPARγ, C/EBPα, and SREBP1a, three transcription factors important for adipogenesis. TSA treatment also reduced mRNA levels for PPARγ, C/EBPα, and SREBP1a, whereas VPA treatment reduced PPARγ and SREBP1a, but not C/EBPα mRNA levels. Based on these results the reduction in C/EBPα protein and inhibition of HDAC activity by VPA are hypothesized to be responsible for the attenuation of adipogenesis by VPA. We also hypothesize that HDAC activity is required for adipocyte differentiation.

Histone Deacetylase (HDAC)

The packaging of DNA into chromatin is strongly influenced by post-translational mechanisms including methylation, phosphorylation, and acetylation of the histone proteins. Acetylation of the histones results from a balance between the activities of histone acetyltransferases (HATs), which increase histone acetylation resulting in a state of hyperacetylation, and HDACs, which decrease histone acetylation and result in a state of hypoacetylation (reviewed in 278). Generally, acetylated histones are associated with transcriptionally active chromatin, thus HAT activity is associated with increased transcriptional activity and HDAC activity is associated with depressed rates of transcription.

The first HDAC was cloned in 1996 and now over 18 human HDACs have been identified and demonstrated to be involved in numerous cellular processes such as transcription, cell cycle progression, gene silencing, differentiation, and DNA replication (reviewed in 280). Functionally, HDACs remove the acetyl group from lysine residues within the N-terminus of histones through a process that requires the presence of cofactors and a zinc ion (reviewed in 278). The HDAC family is categorized into three classes based on their homology to the yeast HDACs (reviewed in 278, 280). The class I HDACs (related to yeast Rpd3; HDAC 1,2,3,8) generally localize to the nucleus and are ubiquitously expressed in many cell lines and tissues. Class II HDACs (related to yeast Hda1; HDAC 4,5,7,9,10) are twice as large as class I HDACs and are cytoplasmic proteins that can be shuttled to the nucleus. The class III HDACs (related to yeast Sir2) which are also called sirtuins (SIR1-7), were more recently discovered and are unique in that their enzyme activity is dependent on nicotinamide adenine dinucleotide (NAD^+). Substrate specificity of HDACs occurs through the large number of HDACs, HDAC splice variants, subcellular localizations, and proteins that complex with the HDACs to form unique multiprotein complexes.

Inhibitors of HDACs have been isolated from a variety of natural sources, as well as produced as synthetic compounds (reviewed in 281, 282). HDAC inhibitors are grouped according to their structure and VPA, like sodium n-butyrate, is a member of the group of short-chain fatty acids that inhibit HDACs. Since HDAC inhibition generally induces cell cycle arrest and differentiation or apoptosis in cancer cells, these compounds have entered clinical trials as anticancer agents and there is a large interest in the development of more efficacious and specific HDAC inhibitors.

HDACs and Adipogenesis

The role of HDACs during adipocyte differentiation remains largely unknown and the literature is contradictory. Two studies have reported that treatment with HDAC inhibitors (TSA and VPA) induce the differentiation of 3T3-L1 preadipocytes (269, 270), which is in contrast to our published results which are also presented in this chapter (283). Fajas *et al.* (269) reported that treating 3T3-L1 cells once with VPA (1.5 mM) at the onset of treatment with F-MDI caused a significant induction of preadipocyte differentiation, as assessed by Oil Red O staining at day 4 of differentiation. In the current study, these results could not be replicated when using F-MDI to induce differentiation (269). Wiper-Bergeron *et al.* (270) also reported that treatment of 3T3-L1 cells at the onset of differentiation with either 10 mM VPA or 400 nM TSA stimulated adipogenesis, as assessed at day 8 of differentiation. However, in contrast to the results of Fajas *et al* (269), Wiper-Bergeron *et al.* (270) reported differentiation was only stimulated by HDAC inhibitors in the absence of DEX, and had no effect in the presence of DEX. I found that in presence of differentiation inducing medium that did not contain DEX (MI), 10 mM VPA treatment did not induce differentiation. Therefore, my attempts to replicate the experiments of others using the protocols described in these manuscripts were unsuccessful. Indeed, VPA inhibited adipogenesis under all conditions tested, as assessed by a variety of outcomes including Oil Red O staining, measuring TAG synthesis, and levels of adipocyte specific genes.

It is unclear why these conflicting results have been obtained; however they may be partially attributable to the difference in the amount of differentiation obtained at different time points. In the results presented in this chapter, at day 4 of differentiation

very few cells have lipid droplets, yet quantification of Oil Red O staining when measured through absorbance readings demonstrate VPA treatment significantly inhibited differentiation. In the work of Fajas *et al.* (269) differentiation was assessed at day 4 by quantification of Oil Red O staining using Adobe Photoshop, and thus it is difficult to assess the magnitude of differentiation or accuracy of this method of quantification (269). Similarly, at day 8, the results presented in this chapter found that almost all cells had differentiated following induction with differentiation medium. By contrast in the study of Wiper-Bergeron *et al.* (270) the representative photographs show that very few cells at day 8 differentiated into adipocytes following induction with the same differentiation inducing medium. Thus, these differences in basal rates of adipogenesis and the amount of differentiation may have contributed to the differences in the results obtained.

An alternative theory to explain these contrasting results may reside in differences in the population of 3T3-L1 cells used for these experiments. The cells used in the experiments presented in this thesis were obtained from stocks purchased from the ATCC. The 3T3-L1 cells used in the experiments by Fajas *et al.* (269) were also reported to come from ATCC, yet in the publication of Wiper-Bergeron *et al.* (270) there is no information about the origin of the 3T3-L1 cells. It is possible that over time in culture 3T3-L1 cells may undergo subtle changes. Indeed, after multiple passages (>20) it was not uncommon for controls 3T3-L1 cells to display < 100% differentiation (data not shown). Moreover, other have also reported variations in results from 3T3-L1 cell lines (284), suggesting these cells may be susceptible to alterations during culturing. In order

to avoid this, cells were maintained for <25 passages and control cells induced to differentiate in MDI were assessed for each experiment.

Another difference between the experiments presented in this chapter and the studies completed by Fajas *et al.* (269) and Wiper-Bergeron *et al.* (270) is that these groups used a single VPA treatment at the initiation of differentiation. In the results presented in this chapter, HDAC inhibition with VPA or TSA attenuated adipogenesis when added once at the initiation of differentiation or chronically throughout the differentiation period. Thus, timing of VPA treatment does not appear to be responsible for the differences in the results obtained. In models of muscle cell differentiation (C2C12 skeletal muscle cells and human skeletal myoblasts) augmentation or suppression of myogenesis is dependent on the time of HDAC inhibition (285). When HDAC inhibitors (including VPA) were added one day prior to the differentiation-inducing medium they enhanced myogenesis, however, when HDAC inhibitors were added simultaneously to the differentiation medium myogenesis was inhibited (285). Thus HDAC activity may provide an important checkpoint to prevent precocious myogenic differentiation. In the results presented in this chapter treatment of 3T3-L1 cells with VPA or TSA one day prior to the initiation of differentiation produces no abrogation in adipogenesis, suggesting HDAC inhibitors have differential effects in adipogenesis and myogenesis.

HDAC inhibitors were suggested previously to promote the differentiation of adipocytes by enhancing the transactivation of PPARγ and C/EBPα (269, 270). Fajas *et al.* (269) suggested that treatment with HDAC inhibitors (including VPA) results in dissociation of a PPARγ/Retinoblastoma/HDAC3 complex, allowing PPARγ

transactivation and stimulation of adipocyte differentiation. Similarly, Wiper-Bergeron *et al.* (269, 270) suggested that treatment with HDAC inhibitors stimulated adipocyte differentiation by promoting the transcription of C/EBPα by releasing a co-repressor complex comprised of C/EBPβ/mSIN3A/HDAC1 from the C/EBPα promoter. In agreement with these studies, this study found that the mRNA for PPARγ and C/EBPα are made in the presence of VPA or TSA, however these transcripts were expressed at a reduced level. Previous studies had not directly assessed the effect of inhibition of HDACs on the protein level for PPARγ and C/EBPα during differentiation (269, 270). The results from this chapter demonstrated that treatment with inhibitors of HDAC caused a reduction in the protein levels for both PPARγ and C/EBPα, which correlates with the limited amount of adipocyte differentiation observed with treatment. Thus, I conclude that HDAC inhibition has the outcome of suppressing PPARγ and C/EBPα protein levels resulting in attenuation of adipogenesis.

In addition to the effect of VPA on PPARγ and C/EBPα, this study demonstrated that HDAC inhibition was associated with a significant reduction in mRNA and protein levels for SREBP1a. It has been recently determined that SREBP1c interacts with another bHLH protein called Twist2 to attenuate SREBP1c transcription, and that this repression can be relieved by treatment with HDAC inhibitors (286). These results suggest that SREBP1c transcription may be dependent on the activity of the SREBP1c/Twist2/HDAC complex (286), however, the role that a similiar interaction may have on adipogenesis and whether this interaction occurs in adipocytes is unknown. Based on the finding of SREBP1c forming complexes with HDACs, HDAC inhibition should promote the expression of SREBP and thus enhance adipogenesis, opposite to the

results presented in this chapter. Thus, the molecular mechanism underlying the ability of HDAC inhibitors to regulate C/EBPα, PPARγ, SREBP1 expression and activity has just begun to be identified. It is likely that regulation of these transcripts is dependent on positive and/or negative factors including corepressor complexes containing the HDACs. Inhibition of HDAC activity may result in blocking transcriptional activity of genes critical for adipocyte differentiation through its actions on these multiprotein complexes in combination with its possible effects on core histone proteins.

Which of the numerous HDACs may be important for adipogenesis? TSA, like most HDAC inhibitors, equally inhibits all known HDACs in a reversible fashion by displacing the requisite zinc ion within the active site (278). In the first report describing VPA effects on HDACs, VPA was reported *in vitro* to inhibit class I HDACs (HDAC 2 $IC_{50} = 0.5$ mM) with five times greater potency than class II HDACs (HDAC 5 $IC_{50} = 2.8$ mM) (40). In this chapter VPA inhibited adipogenesis at doses of 0.5–5 mM; thus at the lower dose of 0.5 mM, VPA would inhibit class I HDACs yet may not significantly affect class II HDACs, leading to the speculation that class I HDACs may be critical for adipogenesis. However, more recent work by Gurvich *et al.* (42) has reported inhibition of class I and class II HDACs *in vitro* by VPA is similar, with the exception that VPA does not inhibit class II, subclass II HDACs including HDAC6 and HDAC10. More specifically VPA inhibited class I HDACs (HDACs 1-3) with IC_{50} values ranging between 0.7-1 mM and class II HDACs (subclass I, HDAC 4,5,7) with IC_{50} values ranging from 1-1.5 mM (42). Although the IC_{50} values vary between these two reports, together these findings support that VPA may have slightly more potent effects on class I HDACs. It has also been recently reported the VPA can also effect HDAC activity by

inducing the proteosome-dependent degradation of HDAC2, a member of class I HDACs (287). However, it is unlikely that the degradation of HDAC2 is responsible for the similar effects of VPA and TSA to inhibit adipogenesis, since TSA does not appear to induce degradation of HDAC2 (287).

It is unknown which HDACs may be present and active during adipogenesis. Wiper-Bergeron *et al.* (270) demonstrated that during the initial 24 hours of 3T3-L1 cell differentiation in the presence of DEX, HDAC1 protein levels are reduced by 50%, without affecting HDAC1 mRNA, when compared to cells differentiated in the absence of DEX. In this chapter HDAC1 protein levels in 3T3-L1 cells were demonstrated to remain unchanged during differentiation in the presence of DEX and absence or presence of HDAC inhibitors. Although HDACs may remain at a steady state level during adipogenesis, this does not rule out the possibility that they may be dynamically regulated by their subcellular localization. In muscle cell differentiation, the shuttling of HDAC 4, 5, and 7 between the cytoplasm and nucleus plays a critical role in myogenesis (275). In order to fully explore the role of HDACs in adipogenesis it will be necessary to identify which HDACs are present throughout adipogenesis and determine if their localization and activity are dynamically regulated.

The Effect of VPA on Transcription Factors Involved in Adipogensis

Three main classes of transcription factors directly influence fat cell development, PPARγ, C/EBP, and SREBP-1 (228, 245, 259). The expression of PPARγ, and most importantly $PPAR\gamma_2$, has been identified to be sufficient to induce adipogenesis (240). Thiazolidinediones are synthetic PPARγ agonists that can induce differentiation of adipocytes, whereas PPARγ antagonists or partial agonists reduce adipogenesis induced

by treatment with either MDI or treatment with thiazolidinediones (288, 289). Chronic treatment of 3T3-L1 preadipocytes with VPA was unable to induce adipogenesis and VPA blocked adipogenesis induced by either TGZ or ROS, suggesting that VPA does not act as a PPARγ agonist. To test whether VPA acted as a PPARγ partial agonist or antagonist reporter assays were conducted using a PPARγ LBD-Gal4 DBD chimeric receptor (PPAR-Gal4) as the activator of transcription. Previously, Lampen *et al.* (290) reported that VPA (0.5-1.5 mM) induced activation of a glucocorticoid receptor DBD-PPARγ LBD hybrid receptor in Chinese hamster ovary (CHO) cells. Similarly, using NIH-3T3 cells expressing endogenous PPARγ, Fajas *et al.* (269, 270) demonstrated that VPA (0.5 – 1.5 mM) activated a reporter gene (PPRE-TK-luc) driven by PPARγ binding elements linked to a minimal thymidine kinase (TK) promoter. In this chapter VPA (1 mM) enhanced TGZ-induced reporter activation at all doses of TGZ tested (1-20 μM), however, the increase in luciferase activity by PPAR-Gal4 in the presence of VPA is equal to the fold increase in activation of the control protein (GAL4 DBD alone, BD-Gal4), supporting the notion that VPA does not specifically activate the PPARγ receptor. VPA has been shown to induce a diverse number of promoters including the simian virus-40 (291), cytomegalovirus (CMV) (41, 292), and Rous sarcoma virus (41) promoter. Furthermore, VPA-mediated activation of the CMV *Renilla* reporter *in vivo* can be reversed with overexpression of HDAC1 (41). These data suggest that VPA has a non-specific ability to indirectly induce gene transcription, likely due to its HDAC inhibitory properties (40, 41).

The attenuation of adipogenesis by VPA does not appear to be mediated by VPA binding directly to PPARγ or preventing PPARγ expression, since treatment with the

HDAC inhibitors can inhibit differentiation following the removal of MDI after PPARγ expression has been upregulated. However, the inhibition of differentiation by HDAC inhibitors, including VPA, was associated with a reduction in PPARγ mRNA and protein levels. It is possible that HDAC inhibitors could cause a direct or indirect reduction in PPARγ mRNA transcription or stability. It is also possible that the down regulation of PPARγ mRNA by VPA treatment results from VPA-induced down-regulation of C/EBPα protein. Several studies have demonstrated that C/EBPα regulates the expression of PPARγ, most notably, *in vitro* C/EBPα is able to directly bind to the $PPAR\gamma_2$ promoter (293) and *in vivo,* mice with disrupted C/EBPα expression shown a reduction in PPARγ levels (232).

VPA and TSA treatment caused a reduction in SREBP mRNA and mature protein levels during adipogenesis, similar to its effects on PPARγ. There is conflicting evidence from different laboratories about whether 3T3-L1 cells predominately express either the SREBP-1a or the SREBP-1c transcript (249, 253-256). Using a monoclonal antibody against SREBP1a, the 3T3-L1 cells in this study are demonstrated to have a significant increase in protein levels for the mature 68-kD form of SREBP1a during differentiation. Surprisingly the precursor form of SREBP1a protein appears to remain at a steady state following treatment with HDAC inhibitors. In liver cells, once the SREBP cleavage-activating protein senses low sterol levels the precursor SREBP translocates from the ER membrane to the Golgi where the mature SREBP is formed by a two step proteolysis via the Site-1 protease and Site-2 protease (250). In adipocytes it is unknown whether this process also controls the maturation of SREBP from its precursor. Inoue *et al.* (255) found that the mRNA levels for SREBP cleavage-activating protein (SCAP), Site-1

protease, and Site-2 protease in adipocytes remain at a steady state throughout adipogenesis, suggesting that in adipocytes, where sterol levels are not depleted, the mechanism producing the proteolytic activation of SREBP may be unique. Similar to our findings with VPA, other have demonstrated that HIV protease inhibitors, which also are known inhibitors of adipogenesis, reduced SREBP1a maturation despite steady state precursor levels (294). Future studies are necessary in order to elucidate the mechanism of SREBP1a processing during adipogenesis and how HDAC inhibitors may affect this process.

VPA treatment induced a striking reduction in C/EBPα protein levels although it did not affect C/EBPα mRNA levels during differentiation. Since this effect is observed with VPA but not TSA, it is unlikely to be mediated by HDAC inhibition. These results also suggest that VPA may have multiple mechanisms of action to abrogate adipogenesis. It remains to be determined how VPA reduces C/EBPα protein levels. C/EBPα mRNA half-life is approximately 5 h and the C/EBPα protein half-life is approximately 1 h (295, 296). In some tissues/cells C/EBPα protein levels are disparate with C/EBPα mRNA levels, suggesting that C/EBPα protein levels may also be regulated at the translation and/or post-translational levels (295, 296). The transcriptional activity of C/EBPα and levels of C/EBPα protein can be modulated through phosphorylation, ubquitination, or sumoylation (296-298). In order to determine the mechanism by which VPA affects C/EBPα protein levels, future work will need to determine whether VPA affects the rate of protein synthesis or degradation. In keratinocytes, treatment with lithium increases C/EBPα protein levels without altering C/EBPα mRNA levels through inhibiting the degradation of C/EBPα by the ubiquitin-dependent proteasomal pathway (296). VPA

induces polyubiquitination and proteasome-dependent degradation of other proteins, such as HDAC2 (287), and thus it is possible that VPA may alter C/EBPα stability.

When preadipocytes were differentiated in the presence of HDAC inhibitors, including VPA, an attenuation, and not complete block of adipogenesis was observed. A reduction in differentiation also occurred when HDAC inhibition was begun after the initial critical steps of differentiation had occurred including clonal expansion and expression of PPARγ and C/EBPα. It is difficult to determine whether the reduction in adipogenesis is due to a decreased level of differentiation occurring in all cells or results from the inability of a proportion of the cells to continue along the path of differentiation. Differentiation of preadipocytes in the presence of HDAC inhibitors resulted in a minority of the cells having an adipocyte morphology characterized by numerous lipid droplets, whereas the majority of the cells appeared similar to confluent preadipocytes (Figure 3.17). These results suggest that a proportion of the cells are susceptible to the effects of HDAC inhibitors and fail to differentiate. However if this was true, then a general reduction in PPARγ, C/EBPα and SREBP1a mRNA levels should have occurred for that proportion of the cells, however levels of C/EBPα mRNA were not altered with HDAC inhibition. In order to understand more about this phenomenon, future work should examine the differences and similarities between those cells that differentiate and cells that do not differentiate in the presence of HDAC inhibition.

VPA effects on adipogenesis were examined with the initial aim of understanding how VPA may induce weight gain in patients. It was hypothesized that VPA would enhance adipogenesis, however VPA did the opposite. It is paradoxical that *in vitro* VPA inhibits adipogenesis yet *in vivo* induces weight gain. During weight gain, hyperplasia

occurs in WAT (193), thus there should be a correlation between enhanced adipogenesis and weight gain. Several drugs have been characterized to have similar effects *in vitro* and *in vivo*, such as the thiazolidinediones, which induce adipogenesis *in vitro* and increase body weight *in vivo* (245). However, there is not always a direct correlation between induction of adipogenesis and weight gain. For example, there is an inverse correlation between the percentage of preadipocytes that differentiate *in vitro* and the percent body fat in the Pima Indians of Arizona, which are a population that is genetically predisposed to weight gain (299). Moreover, other medications have been observed to induce weight gain and have either no effect on adipogenesis, or inhibit adipogenesis. Clozapine, an antipsychotic drug, which induces weight gain in patients, has been demonstrated *in vitro* to have no effect on adipogenesis in cultured human stromal cells obtained from mammary adipose tissue (300). In addition, lithium carbonate, a mood stabilizing drug, induces weight gain *in vivo,* yet inhibits adipogenesis *in vitro* most likely through its effects on Wnt/GSK3ß signaling (298, 301). Similarly, growth hormone (GH) *in vitro* suppresses adipogenesis, yet children with GH deficiencies have increased fat mass with a smaller number of larger adipocytes (150). Moreover, treatment with GH in these subjects results in a reduction in body mass and a corresponding reduction of adipocyte size and increase in number of adipocytes (150). Thus, the *in vivo* effects of treatment are not only dependent on the number but the size of adipocytes. In the next chapter of this thesis, the effects of VPA on TAG synthesis and break down within mature adipocytes are measured *in vitro*, in order to assess whether VPA affects the size of adipocytes. Thus, it may be that VPA could be anti-adipogenic, yet enlarge adipose tissue through increasing fat cell volume.

CONCLUSION

The results presented in this chapter show that VPA *in vitro* has inhibitory effects on rodent and human preadipocyte differentiation. The suppression of adipogenesis by VPA is not mediated by VPA suppressing inositol levels, inhibiting clonal expansion or acting as a ligand for PPARγ. VPA is demonstrated to reduce C/EBPα protein levels in the absence of altering C/EBPα mRNA, suggesting that VPA-induced suppression of differentiation could be partially due to decreased C/EBPα protein levels through an unidentified translation or post-translation mechanism. Independent of the reduction in C/EBPα protein, VPA appears to attenuate adipogenesis through inhibiting HDACs. Treatment with HDAC inhibitors reduced adipogenesis and protein levels for transcription factors responsible for adipocyte phenotype, whereas VPM, an analogue of VPA that does not affect HDAC activity, did not produce these effects. Overall these results highlight a role for HDAC activity in adipogenesis that can be blocked by treatment with VPA.

Chapter 4

VALPROIC ACID DOES NOT ALTER LIPID METABOLISM, BUT INHIBITS LEPTIN SECRETION AND REDUCES LEPTIN mRNA LEVELS IN ADIPOCYTES

Portions of this chapter will appear in the following manuscript:

Lagace DC, McLeod RS, Nachtigal MW. (Submitted) Valproic Acid Inhibits Leptin Secretion and Reduces Leptin mRNA Levels in Adipocytes

Abstract

Treatment of epilepsy or bipolar disorder with valproic acid (VPA) induces weight gain and increases serum levels for the hormone leptin through an unidentified mechanism. In this study we tested the effects of VPA, a short-chain branched fatty acid (C8:0), on leptin biology and lipid metabolism in 3T3-L1 adipocytes. Acute VPA treatment (hours) significantly reduced leptin mRNA and protein secretion in a dose- and time-dependent manner. The reduction in leptin mRNA levels was due to VPA effects on leptin transcription and not associated with VPA enhanced leptin degradation. The decrease in leptin secretion by VPA was not accompanied by alterations in glucose uptake, a known regulator of leptin secretion. Since fatty acid accumulation has been hypothesized to block leptin secretion, we tested the effect of VPA on fatty acid metabolism. Using ^{14}C-radiolabeled VPA, we found VPA catabolites were mainly incorporated into triacylglycerol (TAG). However, VPA did not alter lipogenesis from acetate, nor did it change the amount of intracellular free fatty acids available for TAG synthesis. In addition, levels of transcription factors that are important for leptin expression, including C/EBPα, PPARγ, or SREBP1a were not altered by VPA treatment. VPA altered levels of leptin mRNA independent of *de novo* protein synthesis, suggesting that VPA treatment decreases leptin mRNA levels through modulation of existing adipocyte signaling pathways. This report demonstrates that VPA decreases leptin secretion and mRNA levels in adipocytes *in vitro*, suggesting that VPA therapy may be associated with altered leptin homeostasis contributing to weight gain *in vivo*.

Key Words: Leptin/Adipocyte/Valproic Acid/Lipid Metabolism

INTRODUCTION

As described in Chapter 3, treatment with VPA induces weight gain in patients. The etiology of VPA-induced weight gain is unknown but most likely is multi-factorial and includes VPA affecting peripheral tissues and modulating the production and/or secretion of a variety of factors involved in energy balance. For example, VPA can directly increase insulin release *ex vivo* in human pancreatic islet cells (121). Within WAT, adipocytes contribute to energy balance by changing size and increasing in number in accordance with need by responding to central and peripheral metabolic signals. In addition to storing TAG and secreting free fatty acids, adipocytes can modify circulating steroid hormones leading to the production of cortisol and estrone, as well as synthesize and release a number of bioactive peptides and proteins called adipokines (189). Adipokines include cytokines (interleukin 6 (IL-6) and TNFα), peptides involved in vascular homeostasis (angiotensinogen, plasminogen activator inhibitor 1 (PAI 1)), and a variety of other proteins including leptin, acylation-stimulating protein (ASP), resistin, adiponectin, and complement factor D, which is also known as adipsin (reviewed in 192, 302, 303). These factors can have endocrine, paracrine and/or autocrine effects and are involved in regulating energy, vascular and immune homeostasis. Of these adipokines, leptin is the most well characterized and has been shown to be dynamically regulated by a variety of factors.

Increased serum leptin levels have been reported following VPA treatment (126), which may be a consequence of either 1) VPA increasing the amount of adipose tissue producing leptin, 2) VPA having an effect on leptin secretion from adipocytes, or 3) VPA altering leptin signaling in target tissues. Since VPA is a fatty acid and VPA therapy

modulates weight and leptin levels in humans, this chapter explores whether VPA alters lipid metabolism and/or the production and secretion of leptin from adipocytes *in vitro*. This introduction will explain the basis of TAG and fatty acid metabolism and summarize the mechanism for leptin synthesis and secretion in adipocytes.

Lipid Metabolism in Adipocytes

WAT was previously viewed as an isolated tissue made up of adipocytes that stored energy in the form of TAG and released energy in the form of free fatty acids and glycerol. WAT is now recognized to be a dynamic tissue with altered adipocyte function translating into changes in systemic energy balance. Despite this alteration in perspective on WAT, the most dramatic change that occurs in adipocytes is cell hypertrophy through the accumulation of TAG. Fatty acids are stored in TAG and the main metabolic function of WAT occurs through lipid accumulation via TAG synthesis and lipid mobilization via TAG breakdown, occurring through lipolysis. In addition to TAG, the adipocyte is able to both synthesize fatty acids through lipogenesis and break down fatty acids through β-oxidation. The synthesis and degradation of TAG and fatty acids is controlled by the energy requirements of the body and can be modulated through processes such as substrate availability and rate of enzyme synthesis. Since VPA is an 8-carbon branched chain fatty acid, experiments described in this chapter examined if VPA was metabolized into lipids, affected the synthesis of lipids, or modulated the levels of intracellular free fatty acids. In order to understand these experiments, the basic processes that occur during the synthesis and breakdown of TAG and fatty acids in adipocytes are summarized in Figure 4.1 and explained in detail based on information from reviews and textbooks (304-306).

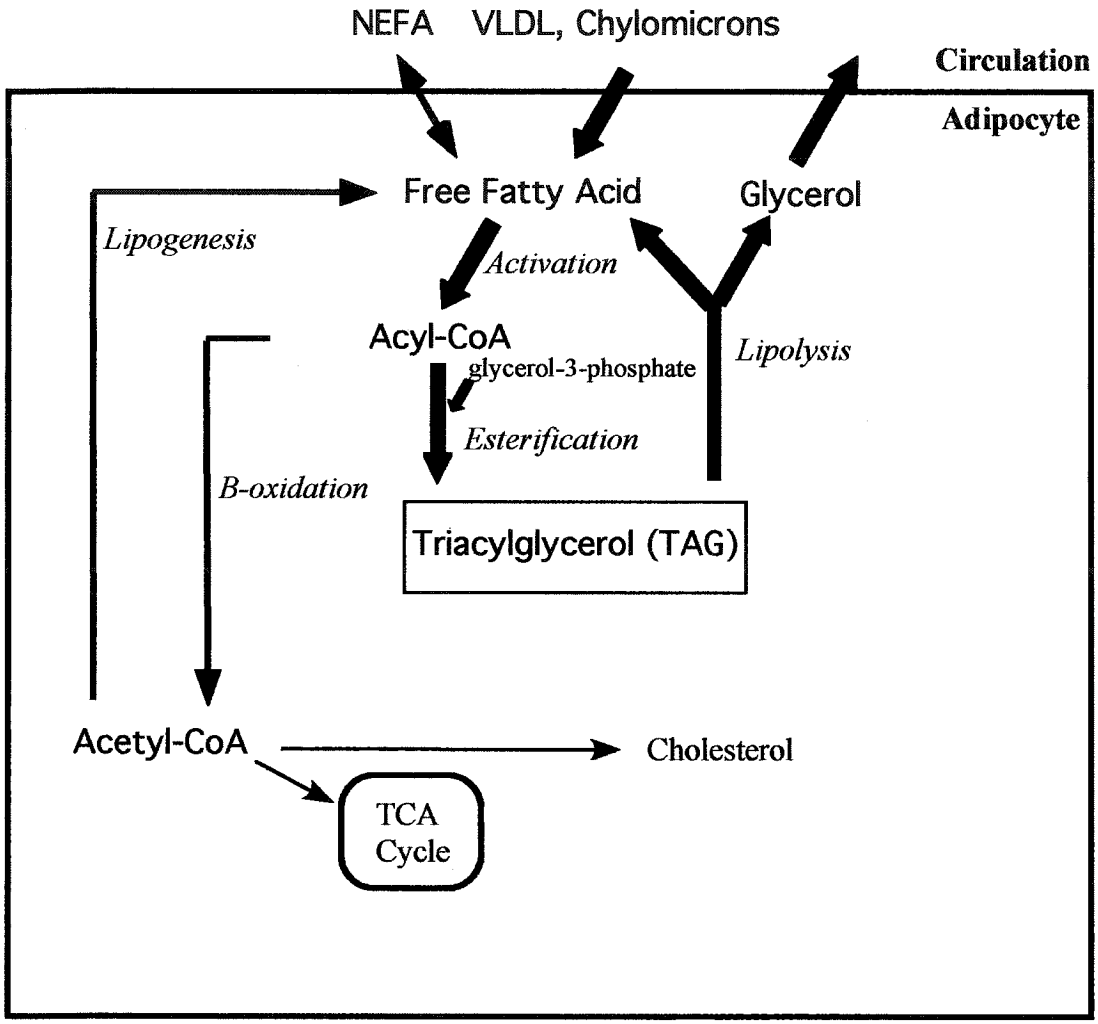


Figure 4.1 Simplified scheme for triacylglycerol (TAG) and fatty acid metabolism in adipocytes

Lipids are mainly presented to the adipocyte in the form of chylomicrons, very low density lipoproteins (VLDL), and non-esterified free fatty acids (NEFA). VLDL and chylomicrons are hydrolyzed into fatty acids by lipoprotein lipase. Fatty acids are activated into acyl-CoA and are incorporated into TAG through esterification. TAG is broken down during lipolysis by the rate-limiting enzyme hormone-sensitive lipase, resulting in the formation of fatty acids and glycerol. *De novo* fatty acid synthesis occurs during lipogenesis by the rate-limiting enzyme fatty acid synthase which uses acetyl-CoA as a substrate to form fatty acids. Fatty acids are broken down through the sequential removal of 2-carbon units by β-oxidation. Acetyl-CoA can be utilized for cholesterol synthesis or can enter the tricarboxylic acid (TCA) cycle for further oxidation and ultimate production of energy in the form of adenosine triphosphate (ATP).

Within the circulation, lipids are presented to the adipocyte mainly in the form of chylomicrons, lipoproteins, and non-esterified free fatty acids (NEFA). The NEFA are fatty acids that have been ionized in the plasma and bound to plasma proteins. Chylomicrons are formed from dietary fatty acids and are lipoprotein complexes made within the intestine. Lipoproteins are formed from lipids that have been synthesized in the liver and include very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The VLDL and chylomicrons are hydrolyzed into free fatty acids in the capillaries of adipose tissue through the action of lipoprotein lipase. The free fatty acids that are utilized to form TAG in adipocytes mainly include those fatty acids that have a chain length of 12-18 carbons and have 1-3 double bonds (306).

In order for the fatty acids to be incorporated into TAG they need to be activated into acyl-CoA. Unlike other tissues, adipocytes lack glycerol kinase and therefore do not use glycerol, but use glycerol-3-phosphate, which is produced from dihydroxyacetone phosphate (DHAP), to make TAG. This results in adipocytes requiring the oxidation of glucose during glycolysis in order to make DHAP and store fatty acids in the form of TAG. TAG synthesis occurs through the esterification of acyl-CoA by acyltransferase enzymes. The newly formed TAG is stored in lipid droplets, which are covered by a phospholipid monolayer and a protein coat containing members of the PAT (perilipin, adipose differentiation-related protein, and TIP47) family (307, 308).

In situations of negative energy balance, such as fasting, fat mobilization and breakdown of TAG occurs through lipolysis. In lipolysis, TAG is hydrolyzed into free fatty acids and glycerol under the control of the rate-limiting enzyme, hormone-sensitive

lipase (HSL). There is a complex series of interrelated cascades that activate HSL, which is largely controlled by the levels of intracellular cAMP. HSL can be activated by a variety of stimuli, such as glucagon or epinephrine, which activate adenylate cyclase, leading to an increase in cAMP, activation of cAMP-dependent protein kinase A (PKA), and phosphorylation of HSL. In contrast, insulin inhibits the activities of HSL by inhibiting the synthesis of cAMP. Within the adipocyte, therefore TAG is formed generally through esterification of fatty acids and hydrolyzed in the process of lipolysis.

De novo fatty acids are synthesized through the process of lipogenesis and broken down and oxidized through the process of β-oxidation. Fatty acid synthesis occurs in the cytoplasm and uses acetyl-CoA as the substrate. Fatty acid synthase (FAS) is a multi-enzyme complex that is responsible for the synthesis of fatty acids from acetyl-CoA and generates the fatty acid palmitate (16:0). Other fatty acids can then be generated from palmitate through elongation and/or desaturation. In contrast to fatty acid synthesis, fatty acids are broken down by β-oxidation, which can occur in either the mitochondria or in peroxisomes. In mammals, the mitochondria oxidizes short, medium, and most long chain fatty acids, while the peroxisomes oxidize some long chains, but mostly oxidize very long chain fatty acids.

Before being oxidized, fatty acids are activated in the cytoplasm by acyl-CoA synthetase (ACS). The transport of long chain fatty acids from the cytoplasm to the inner mitochondria requires an acyl-carnitine intermediate, which is generated through the actions of carnitine acyltransferase. Once inside the mitochondria or peroxisomes, fatty acids are broken down through the sequential removal of 2-carbon units by oxidation at the β-carbon position of the fatty acyl-CoA molecule, hence the name β-oxidation. The

majority of natural lipids contain an even number of carbon atoms and are completely broken down by β-oxidation resulting in the formation of NADH, reduced flavin adenine dinucleotide ($FADH_2$), and acetyl-CoA. In contrast, β-oxidation of odd carbon length fatty acids, leads to the formation of acetyl-CoA and a 3-carbon (propionyl-CoA) residue, which undergoes subsequent conversion into succinyl-CoA. Acetyl-CoA or succinyl-CoA can enter the TCA cycle for further oxidation and ultimate production of energy in the form of adenosine triphosphate (ATP). Acetyl-CoA also provides all the carbon atoms for the formation of cholesterol, which is used in the formation of membranes and synthesis of steroids and bile acids.

During the metabolism of VPA, as described in Chapter 1, VPA can be activated into VPA-CoA, through conjugation with coenzyme A by ACS (22, 23). Moreover VPA has been identified to undergo ß-oxidation in the mitochondria (22, 23). In this chapter, the fate of VPA metabolism in adipocytes is determined and the effect of VPA treatment on lipogenesis and levels of intracellular free fatty acids are measured.

Leptin

In 1953, Kennedy *et al.* (309) first hypothesized that peripheral factors signaled to the hypothalamus and provided information about the fat stores in the body. Subsequent parabiosis experiments demonstrated that it was a blood-borne factor which produced a signal for satiety, since when one rat was made obese through lesioning the ventromedial hypothalamus, the parabiotic intact partner became orexic and lean (310). This blood-born factor was recognized to be the product of the obese (ob) gene by the research group of Coleman *et al.* in the 1970s, who discovered that recessive mutations in the mouse ob gene resulted in obesity, which could be reversed in parabiosis experiments (311).

Twenty years later Jeffrey Friedman's research group characterized the ob gene and it's16 kDa protein product, leptin, which was named based on the Greek word for thin, "leptos" (312). Exogenous leptin administration restored normal body mass and decreased food-intake in the leptin-deficient ob/ob mouse, a finding that identified leptin as a satiety factor (313).

These discoveries stimulated excitement about whether human obesity could be cured by treatment with leptin. However, leptin therapy is beneficial in only a rare number of individuals that are morbidly obese and have deficiencies in leptin due to mutations in the leptin gene (314, 315). Paradoxically, serum leptin levels are increased in humans that are obese, are positively correlated with adipocyte tissue mass, and decrease following weight loss (316-318). Therefore the hypothesis was proposed that leptin resistance occurs in human obesity, comparable to insulin resistance in type 2 diabetes (317). The mechanism for leptin resistance remains undefined, yet current hypotheses suggest that leptin resistance is due to dysregulation of leptin synthesis and/or secretion, abnormalities in brain leptin transport, or abnormalities in leptin receptor and/or post-receptor signaling (reviewed in 189, 319). This chapter describes the effect of VPA on the production and secretion of leptin in adipocytes; thus this review focuses on mechanisms that underlie leptin production and secretion, and will only briefly summarize some of leptin's main effects within the CNS.

The primary site of action for leptin is within the hypothalamus where it binds to leptin receptors, which are members of the class I cytokine receptor family. Multiple splice variants of the leptin receptor mRNA encode at least 6 different receptor isoforms; however the full-length leptin receptor (long isoform, Ob-R) is primarily expressed in the

hypothalamus (319, 320). Ob-R is also the only leptin receptor isoform that contains the intracellular motifs that are involved in leptin signaling by activation of the Janus kinase (Jak) signal transducers and activators of transcription (Stat) pathway (321, 322). In addition to signaling through the Jak-Stat pathway, it has become evident that leptin can signal through the Ob-R via the PI3K-phosphodiesterase 3B (PDE3B)-cAMP pathway (reviewed in 323). Leptin binding to Ob-R can induce the phosphorylation of Jak and subsequent activation of the PI3K- PDE3B-cAMP pathway, which can be inhibited by protein tyrosine phosphatase IB (PTP1B) via dephosphorylation of Jak. Other potential pathways, such as the MAPK-ERK pathway, have also been proposed to be involved in leptin intracellular signal transduction, however the interactions between these pathways and role in regulation of leptin signal is a current area of investigation (reviewed in 323).

The effect of leptin within the hypothalamus has been the subject of many recent reviews (189, 323-325). Briefly, leptin binds to Ob-R on both orexigenic (feeding inducing) and anorectic (appetite suppressing) peptide-producing neurons. Leptin decreases the gene expression, peptide release and/or actions of the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) co-expressing neurons in the arcuate nucleus. Moreover, leptin suppresses the melanin-concentrating hormone (MCH), galanin, orexin, and galanin-like expressing neurons in the lateral hypothalamus. Opposite to the effects of leptin on orexigenic signaling, leptin induces the actions of the anorectic, proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) expressing neurons in the arcuate nucleus. Activation of POMC neurons results in an increase in production of α-melanocyte stimulation hormone (α-MSH), which targets the melanocortin 4 receptor (MC4R) expressing neurons. MC4R

can be activated by α-MSH and inhibited by AgRP producing physiological alterations that favor catabolic changes in energy intake and expenditure. Overall, leptin actions within the hypothalamus result in a suppression of orexigenic and induction of anorectic signals.

In addition to the action of leptin within the hypothalamus, more recent studies have identified that leptin can have direct actions on a variety of peripheral tissues. For example, leptin can directly stimulate fatty acid oxidation in skeletal muscle through activation of the AMP-dependent protein kinase (AMPK) (326). Moreover, leptin binds to Ob-R within pancreatic β-cells and suppresses insulin production (reviewed in 327). Overall, the combination of the hypothalamus and peripheral tissue effects of leptin mediate the role of leptin, not only in energy balance, but also in reproduction, bone formation, and angiogenesis (reviewed in 328, 329).

Leptin is produced primarily from adipose tissue, however subsequent work has shown that a small amount of leptin production occurs in other tissues, including the placenta (330), stomach (331), pituitary and hypothalamus (332). The mouse ob gene has 3 exons and 2 introns, with exon 2 and 3 encoding the 4.5 kB mRNA transcript. During the differentiation of preadipocytes into adipocytes, leptin expression is induced through the demethylation of stretches of CpG sites in the proximal part of the leptin promoter (333, 334). In addition, many known DNA elements within the leptin gene promoter regulate leptin expression in adipocytes including 1) a C/EBPα binding site, which binds C/EBPα/β/δ, (335-337); 2) a Sp1 motif, which is important in *trans*-activating leptin in the absence of C/EBPα binding (335); 3) a TATA-like element, which requires the presence of additional transcription factors in order to activate transcription (338); 4) a

PPARγ binding site (DR+1), which binds PPARγ/RXR heterodimers (339); and 5) a hypoxia response element (HRE), which is responsible for the increase in leptin gene expression in response to hypoxia (340). The leptin promoter also contains multiple recognition sites for other transcription factors including the enhancer factor Ap-1, 3 consensus serum response factor (SRF) binding sites, and a cyclic AMP response element (CRE) (341). Cell-type specific differences in the regulation of leptin gene expression that have been observed in preadipocytes compared to glioma or pituitary cells are most likely due to the use of a variety of these uncharacterized transcription factors (341). Within the human leptin promoter there is also a human polymorphism (-2548G/A) that has been suggested to regulate leptin transcription, with individuals that are homozygous for the AA phenotype having significantly higher serum leptin levels and increased amounts of adipose leptin mRNA (342). Thus, the transcription of leptin gene expression is regulated by methylation, activation by different transcription factors, and may also be effected by DNA polymorphisms.

Like many secreted proteins from adipocytes, leptin can undergo both constitutive and regulated secretion (reviewed in 343). In adipocytes, stimulation of leptin secretion by glucocorticoids or MCH is accompanied by increases in leptin mRNA, suggesting that signals from these molecules can lead to modulation of leptin transcription or degradation (344). Leptin has also been identified to localize within the ER (345), and the secretion of leptin can be inhibited by treatment with brefeldin A or placement of adipocytes at 20^{o}C, which is known to prevent the exit of secretory proteins from the Trans-Golgi network (344, 346, 347). It has been hypothesized that treatment with some agents, such as insulin, can induce leptin secretion from the Trans-Golgi

network, independent of effects on leptin transcription (344). The mechanism for the translational regulation of leptin secretion depends on PI3K and requires a intact mammalian target of rapamycin (mTOR)-mediated signaling pathway (344, 348). These data support the notion that leptin secretion is regulated through transcriptional or translational mechanisms, with secretion occurring through the classical constitutive secretory pathway. However, in sucrose density sedimentation experiments, intracellular leptin secretory vesicles have been identified in adipocytes that do not co-localize with the classical peptide-containing granules of the Trans-Golgi network (349). These findings agree with the hypothesis that insulin and serum can induce regulated secretion of leptin, as well as constitutive secretion (344). When compared to secretion through the constitutive secretory pathway, a relatively smaller amount of transport occurs in these small leptin containing vesicles (344). The identity and components of the leptin-containing vesicles are unknown, yet they do not associate with the GLUT4 glucose transporter, which undergoes translocation in adipocytes in response to insulin (344, 345). Thus, leptin is mainly secreted in a constitutive manner with a small amount undergoing regulated exocytosis.

Leptin expression and secretion is influenced by the status of energy stores; however leptin is neither a satiation nor a meal-satiety factor in humans, because acute changes in food intake do not induce acute changes in plasma leptin levels (reviewed in 324). In rodents and humans, leptin secretion exhibits a diurnal rhythm, with the zenith occuring during the dark cycle and the nadir during the light cycle (350, 351). The diurnal rhythm is associated with meal timing since a 6.5-h delay in meal timing, without alterations in sleep or light cycles, produces a phase shift in leptin levels (351).

Moreover, leptin levels will decrease within hours after initiation of fasting in both humans and rodents (352). The complexity of the regulation of leptin rhythm is under investigation and appears to be under the control of the biological clock in the suprachiasmatic nucleus (SCN) of the hypothalamus (353).

Leptin production in adipocytes is correlated with adipocyte size and this may be one mechanism by which leptin acts as a sensor for energy stores. Leptin mRNA expression was first correlated with cell size in 1995, when elevated leptin mRNA levels were found in *ex vivo* omental adipocytes isolated from morbidly obese humans (317). In mice, adipocyte volume correlates with tissue leptin mRNA levels (354) and human serum leptin levels correlate with abdominal adipose cell weight (355). When examining whether fat cell weight or total number of fat cells contributes to fasting plasma leptin concentrations in humans, both increased cell weight and number were correlated with body weight and fasting leptinemia (355). Moreover, cell weight was reported to be a more significant predictor of increased serum leptin levels when compared to cell number. These findings support the idea that hypertrophy (which is often assessed by increased cell weight) is associated with increased plasma leptin concentrations.

The signal-transduction systems that adipocytes use to alter leptin production based on their size or lipid content are unknown. In adipocytes a variety of factors can increase leptin expression such as insulin, glucocorticoids, glucose and some cytokines, whereas others factors such as androgens, β-adrenergic receptor agonists, cAMP agonists, and catecholamines can decrease leptin expression (reviewed in 319, 343). In association with adipocyte hypertrophy there is increased macrophage infiltrations and cytokine availability (356), as well as alterations in the integrin/ERK signaling pathway

(357), both of which could play a role in modulating leptin secretion in correlation with adipocyte size. In this chapter we explore whether VPA can modulate adipocyte size by altering lipid metabolism, and/or modulate the secretion of leptin.

MATERIALS AND METHODS

Cell culture and differentiation

Mouse 3T3-L1 cells were obtained from the ATCC and cultured as described in Chapter 3. Briefly, two days after reaching confluence preadipocytes were treated with medium to induce differentiation [MDI: DMEM, 10% FBS, 250 nM DEX, 500 μM IBMX, and 100 nM (0.58 μg/ml) human insulin]. After 2 days in MDI preadipocytes were cultured in DMEM containing 10% FBS, and 5 μg/ml insulin. Subsequent medium changes occurred every other day. Valproic acid sodium salt (VPA, Sigma) was dissolved in PBS and added to medium as indicated in the figure legends.

Leptin assay

Leptin concentrations were determined using a radioimmunoassay for mouse/rat leptin produced by Alpco Diagnostics (cat. # 022-LEP-R61) as per manufacturers instructions. The sensitivity of this assay is 6 pg/ml and the intra-assay variation is lower than 5%.

Glucose Uptake

Glucose uptake was determined similar to the method of Yang *et al.* (358). Briefly, differentiated adipocytes in six-well plates were washed twice with serum-free DMEM and serum-starved for 2 h in DMEM containing 25 mM glucose and 2 mM glutamine. Medium was replaced with 1 ml of Krebs-Ringer-HEPES (KRH) buffer (121 mM NaCl, 4.9 mM potassium chloride (KCL), 1.2 mM magnesium sulfate ($MgS0_4$), 0.33 mM calcium chloride ($CaCl_2$), 12 mM HEPES, pH 7.4) per well. Cells were incubated in the presence or absence of 100 nM insulin, and presence or absence of VPA (1 mM), and labeled for the last 10 min of incubation with 2-deoxy-D-[2,6-^{3}H]glucose (43 Ci/mmol; 1

μCi/ml). Uptake was stopped by three rapid washes with ice cold KRH buffer, cells were solubilized in 1 ml of 0.1 M NaOH and radioactivity was measured in acidified liquid scintillation fluid.

Uptake of [^{14}C] radiolabeled VPA

Cells were treated with 10 μM valproic acid (1-^{14}C) sodium salt ([^{14}C]-VPA; 55 mCi/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) in normal growth medium. After treatment cells were washed three times with PBS and then solubilized in 500 μl of 0.1 M NaOH for 1 h at RT. Total cellular radioactivity was determined for a 100 μl aliquot of each sample in acidified liquid scintillation fluid. Radioactivity counts in counts per minute (cpm) were normalized to total protein per sample. Protein was quantified using the Bradford assay (BioRad Laboratories).

Analysis of lipid metabolism

Cells were treated with 20 μM [^{14}C]-VPA (specific activity = 55 mCi/mmol) plus 980 μM VPA to bring the total concentration to 1 mM. For experiment with triacsin C, cells were simultaneously treated with radiolabeled VPA (20 μM [^{14}C]-VPA + 980 μM VPA) and triacsin C (9.6 μM) for 2 h. For control experiment with triacsin C, cells were simultaneously treated with [^{3}H]-oleate (100 mM, specific activity 4.2 disintegrations per minute (dpm)/pmol) and triacsin C (9.6 μM) for 2 h.

For the triacsin C experiments, cells were rinsed twice with ice cold PBS, scraped into 1 ml PBS with a rubber policeman, and sonicated for 1 min. Lipids were extracted by the addition of 4 ml of isopropanol:3N sulfuric acid (40:1, v/v), 2 ml of distilled water, and 5 ml hexane. After 10 min of end-over-end mixing, the upper hexane phase was collected and the solvent was evaporated under nitrogen. The residual lipid phase was

resuspended in chloroform. Samples were resolved by TLC in hexane:diethyl ether:acetic acid (70:30:1, v/v/v).

For analysis of lipogenesis, cells were pulse labeled with [^{14}C] acetic acid (250 nCi/ml, Amersham) for 2 h at 37^{o}C, and incorporation into newly synthesized lipids was measured. Following the labeling period, cells were rinsed twice with ice cold PBS, scraped into 1 ml methanol:water (5:4, v/v) with a rubber policeman, and sonicated for 1 min. Lipids were extracted using chloroform:methanol (1:2, v/v) and phases separated by the addition of 0.58% NaCl. The lower organic phase was washed and extracted three times with ideal upper phase buffer (methanol: 0.58% NaCl:choloroform, 45:47:3, v/v/v), evaporated and then resuspended in chloroform. Radiolabeled lipids were resolved by TLC in petroleum ether:diethyl ether:acetic acid (60:40:1, v/v/v).

On TLC plates lipids were identified using lipid standards (Sigma, 99F7817) and visualized with iodine vapor, as described in Chapter 3. Radiolabeled lipids identified by co-migration with standards were scraped into vials and quantified by scintillation counting.

Analysis of fatty acids

For analysis of total fatty acids, cells were treated for 12 h with 1 mM VPA, rinsed twice with ice cold PBS, scraped into 1 ml NaOH with a rubber policeman, and sonicated for 1 min. Fatty acids were extracted as previously described (359). Briefly, cells were dissolved in 1 ml of 0.5 N NaOH and saponified in 3 ml of ethanol and 0.5 ml of 50% (w/v) KOH for 1 h at 60^{o}C. The fatty acids were extracted from the hydrolysate twice with 4 ml of hexane after acidification (pH<3) with hydrochloric acid (HCL).

For analysis of free fatty acids, cells were treated with 1 mM VPA for 12 h, rinsed twice with ice cold PBS, scraped into 1 ml PBS with a rubber policeman, and sonicated for 1 min. Lipids were extracted into acidified isopropanol and then hexane as described above [*Analysis of lipid metabolism*]. Free fatty acids were then partitioned from the hexane and separated into the aqueous phase through the addition of 1 ml 0.1 N potassium, hydroxide (KOH) and mixed for 10 min. To remove residual neutral lipids, the KOH phase was re-extracted with 5 ml hexane. The KOH phase was then neutralized with 50 μl of glacial acetic acid and free fatty acids were re-extracted with 6 ml chloroform:methanol (2:1, v/v) and 50 μg of internal standard (methyl heptadecanoic acid C17:0) was added to each sample.

Dr. Roger McLeod and Debbie Currie conducted the subsequent analysis of fatty acids. Total or free fatty acids were passed through a sodium sulfate column to remove water and dried under nitrogen. Fatty acids were esterified by incubation with 1 ml of 20% methanol in benzene and 0.5 ml of 0.2 M (trimethylsilyl) diazomethane (Sigma) in hexane for 30 min with occasional shaking. Acetic acid was added as required to neutralize the solution and 2 ml of distilled water and 2 ml of petroleum ether were added to separate the methyl esters. The organic phase was collected and purified on a sodium sulfate column. Effluent samples were evaporated under nitrogen and then fractionated by gas chromatography (360). Standard mixtures of fatty acids were used to identify peaks that were quantified using the C17:0 as internal standard. In Dr. McLeod's laboratory, Debbie Currie performed the lipid extraction, gas chromatography, and quantification of lipids.

Northern blot analysis

RNA was isolated and Northern blot was completed as described in Chapter 3. For experiments using TSA, cells were treated with 3nM TSA or DMSO [vehicle control (1 μl/ml)]. For experiments with actinomycin D (ActD) and cycloheximide (CHX), cells were pretreated with ActD (1 μg/ml), CHX (10 μg/ml), or DMSO [vehicle control (1 μl/ml)] for 30 min and then co-treated in the presence or absence of VPA for 4 h prior to cell harvest. The mouse leptin cDNA was a kind gift from Dr. G.S. Hotamisligil (Harvard School of Public Health, Boston).

Western blot analysis

Protein extracts and western blot analysis were completed as previously described in Chapter 3.

Leptin Promoter Transient Transfection Assay

Reporter gene assays were conducted using the human throphoblast BeWo cell line (ATCC: CCL-98). Cells were maintained in Nutrient Mixture Ham's F-12 (Gibco) medium supplemented with 10% FBS and 2 mM L-glutamine. Cells were plated in 12-well plates at $2x10^5$ cells/well the day prior to transfection. Cells were transfected with 200 ng of internal reference plasmid pCMV β-galactosidase and 1 μg of either the pGL3-Ob1 reporter plasmid containing 3 kB of the human leptin promoter, or the pGL3-Ob2 reporter plasmid containing 0.2 kB of the proximal human leptin promoter. Both pGL3-Ob1 and pGL3-Ob2 were kind gifts from Dr. J. Auwerx (Deparment d'Atherosclerose, Institute Pasteur, France). Cells were transfected in triplicate in normal growth medium for 6 h with the Lipofectamine 2000 transfection reagent (Invitrogen). The subsequent day, cells were treated for 16 h with either 1 mM VPA, 1 mM dicosahexaenoic acid

(DHA) or the DHA vehicle control, 10% bovine serum albumin (BSA). DHA was obtained as a 10 mM stock conjugated with BSA (final BSA concentration 10% (w/v) in 0.15 M NaCl, pH 7.4 (361)) (Atlantic Research Center, Dalhousie University, Halifax). Cells were harvested and luciferase activity was determined using the Enhanced Luciferase Assay kit (BD Pharmingen). Results were normalized using β-galactosidase activity and represent the mean data from three independent experiments.

Statistical Analysis

Values are expressed as mean ± standard error of the mean. For outcome measurements that were assessed at one time point during the study, a one-way ANOVA was used for multiple group comparisons, and paired t-test for comparisons between 2 groups. For outcome measures that were assessed at two or more time points during the study, a repeated measure ANOVA was used to determine differences that occurred over time and between the treatment groups. For subsequent group comparisons, *post hoc* analysis was completed using the Bonferroni test. All tests were considered significant at $p<0.05$. Data analysis was done using SPSS Base 11.0 for Windows.

RESULTS

VPA Reduces Leptin Secretion

Differentiated mouse 3T3-L1 cells were used as an adipocyte model. Adipocytes were incubated *in vitro* with VPA (0.25 mM – 5 mM) for 6-24 h and leptin secretion into the medium was measured by radioimmunoassay. In untreated cells, leptin levels within the medium increased over time, with values ranging from 229 – 497 pg/ml medium (Figure 4.2). Treatment with VPA significantly decreased leptin secretion and there was a significant effect of time and an interaction between time and treatment on leptin secretion.

Post hoc analysis demonstrated treatment with 0.25 mM VPA had no significant effect on leptin secretion, whereas the more therapeutically relevant dose of 1 mM VPA significantly inhibited leptin secretion after 6, 12, and 24 h of treatment. Treatment with higher doses of VPA was associated with a greater reduction in leptin secretion, with treatment with 1 mM and 5 mM VPA being associated with an average reduction in leptin secretion by 38.3 ± 4.5% and 47.3 ± 2.2%, respectively.

VPA Does Not Alter Intracellular Leptin Levels

The secretion of leptin may be regulated by alterations in leptin synthesis or changes in the amount of leptin secretion (346, 362). VPA did not affect the amount of intracellular leptin (ranged from 33 to 76 pg leptin/50 ug of protein) compared to untreated samples (Figure 4.3). Thus decreased leptin secretion was not due to accumulation of intracellular leptin.

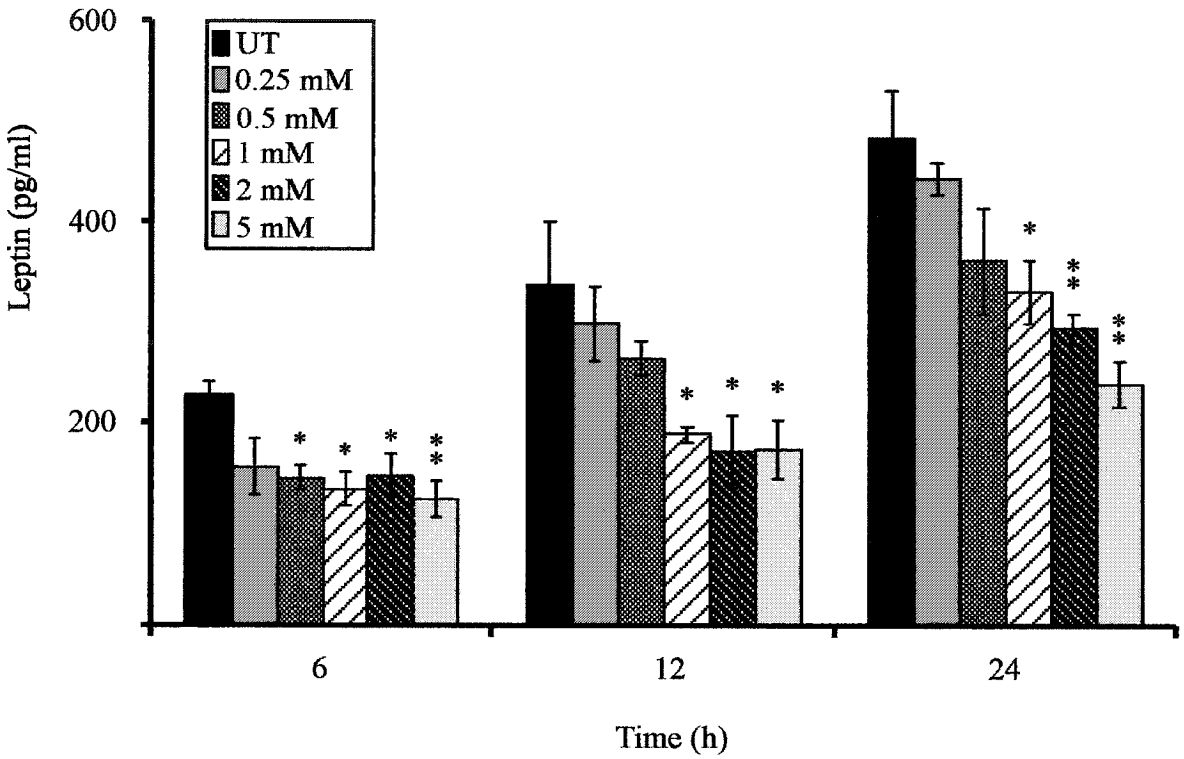


Figure 4.2 VPA decreases leptin secretion
Cells treated with VPA (0.25-5 mM) for 6-24 h have a concentration- and time-dependent decrease in leptin secretion into the medium, as assessed by radioimmunoassay. Data represents mean ± SEM from triplicate samples per time point per group; experiment was repeated with similar results; significantly different from untreated (UT) at each time point as analyzed by post-hoc comparison, * $p<0.05$, ** $p<0.01$.

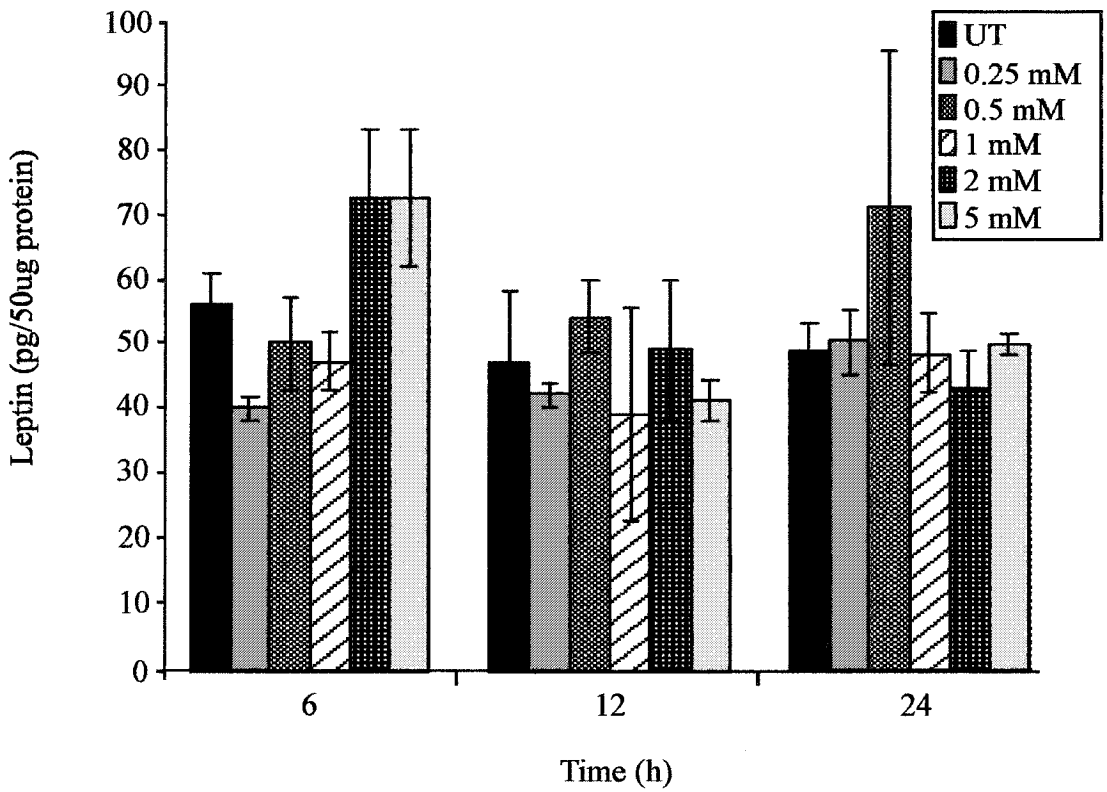


Figure 4.3 VPA does not alter intracellular levels of leptin
Intracellular levels of leptin were quantified from cells treated with VPA (0-5 mM) for 5-24 h. Leptin concentration was determined by radioimmunoassay and normalized against the amount of total protein. Data represents mean ± SEM from triplicate samples per time point per group; experiment was repeated with similar results; no significant differences from untreated (UT) at any time point.

VPA Does Not Alter the Uptake of Glucose

Glucose is one of several circulating factors that can alter leptin secretion from adipocytes. Increased uptake of glucose by adipocytes is associated with decreased leptin secretion (363). We investigated the effect of VPA on basal and insulin-sensitive (358) glucose uptake in differentiated 3T3-L1 cells. There was a significant effect of treatment, time, and an interaction between time and treatment on glucose uptake (Figure 4.4A). Post hoc analysis demonstrated glucose uptake in cells treated with insulin or insulin and VPA was significantly increased compared to untreated (UT) cells at both 30 min and 60 min. Treatment with VPA did not alter the uptake of glucose in either the presence or absence of insulin. Similar results were obtained with cells treated with insulin in the presence of VPA treatment for up to 24 h (Figure 4.4B). These data suggest that the VPA-induced decrease in leptin secretion is not mediated by alterations in glucose uptake.

VPA Incorporation into Adipocytes

VPA has been shown to be actively transported via a carrier mediated system, such as the protein-coupled monocarboxylic acid transporter, in a human placenta trophoblast cell line (14, 364). There are no published studies examining VPA uptake by adipocytes, therefore we used [^{14}C]-labeled VPA to measure uptake into adipocytes. VPA uptake was linear for up to ~30 min following treatment, then reached a plateau at 90 min (Figure 4.5). Levels continued to increase up to 12 h (720 min), most likely due to metabolism of VPA and incorporation of the [^{14}C]-label into intracellular lipids. Of the total amount of [^{14}C]-label added to the medium (1.3×10^6 cpm/ml), on average only 0.2% of the [^{14}C]-label was intracellular after 12 h of treatment.

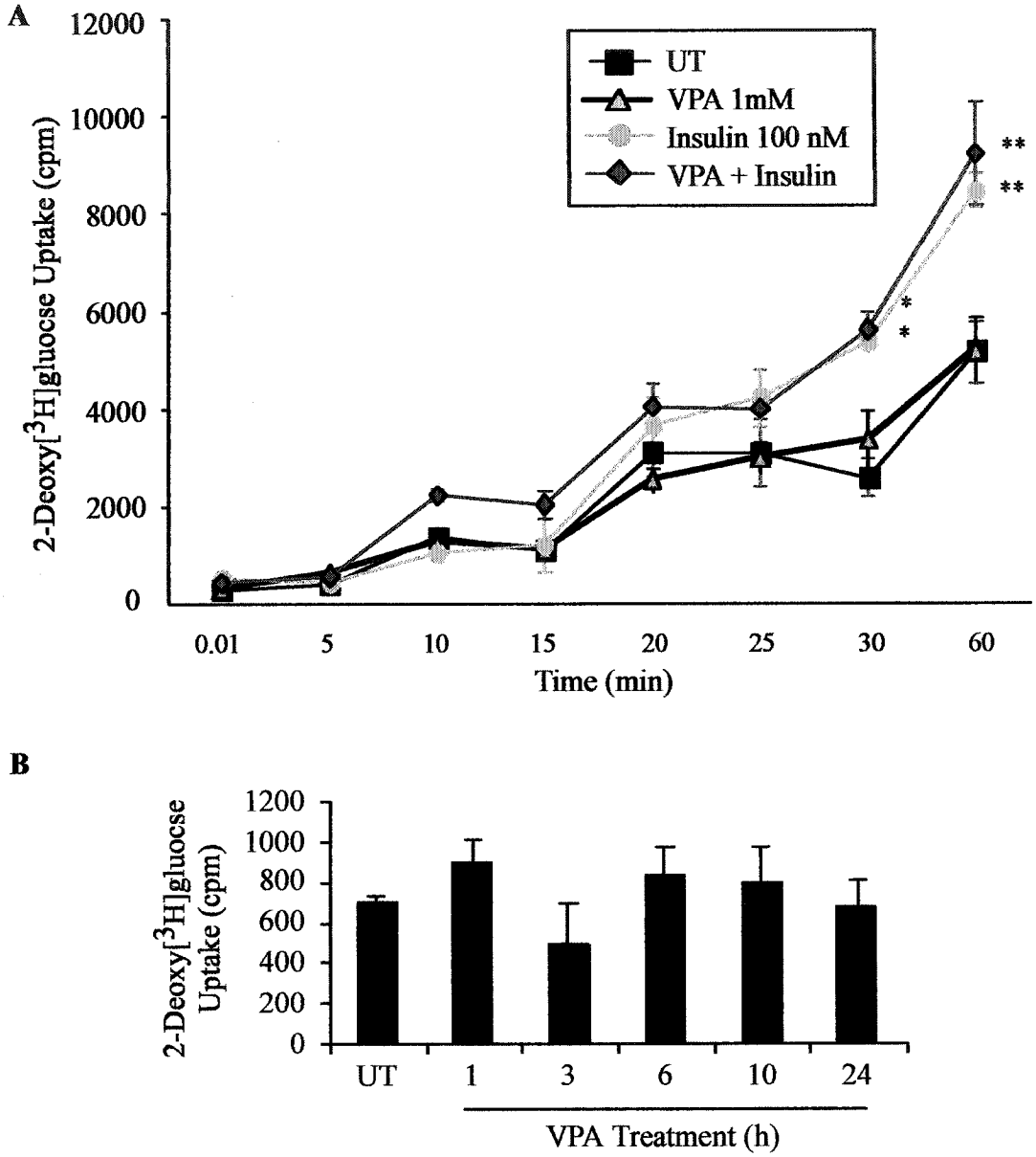


Figure 4.4 VPA does not alter glucose uptake

A) Uptake of 2-deoxy-D-[2,6-^{3}H]-glucose was measured in cells treated with either VPA (1 mM), insulin (100 nM), or VPA and insulin for 0.01-60 min. At 30 and 60 min there is a significant increase in glucose uptake in insulin treated versus (UT) cells. There was also a significant increase in glucose uptake in VPA + insulin versus VPA treated cells. Data represents mean ± SEM from triplicate samples; experiment was repeated three times with similar results; significantly different from UT at each time point as analyzed by post-hoc comparisons based on Bonferroni, * $p<0.05$, ** $p<0.01$. **B)** Uptake of 2-deoxy-D-[2,6-^{3}H]-glucose was measured in cells UT or treated with VPA (1 mM) for 1-24 h. Data represents mean ± SEM from triplicate samples, no significant difference from UT cells.

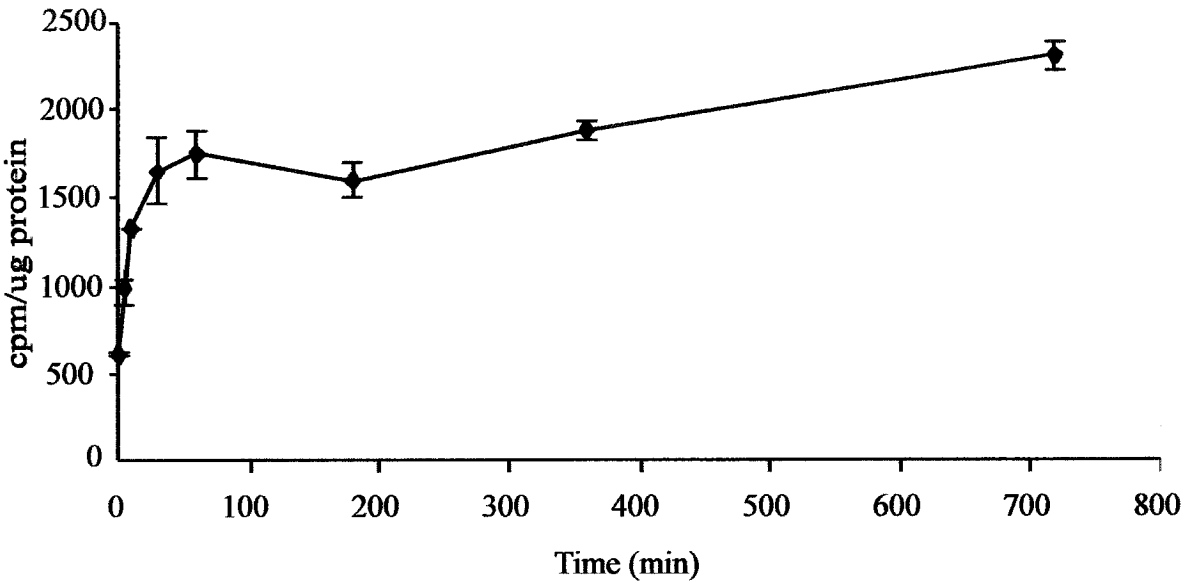


Figure 4.5 Uptake of [^{14}C]VPA into adipocytes
Adipocytes were treated with [^{14}C]-VPA for 1-720 min and uptake of [^{14}C]-label was measured and normalized against intracellular protein content (cpm/μg protein).

These results suggest that a small proportion of VPA enters adipocytes. Previous results in a neuronal cell line (GT1-7) have suggested that within cells VPA is incorporated into phospholipids and neutral lipids (365). Following [^{14}C]-labeled VPA treatment of adipocytes, the [^{14}C]-label was incorporated mainly into TAG, and the general lipid pools of diacylglycerol (DAG), cholesterol, and phospholipids (PL) (Figure 4.6A). The incorporation of [^{14}C]-label into free cholesterol suggests that VPA is oxidized into acetyl-CoA, which is required for cholesterol biosynthesis (Figure 4.1). Greater than 75% of the [^{14}C]-label in the cellular aqueous phase was incorporated into TAG by 6 h (Figure 4.6B).

To be incorporated into TAG, long chain fatty acids are activated by ACS and subsequently metabolized. Previous studies found that VPA was activated into valproyl-CoA and subsequently undergoes complete ß-oxidation (22, 23). To determine if VPA was activated by ACS in adipocytes, cells were co-treated with VPA and triacsin C, a known inhibitor of ACS1 and ACS4 (366) that blocks TAG synthesis (367). Co-treatment inhibited the [^{14}C]-label incorporation into TAG by 57% (2568±142 vs. 1109±154 cpm/ug protein). Similar results were obtained using [^{3}H]-labeled oleate as a control for radiolabel incorporation into TAG (58% reduction). These findings support the notion that VPA undergoes activation by ACS in adipocytes.

To identify whether [^{14}C]-VPA was catabolized and the [^{14}C]-labeled acetate incorporated into the fatty acid side chain of TAG, or whether the activated VPA was incorporated directly as a fatty acid, in collaboration with Dr. Roger McLeod at Dalhousie University, the fatty acids on the glycerol backbone of TAG following VPA treatment were identified (Figure 4.7A).

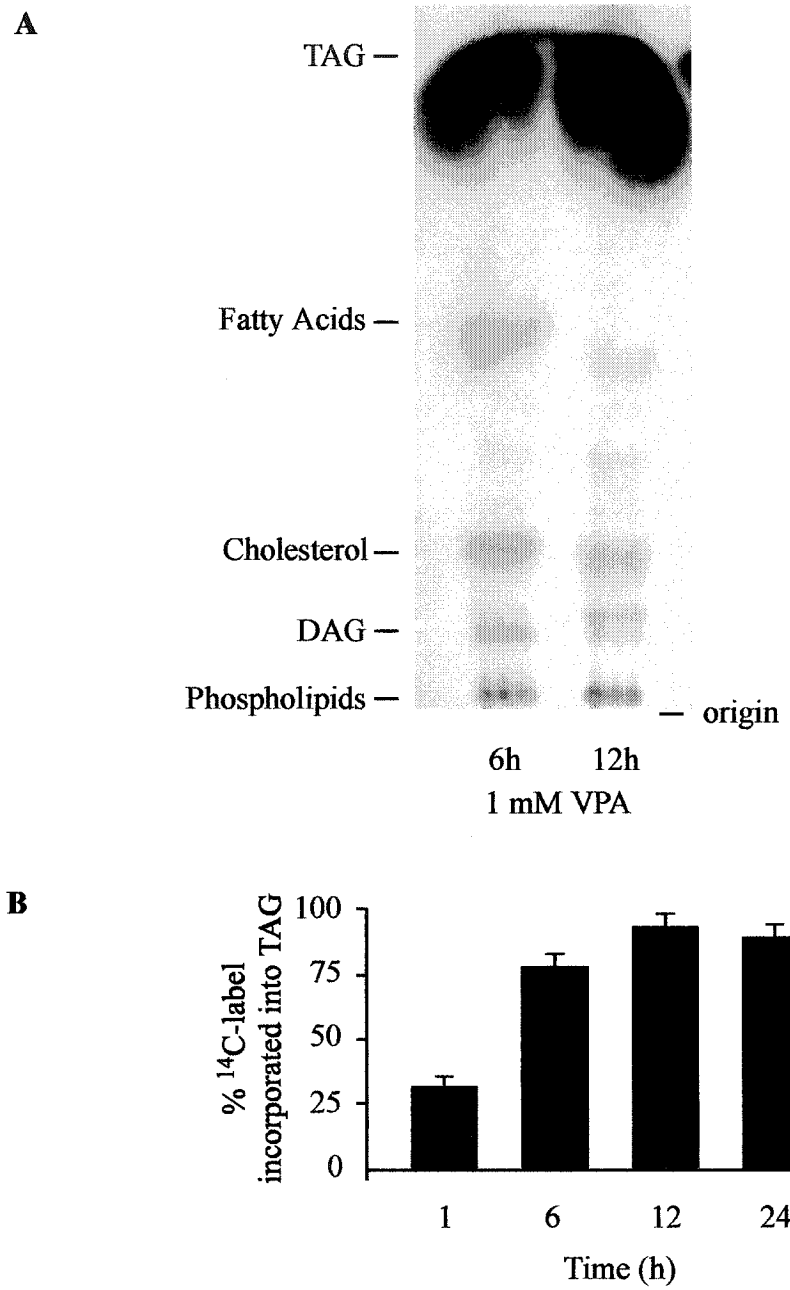
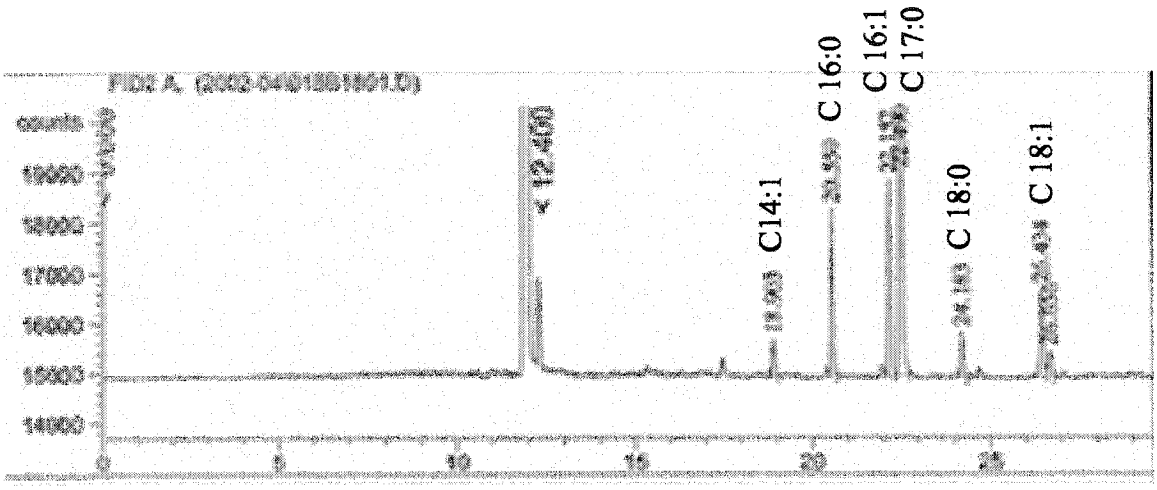


Figure 4.6 [^{14}C]-VPA treatment results in [^{14}C]-label being incorporated into lipid pools

A) Autoradiograph of a representative thin layer chromatography (TLC) plate demonstrating the presence of the [^{14}C]-label in different lipid pools including TAG, fatty acids, cholesterol, diacylglycerol (DAG), and phospholipids, 6 and 12 h after treatment with [^{14}C]-VPA. The lipid standard was visualized with iodine vapor. **B)** Quantification of the percent of [^{14}C]-label that was incorporated into TAG following treatment with [^{14}C]-VPA for 1, 6, 12, and 24 h. Data represents mean ± SEM from triplicate samples; experiment was repeated three times with similar results.

A



B

	Intracellular Free Fatty Acid				
	14:0	16:0	18:0	18:1	20:0
UT	6.3 (0.6)	18.5 (2.0)	16.1 (1.0)	8.8 (2.7)	16.9 (1.2)
VPA	6.1 (0.6)	18.8 (0.6)	15.0 (0.7)	7.1 (0.7)	18.4 (1.0)

Figure 4.7 VPA-CoA is not one of the fatty acids in TAG, and VPA treatment does not alter intracellular free fatty acid levels

A) Example of gas liquid chromatography (GLC) profile of fatty acids that were in TAG from cells treated with VPA treatment. Fatty acids are indicated above peaks and C17:1 was added manually as internal control. VPA has a retention time of 12.58 min and is not one of the fatty acids on TAG. This profile was generated by Debbie Currie for Dr. R McLeod and repeated in two seperate experiments. **B)** Total intracellular free fatty acids (C14:0, C16:0, C18:0, C18:1, C20:0) in adipocytes untreated (UT) or treated with 1 mM VPA for 12 h. Free fatty acids were quantified using GLC and normalized against intracellular protein content (µg free fatty acid/mg protein). Data represents mean ± SEM from triplicate samples; experiment was repeated two times with similar results; no significant differences were observed by ANOVA analysis.

Untreated and VPA treated cells had similar long chain fatty acids including C14:1, C16:0, C16:2, C18:0, C18:1, and VPA was not one of the TAG side chains (Figure 4.7A). This data shows that [^{14}C]-VPA was likely activated, oxidized to acetyl-CoA, and incorporated into naturally occurring fatty acids within TAG. These results strongly suggest that the ability of VPA to decrease leptin secretion is not due to VPA altering TAG composition.

VPA Does Not Alter Intracellular Free Fatty Acid Levels or Lipogenesis

Saturated fatty acids with chain lengths of greater than 7 carbons and a variety of long chain mono- and polyunsaturated fatty acids can attenuate leptin secretion (368-370). The mechanism of fatty acid suppression of leptin secretion remains unknown, but is suggested to be due to increased lipolysis or levels of intracellular free fatty acids (368-370). Since VPA is an 8-carbon (medium length) branched chain fatty acid, the effect of VPA treatment on lipogenesis and lipolysis was measured.

Lipolysis occurs through the metabolism of TAG into free fatty acids, which are transported into the mitochondria to undergo ß-oxidation (Figure 4.1). Carnitine is involved in the transport of activated long chain fatty acids through the inner mitochondria membrane to gain assess to the enzymes responsible for ß-oxidation. VPA treatment in patients is known to cause carnitine deficiency (20, 182-184). Decreased carnitine levels result in accumulation of intracellular free fatty acids due to decreased mitochondrial transport of long chain fatty acids. This led us to hypothesize that VPA may decrease leptin secretion through increasing intracellular levels of free fatty acids. In collaboration with Dr. Roger McLeod at Dalhousie University, intracellular free fatty acids were measured following VPA treatment. Compared to untreated cells, cells

treated with VPA had similar levels of intracellular free fatty acids (Figure 4.7B). Therefore, despite the effect VPA may have on fatty acid transport due to carnitine deficiencies, VPA does not alter intracellular levels of free fatty acids.

In yeast VPA has been shown to significantly reduce phospholipid biosynthesis (371). Therefore, in addition to examining levels of free fatty acids, the effect of VPA on lipid synthesis in adipocytes was measured. Cells were pulse labeled with [^{14}C]-acetic acid, which is subsequently metabolized into acetyl CoA and incorporated into lipids (Figure 4.1). VPA did not alter the synthesis of TAG, free fatty acids, DAG, cholesterol, or phospholipids (Figure 4.8A,B). Therefore the ability of VPA to decrease leptin secretion was not associated with changes in lipid synthesis or the amount of intracellular free fatty acids.

Valproic Acid Reduces Leptin mRNA Levels

Northern blot analysis was conducted to determine whether VPA treatment altered leptin mRNA levels. Leptin mRNA in mature adipocytes was reduced by treatment with VPA (Figure 4.9A). Both between treatment groups and over time there was a significant change in relative leptin mRNA levels (Figure 4.9B). Post hoc tests indicated a significant reduction in leptin mRNA at 6 and 12 h following treatment with 1 mM VPA, and at 3, 6, and 12 h following treatment with 5 mM VPA, when compared to UT.

VPA Reduces PPARγ and SREBP1a mRNA but Does Not Alter C/EBPα mRNA Levels

Daily treatment with 1 mM VPA throughout adipogenesis decreased mRNA and protein levels for PPARγ and SREBP1a, two transcription factors that are required for adipocyte differentiation (Chapter 3).

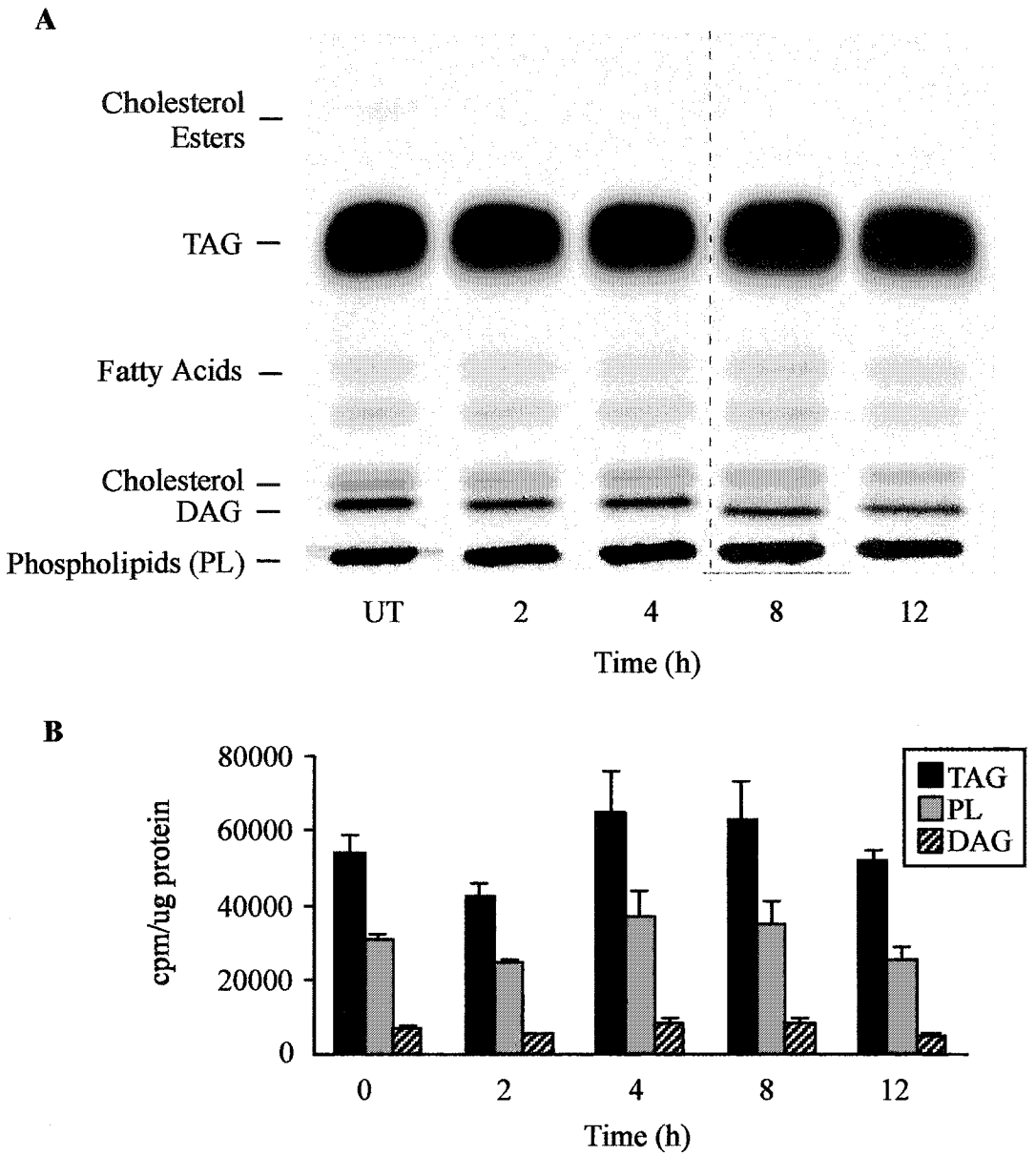


Figure 4.8 VPA does not alter lipogenesis
A) Autoradiograph of two representative thin layer chromatography (TLC) plates (dashed line represents seperate plate) demonstrating the presence of the [^{14}C]-label in different lipid pools including TAG, fatty acids, cholesterol, cholesterol esters, diacylglycerol (DAG), and phospholipids in cells treated with 1 mM VPA for 0-12 h and then pulse labeled with [^{14}C] acetic acid. The lipid standard was visualized with iodine vapor. **B)** Quantification of A, after counting total radioactive [^{14}C] in samples and normalized against intracellular protein content (ug protein). Data represents mean ± SEM from triplicate samples; experiment was repeated three times with similar results; no significant differences were observed using ANOVA analysis.

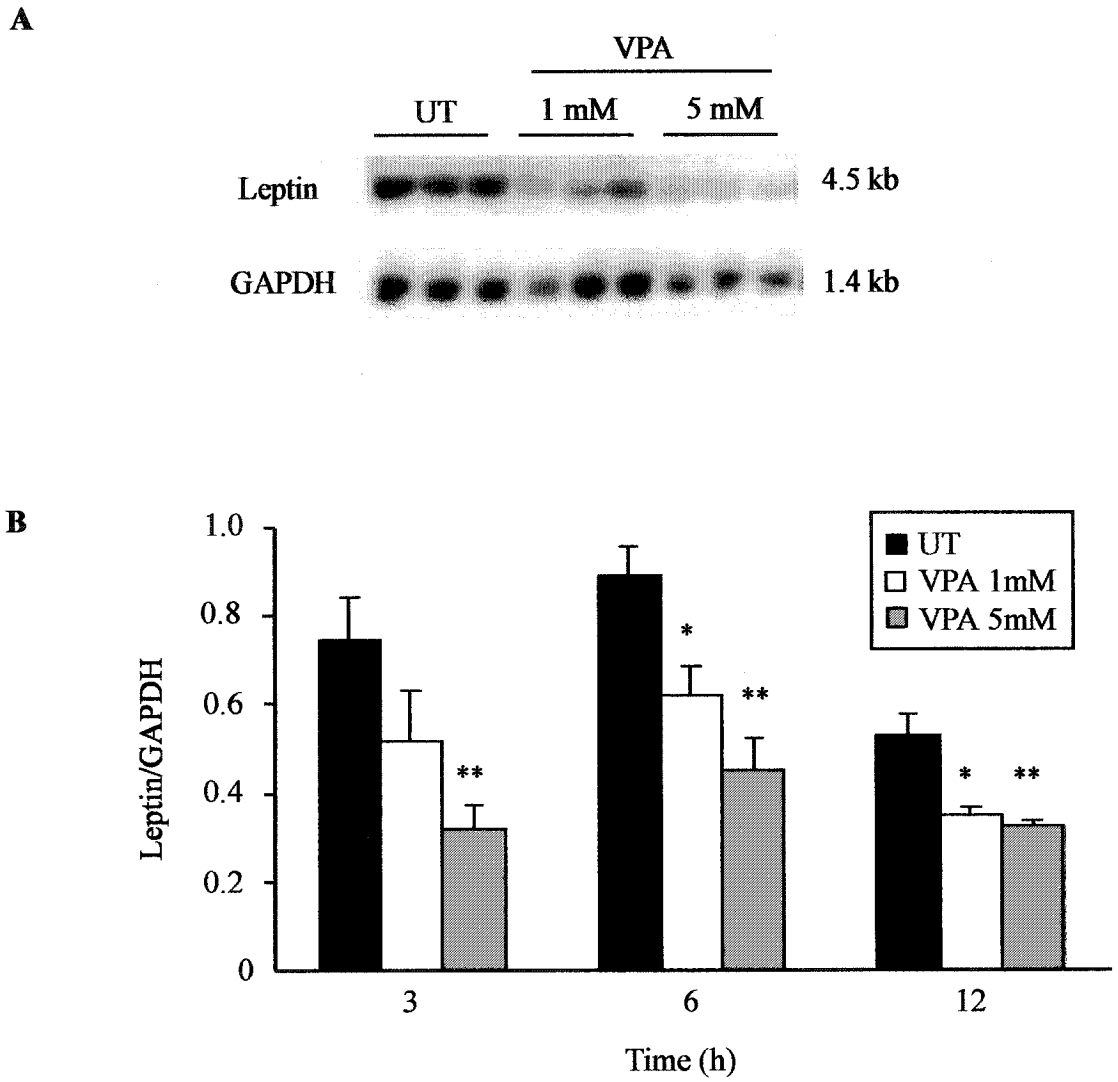


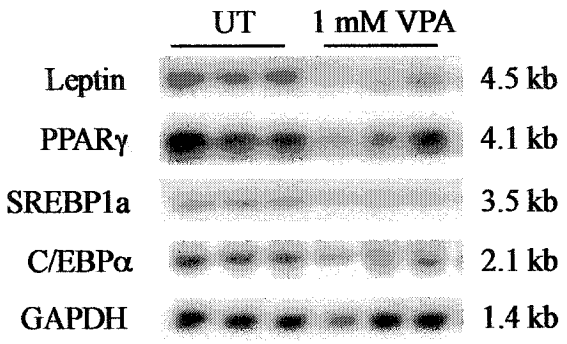
Figure 4.9 VPA treatment decreased leptin mRNA levels.
A) Leptin mRNA levels decrease following 3 h treatment with VPA (1 mM, 5 mM) as shown by Northern Blot analysis. GAPDH is shown as a loading control. **B)** Quantification of leptin mRNA levels normalized against GAPDH levels. Data represents mean ± SEM from triplicate sample collected in two independent experiments; significantly different from untreated (UT) at each time point as analyzed by post-hoc comparisons, * $p<0.05$, ** $p<0.01$.

Transcription of the leptin gene is regulated by C/EBPα, PPARγ, and SREBP1, thus the effect of acute VPA treatment (≤12hr) on expression of these transcription factors was measured in mature adipocytes. Post-hoc analysis indicates that VPA treatment caused a significant decrease in PPARγ and SREBP1a mRNA levels at 3 h, without altering C/EBPα mRNA levels (Figure 4.10A,B). However, at 6 and 12 h, the amount of PPARγ, SREBP1a, and C/EBPα mRNA levels were not significantly different (Figure 4.10B).

VPA Does Not Alter PPARγ, SREBP1a, or C/EBPα Protein Levels

The reduction in PPARγ mRNA levels following 3 h of VPA treatment was not accompanied by changes in the amount of either PPARγ1 or PPARγ2 protein (Figure 4.11A,C). After detection of PPARγ, western blots were stripped and re-probed with an antibody against C/EBPα, which detects both the 42 kDa and 30 kDa alternative translation products. In agreement with the C/EBPα mRNA data, there was no detectable alteration in the amount of 30 kDa or 42 kDa C/EBPα protein following treatment with VPA (Figure 4.11A, C). Furthermore, there was no change in the amount of precursor (P; 125 kDa) or cleaved (C; 68 kDa) form of SREBP1a protein following 1mM VPA (Figure 4.11B, C). Therefore, the decrease in leptin secretion and mRNA levels, following treatment with 1 mM VPA, was not associated with significant decreases in C/EBPα, PPARγ, and SREBP1a protein levels. There are numerous transcription factors that can regulate leptin promoter activity (335, 339, 369, 372), raising the possibility that VPA may alter the production, activity, or stability of these transcription factors ultimately resulting in decreased leptin mRNA levels.

A



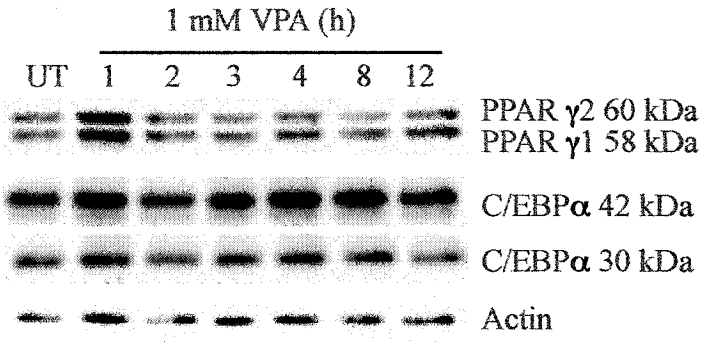
B

	Time (h)	UT	1 mM VPA
PPARγ	3	0.78 (0.04)	0.50 (0.03)*
	6	0.66 (0.03)	0.52 (0.04)
	12	0.60 (0.03)	0.60 (0.05)
SREBP1a	3	0.58 (0.02)	0.41 (0.02) *
	6	0.60 (0.03)	0.53 (0.04)
	12	0.56 (0.04)	0.56 (0.06)
C/EBPα	3	0.28 (0.02)	0.20 (0.02)
	6	0.19 (0.02)	0.21 (0.07)
	12	0.21 (0.04)	0.22 (0.09)

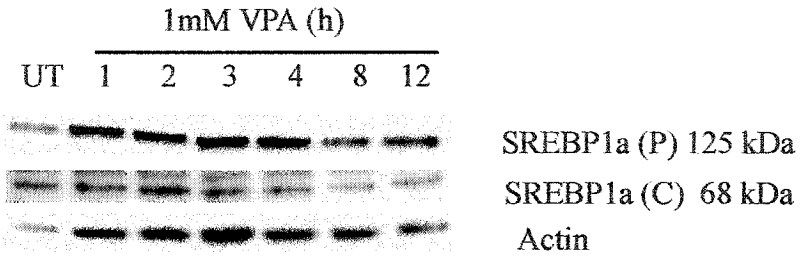
Figure 4.10 VPA treatment reduces PPARγ and SREBP1a mRNA levels but does not alter C/EBPα mRNA levels

A) Northern blot analysis of leptin (as shown in Figure 4.9), PPARγ, SREBP1a, and C/EBPα mRNA levels in adipocytes following treatment for 3 h with 1 mM VPA. GAPDH is shown as a loading control. **B)** Quantification of mRNA levels for PPARγ, SREBP1a and C/EBPα normalized against GAPDH levels. Data represents mean ± SEM from triplicate samples collected in two independent experiments; significantly different from untreated (UT) at each time point, as analyzed by post-hoc comparisons, * $p<0.05$.

A



B



C

	UT	1 h	2 h	3 h	4 h	8 h	12 h
PPARγ2	1.15 (0.47)	1.50 (0.30)	1.44 (0.18)	1.26 (0.13)	1.28 (0.14)	1.02 (0.10)	1.27 (0.32)
PPARγ1	1.20 (0.53)	1.65 (0.34)	1.60 (0.21)	1.56 (0.14)	1.71 (0.25)	1.53 (0.16)	1.70 (0.49)
C/EBPα 42	1.90 (0.40)	2.21 (0.60)	1.86 (0.50)	2.16 (0.22)	2.34 (0.41)	2.15 (0.41)	1.76 (0.21)
C/EBPα 30	1.04 (0.47)	1.12 (0.59)	1.04 (0.50)	0.99 (0.38)	1.02 (0.57)	0.81 (0.27)	0.69 (0.26)
SREBP1a (P)	1.02 (0.14)	0.75 (0.05)	1.09 (0.24)	0.78 (0.05)	0.87 (0.07)	0.92 (0.21)	0.87 (0.08)
SREBP1a (C)	0.83 (0.21)	0.89 (0.12)	1.18 (0.13)	1.37 (0.34)	1.34 (0.30)	0.71 (0.08)	0.92 (0.29)

Figure 4.11 VPA treatment does not alter PPARγ, C/EBPα, or SREBP1a protein levels

A) Western analysis to detect PPARγ1 and PPARγ2, p30 and p41 C/EBPα, from adipocyte whole cell protein extracts treated (1-12 h) with 1 mM VPA. **B)** Cells were pretreated with, and protein samples isolated in the presence of, the proteosome inhibitor N-acetyl-leucine-leucine-norleucinal (ALLN), and western analysis to detect cleaved SREBP1a (C) and the precursor SREBP1a (P) protein. **C)** Quantification of protein levels for PPARγ, SREBP1a and C/EBPα as normalized against actin levels. Data represents mean ± SEM from a minimum of three independent experiments; no significant differences were observed using ANOVA analysis.

Effect of VPA on Leptin Promoter Activity

In order to assess the effect of VPA on leptin promoter activity, transient transfection experiments were completed in human BeWo cells, which have been demonstrated to express leptin (369). Two leptin promoter-luciferase reporter constructs, containing –3 kB to + 30 (Ob1) or –217 to +30 base pair (Ob2) (+1 is start of transcription) of the human leptin gene were used and control cultures were transfected with the promoterless pGL3-basic-luc vector (Promega). Long chain fatty acids, including dicosahexaenoic acid (DHA, 22:6 1mM) have been reported to decrease leptin promoter activity (369) and were used as a positive control.

Leptin promoter activity of the pGL3-Ob1 construct was significantly induced by VPA treatment on average by 2 fold, by contrast DHA treatment significantly reduced pGL3-Ob1 activity on average by 81% (Figure 4.12A). As previously reported pGL3-Ob2 displayed reduced activity when compared to pGL3-Ob1 (336). Treatment with VPA or DHA produced similar results in the presence of pGL3-Ob2 or pGL3-Ob1 (Figure 4.12A). When examining the effect of treatment on pGL3-basic activity, VPA treatment also induced activity by 2.15 fold and DHA treatment reduced activity by 58% (Figure 4.12B). Thus, when comparing the fold induction by VPA treatment and the average reduction by DHA treatment, similar changes in activity occurred with either the pGL3-basic, pGL3-Ob1, or pGL3-Ob2 plasmids (Figure 4.12C). As discussed in Chapter 3, VPA effects in this experiment are likely due to the inhibition of HDAC by VPA, since VPA had a similar effect on the reporters and promoterless plasmid. Therefore this experiment was unable to assess whether VPA had effects on leptin promoter activity, due to the effects of VPA in transient transfection experiments.

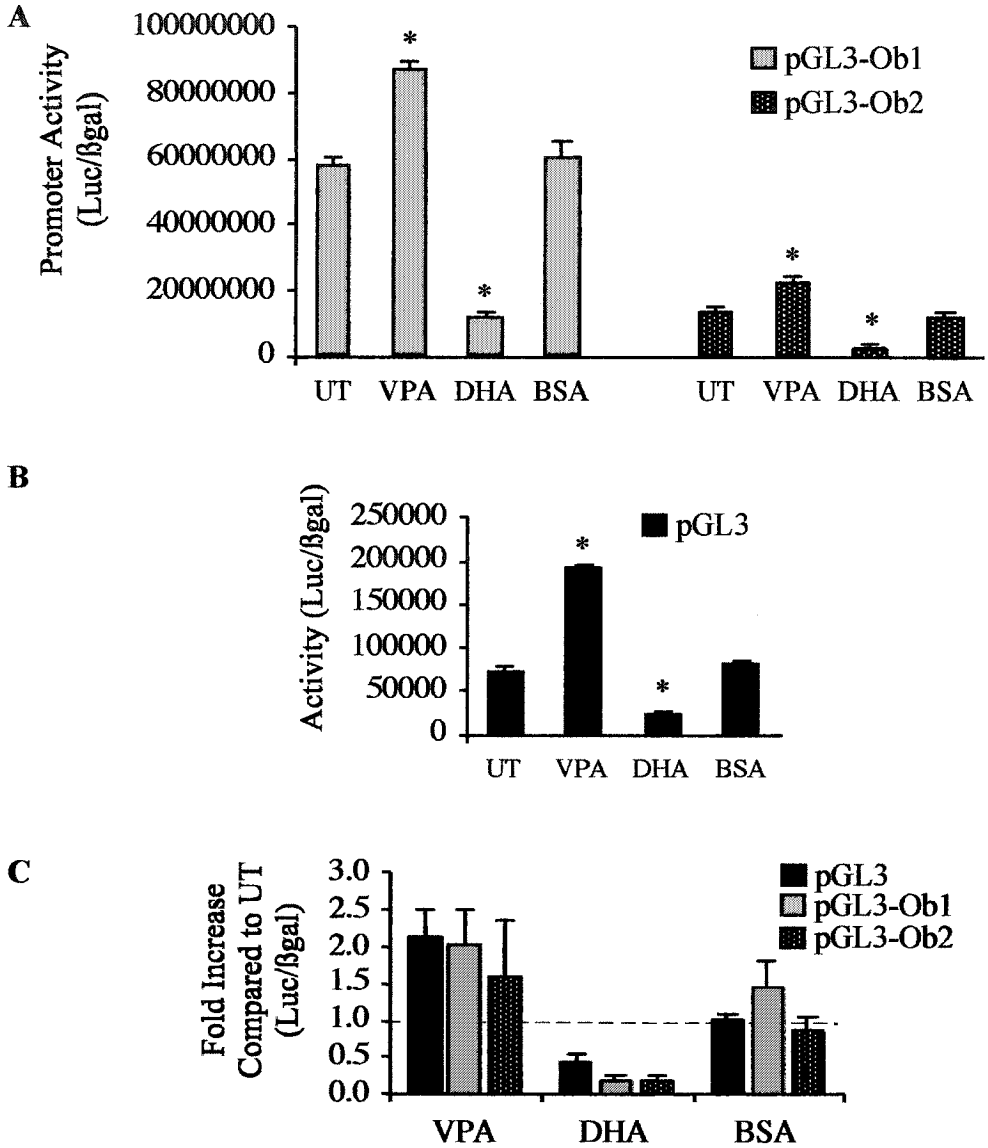


Figure 4.12 Activation of the control pGL3 vector and the leptin promoter constructs by VPA

A) Induction of the pGL3-Ob1.luc or pGL3-Ob2.luc constructs containing 2.9 and 0.2 kb of human leptin promoter, respectively, in BeWo cells following treatment with either VPA (1 mM), DHA (1 mM) or the DHA vehicle control, 10% BSA. VPA induces a significant increase and DHA induces a significant decrease in activity compared to untreated (UT) cells. **B)** Induction of the pGL3-basic.luc vector in cells treated as described in A. VPA induces a significant increase and DHA induces a significant decrease in activity compared to UT cells. Data in A and B represent luciferase activity normalized with ß-gal activity for triplicate wells done in one experiment; similar findings were obtained in three independent experiments. t-test, * $p<0.01$. **C)** Summary of mean fold induction of the pGL3-basic.luc, pGL3-Ob1.luc and pGL3-Ob2.luc reporters from three independent experiments completed as described in A and B. Compared to UT cells (set at 1, noted by dashed line), VPA induces an average 1.9 fold increase in activity, whereas DHA induces a 74% reduction in the activity of all three constructs.

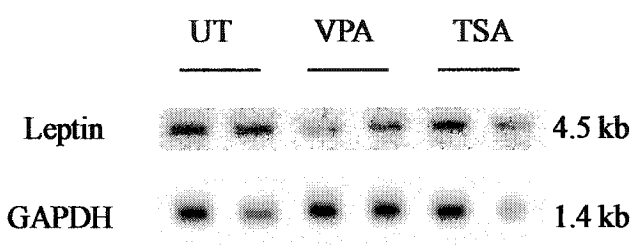
HDAC Inhibition by TSA does not Alter Leptin mRNA Levels

In chapter 3, VPA attenuated adipogenesis through its ability to suppress HDAC activity. The effect of HDAC inhibition on leptin secretion or leptin mRNA levels in adipocytes is unknown. HDAC inhibition is normally associated with activation of gene expression (reviewed in 278), however HDAC inhibition by VPA can indirectly suppress gene expression in adipocytes (Chapter 3). Leptin mRNA levels were measured in cells treated with the commonly used HDAC inhibitor TSA (3 nM) for 6 h (Figure 4.13). Unlike VPA, which produces a significant decrease in leptin mRNA levels, there was no significant difference in leptin mRNA levels in cells treated with TSA compared to untreated cells, suggesting that leptin mRNA levels are unaltered by HDAC inhibition. Moreover, this suggests that alterations in leptin mRNA levels are unrelated to the HDAC inhibitory properties of VPA.

VPA Does Not Enhance Leptin mRNA Degradation

Studies have suggested that leptin expression can be controlled by both transcriptional and post-transcriptional regulation (373). To determine if VPA altered leptin mRNA levels through increasing degradation, leptin mRNA levels from cells co-treated with VPA (1 mM) and the RNA synthesis inhibitor actinomycin D (ActD) were assessed. The half-life of leptin mRNA in 3T3-L1 cells is reported to be approximately 2~4 h (374, 375). In agreement with this data, there was a greater than 50% reduction in leptin mRNA levels in adipocytes 4 h after treatment with ActD (Figure 4.14 A,C). There was no significant difference in the cells treated with ActD or VPA + ActD, suggesting that VPA does not alter leptin mRNA stability (Figure 4.14 A,C).

A

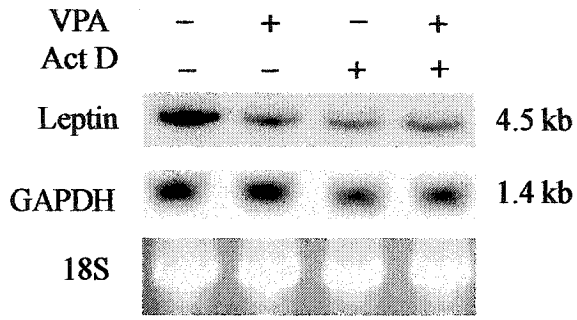


B

	Leptin/GAPDH
UT	0.086 (0.02)
TSA	0.089 (0.02)

Figure 4.13 TSA treatment does not alter leptin mRNA levels
A) Treatment with 3 nM trichostatin A (TSA) for 6 h did not alter leptin mRNA levels, when compared to untreated (UT) cells. Cells treated with 1mM VPA for 6 h had decreased leptin mRNA levels. **B)** Quantification of leptin mRNA levels normalized against GAPDH mRNA. Data represents mean ± SEM from triplicate samples from one experiment; repeated in an independent experiment, analyzed by t-test.

A



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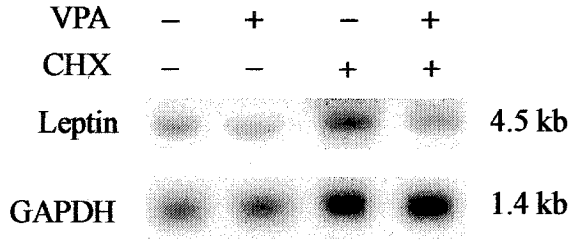

C

	UT	Actinomycin D	Cycloheximide
UT	0.102 (0.030)	0.025 (0.007)	0.099 (0.015)
VPA 1mM	0.044 (0.018)*	0.018 (0.009)	0.041 (0.007)*

Figure 4.14 VPA did not affect leptin mRNA degradation, but reduced leptin mRNA levels independent of protein synthesis inhibition

A) Treatment with 1 μg/ml actinomycin D (ActD) for 4.5 h results in decreased leptin mRNA levels. Co-treatment of ActD + VPA did not alter leptin mRNA levels when compared to ActD treatment alone. ActD treatment decreased GAPDH levels, thus ribosomal 18S RNA is shown as a loading control. **B)** Treatment with 10 μg/ml cycloheximide (CHX) for 4.5 h does not alter leptin mRNA levels. VPA decreased leptin mRNA in the presence of CHX when compared to CHX treatment alone. **C)** Quantification of leptin mRNA levels normalized against GAPDH mRNA. Data represents mean ± SEM from three independent experiments; significantly different from UT as analyzed by ANOVA, *$p<0.05$.

We also found that GAPDH mRNA levels were decreased in adipocytes following ActD treatment, therefore the 18S ribosomal RNA is shown to assess loading (Figure 4.14A). VPA does not affect leptin mRNA degradation suggesting that VPA can affect leptin gene transcription.

VPA Reduces Leptin mRNA Independent of New Protein Synthesis

VPA has been demonstrated to activate a variety of intracellular signaling pathways. Leptin mRNA levels may be reduced by VPA treatment through post-translational effects on transcriptional machinery. Cycloheximide (CHX) is a commonly used inhibitor of protein synthesis. Cells treated with CHX (10 μg/ml) have similar levels of leptin mRNA compared to untreated cells (Figure 4.14B, C). Pre-treatment with CHX followed by co-treatment with CHX and VPA produced a significant reduction in leptin mRNA levels compared to VPA treatment alone (Figure 4.14B, C). These results show that the VPA-induced decrease in leptin mRNA is independent of *de novo* protein synthesis.

DISCUSSION

In this chapter VPA was demonstrated to decrease leptin secretion and mRNA levels in mature adipocytes. Acute treatment (hours) with VPA significantly reduced leptin mRNA and protein secretion in a dose- and time-dependent manner. The reduction in leptin secretion by VPA was not accompanied by alterations in intracellular levels of leptin or uptake of glucose, suggesting that these known regulators of leptin secretion are not affected by VPA. Although VPA was metabolized in adipocytes, VPA treatment was not associated with changes in lipogenesis or levels of intracellular free fatty acids. In addition, VPA treatment of adipocytes did not alter C/EBPα, PPARγ, or SREBP1a protein levels suggesting that reduced expression of these transcription factors was not responsible for the VPA-induced decrease in leptin production. VPA treatment was not associated with enhanced leptin mRNA degradation, rather VPA treatment decreased leptin mRNA levels independent of *de novo* protein synthesis.

Effect of Fatty Acids on Leptin Transcription and Secretion

It has been demonstrated that short-chain fatty acids (C2-C6), unlike VPA and long chain fatty acids, stimulate leptin production through activation of the orphan G protein coupled receptor 41, GPR41 (376). VPA effects on leptin secretion are similar to those of saturated fatty acids (> C8), as well as long-chain mono- and poly-unsaturated fatty acids, which have been demonstrated to inhibit leptin secretion (370). It has been argued that lipids reduce leptin secretion and mRNA levels in both fully differentiated 3T3-L1 cells and in primary cultures of rat adipocytes because of an accumulation of intracellular free fatty acids (368, 377). This argument is based on experimental evidence demonstrating that (i) treatment with triacsin C, which causes the accumulation of free

fatty acids, is correlated with reduced leptin mRNA levels and decreased leptin secretion, and (ii) treatment of cells with 2-bromopalmitate, a poorly metabolized fatty acid that accumulates in the cell, results in reduced leptin mRNA to a greater degree than treatment of cells with palmitate, a more efficiently metabolized fatty acid (377). The ability of fatty acids to suppress leptin secretion does not appear to depend on fatty acid oxidation since inhibitors of oxidation were unable to reverse the effects of fatty acids on leptin secretion (370). These experiments indirectly suggest that increased free fatty acid levels correlate with decreased leptin mRNA levels and/or protein secretion. The results in this chapter demonstrate that the VPA-induced decrease in leptin secretion was not due to altered total intracellular free fatty acid levels.

Additionally, there is evidence indicating that some, but not all, long chain fatty acids inhibit leptin production by altering leptin gene transcription (369, 370, 378). VPA reduces leptin mRNA levels as early as 3 h following treatment, and this reduction was not due to enhanced leptin mRNA degradation. An isomer of conjugated linoleic acid (t10c12 CLA) has also been shown to reduce leptin mRNA levels in both 3T3-L1 cells (24 h treatment) and mouse adipose tissue *in vivo* after diet supplementation with 0.5% t10c12 CLA for 4 weeks (378). Similarly, polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA, 22:6) and eicosapentanenoic acid (20:5), can decrease leptin mRNA in rat adipose tissue (diet supplementation for 3 weeks), as well as in 3T3-L1 and the human BeWo cell lines (24-72 h). In addition, PUFA can also decrease leptin promoter reporter activity in BeWo cells (378). In contrast, saturated [palmitic acid (16:0)] and mono-unsaturated [oleic acid (18:1)] fatty acids did not effect leptin mRNA levels or leptin promoter activity in rat adipose tissue, 3T3-L1 or BeWo cell lines (369).

Using leptin promoter reporter constructs transfected into BeWo cells, we were unable to determine if VPA directly modulated leptin reporter activity. VPA induced a significant increase in both the activity of the control vector and in vectors containing the leptin promoter constructs. As discussed in detail in Chapter 3, VPA produces non-specific effects on promoter activity in numerous reporter gene assays, likely due to the HDAC inhibitory properties of VPA (40, 41). In order to address whether VPA affects leptin promoter activity and possibly avoid the confounding effects of HDAC inhibition by VPA, future experiments could be performed in an Ob-Luc cell line which has one allele of the leptin gene knocked in with a luciferase cassette, allowing endogenous leptin gene expression to be monitored by luciferase activity (376).

When replicating the findings of Reseland *et al* (369), DHA was demonstrated to significantly decrease leptin reporter activity, however unexpectedly DHA treatment also significantly reduced the activity of the pGL3-basic control plasmid. Previous work has not documented the effects of DHA on the pGL3-basic control plasmid (369). Although not quantified in this experiment, 1 mM DHA treatment for 15 h was associated with cell death as noted by cells appearing unhealthy and detaching from the culture plate. Thus, in the experiments presented in this chapter, the effects of DHA cannot be specifically attributed to the effects of DHA on the leptin promoter, but may result from a general effect of DHA on cell viability. Previous work had documented that DHA treatment (1mM for 72 h) did not result in decreased viability in the BeWo cells (369). In order to ascertain the effects of DHA and other fatty acids on leptin promoter activity, future work should use a dose-response curve and determine if DHA and other fatty acids can

modulate activity of the leptin promoter constructs independent of effects on the control pGL3 plasmid.

VPA Reduces Leptin mRNA Levels

Expression of leptin mRNA during the formation of adipocytes is highly dependent on the DNA methylation pattern of the leptin gene (333, 334). VPA has also been recently identified to reduce DNA methylation (379, 380). In preadipocytes leptin gene expression is silenced through methylation of the leptin promoter, and is activated by demethylation during the differentiation of preadipocytes into adipocytes (333, 334). Since VPA induces demethylation and demethylation of the leptin promoter is associated with leptin expression, it is unlikely that VPA's ability to decrease leptin expression is through leptin demethylation. Moreover since VPA was able to decrease leptin mRNA expression as early as 6 h, it is unlikely that methylation status of the promoter would be altered in this time period.

This study examined VPA effects on the production of transcription factors that are known regulators of the leptin promoter, including C/EBPα. The leptin promoter contains a C/EBP binding motif, and a number of studies have identified that C/EBPα functions as a transactivator of the leptin gene promoter (335, 336, 339). VPA treatment reduced leptin mRNA levels without altering the level of C/EBPα mRNA or protein. However, leptin promoter activity can be modulated by mechanisms that change C/EBPα activity but not expression (381). For example, in response to transforming growth factor beta (TGFβ), the Smad3-Smad4 heteromeric complex interacts with C/EBPα and inhibits transactivation of the leptin promoter (382). Altering phosphorylation of C/EBPα has also been implicated in the ability of insulin to repress insulin-response genes, such as

GLUT-4 (383). Whether leptin transcription is modulated by C/EBPα phosphorylation status is unknown. Glycogen synthase kinase 3 (GSK-3) is an insulin-regulated kinase that can phosphorylate C/EBPα on two threonine residues (298). VPA has been suggested to be an inhibitor of GSK3 (70, 71, 73), raising the possibility that VPA could alter the phosphorylation of C/EBPα through inhibiting GSK3. Future work will need to identify whether VPA modifies GSK3 activity in adipocytes, and whether this modification alters C/EBPα phosphorylation status and ultimately affects leptin promoter activity.

Acute treatment with VPA did not alter PPARγ protein levels or SREBP1a processing. Synthetic ligands of PPARγ, such as antidiabetic thiazolidinedione agents, or endogenous ligands, such as prostaglandins (384), decrease leptin secretion and mRNA levels in 3T3-L1 cells (385), and in rodent (386) and human adipocyte tissue (387). Regulation of the leptin promoter by PPARγ ligands occurs through functional antagonism of C/EBPα by PPARγ (339). Many fatty acids are PPARγ ligands and while some investigators suggest that VPA is a PPARγ ligand (269, 290), at a dose of 1 mM, VPA does not act as a PPARγ ligand in reporter gene assays, as shown in Chapter 3. Therefore it is unlikely that VPA decreases leptin mRNA levels through a direct effect on PPARγ expression or activity.

SREBP1a is a transcription factor that transactivates the leptin promoter by binding to the SRE-1 sequence (252). Reseland *et al.* (369) proposed that PUFA decreased leptin secretion by inhibiting SREBP processing, and that activation of SREBP1 protein processing through cholesterol starvation, prevented the decrease in leptin mRNA by PUFA. In this chapter, 1 mM VPA reduced SREBP1a mRNA levels at

3 h, but not at later time points. This change in mRNA levels did not result in alterations in SREBP1a protein levels or processing into its active form, suggesting that VPA does not affect leptin mRNA or secretion through SREBP1a activity. These results demonstrate that VPA effects on leptin production occur independent of alterations in PPARγ activity or SREBP1a processing.

Mechanism for Reduced Leptin Secretion and mRNA Levels by VPA

How does VPA decrease leptin mRNA and subsequently decrease leptin secretion? The results presented in this chapter rule out several possibilities including increasing the intracellular pools of leptin, increasing the level of intracellular fatty acids, or promoting the degradation of leptin mRNA. Moreover the attenuation of leptin mRNA does not appear to be due to HDAC inhibition, since treatment of adipocytes with another HDAC inhibitor, TSA, did not produce a reduction in leptin mRNA. Experiments with CHX suggest that VPA reduces leptin mRNA levels independent of new protein synthesis. In other cell types, VPA can have multiple affects on a variety of cell signaling pathways including, and not limited to, increased GABA signaling, activation of the MAPK pathway, and inhibition of GSK-3 activity. VPA has also been demonstrated to alter the expression of a variety of intracellular signaling molecules such as protein kinase C (reviewed in 49, 51). We hypothesize that the ability of VPA to alter intracellular signaling pathways may be one mechanism by which VPA can affect leptin transcription. Understanding the different regulatory elements in the leptin promoter that are regulated by VPA treatment will help to elucidate whether the activity of specific transcription factors, such as C/EBPα, contribute to the reduction in leptin expression.

CONCLUSIONS

A majority of patients treated with VPA have significant weight gain that is often associated with increased levels of serum leptin. Increased serum leptin levels are associated with human obesity, suggesting a state of leptin resistance occurs in obesity (316-318). It is unclear whether increased leptin levels are due to a direct effect of VPA on adipocytes *in vivo*, or whether increased serum leptin is simply a consequence of increased adipose mass. This chapter identifies that VPA can decrease leptin secretion and mRNA levels in cultured adipocytes. As described in Chapter 2, there was a loss of correlation between leptin levels and body mass in female rats treated with VPA, despite VPA having no direct effect on body mass.

One simple explanation for the difference seen in patients and in rats or isolated mouse adipocytes could be due to species-specific effects of VPA. Since the literature supports the validity of 3T3-L1 adipocytes as a model for human primary adipocytes and the results in Chapter 3 illustrate inhibition of adipogenesis by VPA in both 3T3-L1 and primary human adipocytes, species differences seem unlikely to account for this paradox. To clarify this issue it will be necessary to examine the effect of VPA in human primary adipocyte cultures. In order to assess the effect of VPA on adipocyte biology *in vivo*, it would also be necessary to obtain tissue biopsies from patients that are receiving VPA and examine leptin secretion and leptin mRNA levels in individual adipocytes.

Another possible explanation for the paradox could be that acutely VPA treatment *in vivo* may decrease leptin mRNA and secretion from adipocytes yet chronically this may lead to an increase in adipocyte size and subsequent increase in leptin secretion through compensatory mechanism that are designed to maintain energy balance. If this

hypothesis is correct, early during treatment with VPA (days) patients should have reduced serum leptin levels, followed by an increase in serum leptin levels accompanying weight gain. To our knowledge there are no clinical studies that have examined serum leptin levels in days following the onset of VPA treatment. It has however been shown that leptin levels increase in only those patients that gain weight on VPA, suggesting that serum leptin levels are associated with increases in body mass (144). The data presented in this chapter also advocate that the increase in leptin secretion observed *in vivo* is not directly correlated with VPA inducing leptin expression, but may result from the increase in weight observed in patients treated with VPA.

Chapter 5

General Discussion and Significance

The work presented in this thesis began with a quest to understand more about how VPA induces PCOS and obesity in patients. This is an ambitious task considering the mechanism of action for VPA therapy remains unknown and both PCOS and obesity are complex pathological conditions with unknown etiologies. My results demonstrate that VPA does not induce PCOS in rats and VPA inhibits preadipocyte differentiation and leptin secretion from adipocytes. These findings are unable to answer how VPA induces PCOS or weight gain, but provide information about using a rodent model to study these side effects and demonstrate that VPA can directly affect adipocyte biology.

Modeling VPA-Induced PCOS or Weight Gain

When attempting to model the induction of PCOS by VPA, different observations have been reported in studies with female rats or monkeys. VPA treatment in rats increased the formation of cystic follicles with no accompanying alterations in other PCOS-like symptoms, yet no cystic follicles or PCOS-like symptoms were observed in monkeys (79, 133-135). VPA treatment is not associated with weight gain in rodents yet was associated with weight gain in monkeys (79, 133-135, 154). Since VPA is unable to induce PCOS or weight gain in rodents, does this suggest that VPA is not responsible for the increased rates of PCOS or weight gain observed in women treated with VPA? This generalized conclusion is not supported by the current literature, which has simply demonstrated that VPA treatment in rodents does not produce symptoms of PCOS or weight gain that are observed in patients, with the exception of cystic follicles being observed in rats and weight gain observed in monkeys treated with VPA.

There are multiple reasons why VPA could induce PCOS and weight gain in patients, yet not induce these effects in rodents or non-human primates. One possibility could be the development of PCOS or weight gain by VPA treatment may require drug treatment plus a genetic predisposition to either PCOS or weight gain. This might also explain why only a proportion of individuals treated with VPA experience these side effects. No rodent models exist for PCOS that fully mimics PCOS in humans and it has been suggested that there may not be a possibility for creating a "universal" PCOS model due to the multifactorial and genetic etiology associated to this disorder (388). In agreement with the idea that a genetic predisposition to PCOS may alter VPA effects, VPA has been demonstrated to augment androgen release in thecal cells from PCOS patients but not in thecal cells from controls who do not have PCOS (130). Weight is also known to be largely genetically determined and the heritability of obesity is equivalent to that of height and exceeds that of many other disorders for which a genetic basis is accepted (reviewed in 188). In agreement with the hypothesis that genetics may influence VPA-induced weight gain, a polymorphism in the promoter of the leptin gene (389) as well as a polymorphism in the serotonin $(5\text{-HT})_{2C}$ receptor gene (390) have been identified as genetic risk factors for weight gain in patients on antipsychotic medications. These findings suggest that individual differences in body weight following treatment with psychotropic medications may be determined in part by allelic variations in genes, however it is unknown whether these polymorphisms have functional effect on weight, or simple co-segregate with other genes that contribute to obesity. The identification of these polymorphisms may provide a marker that could identify which patients may be at increased risk for gaining weight on VPA therapy.

Physiological differences between species may also affect the ability of VPA to induce PCOS or weight gain. In terms of PCOS, female reproductive-endocrine function in rodents differs from humans, with rodents having a much shorter estrous cycle length, producing multiple offspring in one pregnancy, and exhibiting seasonal breeding in the wild. Species differences in reproductive functioning seems less likely to account for the lack of induction of PCOS in female monkeys treated with VPA, since endocrine function is relatively similar in monkeys and humans (135). In terms of modeling VPA-induced weight gain the physiological control of energy balance also differs between species, which may contribute to the inability of VPA to induce weight gain in rodents. As described in the introduction to Chapter 3, BAT in rodents is important for the maintenance of energy homeostasis and body weight regulation, yet in humans WAT is mainly responsible for energy homeostasis. Therefore, it is possible that VPA may not affect body weight in rodents, if VPA altered energy expenditure by BAT. There are also important differences in leptin biology between mice and humans. Obesity develops without overeating in leptin deficient (ob/ob) mice due to reduced energy expenditure, whereas obesity develops primarily due to hyperphagia in humans with leptin deficiencies (reviewed in 189). Therefore the fact that VPA-induced weight gain occurs in monkeys and not rats supports the notion that physiological differences in the control of energy balance in rodents may contribute to the inability to model VPA-induced weight gain in rodents.

Differences in the pharmacokinetic properties of VPA between species may also account for VPA actions in these different models. The occurrence of VPA-induced PCOS or weight gain is suggested to occur independent of VPA dosage or serum levels

in humans (105, 391). Dosages of VPA treatment in patients range from 20-50 mg/kg/day, resulting in trough serum levels of VPA ranging between 45-120 μg/ml (25, 391). In rodent studies examining VPA-induced PCOS, we and others, have treated rats with VPA dosages of 50-600 mg/kg/day, resulting in average trough serum levels of VPA between 3 - 74 μmol/L (0.4-10.6 μg/ml) (79, 133, 134, 154). Thus, in rodents a lack of PCOS symptoms and weight gain may be partly attributable to lower trough drug levels. Similarly in female monkeys the mean VPA serum level of 88.7 μg/ml reported 2 h following treatment suggests that trough levels 17 h after treatment, would be below the therapeutic human range and may account for the lack of VPA-induced PCOS (135). Although the serum levels of VPA are not maintained at human therapeutic ranges in rodents or monkeys, VPA treatment in these species can model the anticonvulsant and teratogenic properties of VPA (reviewed in 11).

Within the class of psychotropic medications, weight gain is associated with treatment with lithium, some of the antidepressant medications, and especially the atypical antipsychotics (e.g. olanzapine, risperidone, clozapine) (reviewed in 165, 392). Others have tried to establish animal models for weight gain induced by other psychotropic medications (393-397). Lithium, risperidone and olanzapine treatment have been demonstrated to be associated with weight gain in rats. Surprisingly however, 4/5 studies report this weight gain only occurs in female, and not male rats (393-397). Since these medications can induce weight gain in both men and women, it appears that there are no existing models that mirror the human response to these compounds. In contrast, however, the newer anticonvulsant and mood-stabilizing drug, topiramate has been demonstrated to induce weight loss both in clinical patients, and in lean and obese rats

(398-400). This suggests that the effect of topiramate on energy balance has a similar outcome in rodents and humans, which has not been demonstrated to be true for those medications in psychiatry that induce weight gain.

VPA Effects on Adipocyte Development and Leptin Production

In trying to elucidate VPA effects on energy balance, I examined the affects of VPA in adipocyte development and mature adipocyte biology and demonstrated that VPA attenuates adipogenesis and significantly reduces leptin production and secretion. The attenuation of adipogenesis by VPA was associated with a reduction in C/EBPα protein expression and inhibition of HDAC activity. In adipocytes acute VPA treatment also reduced leptin production and secretion through an unknown mechanism that was not associated with alterations in lipid metabolism, leptin mRNA degradation, or expression of specific transcription factors known to effect leptin production. These findings were obtained using a dosage of 1 mM (143 μg/ml) VPA, which is commonly used in experiments examining the *in vitro* effects of VPA (31, 40, 61, 63) and is generally accepted to be within a reasonable therapeutic range.

Since VPA treatment in humans is associated with weight gain and an increase in serum leptin levels, the results obtained were opposite to the original hypothesis that VPA would induce adipogenesis, increase adipocyte size, and increase leptin production and secretion. VPA *in vitro* was demonstrated to attenuate the development of new adipocytes and decrease leptin production and secretion. Although I did not directly measure the size of VPA-treated adipocytes there were no alterations in the levels of TAG or intracellular free fatty acids following treatment, indicating these cells did not have increased lipid storage, which is characteristic of larger adipocytes. Also, rats

treated with VPA did not exhibit increased weight when compared to untreated animals. These results support the theory that VPA treatment does not alter the size or number of adipocytes.

No published studies have examined whether there is a significant alteration in the number or size of adipocytes, or the amount of leptin secretion from adipocytes in WAT in patients following VPA treatment. In patients that gain weight on VPA, I would speculate that in WAT there would be an increase in the number or size of adipocytes, as well as an overall increase in leptin secretion. This prediction is based on the evidence that VPA induces weight gain in humans, and models of human obesity associating weight gain with an increase in the number and/or size of adipocytes, as well as an increase in serum leptin levels. The correlation between number and size of adipocytes and weight gain in patients treated with lithium demonstrated that only the number of adipocytes is significantly correlated with weight gain, with individuals that gain more weight having more adipocytes within their WAT (401). In order to assess whether VPA also alters the number or size or adipocytes or leptin secretion from adipocytes, future studies obtaining adipose tissue biopsies could measure adipocyte cell number, size, and leptin secretion in patients treated with VPA, as compared to weight-matched controls.

If the prediction is true that those who gain weight with VPA therapy have an increase in adipocyte cell number, size, and/or levels of leptin, how can one rationalize this with the attenuation of adipocyte development and leptin secretion by VPA observed in the experiments presented in this thesis? One possibility could be that the inhibition of leptin secretion by VPA can acutely result in enhanced appetite that, over time, could produce a gain of adiposity leading to enhancement of leptin secretion. To test this

hypothesis it would be necessary to measure serum levels for leptin in hours to days following the onset of VPA treatment, to observe whether there is an initial reduction in leptin serum levels followed by a rise in leptin serum levels. Another possibility could be that weight gain and alterations in leptin are due to the effects of VPA on other tissues (e.g. CNS, pancreas, gut), which may over compensate for the effect that VPA has within the adipocyte, resulting in the net balance of weight gain. The effects of VPA within the hypothalamic areas of the brain that control energy balance have not been examined and it is possible that VPA could directly modulate signals that control energy balance. Learning more about the effects of VPA on the different areas of the body controlling energy balance will help clarify how the peripheral and CNS effects of VPA contribute to the development of weight gain. For example, VPA has direct effects on increasing insulin secretion from pancreatic β-cells, which has led to the hypothesis that VPA may increase weight by increased serum insulin levels (131). In support of this theory insulin has been demonstrated to be adipogenic, a potent stimulator of leptin production and secretion (327), and a known inducer of weight gain in diabetic patients (402). Current work in our laboratory is now examining whether VPA is able to attenuate leptin secretion in the presence of higher dosages of insulin, in order to explore what happens to leptin production and secretion in the presence of increased levels of insulin and VPA.

Since weight is the outcome of energy balance (reviewed in 155), in humans that gain weight with VPA therapy, a disruption of energy balance has occurred and the increase in weight is due to either an increase in energy intake or decrease in energy expenditure. This point raises the question of whether VPA affects either energy intake or expenditure, or both? No prospective studies have addressed VPA effects on these

two separate indices and although some data suggests that patients do not eat more, even a small alteration in food intake with no alterations in expenditure can lead to alterations in weight. For example, ingestion of around 500 kcal/day could account for a gain of approximately 4.5 kg in 10 weeks, which is comparable to the weight gain reported VPA-treated patients (reviewed in 165). This intake could represent eating one chocolate bar and a soft drink every day, thus small changes in appetite or diet may not have been detected in previous studies, yet these small changes could induce a significant increase in weight over time if they occur with no alterations in energy expenditure. VPA effects on energy expenditure have not been rigorously tested. Only one case report describes that VPA therapy in children does not alter resting energy expenditure (403, 404). It is possible to predict that energy expenditure may be decreased in patients receiving VPA, due to the side effects of sedation or lack of energy. However, these are not common side effects of VPA therapy, and patients may become more active following effective therapy since they may reengage in work or other activities (reviewed in 8). In order to clarify these hypotheses, the relative contribution of energy intake versus expenditure will need to be measured in prospective long-term trials with patients on VPA.

Treatment for Patients Experiencing VPA-Induced PCOS or Weight Gain

For clinicians and their respective patients who experience PCOS following the onset of VPA treatment, it is difficult to determine the best treatment plan. It appears that in order to reverse PCOS occurring with VPA therapy, it may be necessary to discontinue VPA treatment (reviewed in 103, 405). However, current clinical guidelines support that a decision to switch medications must take into consideration the beneficial effects of VPA therapy in individual patients, weighted against the risks associated with

discontinuation of VPA, balanced with the risks associated with PCOS in individual patients (103). For patients that decide to switch medications there is evidence supporting the reversibility of PCOS symptoms. Favorable clinical outcomes including sustained antiepileptic effects and discontinuation of PCOS symptoms, have been documented for patients with epilepsy that discontinue VPA therapy and switch to treatment with lamotrigine following the development of PCOS (107, 142). In one preliminary report substitution of lamotrigine for VPA therapy in epileptic patients led to an immediate change in endocrine hormone levels (reduction in testosterone and LH) even before the VPA was withdrawn (107, 142), suggesting that adjunctive lamotrigine treatment may also be useful in reducing PCOS symptoms following VPA therapy.

For patients that gain weight in association with VPA therapy most treatment plans aim to minimize weight gain while maintaining therapy (reviewed in 165). Add-on pharmacotherapy is one method that has been used to achieve weight loss with psychotropic medication such as VPA. For example, there is a case study reporting adjunctive therapy with metformin, an antidiabetic agent, can reduce weight gain occurring during VPA treatment (406). However strategies to counteract drug-induced weight gain by treatment with other medication are poorly developed and may even induce other more serious side effects (reviewed in 165). For some patients, the discontinuation of VPA treatment may be an acceptable solution for the management of VPA-induced weight gain, especially if VPA treatment can be replaced with other therapeutic agents, such as topiramate, which is being described as an effective anticonvulsant and mood stabilizer, and appears to induce weight loss instead of weight gain (407). One disadvantage of this option for patients with bipolar disorder, is that

within the class of mood stabilizers both of the first-line recommended medications, VPA and lithium induce weight gain (407), therefore if patients choose to discontinue VPA or lithium therapy they will be accepting in most cases to receive a medication that has not been as well documented to be as effective in treating the symptoms associated with bipolar disorder.

Common sense also suggests that more education about the side effects induced by VPA prior to treatment onset may help reduce there rate of occurrence, especially for weight gain. Currently, the drug information that is supplied by Abbott for VPA states "Both anorexia with some weight loss and increased appetite with weight gain have also been reported" (408). Moreover, under metabolic side effects the product insert states: "There have been rare spontaneous reports of polycystic ovary disease. A cause and effect relationship has not been established". These statements are in disagreement with the clinical literature that is reviewed in the introduction and supports that VPA treatment is often associated with significant weight gain in at least 20% of patients and the induction of PCOS in female patients more often then one would consider to be "rare".

When VPA was introduced as a therapeutic drug, the ability of a medication to reduce seizure frequency associated with epilepsy or mood swings associated with bipolar disorder were striking advances in medicine. Now there are multiple medications available that are able to produce these efficacious properties. Within the last two decades, there is more awareness about the side effects that are associated with VPA. This is clearly not due to the marketing of VPA, but is partially a result of more people receiving VPA and therefore experiencing these side effects associated with VPA treatment. Moreover, since multiple medications are now available to treat epilepsy,

bipolar disorder, or migraine headaches, there is competition between pharmaceutical companies to demonstrate that other medications have better side effect profiles compared to VPA. Although there are other options for therapeutic agents, VPA is considered to be a highly beneficial and safe to use medication, as proven by its sales. As research continues to unravel the mechanisms by which VPA induces side effects such as PCOS or weight gain, it is hoped that less patients will experience these side effects and that future drug development will produce agents that are as safe and effective therapeutically, yet not associated with the induction of PCOS or weight gain.

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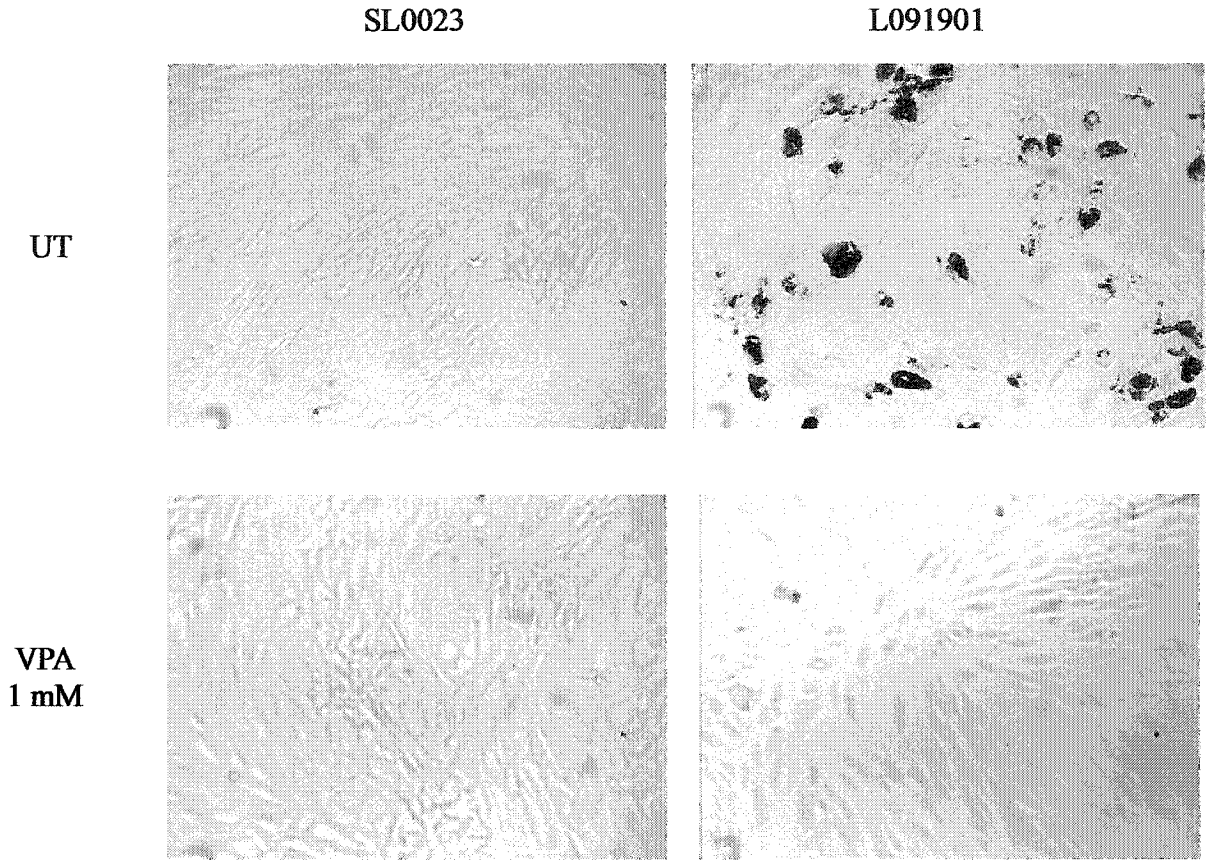
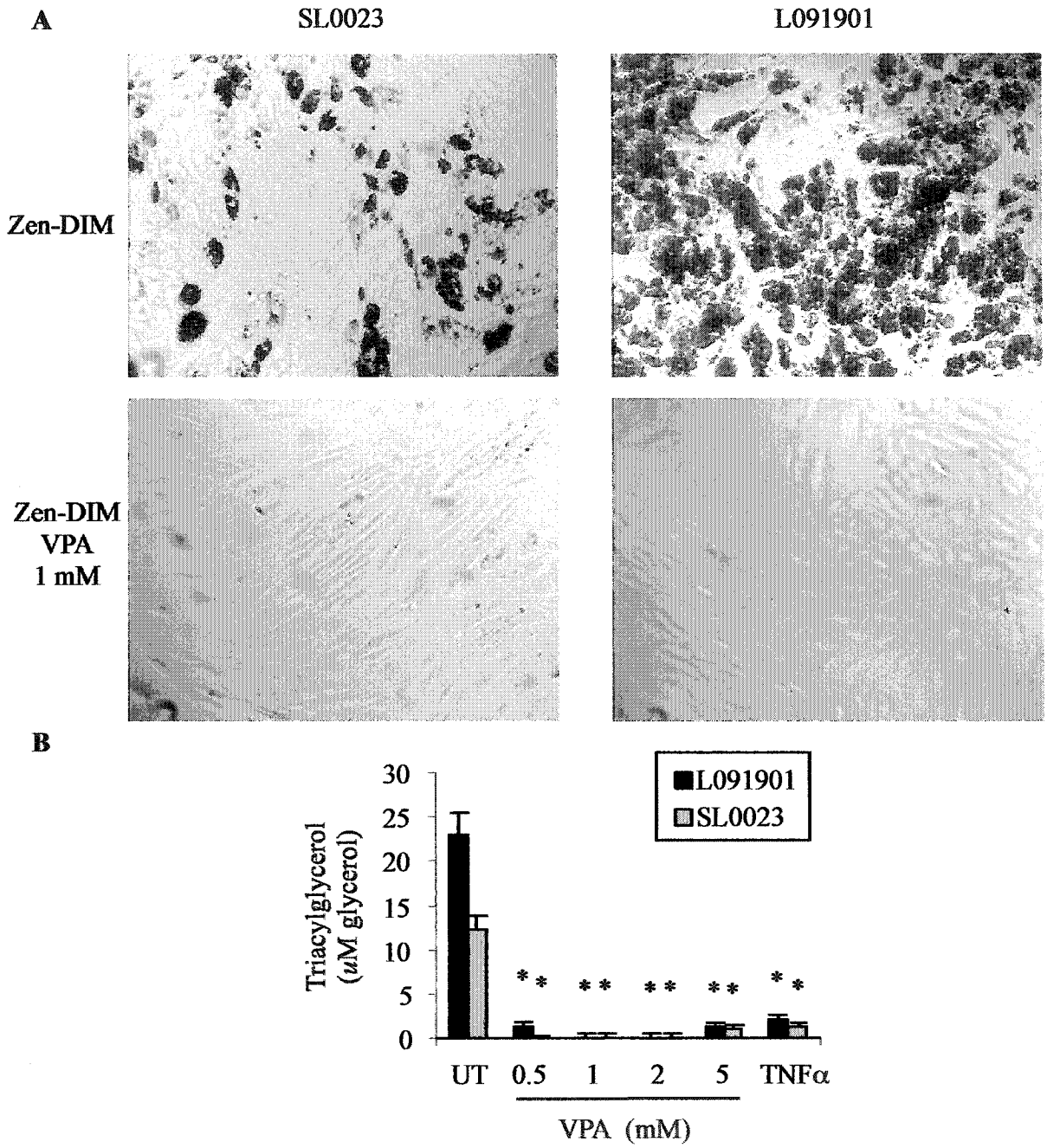
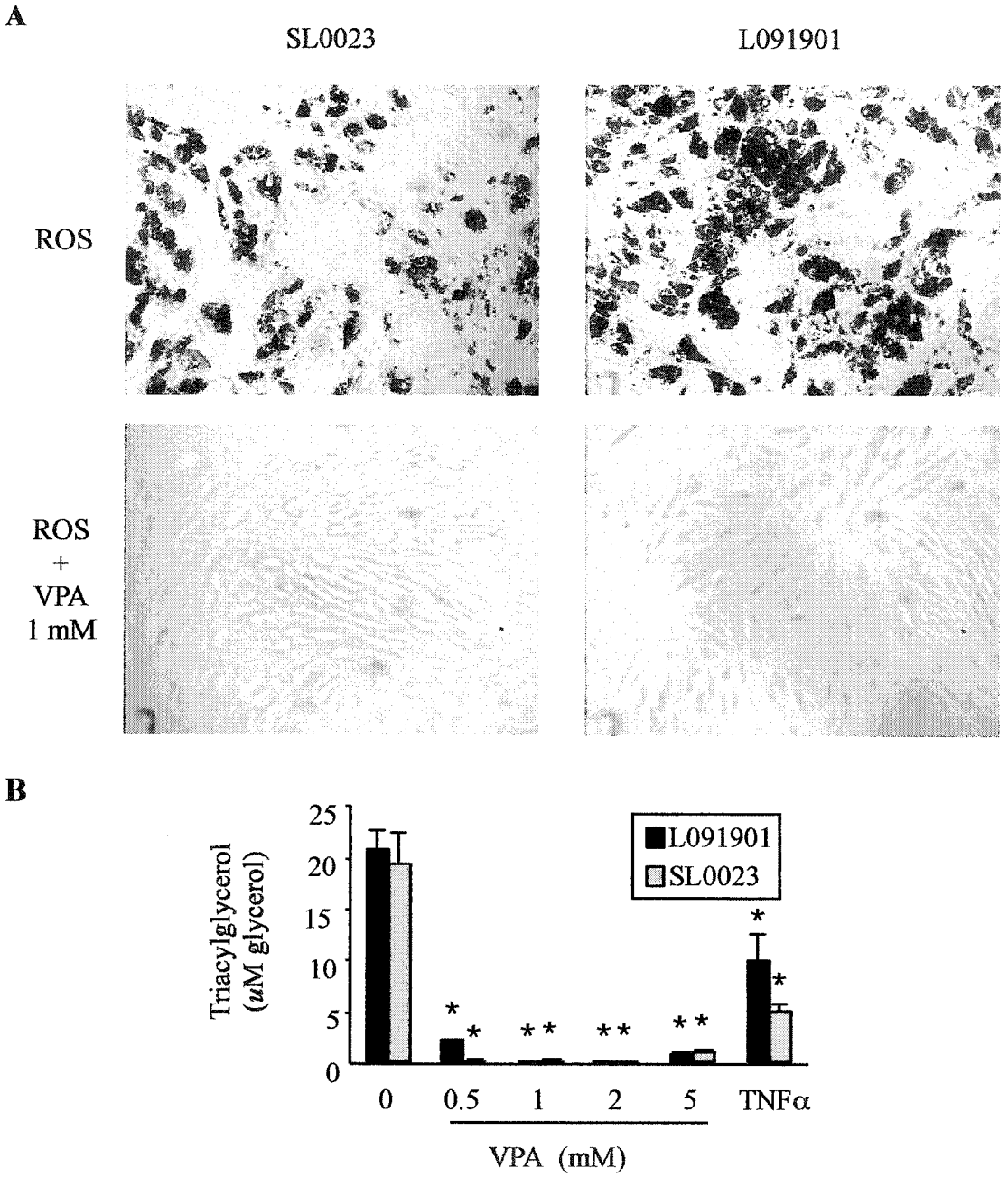


Appendix A VPA does not induce differentiation of human preadipocytes
Representative photomicrographs of two lots of subcutaneous preadipocytes from one individual (L091901) and cells pooled form six individuals (SL0023) stained with Oil Red O 12 days after initiation of differentiation. Preadipocytes did not differentiate in the presence of normal growth medium in the absence (UT) or presence of 1 mM VPA, with the exception of a few cells in the L091901 sample differentiated into adipocytes.



Appendix B VPA inhibits Zen-DIM induced differentiation of human preadipocytes **A)** Representative photomicrographs of two lots of subcutaneous preadipocytes from one individual (L091901) and cells pooled from six individuals (SL0023) stained with Oil Red O 12 days after initiation of differentiation in the presence of Zen-Bio's differentiation medium (Zen-DIM), and/or daily treatment with 1 mM VPA. VPA did not induce adipocyte formation in absence of Zen-DIM, and inhibited adipocyte formation in the presence of Zen-DIM. **B)** Quantification of TAG levels from cells described in (A) that were treated daily with VPA (0-5 mM) or 4 ng/ml of tumor necrosis factor α (TNFα). Data represents mean from one experiment performed in triplicate, post-hoc compared to UT, * $p<0.01$.



Appendix C VPA reduces PPARγ-induced differentiation of human preadipocytes
A) Representative photomicrographs of two lots of subcutaneous preadipocytes from one individual (L091901) and cells pooled from six individuals (SL0023) stained with Oil Red O 12 days after initiation of differentiation with the PPARγ agonist, rosiglitazone (ROS, 1 μM). Daily VPA (1 mM) inhibited differentiation of both L091901 and SL0023 cells. **(B)** Quantification of triacylglycerol levels demonstrating cells differentiated with 1 μM ROS had more triacylglycerol than cells that were co-treated daily with VPA (0-5 mM). Cells treated with 4 ng/ml of TNFα at each medium change are shown. Data represent mean values from one experiment performed in triplicate, post-hoc compared to UT, * $p<0.001$.

Appendix D

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