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LEPTIN RECEPTORS IN CAVEOLAE: REGULATION OF LIPOLYSIS IN 3T3-L1 ADIPOCYTES

Gentle P. Chikani
University of Kentucky, gpchik2@uky.edu

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ABSTRACT OF THESIS

LEPTIN RECEPTORS IN CAVEOLAE: REGULATION OF LIPOLYSIS IN 3T3-L1 ADIPOCYTES

The present study has tested the hypothesis that leptin receptors are localized in caveolae and that caveolae are involved in the leptin-induced stimulation of lipolysis in 3T3-L1 adipocytes. Leptin, a peptide hormone, is secreted primarily by adipocytes and has been postulated to regulate food intake and energy expenditure via hypothalamic-mediated effects. Exposure to leptin increases the lipolytic activity in 3T3-L1 adipocytes. We isolated caveolae from 3T3-L1 adipocytes using a detergent free sucrose gradient centrifugation method. Leptin receptors were localized in the same gradient fraction as caveolin-1. Confocal microscopic studies demonstrated the colocalization of leptin receptors with caveolin-1 in the plasma membrane, indicating distribution of leptin receptors in the caveolae. We disrupted caveolae by treating cells with methyl-β-cyclodextrin and found that leptin induced lipolytic activity was reduced after caveolae disruption, indicating an important role of caveolae in the signaling mechanism of leptin.

KEY WORDS: leptin, leptin receptors, caveolae, caveolin-1, lipolysis.

Gentle P. Chikani

11/07/04

LEPTIN RECEPTORS IN CAVEOLAE: REGULATION OF LIPOLYSIS IN 3T3-L1 ADIPOCYTES

By
Gentle Chikani

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Director of Thesis

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THESIS

Gentle Chikani

The Graduate School

University of Kentucky

2004

LEPTIN RECEPTORS IN CAVEOLAE: REGULATION OF LIPOLYSIS IN 3T3-L1 ADIPOCYTES

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Nutritional Sciences at the
University of Kentucky.

By

Gentle Chikani

Lexington, Kentucky

Director: Dr. Eric J Smart, Professor of Pediatrics and Physiology

Lexington, Kentucky

2004

MASTER'S THESIS RELEASE

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INTRODUCTION

Background

Obesity is a major health risk in much of the human population. It is now recognized that obesity is a serious, chronic disease. The prevalence of obesity is increasing at an alarming rate in developed and developing countries. Obesity is a disease that affects nearly one-third of the adult American population (approximately 60 million). Each year, obesity causes at least 300,000 excess deaths in the U.S., and healthcare costs of American adults with obesity amount to approximately $100 billion. Environmental and behavioral changes brought about by economic development and urbanization are linked to the rise in global obesity. The morbidities associated with obesity, such as cardiovascular disease, type 2 diabetes and osteoarthritis, represent a major health risk to the obese population. Physiologically, obesity is a disorder of energy balance. Whenever energy intake exceeds energy expenditure, the excess of energy is stored as fat. Energy storage and energy expenditure are highly regulated by the complex interactions between hormone axes in the periphery, which is ultimately controlled at the level of the central nervous system (CNS). Leptin is one of the important regulators in the CNS and the periphery.

Leptin is a 16 kD peptide hormone, synthesized and secreted primarily by adipocytes. The word leptin, meaning thin, refers to its anti-obesity effect by regulating food intake and energy balance, which is believed to be the primary physiological function of the hormone. Its circulating levels show correlation with body mass index and the amount of body fat stores (31, 39, 83). Both leptin deficiency (*ob/ob* mice) and leptin

resistance (*db/db* mice, having a defective leptin receptor) lead to an obese phenotype, characterized by hyperphagia and reduced energy expenditure (75, 143). The weight of these obese animals stabilizes at 60-70 g, compared with 25-30 g in control littermates (figure 1).

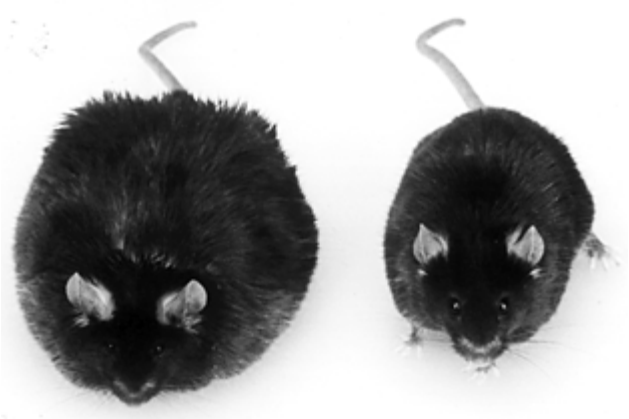

Figure 1. Both of these mice (C57BL/6J *ob/ob*) have mutation in the *ob* gene. The mouse on the right, who received daily injections of leptin, weighed 35 g. while the mouse on the left, who did not receive leptin, weighed 67 g. (Adapted from Glick) (160).

Leptin synthesis and its physiological role:

Leptin is the product of the *ob* gene that was discovered by Zhang et al. (1994) using positional cloning technique (156). The gene is located on chromosome 7 in humans and on chromosome 6 in the mouse. The ob gene encodes a protein that shows a high degree of homology between these two species. Leptin is primarily synthesized by adipocytes, but it is also produced by gastric mucosa, skeletal muscle, mammary epithelium, placenta, bone marrow and pituitary (2, 151). Leptin is translated as 167 amino acid protein with an amino terminal secretory signal sequence of 21 amino acids.

This signal sequence is truncated in microsomes and thus leptin circulates in blood as a protein of 146 amino acids (156).

The adipose-derived hormone, leptin, is an important regulator of energy homeostasis (41, 135). Hypothalamic stimulation by leptin regulates the expression of a number of orexigenic and anorexigenic neuropeptides, which in turn, results in decreased food intake and increased energy expenditure. It has been postulated that leptin functions as a sensor of fat mass, indeed plasma leptin levels are correlated with body weight and fat mass in rodents and human (31, 39). Apart from its pivotal role in energy homeostasis, body weight, appetite and fat stores, leptin has diverse effects on the neuroendocrine axis. Leptin interacts with hypothalamic-pituitary-adrenal axis and influences sexual maturation. Leptin accelerates puberty in wild type mice; facilitate reproductive behavior in rodents, and restores puberty and fertility in *ob/ob* mice (1, 19, 20, 149).

Leptin may play an important role in development, as evidenced by formation of leptin in placenta, widespread expression of leptin and its receptors in the fetal tissues, and stimulation of hematopoiesis and angiogenesis by leptin (47, 63, 86, 126). It also affects bone formation, phagocytic activity of macrophages, and liver function (34, 151).

Leptin exerts several acute metabolic effects. It decreases glucose and insulin levels acutely in *ob/ob* mice before detectable weight loss (3), and also stimulates gluconeogenesis and glucose metabolism in wild type rodents (29, 70, 111). Leptin alters lipid partitioning in skeletal muscle and stimulates lipolysis and fatty acid oxidation in adipocytes, (29, 101) which is discussed later in detail.

Regulation of leptin expression:

Obese humans and other mammals have increased adipose *ob* mRNA and serum leptin levels, suggesting that leptin expression is influenced by the status of energy stores in fat (31, 39, 56, 82, 83). Plasma leptin levels are directly related with the total body fat stores (31, 39, 83); however it's still not known whether increased triglyceride levels or lipid metabolites associated with increased adipocyte size influence leptin expression. Leptin levels increase after several days of overfeeding in humans and within an hour after a meal in rodents (57, 72, 115). Leptin levels decrease within hours after initiation of fasting in both species (12, 40, 115). Regulation of leptin expression by nutrition is partly regulated by insulin. Leptin expression is directly stimulated by insulin in isolated adipocytes (109) and also increases after peak insulin secretion during the feeding cycle (109, 115). Insulin increases leptin levels when injected into rodents (115).

Controversy exists over whether exercise has any effect on leptin levels. Hypoleptinemia was detected in female and male elite gymnastics of pubertal age (152). Leptin gene expression was decreased after 4 weeks of exercise training in Sprague-Dawley rats (157). Hicky et al. reported that moderate intensity aerobic exercise in women reduced leptin levels by 17.5 % after 12 weeks (62). However, other studies have shown that moderate intensity aerobic exercise and acute and chronic exercise in women and men do not affect the leptin levels (61, 71, 102, 104).

Leptin levels are regulated by other factors. Leptin synthesis is also stimulated by infection, endotoxins and cytokines such as tumor necrosis factor (TNF), leukemia inhinitory factor (LIF), and interleukin-1 (IL-1) in humans and rodents, indicating that the rise in leptin as a result of increased cytokine levels may also contribute to the anorexia and weight loss in these inflammatory conditions (13, 52, 69, 117).

The prepubertal increase in the leptin expression precedes the rise in estradiol and testosterone and is believed to be involved in the maturation of gonadal axis (3, 84). Females have higher leptin levels than males when matched by age, weight, or body fat (110, 114). This could be due to the difference in body fat distribution and testosterone levels in both sexes (11, 110).

Leptin receptors:

Leptin exerts its hormonal effects by binding to its specific transmembrane receptor known as ObR or leptin receptors. Leptin receptors are the products of the diabetes (*db*) gene (64), which was discovered by Tartaglia et al. using an expression cloning strategy in mouse (156). Leptin receptors belong to the class I cytokine family (142) which is characterized by a single membrane spanning domain that associates with a class of protein tyrosine kinase known as Janus kinase (JAK).

Alternative splicing:

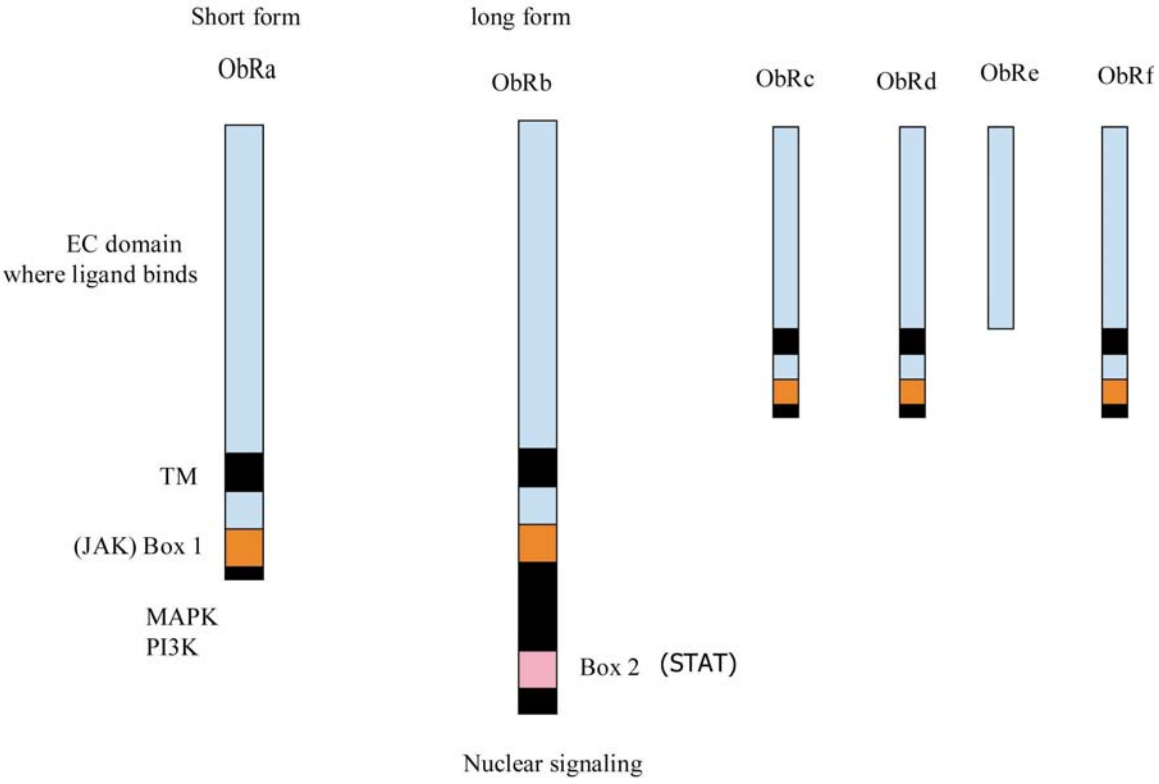

Figure 2. Leptin receptor isoforms. EC – extracellular domain, TM – transmembrane domain.

The leptin receptor gene is alternatively spliced to produce at least six different isoforms (ObRa – ObRf). All isoforms of leptin receptor share an identical extracellular, ligand binding domain (at amino terminus) of 816 amino acids, while the intracellular domain at the C – terminus is distinct (75, 143). ObRa, ObRb, ObRc, ObRd and ObRf contain transmembrane domains of 34 amino acids, while ObRe is truncated before the membrane spanning domain and is therefore likely to be secreted (75). The secreted extracellular domains of other cytokine receptors are known to function as specific

binding proteins (59). ObRb is the only long form of leptin receptor which has a long 302-residue intracellular domain, while the other isoforms have a short (32-97 amino acids) intracellular domain (41). ObRb is predominantly expressed in the brain but, it is also present at a lower level in other peripheral tissues like skeletal muscles, adipocytes and liver. The short leptin receptors are expressed in choroids plexus, vascular endothelium, and peripheral tissues like kidney, liver, lung, and gonads, where they may serve a transport and/or clearance role (8, 37). In extra-brain tissues, expression levels of the total ObRb account for only a small part of the total ObR expression (49, 66, 67).

The ObR does not have an intrinsic tyrosine kinase domain, therefore binds cytoplasmic kinases, mainly, JAK2, a member of JAK family (48). The short form of leptin receptor has the JAK binding site, but only the long form of leptin receptor, ObRb contains intracellular motifs required for the binding and activation of STATs (signal transducers and activators of transcription) (9, 24, 49, 145). Activation of ObRb, and to a lesser extent ObRa, activates JAK dependent signaling to pathways other than STAT, such as MAP kinase (9). The relative importance of these different signaling pathways in leptin action is still unknown.

ObR induced signal transduction:

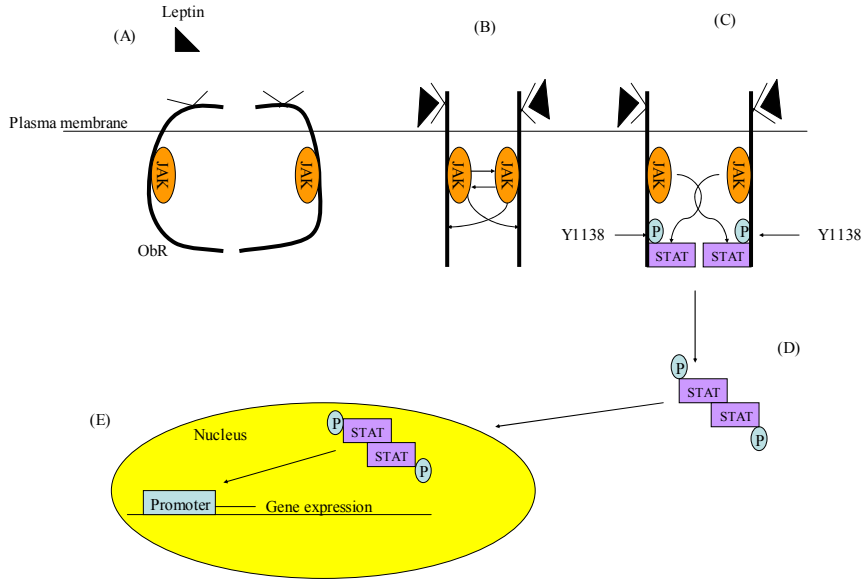

Figure 3. ObR-induced signaling mechanism in the hypothalamus.

The figure shows a defined JAK/STAT signaling pathway in the hypothalamus. When leptin binds to its receptor, it causes receptor dimerization. Both the long and short form of leptin receptors are capable of forming homodimers even in the absence of the ligand, and the extent of this association does not seem to change after leptin stimulation. (33, 92, 154), yet dimerization is necessary for the leptin signaling (153). Leptin receptor binds to leptin in a 1:1 stoichiometry. Thus each receptor binds to one molecule of leptin forming a tetrameric complex (33). Cytoplasmic tyrosine kinases, members of the JAK family, recognize and associate with a specific membrane-proximal domain of the receptor upon ligand binding (60). JAK2 associates with leptin receptor via the box 1

motif common to all receptor isoforms (except ObRe). Leptin receptor may form dimers under nonstimulated conditions, yet the conformation of the receptor is likely to prevent close proximity of JAKs. Upon ligand binding the number of receptor dimmers does not change appreciably, yet, a conformational change allows the juxtaposition of JAKs, which then become activated. The activated JAKs can tyrosine phosphorylate each other and the tyrosine residues on ObRb. Phosphorylation of tyr-1138 on ObRb allows binding of the STATs, which then become phosphorylated by JAKs. The activated STATs dissociate from the receptor, dimerize and translocate to the nucleus and bind with specific DNA elements in the promoters of target genes to regulate gene expression (32).

It has been reported that the MAPK (ERK1/ ERK2) pathway can be stimulated by both long and short forms of leptin receptors (7, 9). Murakami et al. have also reported the mRNA expression of immediate early genes c-fos, c-jun and jun-B which are induced by leptin addition in CHO cells expressing ObRa (90). Thus it appears that short forms of leptin receptors also have the potential to mediate signal transduction. Although the short forms have an attenuated signal, their relative abundance in peripheral tissues may mediate specific effects of leptin on those tissues.

Caveolae:

Caveolae were initially described as 50-100 nm smooth surfaced omega or flask-shaped invaginations of the plasma membrane (97). Caveolae can fuse to form grape-like structures (119) and tubules (99) that are significantly larger than 100 nm. Morphologically, caveoale are abundant in adipocytes, endothelial cells, muscle cells and lung epithelial cells (38, 94). Caveolae have a unique lipid composition and are mainly

composed of cholesterol and sphingolipids (sphingomyelin and glycosphingolipids). Cholesterol is crucial to the structure and function of caveolae. Depleting cells of cholesterol reduces the number of caveolae at the cell surface (54, 113). Caveolins are the defining protein components of the caveolae. Caveolin is believed to encase and cover the surface of caveolae by the means of its ability to form homo and oligomeric complexes (112).

Functional role of caveolae:

Caveolae are involved in a variety of cellular activities such as transcytosis, potocytosis (5), uptake of cholesterol (128) and signal transduction. Caveolae also play a significant role in cholesterol trafficking. Caveolin is a cholesterol binding protein that forms a chaperone complex with heat shock protein-56 (HSP-56), cyclophilin A, and cyclophilin 40 to bind the cytosolic pool of cholesterol, presumably with the function of trafficking cholesterol between membrane compartments (91). Apart from this, newly synthesized cholesterol translocates from the ER to caveolae before diffusing into the bulk plasma membrane in a caveolin dependent manner. This suggests that caveolin may translocate cholesterol from ER to the cell surface independent of vesicles (130).

Recent research has indicated that caveolae serve to compartmentalize various signaling molecules (76, 79). Caveolin-1 interacts with various signaling molecules like, H-Ras, c-Src, insulin receptor, endothelial nitric oxide synthase (eNOS), G_{α} subtypes, protein kinase A (PKA), and inhibits their catalytic activity (77, 100). Thus the interaction of the caveolin-1 with the caveolin binding domain of that protein can further inhibit the downstream signaling events.

Caveolae are enriched in molecules that play an important role in intracellular signal transduction. These molecules are G-protein coupled receptors, heterotrimeric G proteins, protein kinase C's (PKCs), receptor tyrosine kinases, nitric oxide synthase (NOS), components of Ras-mitogen activated protein (MAP) kinase. As a consequence, these structures participate in the cross talk between different signaling molecules. Thus caveolae function to integrate numerous signaling events at the cell surface. Disruption of caveolae causes a wide range of disorders (136) – such as cancer (129), Alzheimer's disease (68), and muscular dystrophy (87).

Lipid metabolism:

Triglycerols constitute both ~ 90 % of the dietary lipid and the major form of metabolic energy storage in humans. Triglycerols consist of glycerol triesters of fatty acids such as palmitic and oleic acids. Like glucose, they are metabolically oxidized to CO_2 and H_2O. The lipid digestion products absorbed by the intestinal mucosa are converted by these tissues to triglycerols and then packaged into lipoprotein particles called chylomicrons. These, in turn, are released into the blood stream via the lymph system for delivery to the tissues. Mobilization of triglycerols stored in adipose tissue involves their hydrolysis into glycerol and free fatty acids by hormone-sensitive lipase (HSL). The free fatty acids are released into the blood stream, where they bind to the albumin. Fatty acid oxidation is regulated largely by the concentration of free fatty acids in the blood stream, which in turn is controlled by the rate of hydrolysis of triglycerols in the adipocytes by HSL. The enzyme is so named because it is highly regulated by phosphorylation and dephosphorylation in response to the hormonally controlled cAMP

levels in the cell. Epinephrine, norepinephrine and glucagons act on adipocytes to increase the cAMP levels, which in turn, activates cAMP dependent protein kinase (PKA). PKA increases phosphorylation of HSL. Phosphorylation activates HSL and thus increases the lipolysis in the adipocytes, raising the fatty acid concentration in the blood stream, and ultimately activating ß–oxidation pathways in other tissues like liver and muscle. Insulin has the opposite effect of glucagon and epinephrine. It stimulates the formation of glycogen and triglycerols. It is secreted in response to the high blood glucose levels and decreased cAMP levels. This, in turn, dephosphorylates the HSL and inactivates the enzyme, thereby reducing the rate of lipolysis and the amount of fatty acids available for oxidation.

Leptin and lipid metabolism/ lipolysis:

Lipolysis is the process of triglyceride hydrolysis, via mono- and diglyceride intermediates, to free fatty acids and glycerol (108). It is a critical event controlling lipid storage and lipid mobilization. Lipolysis occurs in all triglyceride-storing tissues, the most important ones are adipose tissue, liver and muscle (both skeletal and cardiac). The vast majority (>95 %) of body's triglycerides are found in the adipose tissues (108). Lipolysis occurs in adipocytes, releasing free fatty acids into circulation. Thus adipose tissue lipolysis is the major regulator of the body's supply of lipid energy as it controls the release of fatty acids into the plasma.

Regarding body composition, leptin acts as a sensing hormone or "lipostat" in a negative feedback control from adipose tissue to hypothalamic receptors. Plasma leptin levels are directly related with the total body fat stores (31, 39, 83). In other words, leptin

informs the brain about the body fat storage and thus controls the feeding behavior, metabolism, and endocrine physiology to be coupled with nutritional status of the organism. Leptin acts via centers in the hypothalamus, and suppresses appetite by inhibiting orexigenic factors such as Neuropeptide Y and by increasing thermogenesis via sympathetic innervation of brown adipose tissue (118). The functional leptin receptor is highly expressed in the hypothalamus, but it has also been found in other organs and tissues like liver, heart, lungs, kidneys, the small intestine, spleen, testes, and in adipose tissue (49, 75, 143). This suggests that leptin can act directly on peripheral tissues, independently of any hypothalamic mediation.

Physiological studies in mouse models have shown that leptin administration to the *ob/ob* mice lowered their body weight, percent body fat and food intake and also enhanced their energy expenditure (17, 55, 101). These results strongly implicated leptin as a negative feedback signal, which reflects body adiposity, by its action on hypothalamus (17, 55, 101). The in-vitro lipolytic effects of leptin on white adipose tissue, provides strong evidence for the autocrine/paracrine role of leptin (43, 122). Thus leptin regulates fat accretion by influencing a combination of central and peripheral pathways. Siegrist-Kaiser et al. have shown the direct effects of leptin on the white and brown adipose tissue (WAT & BAT respectively) at the metabolic and molecular levels (125). They observed a 9-fold and 16-fold increase in the rate of lipolysis in WAT fat pads from lean Zucker *Fa/fa* rats, after incubation with 0.1 and 10 nM leptin for 2 hours, respectively. At the level of gene expression, they found, leptin treatment for 24 hours increased malic enzyme and lipoprotein lipase RNA 1.8±0.17 and 1.9±0.14-fold, respectively. The chronic peripheral intravenous administration of leptin in vivo for four

days increased glucose utilization index by 1.6 fold in BAT. These studies suggest that leptin has a direct effect on BAT and WAT, resulting in the increased expression of certain genes that are responsible partially in glucose utilization and lipolysis in leptin treated adipose tissue (125).

Leptin stimulates fatty acid oxidation in vivo (22, 23) and in vitro (150). Gema et al have examined the effects of leptin, on lipolysis in fat cells of different types of mice (43). Exposure to leptin increased the lipolytic activity of fat cells obtained from lean mice in a dose independent manner. A greater stimulation was observed when adipocytes from *ob/ob* mice were examined. Adipocytes derived from *ob/ob* mice responded in a dose dependent manner to leptin (43).

Adenoviral transfer of the leptin gene into normal rats causes rapid loss of all visible body fat within 7 days (21). Hyperleptinemic fat loss is not accompanied by elevations in plasma free fatty acid (FFA) levels, or ketones or by ketonuria, unlike the ketonic fat loss in starvation or insulin deficiency, in which fatty acids and glycerol are released proportionately from the adipocytes (122). One possible explanation could be the fatty acids released by adipocytes are oxidized inside the adipocytes. This is supported by the demonstration that the expression of two major enzymes of long chain fatty acid oxidation, acyl CoA oxidase (ACO), and carnitine palmitoyl transferase-1 (CPT-1) are strikingly increased in the adipocytes of the hyperleptinemic rats during the disappearance of their fat (158, 159). Fruhbeck et al. have shown that in adipocytes from lean rats, preinculated with adenosine deaminase (ADA), leptin caused a concentration-related stimulation of lipolysis (45). These results suggest that the lipolytic effect of leptin is located at the adenylate cyclase/G_i proteins step (45). Overall, the mechanism of

leptin induced lipolysis has not been precisely described. It may involve regulation at the level of adenylate cyclase and subsequent stimulation of hormone sensitive lipase (44, 45).

Hypothesis and objectives of the present study :

Caveolins and caveolae play a role in many aspects of cellular biology, including signal transduction. Within this framework, caveolins act as scaffolding proteins to regulate the activity of numerous signaling molecules, for example caveolin-1 has been shown to have an inhibitory interaction with PKA (105). Recent studies have shown that caveolin-1 can be redirected from the caveolae to intracellular lipid droplets in a variety of cell types (46, 96, 103). Cohen et al. have addressed the role of caveolin-1 in lipid droplet breakdown, showing that caveolin-1 null mice exhibit markedly attenuated lipolytic activity (28). Lipolysis normally occurs through the stimulation of ß-adrenergic receptors and the subsequent activation of PKA (28). HSL is considered as a major lipolytic enzyme in the adipocytes, and it is the only known neutral lipid lipase regulated by PKA mediated phosphorylation (6, 81). This activation of lipolysis is strictly dependent on the PKA-mediated phosphorylation of perilipin A (81, 139, 141). Perilipin A (lipid droplet-associated phosphoprotien) functions as a protective coat (16, 26, 85, 141) surrounding the lipid droplet until phosphorylated by PKA. Phosphorylated perilipin undergoes a conformational change, leaving the lipid droplet as an open target for HSL (81, 95, 120, 134, 139, 140, 155). When HSL is phosphorylated by PKA, it translocates to the lipid droplets via an interaction with perilipin, where it acts on the stored triglycerides (15, 25, 36, 137-139). Alex et al. have shown that treatment with $ß_3$- adrenergic receptor agonist

results in a ligand induced complex formation between perilipin, caveolin-1 and the catalytic subunit of PKA in the wild type but not in caveolin-1 null fat pads (28). Thus caveolin-1 plays a crucial role in the regulation of lipolysis in adipocytes.

Various studies suggest that in addition to PKA, GTP binding protein (G-protein)-coupled receptors and cAMP can also activate mitogen-activated protein kinase (MAPK) pathways (53, 146, 148) and one of the MAPK pathways, identified in mammalian cells, is extracellular signal-regulated kinases (ERKs), p44 MAPK (ERK1) and p42 (ERK2). ß-adrenergic agonists are capable of activating ERK in adipocytes (78, 123, 133). One such study has shown that activation of the ERK pathway appears to be able to regulate adipocytes lipolysis by phosphorylating HSL on Ser^{600} and increasing the activity of HSL (51). Thus the control of lipolysis is complex and involves multiple mechanisms.

The signaling mechanism involved in leptin-induced lipolysis is still not clear. As we discussed in previous sections, when leptin binds to its receptors, it activates JAK/STAT pathway and regulates gene expression. However, for short term (acute) effects of leptin, like lipolysis in adipocytes, the mechanism or the signaling pathways involved are not understood. As discussed before, the short form of the leptin receptors can activate MAPK (ERK1/ERK2) pathway and can also induce certain gene expression (7, 9). From these studies, we speculate that the short form of leptin receptor may be involved in regulating acute effects of leptin such as lipolysis. As caveolae are enriched in various signaling molecules which play a significant role in the regulation of lipolysis, such as insulin receptors, ß-adrenergic receptors, heterotrimeric G_α and $G_ß$, MAPK, adenylyl cyclase (18, 35, 50, 65, 80, 107, 116, 131, 132), we also speculate that these structures (caveolae) may play a role in the regulation of leptin-induced lipolysis.

Thus the central hypothesis of this study is that caveolae regulate leptin-induced lipolysis in 3T3 L1 adipocytes.

In order to experimentally test this hypothesis, the following specific aims were studied.

1. To determine if leptin receptors are localized in caveolae.
2. To determine if the disruption of caveolae inhibits leptin-induced lipolysis.

MATERIALS AND METHODS

Chemicals:

Dulbecco's modified eagle's high glucose medium, calf serum, fetal bovine serum, glutamine, trypsin-EDTA and penicillin/streptomycin were from Invitrogen (Carlsbad, CA). The Bradford assay kit was from Bio-Rad (Hercules, CA). Percoll, PVDF membrane and Tween 20 were from Sigma (St. Louis, MO). Optiprep was from Invitrogen. Insulin, dexamethasone and IBMX (3-isobutyl-1-methylxanthine) were from Sigma. The anti-caveolin IgM was from BD Transduction Laboratories™ (San Jose, CA). The anti-leptin receptor was from Affinity Bioreagents (Golden, CO). Horseradish peroxidase-conjugated IgGs were supplied by Cappel (West Chester, PA). Super Signal® chemiluminescent substrate was purchased from Pierce (Rockford, IL). Isopropanol was purchased from Fisher (Hampton, NH), and hexane was from EM Science (Darmstadt, Germany). Leptin was from Calbiochem (San Diego, CA).

Cell Culture and experimental media:

3T3 L1 cells (mouse fibroblasts) were cultured in DMEM high glucose medium containing 10 % calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin and 1mM sodium pyruvate in 5 % CO_2 and 95 % air /humidified atmosphere at 37^0 C, and passaged at ~ 75 % confluence. The medium was changed every 2-3 days.

Differentiation of 3T3 L1 cells to adipocytes:

The cells were grown in 10 cm dishes (or glass cover slips for immunofluorescence studies) and were used for differentiation 2 days after they reached confluence. The differentiation was induced by treating the confluent cells with adipogenic reagents such as insulin 0.85 μM, 3-isobutyl-1-methylxanthine 0.5 mM , and dexamethasone 0.25 μM, in DMEM high glucose media containing 20 % FBS, for 3 days. After 72 hours, on day 3, the cells were incubated for additional 2 days with insulin only media (DMEM containing 0.85 μM insulin) for 2 days. And then media was changed on day 5 and 7 with FBS only media (DMEM containing 10 % serum) in order to attain maximum differentiation. The cells were used between 9 to 13 days. Approximately 90 % cells were differentiated into adipocyte phenotype as determined by accumulation of lipid droplets (58, 147).

Protein determination:

Protein content was determined by Bradford assay (14, 30). The standard curve was prepared from bovine serum albumin (1mg/ml water). The absorbance was determined at 595 nm, and the protein concentration was calculated from the standard curve.

Isolation of Caveolae:

Caveolae were isolated as described previously (131, 144).

The following buffers were used.

Buffer A: 0.25 M sucrose, 1mM EDTA, 20 mM tricine, pH 7.8.

Buffer B: 0.25 M sucrose, 6mM EDTA, 120 mM tricine, pH 7.8.

Buffer C: 50 % Optiprep (v/v) in Buffer B.

Purification of caveolae: All steps were carried out at 4^0C. A plasma membrane fraction was prepared from ten 100-mm dishes of confluent tissue culture cells (7-8 mg of total protein). Each dish was washed twice with buffer A and the cells were collected by scraping in 3 ml of buffer A. the cells were pelleted by centrifugation for 5 min, 1000 X g. The cells were resuspended in 1 ml of buffer A homogenized. We transferred the suspension to a 1.5 ml centrifuged tube and centrifuged at 1000 X g for 10 min in an Eppendorf centrifuge tubes. The post nuclear supernatant fraction (PNS) was removed and stored on ice. The pellet from each tube was resuspended in 1 ml of buffer A, homogenized and centrifuged at 1000 X g for 10 min again. The two PNSs were combined, layered on the top of 23 ml of 30 % Percoll in buffer A, and centrifuged at 84,000 X g, for 30 min in a Beckman Ti 70 rotor. The plasma membrane fraction was a visible band ~ 5.7 cm from the bottom of the centrifuge bottle. The membrane fraction was collected and placed in a SW41 tube on ice and sonicated. An aliquot of the sonicate was saved before mixing the remainder with 1.84 ml of buffer C and 0.16 ml of buffer A. After vortexing well, a linear 20 % and 10 % Optiprep gradient (prepared by diluting buffer C with buffer A) was poured on the top of the sample and then centrifuged at 52000 X g for 90 min at 4 °C. The top 5 ml of the first OptiPrep gradient was collected, placed in a fresh SW41 centrifuge tube, and mixed with 4 ml of Buffer B. The sample was overlaid with 1 ml of 15% (v/v) OptiPrep and 0.5 ml of 5% (v/v) OptiPrep (prepared by diluting Buffer B with Buffer A) and centrifuged at 52,000 × *g* for 90 min at 4 °C. A distinct opaque band was present at both interfaces. The band at the 5% interface was

collected and designated caveolae membranes. We typically obtained 10-20 μg of protein in this band (131).

Electrophoresis and Immunoblots:

Buffers

Buffer D consisted of:

20 mM Tris, pH 7.6,

137mM NaCl,

0.5 % (v/v) Tween 20.

5X sample buffer:

0.31 M Tris, pH 6.8,

2.5 % (w/v) SDS,

50 % (v/v) glycerol, and

0.125 % (w/v) bromophenol blue.

Lower tris: 1.5 M Tris, 0.4 % SDS, pH 8.8.

Reagents used for acrylamide gel preparation:

30% Acrylamide: bis	6 ml
H_2O	7.5 ml
Lower tris	4.5 ml
TEMED	20 μl
10 % Ammonium persulfate	70 μl

The samples were concentrated by trichloroacetic acid precipitation and washed in acetone (131). Pellets were suspended in 1X sample buffer that contained 1.2 % (v/v) β-mercaptoethanol and heated at 95 °C for 3 min before being loaded onto gels. The proteins were separated in a 10 % SDS-polyacrylamide gel using the method of Laemmli (74). The separated proteins were then transferred electrophoretically to polyvinylidene difluoride (PVDF) blotting membrane. The PVDF was blocked in Buffer D that contained 5% dry milk for 1 h at room temperature. The primary antibodies were diluted in Buffer D that contained 1% dry milk and incubated with the PVDF for 1 hour at room temperature. The PVDF was washed four times for 10 min each time in Buffer D with 1% dry milk. The secondary antibodies (all conjugated to horseradish peroxidase) were diluted 1:20,000 in Buffer D with 1% dry milk and incubated with the PVDF for 1 hour at room temperature. The PVDF was then washed, and the bands were visualized by chemiluminescence.

Gas Chromatography and Mass Spectrometry:

The 3T3-L1 adipocytes were prepared as described in the methods (58, 147) and were maintained in 1 % serum for 20 hours before the experiment. The 3T3 L1 adipocytes were treated with different concentrations of leptin as described in the results. To disrupt the caveolae structure, we treated the cells with 5 mM methyl-β-cyclodextrin (10, 98) for 1 hour at 37^0C. After treatment, the cells were washed with 1X PBS. The lipids were extracted twice from cell layer with isopropanol-hexane (2:3). 150 ng of 5α-Androstane-3α-17βDiol was added as an internal standard. Samples were dried under the nitrogen stream. 15 µl of Bis(trimethylsisyl)trifluoroacetamide

(BSTFA)/trimethylchlorosilane (TMCS)/acetonitrile (100:1:10) solvent was added and the solution was incubated for 30 min at room temperature before injecting into the gas chromatography system. Individual fatty acids were identified by their mass spectra (library Nist98) and their retention time. The retention time for each fatty acid of interest was determined after gas chromatography of TMS derivative of the fatty acid.

Agilent 6890 GC G2579A system (Agilent, Palo Alto, CA) equipped with a silica capillary column (15 m × 0.25 mm × 0.1 μm; Supelco, Bellefonte, PA) was used for gas chromatography. The temperature program was as follows: 139 °C for 3 min, to 380 °C with 20 °C/min, to 390 °C with 2 °C/min, held for 0.25 min. A model 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA) was used in both scan and selected ion monitoring modes to identify the samples.

Immunofluorescence:

Blocking buffer: 1.5 g BSA (1.5 %) in 100 ml of 1X PBS-$MgCl_2$.

Antibody dilution buffer: 0.5 g BSA (0.5 %) in 100 ml of 1X PBS-$MgCl_2$.

Wash buffer: 0.5 g BSA (0.5 %), ml 0.1 % TritonX-100 in 100 ml of 1X PBS-$MgCl_2$.

The 3T3 L1 adipocytes, grown on cover slips, were fixed with 2 % paraformaldehyde (PFA) (prepared in 1X phosphate buffer saline), for 15 minutes on ice and then treated with 100 mM ammonium chloride for 5 min. The non-specific binding was blocked with blocking buffer, for 1 hour at room temperature. The cells were then incubated with the polyclonal primary antibody against leptin receptor (rabbit polyclonal

IgG from Affinity Bio Reagents, prepared in antibody dilution buffer), for 1 hour at room temperature. The cells were rinsed with PBS-$MgCl_2$ and then permeabilized by treating with 0.1 % TritonX-100 for 5 minutes on ice and then blocked again with 1.5 % BSA. The cells were incubated with monoclonal primary antibody against caveolin-1 (monoclonal mouse IgM, 10μg/ml), for 1 hour at room temperature. The cells were rinsed with wash buffer. The primary antibodies were detected with 15 μg/ml cy2 goat anti rabbit IgG (green) and 15 μg/ml cy3 donkey anti mouse IgM (red), for 1 hour at room temperature. The cells were rinsed with wash buffer, and the cover slips were mounted with Gel / MOUNT^{TM} with antifading agents (Bio Meda corp.), before examination of cells with a 100X objective (aperture = 1) in the Leica laser scanning confocal microscope. The leica was equipped with argon and krypton lasers.

The negative controls were prepared by the incubating the cells with rabbit IgG (instead of leptin receptor antibody) and mouse IgM (instead of caveolin antibody) for 1 hour at room temperature.

Also live cell staining was used as a control to show the staining for leptin receptor only in the plasma membrane. Live 3T3-L1 adipocytes were blocked and treated with primary antibody against leptin receptor before fixation. The cells were then fixed with 2 % PFA, permeabilized with 0.1 % TritonX-100, blocked with 1.5 % BSA, and treated with the antibody against caveolin-1. The cells were rinsed with wash buffer, and the cover slips were mounted with Gel / MOUNT^{TM} with antifading agents. This experiment was done to show the staining of leptin receptor only in the plasma membrane and not in the intracellular part of the cell.

RESULTS

Differentiation of 3T3-L1 fibroblasts to adipocytes:

Because we wanted to test our hypothesis in the adipocyte model, we used the 3T3-L1 cell line for this project. The 3T3-L1 cell line, derived from the established mouse line 3T3, is extensively used as an adipocyte model for investigation of structural and functional aspects of adipocyets *in-vitro*. The 3T3-L1 cell line can differentiate into mature, lipid droplet containing adipocytes when stimulated with an appropriate adipogenic regimen. During the process of adipocyte differentiation, these preadipocytes lose their primitive mesenchymal character, assume a rounded morphology and acquire many of the enzymatic and biochemical properties of adipocytes (58, 147).

We used an *in-vitro* adipocyte model system by treating the 3T3-L1 cells with adipogenic factors such as insulin (0.85 μM), dexamethasone (0.25 μM), and 3-isobutyl-1-methylxanthine (0.5 mM) for 72 hours. The cells were then incubated in insulin only media (high glucose DMEM media containing 0.85 μM insulin) for 48 hours and after that maintained in DMEM media containing 10 % FBS. Figure 1 (a) shows the phase contrast image of undifferentiated 3T3-L1 cells. These cells have elongated spindle shape morphology. Figure 1 (b) shows the phase contrast image of differentiated cells. 3T3-L1 cells have accumulated numerous lipid droplets after differentiation. We achieved approximately 90 % differentiation.

Expression of leptin receptors in the 3T3-L1 adipocyte model:

Leptin exerts its effects through leptin receptors. To examine the effects of leptin in adipocytes, we first looked at the expression of leptin receptors in the differentiated

3T3-L1 cells, which we have used as a model system of adipocytes. To demonstrate the expression of leptin receptors, we resolved the lysates of differentiated cells by SDS-PAGE. The material was transferred to PVDF membrane and immunoblotted with the leptin receptor antibody. The figure 2 shows a 100 kD band for the short form of leptin receptors. The wild type mouse brain was used as a positive control for the leptin receptor.

Localization of leptin receptors in the caveolae:

Caveolae play an important role in numerous signaling mechanisms at the cell surface. We were interested in looking at the role of caveolae in regulating leptin signaling or its function (e.g. lipolysis) in adipocytes. For this specific aim, we first looked at the localization of leptin receptors, as many receptors and signaling molecules reside in caveolae. We used two approaches to determine if leptin receptors are localized to caveolae: subcellular fractionation and immunocytochemistry.

(a) Subcellular fractionation:

We subfractionated the differentiated 3T3-L1 adipocytes using a detergent free method to isolate caveolae (as described in methods) and resolved 20 μg of plasma membrane fraction (PM) and the caveolae fraction (CM) by SDS-PAGE. The material was transferred to the PVDF membrane and immunoblotted with the antibodies against leptin receptor and caveolin-1 (Figure 3). As equal amount of protein was loaded, the dense band of ObR in the CM fraction indicates that the caveolae membranes are enriched in leptin receptors.

(b) Immunocytochemistry

To further support the results of subcellular fractionation, we performed immunocytochemistry of 3T3-L1 adipocytes with anti leptin receptor and anti caveolin-1 antibodies. The 3T3-L1 cells were differentiated as described in the methods and were fixed with 2 % paraformaldehyde (PFA). To show the staining of leptin receptors only at the cell surface, we treated our cells with the leptin receptor antibody before permeabilizing the membrane. The cells were then permeabilized and treated with anti caveolin-1 antibody. The confocal microscopic examination (100X) showed very strong staining for the leptin receptors at the cells surface as shown in figure 4a (green). Figure 4b (red) shows the staining for caveolin-1. Figure 4c represents the merged image of figures a and b which shows the co-localization of leptin receptors and caveolin-1 which is the defining protein of caveolae.

Omission of primary antibodies served as a negative control. We prepared these controls by using rabbit IgG (instead of leptin receptor antibody) and mouse IgM (instead of caveolin-1 antibody) (figure 5).

Also to show the staining of leptin receptors only in the plasma membrane, we did the same experiment with live 3T3-L1 adipocytes. In this experiment, we stained the live cells with anti-leptin receptor antibody before fixation. Then we fixed the cells, permeabilized and stained with caveolin-1 antibody. These controls showed positive staining for leptin receptors in the plasma membrane (figure 6a) and caveolin-1 (figure 6b). The merged image (6c) of figures a and b shows the co-localization of leptin receptors and caveolin-1 in the caveolae. Omission of primary antibodies served as a negative control for live cell staining (figure 7).

Thus our second approach supports the data from subcellular fractionation and we conclude that leptin receptors are localized in caveolae.

Lipolytic activity of leptin:

To demonstrate the effect of leptin on triglyceride hydrolysis, we treated the 3T3-L1 adipocytes with 10 nM leptin for 2 hours at 37^0C. The media was removed after treatment and the cells were washed with PBS. The lipids were extracted from the cells by adding hexane: isopropanol (3:2). The unsaturated free fatty acids were measured by gas chromatography/mass spectrometry. The basal lipolytic activity was measured in the absence of leptin. We measured only unsaturated FFAs as an index of lipolysis because saturated FFA are the common contaminants in this method (121). Leptin caused a three fold increase ($P<0.005$) in lipolytic activity as compared to the basal lipolytic activity. We also treated our cells with 10 nM, 100 nM and 1 μM leptin and the amount of lipolysis remained same with increasing concentrations, indicating saturation (data not shown). We also tested the effect of other lipolytic agents like norepinephrine in 3T3-L1 adipocytes (figure 8). As expected, when 5 μM of norepinephrine was added to the incubation media, the unsaturated FFAs increased markedly ($P<0.05$).

Disruption of caveolae with cyclodextrin treatment inhibits leptin induced lipolysis:

To demonstrate if caveolae disruption plays any significant role in the leptin induced lipolysis, we pretreated the cells with or without 5 mM ß-trimethyl cyclodextrin for 1 hour at 37^0C and then treated them with 10 nM leptin or 5 μM norepinephrine for 2 hours at 37^0C. The cells treated only with leptin showed a three fold increase in the

lipolysis, as compared with the basal activity ($P<0.005$). While the cells treated with cyclodextrin prior to leptin, showed only 1.5 fold increase in the lipolysis ($P<0.05$). We found the same results with norepinephrine treatment. Norepinephrine treatment induced a seven fold increase in the lipolysis ($P<0.05$) as compared with the basal activity, while in the cells pretreated with ß-trimethyl cyclodextrin, norepinephrine treatment induced only a three fold increase in the lipolysis ($P<0.05$). These results suggest that disruption of caveolae with cyclodextrin treatment inhibits leptin and norepinephrine induced lipolysis in 3T3-L1 adipocytes

Undifferentiated 3T3-L1 cells

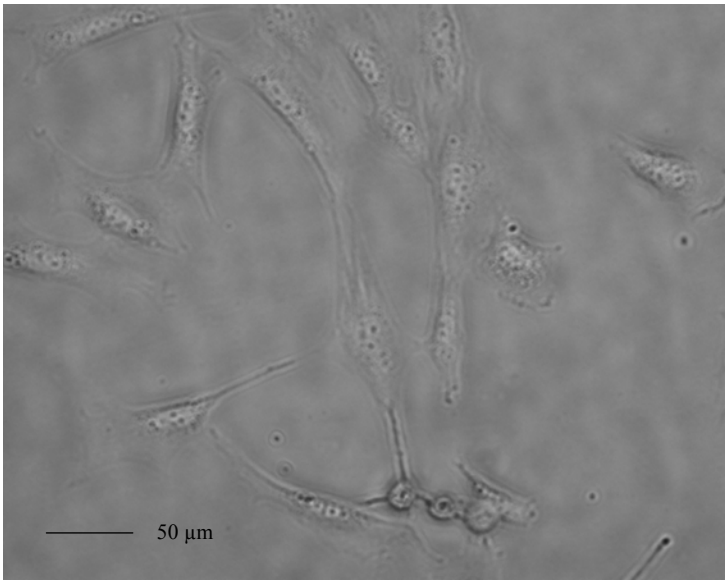

Figure 1(a)

Differentiated 3T3-L1 adipocytes

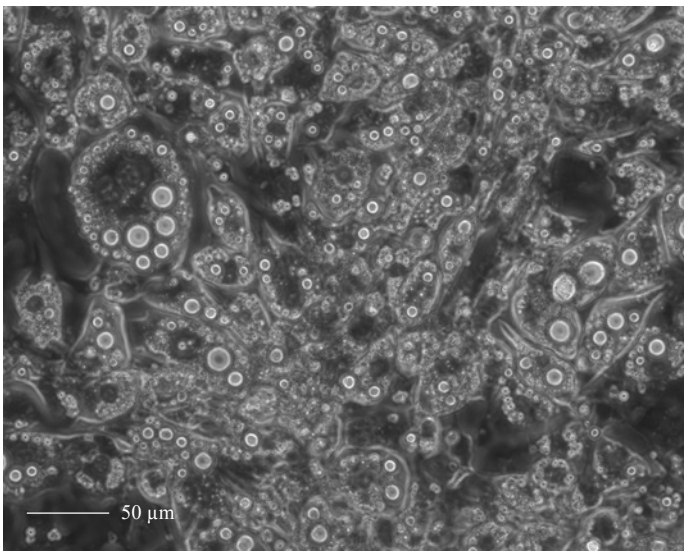

Figure 1(b)

Figure 1. Differentiation of 3T3-L1 mouse fibroblasts. Figure 1 a and b shows the phase contrast images (20X) of undifferentiated and differentiated 3T3-L1 cells respectively. The elongated fibroblasts assumed a rounded morphology and accumulated numerous lipid droplets when stimulated with adipogenic factors such as insulin, dexamethasone, and 3-isobutyl-1-methylxanthine as described in the method section. Almost 90 % of cells differentiated into adipocyte phenotype as seen in figure 1(b).

Expression of leptin receptors in 3T3-L1 adipocytes

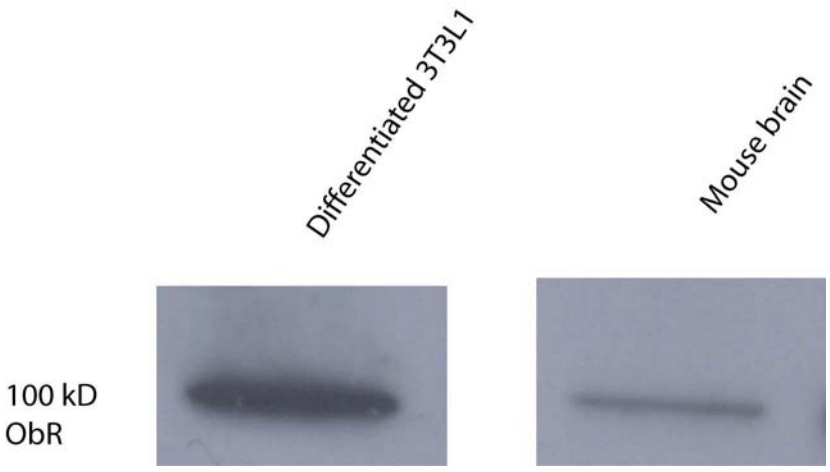

Figure 2

Figure 2: Expression of the short form of leptin receptors in differentiated 3T3-L1 adipocytes. Lysates of differentiated 3T3-L1 cells were prepared and subjected to SDS PAGE and immunoblotted with anti leptin receptor antibody. A wild type mouse brain tissue lysate was loaded as a positive control.

Leptin receptors are co-purified with caveolin-1

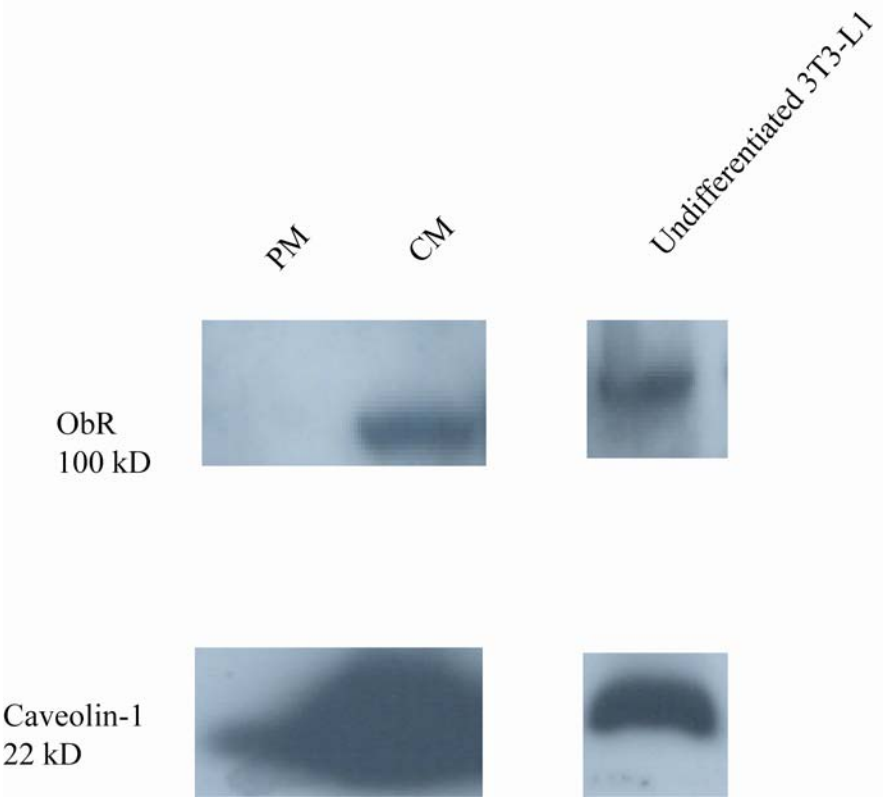

Figure 3

Figure 3. Immunoblot for leptin receptor with the plasma membrane fraction and caveolae fraction of differentiated 3T3-L1 adipocytes. The differentiated cells were subfractionated using a detergent free method to isolate caveolae. 20 μg of each fraction was resolved by SDS PAGE and immunoblotted with anti-leptin receptor and anti-caveolin-1 antibodies. The immunoblots were developed by the method of chemiluminescence. Because equal amount of proteins were loaded in both fractions, the greater intensity associated with the caveolae fraction indicate that caveolae are enriched in leptin receptors compared with plasma membrane. PM: plasma membrane, CM: caveolae membrane. Figure shows representative data from four independent experiments.

Colocalization of ObRs and caveolin-1

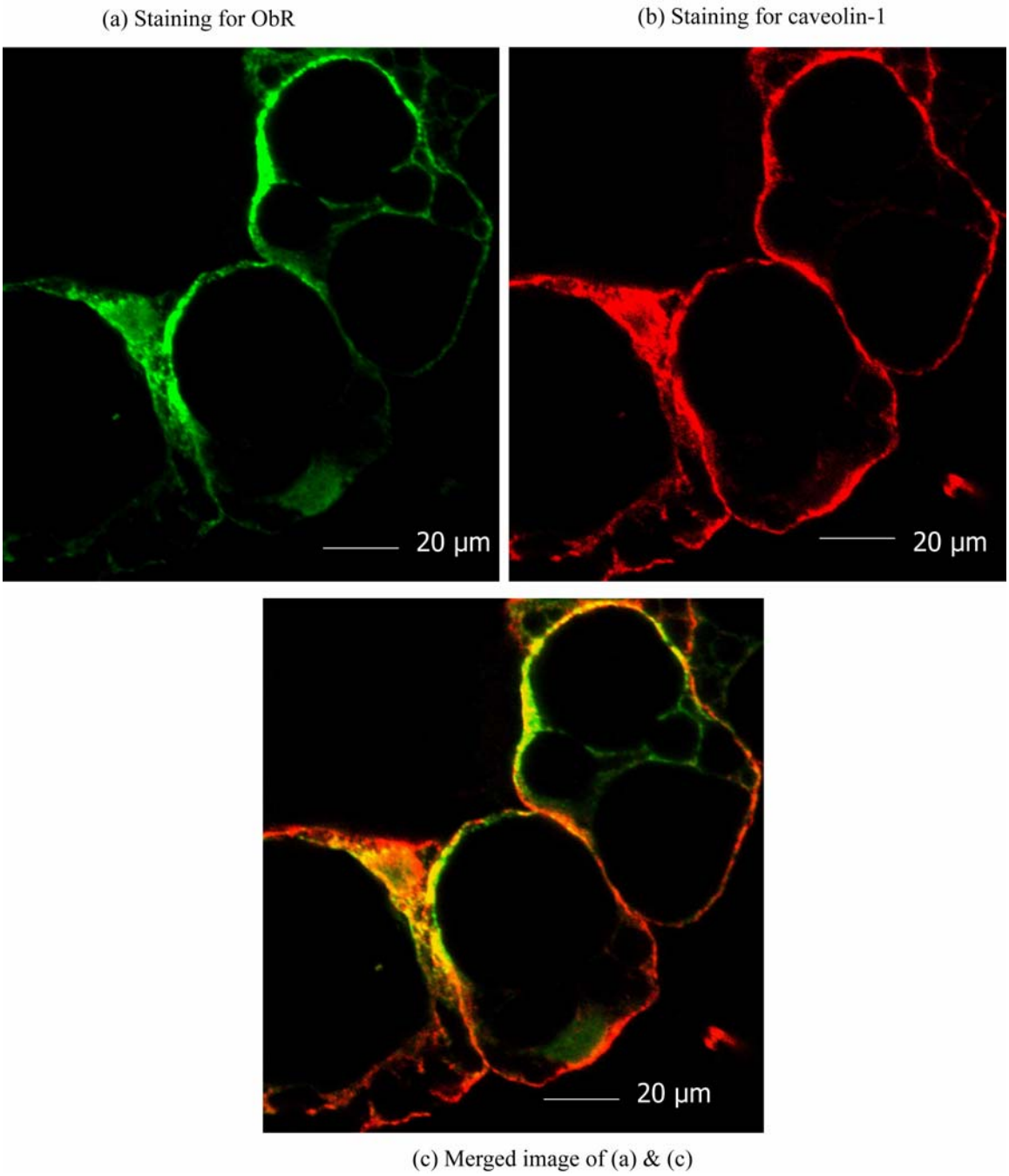

Figure 4

Figure 4. Co-localization of caveolin-1 and leptin receptors in the differentiated 3T3-L1 adipocytes. The differentiated adipocytes were fixed with 2 % PFA and immunolabeled with antibody against leptin receptor (a) before permeabilization. The cells were then permeabilized with 0.1 % TritionX-100 and incubated with antibody against caveolin-1 (b). The primary antibodies were detected with cy2 goat anti rabbit IgG (green) and cy3 donkey anti mouse IgM (red). The cells were rinsed with wash buffer, and the cover slips were mounted with mounting media with antifading agents. The slides were examined by laser scanning confocal microscopy (100X). Figure (c) represents the merged image of (a) and (b).

Negative control without primary antibodies

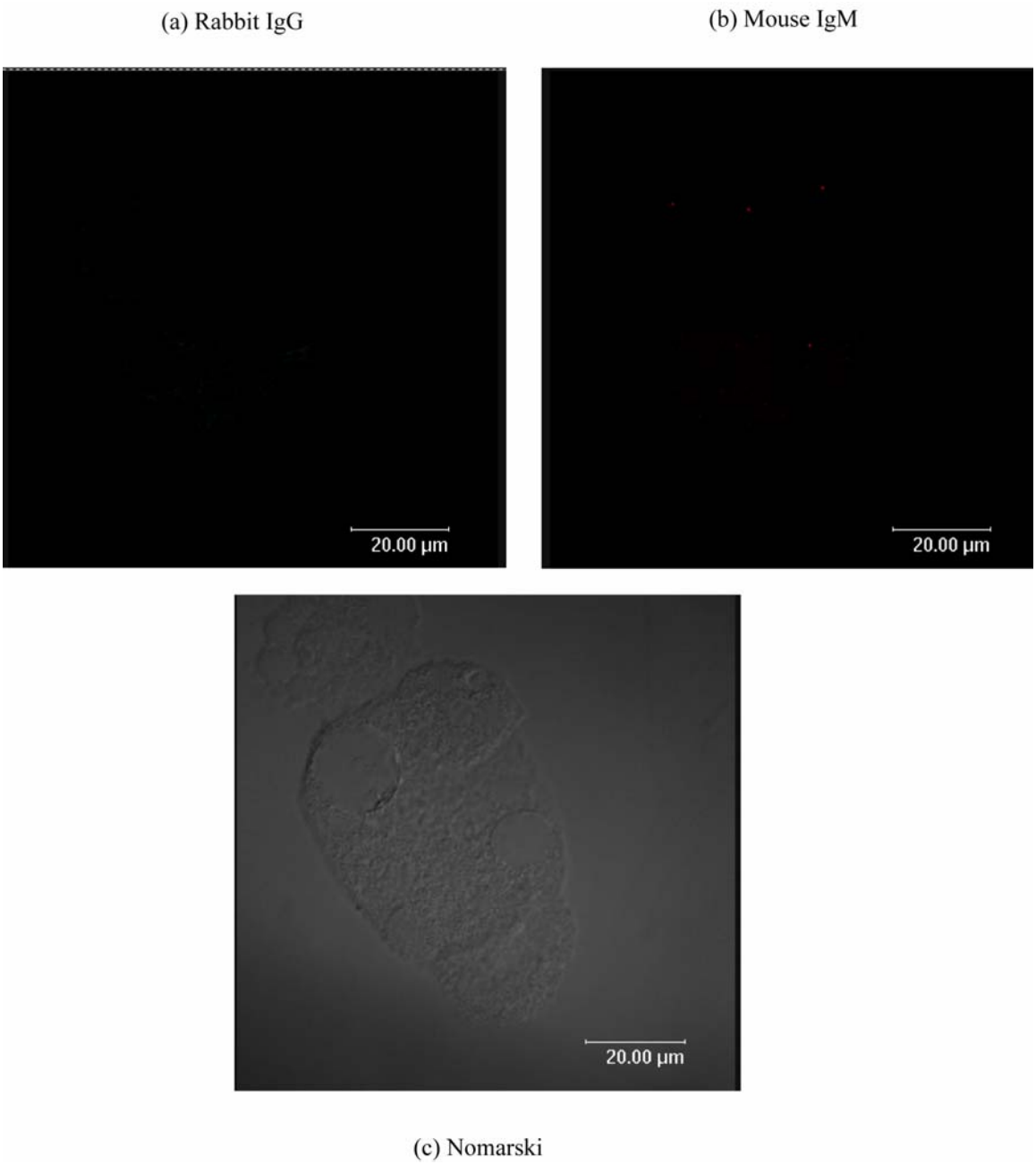

Figure 5

Figure 5. Control experiment without primary antibody. The adipocytes were fixed and incubated with rabbit IgG instead of primary leptin receptor antibody (a). The cells were then permeabilized and incubated with mouse IgM instead of primary caveolin-1 antibody (b). The slides were examined by laser scanning confocal microscopy (100X). Figure (c) represents normarski.

Control experiment with live cell staining

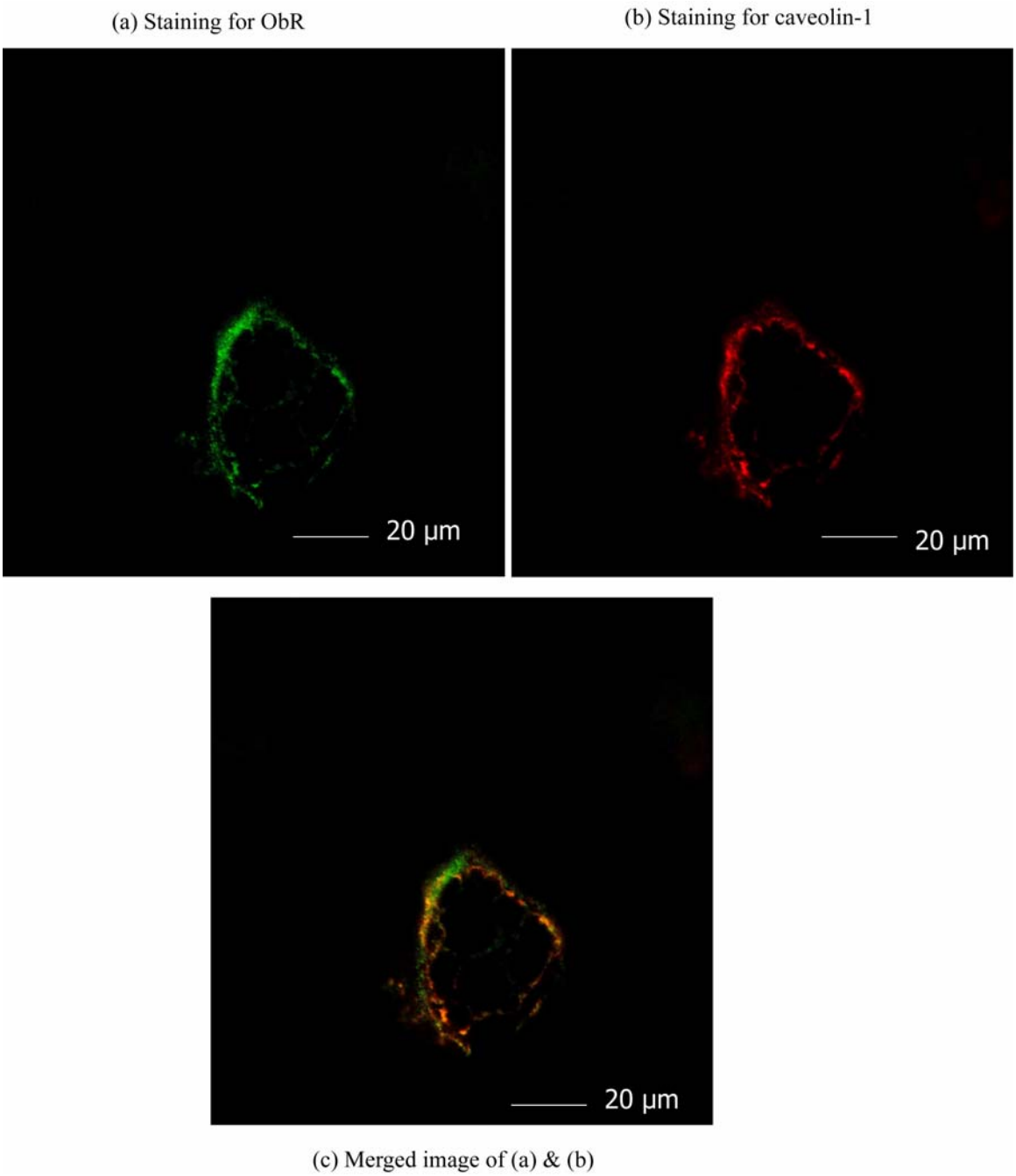

Figure 6

Figure 6. Control experiment with live cell immunostaining. To show the ObR staining only in the plasma membrane, we treated the live cells with the primary antibody against leptin receptor (a) and then fixed with 2 % PFA. We then permeabilized the cells with 0.1 % TritonX-100 and incubated them with primary antibody against caveolin-1 (b). The primary antibodies were detected with cy2 goat anti rabbit IgG (green) and cy3 donkey anti mouse IgM (red). The cells were rinsed with wash buffer, and the cover slips were mounted with mounting media with antifading agents. The slides were examined by laser scanning confocal microscopy (100X). Figure (c) represents the merged image of (a) and (b).

Negative control without primary antibodies

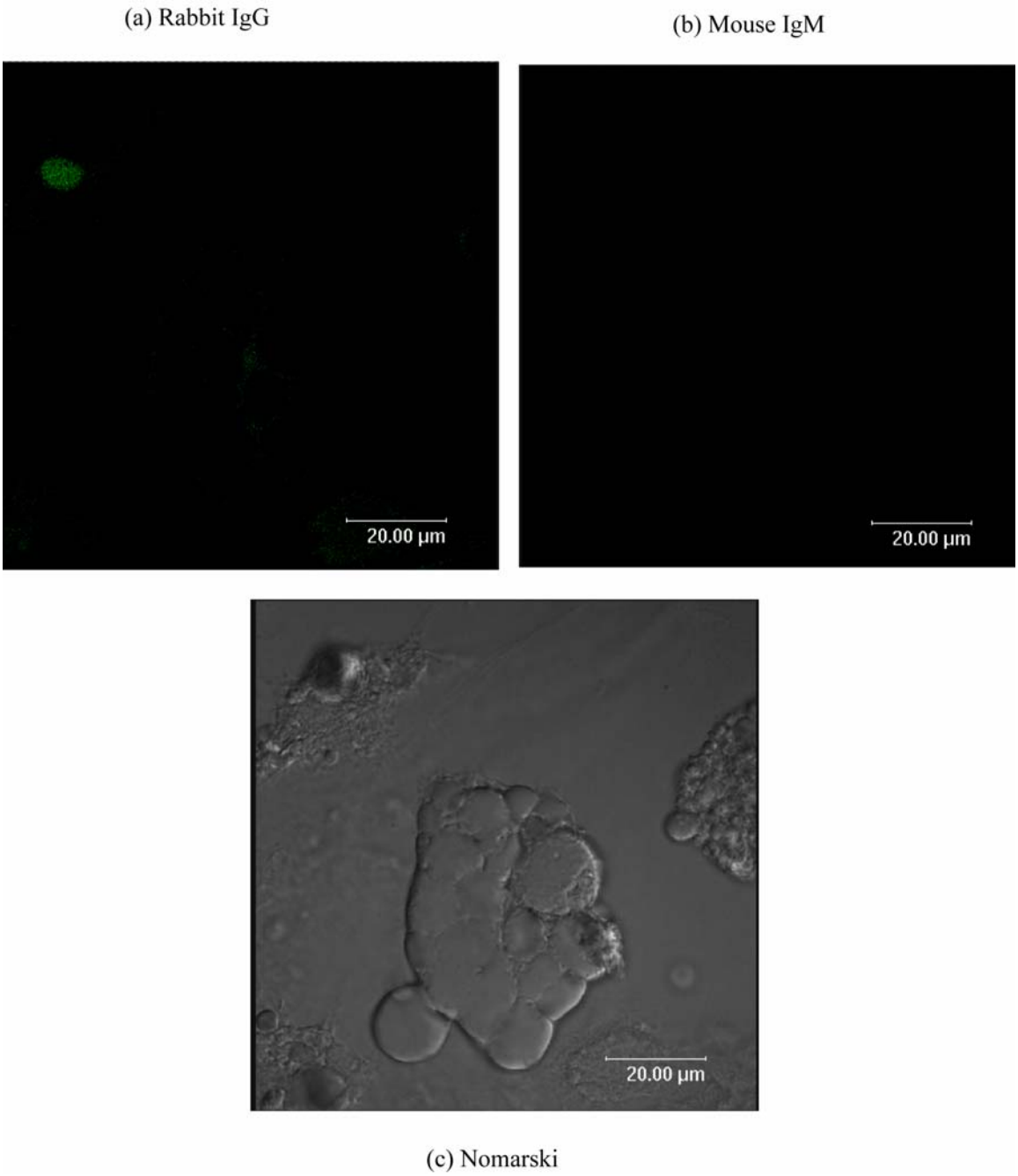

Figure 7

Figure 7. Negative control for live cells. The live cells were incubated with rabbit IgG instead of primary antibody against leptin receptors (a) for 10 minutes and then fixed with 2 % PFA. The cells were then permeabilized with 0.1 % TritionX-100 and incubated with mouse IgM (b) instead of primary antibody against caveolin-1. The slides were examined by laser scanning confocal microscopy (100X). Figure (c) represents the normarski.

Leptin induced lipolysis in 3T3-L1 adipocytes

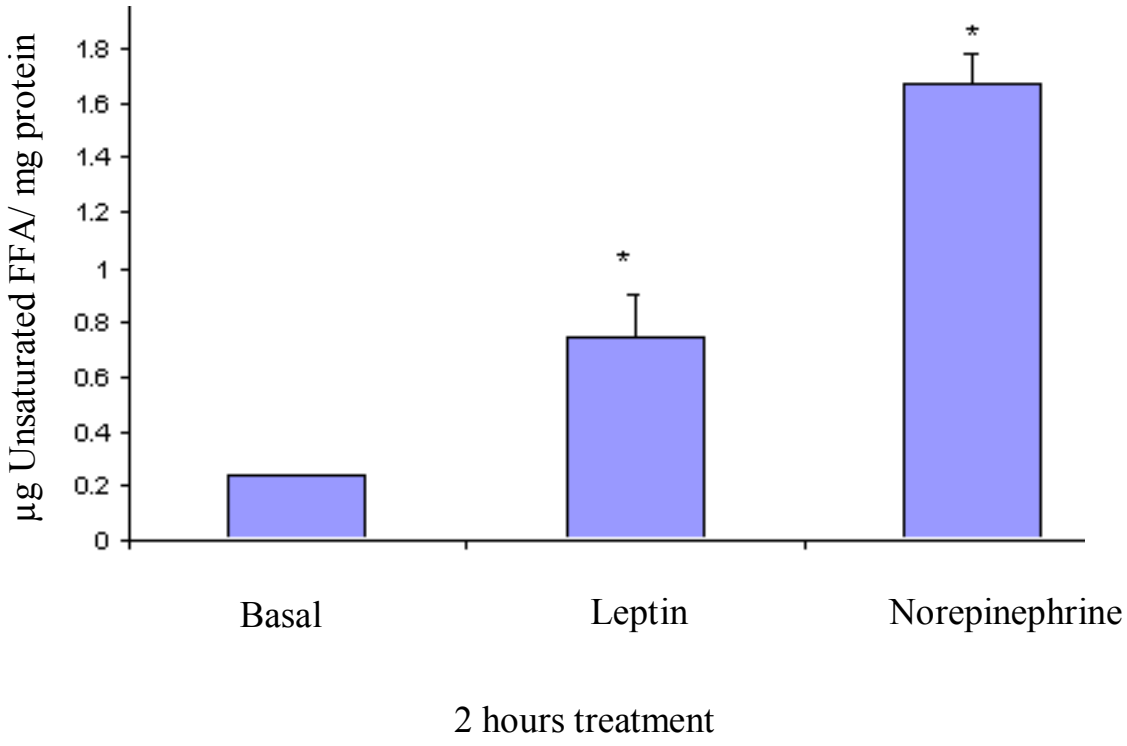

Figure 8

Figure 8. Leptin induced lipolysis in 3T3-L1 adipocytes. The figure shows the lipolytic activity induced by leptin and norepinephrine. The 3T3-L1 cells were incubated in media with 1 % serum for 20 hours before experiment, and treated with 10 nM leptin and 5 μM norepinephrine for 2 hours at 37^0C. To measure the amount of lipolytic activity, the lipids were extracted from the cells and unsaturated free fatty acids were measured, by gas chromatography/mass spectrometry. 10 nM leptin caused a three fold increase in the lipolysis compared with the basal lipolytic activity ($P<0.005$). The positive control norepinephrine caused a seven fold increase in the lipolysis compared with the basal activity ($P<0.05$). Data are representative of 3 independent experiments. Each experiment is done in triplicates. The results are expressed as mean ± S.E. Data were analyzed statistically using a one way analysis of variance (ANOVA). Statistical probability of $P<0.05$ was considered to be significant. * Significantly different from basal value.

Effect of cyclodextrin on leptin induced lipolysis

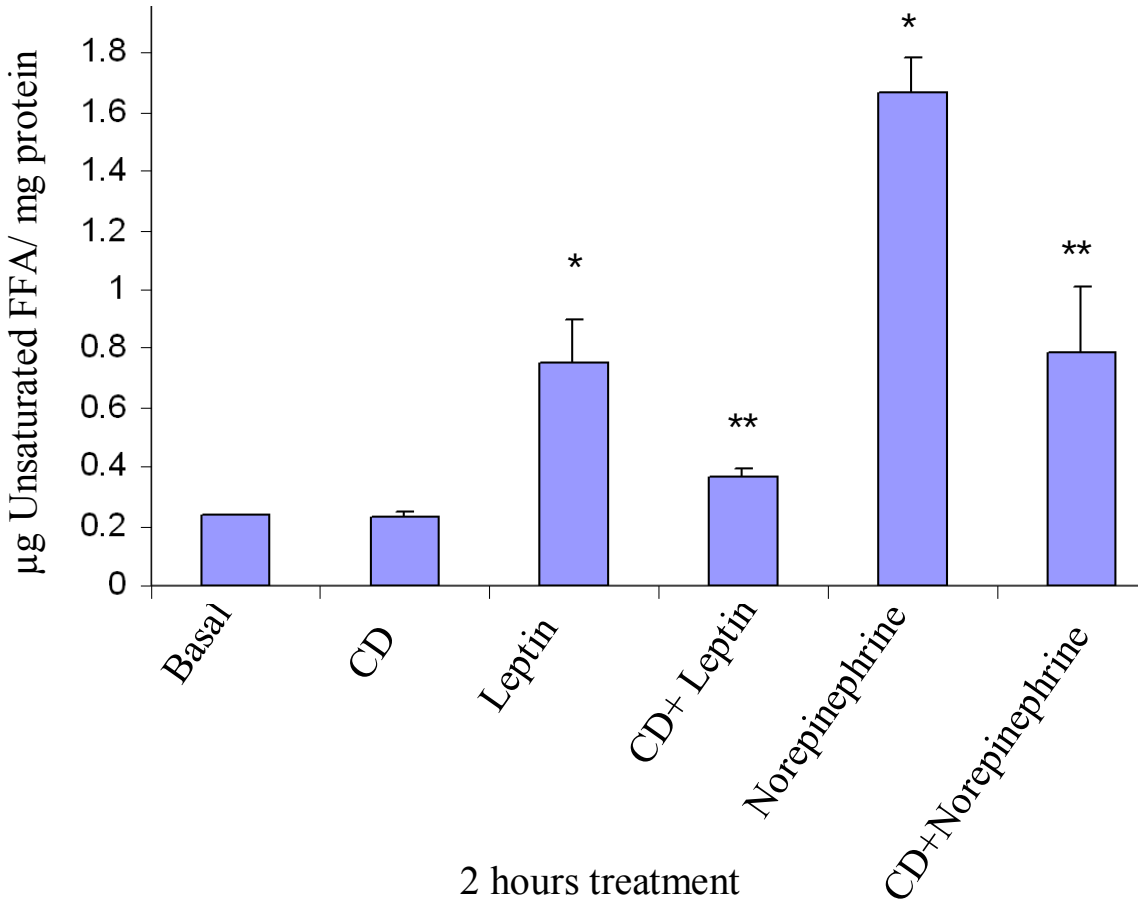

Figure 9

Figure 9. Effect of methyl-β-cyclodextrin on the lipolytic activity of leptin and norepinephrine. Two hours leptin treatment showed a three fold increase in the lipolytic activity as compared to the basal lipolytic activity ($P<0.005$). When the cells were pretreated with 5 mM cyclodextrin for 1 hour and then treated with leptin for 2 hours, the increase in lipolysis was only 1.5 fold ($P<0.05$). Norepinephrine showed a seven fold increase in lipolytic activity compared to the basal level ($P<0.05$). When the cells were pretreated with cyclodextrin for one hour, and then treated with norepinephrine, the increase in the lipolysis was only three fold as compared with the basal activity ($P<0.05$). Data are representative of 3 independent experiments. Each experiment is done in triplicates. The results are expressed as mean ± S.E. Data were analyzed statistically using a one way analysis of variance (ANOVA). Statistical probability of $P<0.05$ was considered to be significant. * Significantly different from basal value. ** Significantly different from stimulated conditions.

DISCUSSION

Obesity, is a major risk factor for the development of type II diabetes, cardiovascular disease and some cancers (88, 89). Obesity occurs as a result of many factors which lead to the storage of lipids in the body which causes enlargement of the adipocytes as well as an increase in the mass of adipose tissue. There is a centrally controlled mechanism, residing in the adipocytes, that regulates the breakdown and storage of lipids. But almost always in obesity there is a dysregulation in this mechanism. Adipose tissue over the last few years has emerged as an endocrine organ that is central to the regulation of energy homeostasis (2, 124). Leptin, the adipocyte-derived hormone, appears to be an important player in the above mentioned mechanism (135). Leptin has been studied extensively in the *ob/ob* mouse model. Leptin deficiency in *ob/ob* mice leads to an increased appetite and decreased energy expenditure leading to an obese phenotype (75, 143). Leptin, meaning thin, refers to the hormone's antiobesity effects. Leptin stimulates lipolysis in isolated adipocytes, implicating its autocrine/paracrine role (43). The largest energy reserves in mammals are the triglycerols housed within the intracellular lipid droplets in adipocytes. Thus adipose tissue lipolysis is a major regulator of the body's supply of lipid energy. Although leptin induced-lipolysis in adipocytes has been previously demonstrated, the signaling mechanism causing this effect is not well understood.

To study the lipolytic effect of leptin in adipocytes, we have used 3T3-L1 cells as an adipocyte model. As stated in the results section, the 3T3-L1 cell line can differentiate into mature, lipid droplet containing adipocytes when stimulated with an appropriate adipogenic regimen. We first conducted an experiment to detect the presence

of leptin receptors in the 3T3-L1 cell system. We observed that the short form of the leptin receptor was the predominant form expressed. We have shown that 10 nM leptin treatment for two hours induced a 3 fold increase in lipolytic activity in 3T3-L1 adipocytes, as compared with basal lipolytic activity. As expected, norepinephrine, a potent lipolytic stimulus, increased the lipolytic activity by 7 folds as compared to the basal activity. Also, in agreement with our findings, Fruhbeck et al. have shown that exposure to leptin increased ($P < 0.01$) the lipolytic activity of the fat cells derived from lean mice (43). These *in-vitro* results suggest an autocrine/paracrine action of leptin on white fat cells and envisage the involvement of leptin, not only in centrally mediated pathways, but also in physiological functions that take place at the periphery (43). The lipolytic activity of leptin was more pronounced in the fat cells of *ob/ob* mice compared to lean mice (43). These findings are supported by smaller reduction in weight loss, food intake and body fat observed in lean as compared to *ob/ob* mice, when they were treated with leptin (17, 55, 101). The lipolytic activity of leptin has also been demonstrated by *in-vivo* studies (42). In one such study the basal lipolytic activity was significantly ($P < 0.001$) increased in lean mice that received 10 mg leptin per kg body weight when compared with the animals that received PBS. Administration of 1.0 and 10.0 mg leptin per kg body weight increased the basal lipolytic activity by two fold and almost three fold respectively (42). Thus our data are in agreement with other reports demonstrating lipolytic effects of leptin in adipocytes.

Although leptin-induced lipolysis has been previously described, the underlying mechanism still remains to be elucidated. Fruhbeck et al suggested that the lipolytic effect of leptin is located at the adenylate cyclase/G_i protein step (45). Another group

suggested that leptin significantly increased HSL activity in J774.2 macrophages, and these effects were additive with the effects of cAMP (93). The signaling pathway of leptin induced lipolysis is still not well understood but considering the above two results we can say that it may involve regulation at the level of the enzyme HSL. HSL plays a crucial role in the release of fatty acids from the triglyceride-rich lipid droplets within the adipocytes which contain the body's major energy storage. The hormonal stimulation of the cAMP cascade and the consequent phosphorylation and activation of HSL leads to a large increase in lipolysis in adipocytes. The critical step after the phosphorylation of HSL following stimulation of adipocytes is the translocation of the lipase from the cytosol to the surfaces of lipid droplets as shown by immunoflorescence studies of stimulated and unstimulated 3T3-L1 cells (15). Brasaemle et al have shown that in unstimulated 3T3-L1 adipocytes, the hormone sensitive lipase is diffusely distributed throughout the cytosol (15). But stimulation with the ß-adrenergic receptor agonist, isoproterenol, causes translocation of HSL from the cytosol to the surfaces of the intracellular lipid droplets concomitant with the onset of lipolysis in the 3T3-L1 adipocytes (15). Greenberg et al have shown that activation of ERK pathway regulates lipolysis in 3T3-L1 adipocytes by phosphorylating HSL on Ser^{600} and increasing the lipolytic activity of HSL (51). Not much is known about leptin signaling in lipolysis at this time though scientific efforts are underway.

Cellular signaling occurs through a number of different processes in the cell and one of the very important cellular structures that play a role in various signaling pathways are the caveolae. Caveolae are involved in cellular processes of receptor mediated uptake, receptor mediated signaling, and vesicular trafficking (106). A number of proteins

involved in signal transduction like ras, raf1, eNOS and others have been found in the caveolae (4, 27, 73, 127, 129). Thus caveolae provide a structural platform and a suitable environment for the interaction of different proteins and their signaling mechanism. Because caveolae serve to compartmentalize and integrate numerous signaling events at the cell surface we tried to find out if there was a role that caveolae played in leptin induced lypolysis. Caveoin-1 is the marker protein for caveolae. Using a subcellular fractionation technique (131), we demonstrated that leptin receptors were co-purified with caveolin-1 suggesting that leptin receptors were localized to same membrane fraction as caveolin-1. Confocal microscopic examination confirmed this colocalization. Caveolin-1 was co-purified with leptin receptors, suggesting that the two molecules (leptin receptors and caveolin-1) can directly interact with each other. In our experiment for studying lypolytic activity of leptin we showed that 10 nM leptin treatment for two hours induced a three fold increase when compared with basal activity. However, in a similar experiment disruption of caveolae by cyclodextrin pretreatment caused only 1.5 fold increase in the lipolysis compared with basal indicating the inhibition of leptin induced lipolysis. We have also shown that cyclodextrin interferes with the lipolytic activity of norepinephrine. Though we know that disruption of caveolae leads to an inhibition of the lipolytic activity of leptin, the entire mechanism remains to be elucidated. From our work it can be speculated that the short form of leptin receptor can partially regulate lipolysis because it was the predominant form of leptin receptor found in our differentiated adipocytes as shown by immunoblotting. The exact role of the short form of leptin receptor is not known though there are reports suggesting that the short form of leptin receptor can stimulate ERK1/ERK2 pathway. It also has been shown that

the activation of the ERK pathway regulates lipolysis in 3T3-L1 adipocytes by phosphorylating HSL on Ser^{600} (51). The work of Shima et al. have shown that in their studies with CHO cells transfected with the long or short forms of leptin receptor, the short form of leptin receptor induced the transcription of mRNA of immediate early genes like c-fos, c-jun and jun-B which are expressed upon leptin addition (90). It was also shown that the long form of the receptor had the same effect on the expression of the mRNA (90). Thus it could be said that the short form of the receptor takes part in cellular signaling.

In summary, we have shown that leptin receptors are localized in the caveolae. Leptin induced statistically significant lipolysis in the 3T3-L1 adipocytes and caveolar disruption reduced this lipolytic effect of leptin suggesting that caveolae might play a significant role in leptin-induced lipolysis though the entire mechanism remains to be elucidated. The signaling pathway of leptin induced lipolysis is a complex mechanism and presents an enormous opportunity for further investigation.

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VITA

Name : Gentle Chikani

Date of birth : 10/20/1977

Place of birth : Mumbai, India

Academic qualifications

- Bachelor of Ayurvedic Medicine and Surgery

  K.G.M.P. Ayurvedic College, Mumbai, India.

Work experience

- Worked as a graduate research assistant in the department of Nutritional Sciences under Dr. Smart at the University of Kentucky from Aug 2002 to Dec 2004.
- Worked as a resident medical doctor in the Ever Shine Nursing Home, Mumbai, India from March 2001 to May 2001.
- Worked as an intern in rural and urban hospitals from Feb 2000 to Feb 2001.

Volunteer experience

- Practice concepts of Preventive and community medicine
- Observation and participation to functioning of Rural Health Center
- Participation and active role play in national tuberculosis and malaria prevention and control program

Seminars

- Gave a seminar on ‘ Leptin: A link between obesity, diabetes and cardiovascular diseases’ at the University of Kentucky in Feb 2004.

Publications

- Chikani G, Zhu W, Smart E.

  Lipids: potential regulators of nitric oxide generation.

  Am J Physiol Endocrinol Metab. 2004; 287(3): E386-389.

Awards and honors

- Award received from the Mayor of Mumbai, India for achieving 6th rank in general merit list and 4th rank among female candidates of Mumbai division of Maharashtra Secondary School Certificate Examination
- 5th rank in Final year of Medicine, K.G.M.P. Ayurvedic Medical College, Mumbai, India.