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THE ROLE OF β3-ADRENERGIC RECEPTORS IN CONTROL OF BROWN AND WHITE ADIPOSE TISSUES AND OF ENERGY BALANCE: REVERSAL OF OBESITY BY CL 316,243, A NEW β3-ADRENERGIC AGONIST

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ABSTRACT

Obesity is a prevalent health hazard that is associated with other metabolic disorders, in particular with insulin resistance and non-insulin-dependent diabetes mellitus. Treatment of obesity requires an increase in energy expenditure or a decrease in energy intake or both of these. One target for raising energy expenditure in the treatment of obesity with drugs is the β3-adrenergic receptor in brown and white adipose tissues. Selective β3-adrenergic receptor agonists have been developed that will stimulate both the mobilization of fat from white adipose tissue, the site of triacylglycerol storage in the body, and the oxidation of this fat in brown adipose tissue, a site of thermogenesis. The principal objectives of the work described in this thesis were to find out whether a selective β3-adrenergic receptor agonist could reverse obesity and insulin-resistance in rats and to elucidate the mechanisms involved.

Two models of obesity were studied: young Sprague-Dawley rats which became obese because they were eating a high-fat diet and continued to eat this diet during the treatment (diet-induced obesity) and old Zucker fa/fa rats (genetic obesity). Rats were treated by continuous subcutaneous infusion of the selective β3-adrenergic receptor agonist, CL 316,243; control rats received infusion of saline. Lean rats of the same age and strain were similarly treated. Rats with diet-induced obesity had a hypertrophic obesity (enlarged white adipocytes but no increase in number of adipocytes) while rats with genetic obesity had a hyperplastic obesity (increase in

number of white adipocytes). Treatment with CL 316,243 reversed obesity in both animal models. Reduction in fat stores was associated with shrinking of enlarged white adipocytes but no reduction in their number, even when this was elevated as in the genetically obese fa/fa rats. Reversal of obesity was associated with a large increase in energy expenditure. This was associated not only with growth of the presumed site of this increase in energy expenditure, brown adipose tissue, but also with appearance of abundant brown adipocytes in white adipose tissues, a site in which they do not normally occur. Reversal of obesity was not associated with any reduction in energy intake, except in the genetically obese rats in which the hyperphagia was reversed. Treatment reduced the concentration of leptin in serum when this was elevated, as in the rats with diet-induced obesity and in the moderately obese old control Zucker rats. Treatment did not, however, reduce the elevated level of leptin in the blood of fa/fa rats. There was thus no correlation of drug-induced changes in leptin concentration with changes in food intake.

Studied only in fa/fa rats, treatment improved insulin resistance, decreasing both hyperglycaemia and hyperinsulinaemia. This improvement was correlated with a reduction of expression of a suggested mediator of insulin resistance, tumour necrosis factor α, in white adipose tissues.

Results show that CL 316,243, a selective agonist for rodent β3-adrenergic receptors, is an effective anti-obesity agent in rat models of obesity. Future development of similar compounds that are selective for human β3-adrenergic

receptors will be needed before this approach to treatment of obesity and insulin resistance can be useful in humans.

DEDICATION

This work is dedicated to my precious wife Fatemeh and my wonderful children Peyman, Sepideh, and Negin and to my father, sisters and brothers for all their support and encouragements

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ABBREVIATIONS

β-AR	β-adrenoceptor
β-ARK	β-adrenoceptor kinase
β3-AR	β3-adrenoceptor
β3-ARs	β3-adrenoceptors
μg	microgram
μM	micromolar
A	adrenaline
ADP	adenosine 5'-diphosphate
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
BAT	brown adipose tissue
BRL	BRL 37,344
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-phosphate
CL	CL 316,243
cm	centimetre
COX	cytochrome oxidase
Cyt c	cytochrome c
Da	a unit of mass nearly equal to that of a hydrogen atom

DAB	3, 3'-diaminobenzidine tetrahydrochloride
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DIO	diet-induced obesity
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dTTP	2'-deoxythymidine 5'-triphosphate
EC_{50}	concentration exerting 50% of maximum response
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetate
EWAT	epididymal white adipose tissue
FFA	free fatty acid
g	gram
G-protein	guanosine nucleotide-binding protein
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
H-33258	Hoechst-33258
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IBAT	interscapular brown adipose tissue

IgG	immunoglobulin G
IP3	inositol trisphosphate
IRS-1	insulin-receptor substrate-1
ISO	isoproterenol
kb	kilobase pair
kDa	a unit mass equal to 1000 daltons
kg	kilogram
KRB	Krebs-Ringer bicarbonate
LB	Luria-Bertani medium
log	logarithm (base 10)
M or mol/l	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM or mmol/l	millimolar
MOPS	3-[N-morpholino]propanesulfonic acid
NA	noradrenaline
NSB	non-specific binding
NCM	nitrocellulose membrane
NIDDM	non-insulin-dependent diabetes mellitus

NPY	neuropeptide Y
PBAT	perirenal brown adipose tissue
PBS	phosphate buffered saline
PIP2	phosphatidyl inositol-4,5-bisphosphate
PKA	protein kinase A
PMSF	phenylmethylsulfonyl fluoride
PPARγ	peroxisome proliferator activator receptor γ
RIA	radioimmunoassay
RMR	resting metabolic rate
RNA	ribonucleic acid
RNAase	ribonuclease
RPM	revolutions per minute
RWAT	retroperitoneal white adipose tissue
S	saline
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SLB	sample loading buffer
SOC	liquid medium containing (2% bacto-tryptone, 0.5% bacto-yeast extract, 0.05% NaCl and 20 mM glucose in deionized H_2O, pH 7.0)
SSC	sodium chloride-sodium citrate

TBS	Tris buffered saline
TCA	trichloroacetic acid
TCT	Tris buffer, carageenan and Triton-X-100
TE	Tris-EDTA
TNF-α	tumour necrosis factor-α
TRI	RNA/DNA/protein isolation reagent
Tris	2-amino 2 hydroxy-methylpropan-1,3-diol
UCP	uncoupling protein
V	volt
WAT	white adipose tissue
°C	degree Celsius

CHAPTER I

INTRODUCTION

OBESITY AND ITS TREATMENT

One disorder involved in human health is an excessive amount of body fat or obesity. Obesity is a complex disease with multiple causes and metabolic consequences. It results from an imbalance between energy intake and energy output for prolonged periods.

The metabolic complications of obesity include non-insulin dependent diabetes mellitus (NIDDM) and dyslipidemia; both are significantly greater in visceral obesity (Kahn 1994).

It has been shown that one of the choices for treatment of NIDDM is weight loss. Weight loss will improve glycemic control and serum lipid level and reduce insulin resistance, and lower blood pressure.

In spite of the undoubted benefits of reducing body weight the results of dieting are often disappointing, one main reason being that reducing energy intake brings about a compensatory decrease in energy expenditure (Ravussin et al., 1988) to a level that matches the low intake, thus preventing further weight loss. Body weight is maintained constant when energy intake is equal to energy expenditure. To lose weight, one must either reduce energy intake or increase energy expenditure. Increasing energy expenditure or preventing the compensatory decrease in energy expenditure during dieting might reverse obesity.

One of the most obvious ways to increase energy expenditure is to increase physical activities like exercise. The most successful form of exercise for body weight reduction would be daily aerobic exercise, but this is almost impossible in severely obese patients due to cardiovascular and/or mechanical problems. It is therefore necessary to look for safe and effective agents to increase energy expenditure in obese patients without side effects.

One potential target for agents to increase metabolic rates in obese subjects is the β3-adrenoceptor (β3-AR) in brown and white adipose tissues (Himms-Hagen and Danforth, 1996). The effect of drugs targeted to this receptor is the principal topic of this thesis. Background information is, therefore, presented about β-adrenoceptors (β-ARs) in the control of metabolic functions of adipose tissue. The physiological functions of white adipose tissue (WAT) and brown adipose tissue (BAT) in the mammalian body are quite different. WAT is the main site of stored energy particularly in obese mammals. However BAT, which is present in most mammals, including humans, has a specific function of heat production or thermogenesis.

In general there are two types of heat production, obligatory thermogenesis, a by-product of all metabolic reactions necessary to keep the body alive, and facultative thermogenesis which usually occurs in two organs, skeletal muscle and BAT. In BAT, this heat production is stimulated by activation of the sympathetic nervous system and release of the neurotransmitter, noradrenaline (NA), from the nerves which supply BAT. Thermogenesis in BAT is independent of muscle

contracture and therefore it is called non-shivering thermogenesis. Physiological regulation of BAT thermogenesis involves the sympathoadrenal system and a complex system of neuroendocrine mechanisms (Trayhurn, 1986). Several drugs have been used to manipulate BAT function experimentally, among them some drugs acting specifically on BAT. The assessment of BAT function in humans is important to find a better way to control obesity and type II diabetes.

BROWN ADIPOSE TISSUE

BAT morphology and location

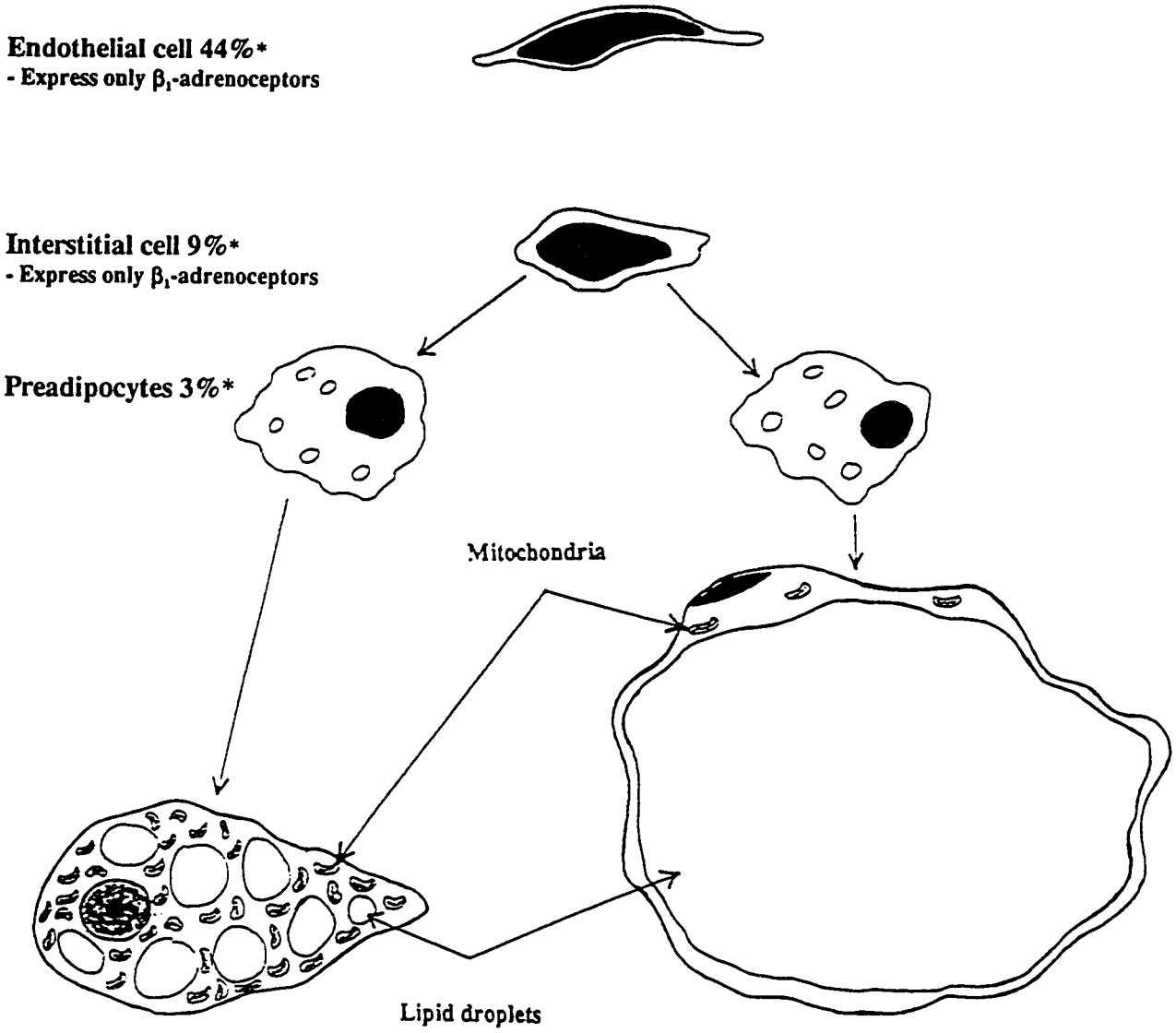
BAT is distributed in small deposits located in different sites in the body. The major locations for BAT are interscapular, axillary, perirenal and pericardiac regions (Néchad, 1986). In different species, the proportions of various deposits are different. In the rat the main deposit of BAT is interscapular BAT, which is a paired organ. Each side of interscapular BAT receives blood from a separate thoracodorsal artery and drains into a separate thoracodorsal vein that together make a plexus to allow a continuous heat exchange. Both sides also drain into one large central Sulzer's vein. Sulzer's vein drains directly into the azygous vein and, then, into the inferior vena cava and directly into the heart (Smith and Roberts, 1964). The internal vasculature of BAT includes an extensive capillary bed and arterio-venous anastomoses (Lever et al., 1985). Since the vasculature in BAT is very extensive and is served by vessels derived from the major arteries and veins, it is able to transfer the heat produced by

activated brown adipocytes to the heart where the warm blood is distributed throughout the body and skin surface.

The cell population in BAT consists of brown adipocytes, interstitial cells, preadipocytes, mast cells, and endothelial cells. The proportion of mature brown adipocytes is only 40% of the total cells in the tissue (Bukowiecki et al., 1986) (Figure 1). Mature brown adipocytes contain numerous small lipid droplets and are therefore called multilocular cells. Brown adipocytes contain abundant large mitochondria equipped with a unique protein, called uncoupling protein (UCP1) which is a proton translocator in the inner mitochondrial membrane and functions as an uncoupler of the mitochondrial respiratory chain (Ricquier et al., 1990). Brown adipocytes also contain other cytoplasmic organelles including a very big, lightly-stained nucleus which is often spherical and centrally located and has a darkly-stained nucleolus.

BAT hyperplasia occurs by proliferation of precursor interstitial cells which contain β1-adrenoceptors (Bukowiecki et al., 1982, 1986; Géloën et al., 1988). As the cells go on to develop, small lipid droplets form. When the hyperplasia process is stimulated, the preadipocytes and interstitial cells start differentiation into mature brown adipocytes with an increase in the size and number of their mitochondria and progressive loss of their ability to divide. At this point the mature brown adipocytes appear as multilocular cells with many mitochondria. As BAT grows the vasculature system is also augmented by recruiting endothelial cells (Himms-Hagen, 1990;

FIGURE 1. **Major cell types in BAT and WAT**



Brown adipocytes

- Express β_3-adrenoceptors as well as β_1-and β_2-adrenoceptors
- Contain several lipid droplets (so-called multilocular cell).
- Innervated by many sympathetic nerves.
- Supplied by many capillaries.
- Respond to catecholamines and β_3-adrenergic agonist by increasing thermogenesis.
- Contain uncoupling protein (UCP) in mitochondria.
- Abundant mitochondria with densely packed cristae.

White adipocytes

- Express β_3-adrenoceptors as well as β_1- and β_2-adrenoceptors.
- Contain single lipid droplet (a so-called unilocular cell).
- Contain few mitochondria.
- Respond to catecholamines and β_3-adrenergic agonists by increasing lipolysis.
- Do not contain UCP.

* The proportion of cells applies only to BAT. The contribution of various cell types in WAT is discussed in the text in chapters I and VI.

Schneider-Picard and Girardier, 1982; Bukowiecki et al., 1982,1986; Géloën et al., 1988). Since BAT possesses a very rich vasculature including arteriovenous anastomoses, it is able to sustain extremely high rates of blood flow when stimulated (Foster 1984; Foster and Frydman, 1979). During differentiation of brown adipocytes, the sympathetic nerve fibres, containing high NA content, grow into the tissue and functionally innervate it (Young et al., 1982; Foster et al., 1982).

The sympathetic innervation of BAT contains two type of nerves identified on the blood vessels of BAT, nerves containing only NA and nerves containing neuropeptide Y (NPY) and NA (Himms-Hagen 1989; Néchad, 1986). However, sympathetic noradrenergic axons on parenchymal cells do not contain NPY (Norman et al., 1988). The origin of the sympathetic nerves is derived from the middle and inferior cervical ganglia and the first five thoracic ganglia (Girardier and Seydoux, 1986). BAT also contains sensory nerves which also play a role in the maintenance of BAT function (Himms-Hagen, 1991).

BAT growth with sympathetic stimulation

Stimulation of the sympathetic nervous system results in the release of NA. A chronic release of NA cause a series of reactions which results in hypertrophy and hyperplasia of the BAT (Himms-Hagen ,1991), accompanied by an overall increase in gene expression and protein synthesis that increases BAT capacity for a thermogenic response to NA. The growth of BAT during cold exposure is thus

brought about by the increase in NA secretion due to the stimulation of sympathetic nervous system activity.

Brown adipose tissue atrophy in obese animals

It is suggested that a reduction in the activity of the sympathetic nervous system could be one of the initial defects in rodent obesity (Bray and York, 1979; Cousin et al., 1993b). A decrease in sympathetic tone decreases the activity of BAT, thus leading to atrophy of BAT and a lower energy expenditure which may result in the development of obesity (Himms-Hagen 1989). BAT is generally thermogenically inactive in an atrophied state in obese rats and mice.

WHITE ADIPOSE TISSUE

WAT morphology and location

WAT is distributed in large deposits at numerous locations in the body including retroperitoneal, inguinal, and parametrial or epididymal WAT depending on the gender of the animal. The size of WAT in different depots varies from species to species. One of the major depots in rats is retroperitoneal WAT. This tissue is a bilateral, symmetrical depot extending caudally from the diaphragm to the inguinal region in the groove formed on the dorsal wall of the abdominal cavity by the convergence of the spinalis and flank muscles. In large rats, these depots are often confluent with the deposits of white fat which surround the kidney capsule and renal

vasculature. White fat cells are very big (80-120 μm in diameter) compared to brown adipocytes. They are filled with a single triacylglycerol droplet and thus described as unilocular cells. White adipocytes contain only a few mitochondria with a few cristae (Figure 1). WAT contains three major cellular types, white adipocytes, interstitial cells, and endothelial cells (Géloën et al., 1989a, 1989b; Cleary et al., 1979).

WAT growth with age and in obesity

As mentioned before, white adipocytes are the major site for storing energy in all species. As the animals or humans grow, the energy intake in excess of energy expenditure is deposited in WAT in different locations in the body. In many models of obesity, both hypertrophy and hyperplasia occur in WAT (Di Girolamo and Mendlinger, 1971). It is suggested that the growth of WAT could be as a result of a decrease in sympathetic tone (Cousin et al., 1993b; Bray and York, 1979).

THERMOGENESIS

Categories and definitions

Shivering Thermogenesis:

Shivering is defined as a rhythmic contraction of the skeletal muscles induced by cold exposure and regulated by the central nervous system. When shivering is activated, ATP utilization increases and muscles start oxidising free fatty acids as the main fuel. The goal of this oxidation is the formation of ATP which is a usable form

of energy for cell functions. This ATP production and utilization is accompanied by heat production. It is interesting that although shivering is an involuntary response, it takes place in the skeletal muscles under control of motor nerves. Shivering increases the metabolic rate up to five times the basal level, depending on the species of animals (Hemingway, 1963). The heat production during the voluntary muscle contractions of light exercise is similar to shivering.

Nonshivering Thermogenesis

Animals exposed to cold are able to maintain a constant body temperature during long term exposure by increasing heat production as well as preventing heat loss. The principal mechanisms of increasing heat production in the cold exposed animals are shivering and nonshivering thermogenesis.

Nonshivering thermogenesis is defined as a heat-production mechanism liberating chemical energy due to processes which do not involve muscular contraction (Jansky, 1973). When a warm adapted animal, such as a rat or a guinea pig, is placed in a cold room (4°C) for a relatively long period of time, shivering gradually disappears and the animal maintains its body temperature by nonshivering thermogenesis. The relative proportion of shivering and nonshivering thermogenesis during the cold exposure depends on the age and the species of the animal and the duration of the exposure. The newborn of many species possess a high capacity for non-shivering thermogenesis. In most newborn mammals, including humans, this

capacity is present only during the early stages of life and gradually disappears during the first few month of life. However in adult rats and mice, the capacity for non-shivering thermogenesis remains high as when they stay in a cold environment for extended period of time. On the other hand, in hibernators, non-shivering thermogenesis is generally greater than in other mammals whether acclimated to cold or not (Himms-Hagen, 1976).

Thermogenesis is divided into two major components, obligatory thermogenesis and facultative thermogenesis (Himms-Hagen, 1989). Obligatory thermogenesis, a by-product of all metabolic reactions, does not include thermoregulatory thermogenesis (controlled by sympathetic nervous system). There is an increase in obligatory thermogenesis induced by thyroid hormone but this is very small compared with facultative thermogenesis. The difference between obligatory and facultative thermogenesis is that obligatory thermogenesis is always switched on whereas facultative thermogenesis can be switched on and off.

Facultative non-shivering thermogenesis is a process under the influence of stimulated sympathetic nervous system on acute exposure to cold. This is principally brought about by the secretion of NA from sympathetic nerve endings, although catecholamines secreted by the adrenal gland may also be significant (Himms-Hagen, 1990). The secreted catecholamines induce mobilization of substrates, mainly free fatty acids from WAT, required to maintain the high metabolic rate.

Role of brown adipose tissue in thermogenesis

The principal site of cold-induced facultative thermogenesis is BAT (Foster and Frydman,1979; Foster, 1984). BAT is known to be an important source of heat production in the newborn, in arousal from hibernation and in small animals living in a cold environment. Chronic stimulation of BAT, caused by cold-acclimation or β-AR agonists, induces hypertrophy of brown adipocytes that may also be accompanied by hyperplasia and a large increase in mitochondrial mass resulting in an increase in capacity for non-shivering thermogenesis in the tissue. Suppression of facultative BAT thermogenesis occurs in accordance with the needs of thermal balance in the body; when there is no need for thermogenesis, such as at thermoneutrality, BAT is suppressed. Respiration and heat production are closely related in BAT mitochondria, brown adipocytes and the whole tissue, suggesting that mitochondrial respiration is responsible for the thermogenic capabilities of BAT (Nicholls, 1983).

Mechanisms of thermogenesis

Heat is produced in all cells as a by-product of the oxidation of the carbohydrates, lipids and proteins derived from ingested nutrients. The major site of heat production in the cell is the mitochondrion; many reactions of metabolic pathways, including electron transport system and oxidative phosphorylation occur in this component. ATP synthesis normally happens in most cells and ATP is the

major source of energy that drives reactions that serve to transport molecules, maintain concentration gradients and muscle tone, accomplish synthesis and secretion, and perform the biological work required for respiration and blood circulation. Thermogenesis is an obligatory part of metabolism. In most cells, control of cellular thermogenic processes is dependent on the coupling of electron transport to ATP synthesis and the level of phosphate acceptor (ADP).

During physical exercise and shivering, ATP utilization increases. Resynthesis of ATP from ADP adjusts itself to the rate of ATP utilization as ADP is supplied to the proton translocating ATP synthetase. Metabolic rate and heat production increase. Synthetic pathways that utilize large amounts of ATP have also often been implicated in heat production, especially if coupled with the corresponding catabolic pathway in order to provide a futile cycle or net ATPase. The intracellular features of energy metabolism are complemented by systemic and local regulation of blood flow to maintain tissue oxygenation and by a hormonal regulatory system capable of controlling the mobilization, distribution, and storage of metabolic fuels.

When BAT is in a thermogenically inactive state, the uncoupling process is inhibited by binding of purine nucleoside diphosphate (ADP) or triphosphate (ATP) to a specific site on the UCP. The functioning of the UCP occurs only when the protein dissociates from the purine nucleotides (LaNoue et al., 1982; Himms-Hagen, 1989; Klingenberg, 1988). It has been shown that the binding of purine nucleotides to the UCP is pH dependent, and a very small increase in intracellular pH during the

stimulation of BAT by NA brings about the dissociation of purine nucleotides from the UCP and activation of a proton translocating system (Klingenberg, 1984, 1988).

In most cells, such as muscle and liver cells, the proton gradient produced by the electron transport chain in mitochondria is coupled to the synthesis of ATP. However in brown adipocytes the mitochondria have a high capacity for heat production that is not coupled to ATP synthesis. In this tissue the proton gradient produced by the mitochondria is allowed to dissipate by re-entry of protons into the mitochondria to produce heat rather than trapping the energy as ATP (Nicholls, 1983; Nicholls et al., 1986). This phenomenon is mediated by a mitochondrial inner membrane protein with a molecular weight of 32,000 Daltons. This protein is unique to BAT and is called uncoupling protein (UCP); it is also known as thermogenin (Cannon et al., 1982). Control of the proton-translocating function of the UCP is probably regulated by the changes in intracellular concentration of free fatty acids which interact with the UCP to activate it, probably via a conformation change which lowers the membrane potential at which proton translocation occurs (LaNoue et al., 1986; Nicholls et al., 1986; Himms-Hagen, 1992). The UCP gene of BAT mitochondria has been cloned and the protein has been purified and sequenced (Rabelo et al., 1995; Bouillaud et al., 1988). The UCP protein is a polypeptide with 306 amino acids (Lin and Klingenberg, 1982). UCP serves as a fatty acid anion transporter. It is able to transfer the proton directly or by catalysing fatty acid anion across the mitochondrial inner membrane. Fatty acids uncouple all mitochondria

when the concentration is high enough but their effect on BAT mitochondria is specific and requires UCP (Jezek et al., 1996).

Very recently, two other forms of UCP have been reported (Fleury et al., 1997; Gimeno et al., 1997; Boss et al., 1997; Vidal-Puig et al., 1997) which are expressed not only in BAT, but also in other tissues such as brain, kidney, liver, WAT, heart, pancreas, and skeletal muscles. These proteins are referred to as UCP2 and UCP3 and the longer known UCP, is now referred to as UCP1. In this thesis, the abbreviation UCP refers to UCP1.

SIGNAL TRANSDUCTION IN BAT AND WAT

Adenylate cyclase activation and cAMP formation

Thermogenesis in BAT is initiated by the interaction of NA at β-ARs on the plasma membrane, which stimulates lipolysis through activation of adenylate cyclase, increased cAMP concentration, activation of cAMP-dependent protein kinases and hormone sensitive lipase. As in other tissues, the fatty acids are then activated to acyl CoA and transported into the mitochondria via the carnitine shuttle and oxidized by β-oxidation (Walsh and Van Patten, 1994). The product, acetyl CoA, then enters into the tricarboxylic acid cycle.

After binding the hormone, adrenaline, or the neurotransmitter, NA to the β-AR subtypes, the receptors become able to activate the stimulatory G-protein (Gs) and cause a stimulation of cyclic AMP accumulation (Palczewski and Benovic, 1991).

The G protein itself is a heterotrimer protein consisting of α-, β-, and γ-subunits, which dissociate after GTP binding to the α-subunit (Sternweis and Smrcka 1992). The free α-subunit activates the catalytic subunit of adenylate cyclase, which forms cAMP from ATP.

Fuels for thermogenesis

Fatty acids, derived from the lipolysis of triacylglycerol are the immediate and major fuel for thermogenesis in BAT. NA-stimulated thermogenesis in BAT is totally dependent on fatty acid oxidation (Assimacopoulos-Jeannet et al., 1992; Vallerand et al., 1990; Marette and Bukowiecki, 1986). Although glucose has also been found to be used rapidly by BAT, it is not a major fuel for stimulated thermogenesis in this tissue (Isler et al., 1987). The fatty acids used by BAT can be derived from different sources in the body based on the nutritional state of the animal. The stores of triacylglycerol in BAT itself can support thermogenesis only for a short period of time. Thereafter, the fuel required for thermogenesis must be provided from triacylglycerol in blood chylomicrons from the food that is consumed or from very low density lipoproteins derived from de novo fatty acid synthesis (Himms-Hagen 1989). Lipid uptake requires lipoprotein lipase. Regulation of this enzyme is quite different in WAT and BAT. Stimulation of thermogenesis in BAT by NA is associated with a decrease in lipoprotein lipase activity in WAT, whereas activity is increased in BAT(Carneheim et al.,1988). During persistent stimulation by NA, the

synthesis of lipoprotein lipase, the enzyme present on the capillary endothelium at the surface of BAT, is increased which allows the tissue to obtain fatty acids from triacylglycerol in blood lipoproteins.

Glucose utilization also increases in stimulated BAT although glucose is not the main fuel for thermogenesis (Greco-Perotto et al., 1987; Isler et al., 1987; Gibbins et al., 1985). Stimulation of glucose uptake occurs in response to NA when thermogenesis is stimulated. Insulin can also increase glucose uptake by BAT but this is not how cold-induced increase is mediated. It has been shown that the major product of stimulated glucose metabolism in BAT is lactate plus pyruvate (Isler et al., 1987; Ma and Foster, 1986). The occurrence of this phenomenon in BAT is probably important to provide ATP generated by substrate level phosphorylation in reactions of glycolosis to sustain ATP-requiring processes such as fatty acid activation and ion pumping through the mitochondrial membrane by UCP (Himms-Hagen, 1989). The pyruvate produced could be converted to oxaloacetate to allow the operation of tricarboxylic acid cycle (Himms-Hagen et al., 1989).

STRUCTURE, FUNCTION, AND REGULATION OF ADRENOCEPTORS

Members of the adrenoceptor family

NA, secreted by sympathetic nerves, exerts its effects on brown and white adipocytes via a family of receptors called adrenoceptors (Himms-Hagen 1990).

In a variety of tissues and organs, the action of catecholamines is mediated by

two major classes of receptor, α and β. The characteristics of α1- and α2-receptors are pharmacologically distinguishable from that of β-receptors (Strosberg, 1993). Cloning and sequencing of the corresponding genes and the pharmacologic analysis of their products expressed in transfected cells have shown the existence of families of receptors, now categorized as: α1A, α1B, α1C; and α2A, α2B, α2C; and β1, β2, β3 (Strosberg et al., 1993; Milligan et al., 1994).

α_1-adrenoceptors

In brown and white adipocytes, α1-mediated effects operate through a transducing pathway involving operation of the phosphatidyl inositol bisphosphate (PIP2) cycle and activation of phospholipase C, which in turn increases the production of second messengers such as inositol tri-phosphate (IP3), Ca^{2+} ion, and diacylglycerol (Nånberg and Putney, 1986). The whole process in this case depends on the translocation and activity of protein kinase C (Lafontan and Berlan, 1993). It seems that stimulation of α1-adrenoceptors has a potentiating effect on thermogenesis and blood flow to BAT stimulated by β-adrenergic agonists.

α_2-adrenoceptors

α_2-adrenoceptors have been investigated extensively in fat cells of various species. The functional and pharmacological properties of the α_{2A}-adrenoceptors have been identified in fat cells of human and other animal species (Lafontan and Berlan,

1993).

Unlike β-adrenoceptors, α_2-adrenoceptors probably have an inhibitory effect on adenylate cyclase activity mediated by G_i-proteins (Strosberg, 1992, 1993; Himms-Hagen 1989). α_2-adrenoceptors may also be involved in the regulation of cytosolic pH in fat cells. Their stimulation induces cytosolic alkalinization and a stimulation of a Na^+/H^+ exchange mechanism (Himms-Hagen 1989; Lafontan and Berlan, 1993).

β1- and β2-adrenoceptors

β-adrenoceptors (β-ARs) are members of the family of G protein-coupled receptors, which all contain the hallmark of seven transmembrane regions (Emorine et al. 1991). These receptors are integral membrane glycoproteins mediating a wide variety of physiological actions of catecholamines, through coupling to G proteins and activation of adenylate cyclase (Emorine et al. 1991; Raymond et al. 1990).

The classic members of this family, introduced as β1-adrenoceptor (β1-AR) and β2-adrenoceptor (β2-AR), were originally classified according to tissue localization and ligand binding properties. Over the last few years, both β1- and β2-AR subtypes have been cloned and the primary sequences determined in human and other species (Frielle et al., 1988; Emorine et al., 1991). Pharmacological studies have shown that they are biochemically and functionally similar (Frielle et al., 1988). Both receptors mediate the catecholamine-induced activation of adenylate cyclase through the GTP-binding protein G_s. The two receptors can be distinguished on the basis of

their interactions with the natural agonists, adrenaline and NA, as well as several selective antagonists. The β1-AR binds adrenaline and NA with approximately equal affinities, whereas the β2-AR binds adrenaline much more than it does NA (Arch, 1989). In addition to the existence of distinct pharmacological differences, the β-AR subtypes also show a characteristic tissue distribution. It has been shown that both subtypes are often present in a tissue although one subtype may predominant. For instance, the β1-AR appears to predominate in heart and adipose tissue, whereas the β2-AR predominates in lung, liver and smooth muscle (Milligan et al., 1994).

Adrenoceptors are proteins with extracellular amino acids which often contain sites for asparagine-linked glycosylation. There are also seven clusters of hydrophobic-rich amino acids which present transmembrane segments within each receptor (Strosberg, 1993). Each of these transmembrane segments is connected by extracellular and intracellular loops. The carboxyl terminus is intracellular. The third intracellular loop and carboxyl terminus can be highly variable in length and amino acid composition between different adrenoceptors. The similarities between amino acid sequences in adrenoceptors are greatest in the transmembrane segments which are the regions that comprise the agonist binding domains (Strosberg, 1993; Dohlman et al., 1988). Different studies have demonstrated that the adrenoceptor binding pocket resides within the transmembrane clusters of hydrophobic residues (Dohlman et al., 1988). Site-directed mutagenesis studies further confirmed that the hydrophobic regions are very important in ligand binding (Dohlman et al., 1990). Structural

determinants important for distinguishing agonists and antagonists are localized on transmembrane segments 6 and 7 (Frielle et al., 1988).

Desensitization is a common feature of β1- and β2-ARs. This phenomenon is defined as a reduction of a response in spite of the continuous presence of a constant stimulation (Dohlman et al., 1991; Carpéné et al., 1993; Hausdorff et al., 1990a). Desensitization has been observed in many G-protein coupled receptor systems and particularly studied for the stimulation of cAMP levels by plasma membrane β1- and β2-ARs (Liggett and Schwinn 1991; Liggett and Raymond, 1993). The desensitization phenomenon includes two mechanisms: short term desensitization and long term desensitization. There is a rapid evolving desensitization that is essentially completed after exposure of cells to micromolar agonist levels for 30 min (Hausdorff et al., 1990a). During this time, receptor-mediated stimulation of adenylate cyclase activity is reduced with a decrease in the ability of the receptor to form the high affinity agonist-receptor-G_s complex. The beginning of the short term agonist-promoted desensitization of the β2-AR is an uncoupling of the receptor from G_s. This uncoupling phenomenon involves phosphorylation of β-AR by at least two kinases, protein kinase A (PKA) and β-AR kinase (β-ARK), which are activated under several desensitizing conditions (Hausdorff et al., 1990a; Carpéné et al., 1993; Liggett and Raymond 1993). It has been shown that the targets of phosphorylation by PKA and β-ARK in β2-AR are located in the third cytoplasmic loop and the carboxyl terminus of the receptor (Carpéné et al., 1993).

It has been shown that purified β-ARs derived from desensitized cells are functionally impaired in their ability to couple to purified G_s proteins in a reconstituted phospholipid vesicle system, due to the phosphorylation of β-AR by PKA and β-ARK (Sibley et al., 1987). Another mechanism which is involved in functional uncoupling is a very rapid sequestration and internalization of β-AR away from plasma membrane which happens after exposure of cells to micro-molar levels of agonist (Liggett and Raymond, 1993).

Down-regulation is considered to be a separate component of agonist-promoted desensitization (Liggett and Raymond, 1993). A decrease in transcription of β2-AR mRNA, a decrease in the stability of β2-AR mRNA, and an increase in degradation of β2-AR expressed at the cell surface could be causes of receptor down-regulation (Hausdorff et al., 1990; Liggett and Raymond 1993). It appears that the processes involved in degradation of β2-AR mRNA are critical to the receptor down-regulation process (Hadcock et al., 1989).

β3-adrenoceptors

During the past few years, investigators have suggested that there is/are one or more additional β-AR subtypes atypical in nature (Arch, 1989; Arch and Kaumann 1993; Bloom et al. 1992; Zaagsma and Hollenga, 1991). These atypical receptors, later called β3-adrenoceptors (β3-ARs), like other members of the β-AR family, are found on the cell surface of both white and brown adipocytes where their stimulation

promotes both lipolysis and energy expenditure in BAT and lipolysis in WAT. The characterization of human and rodent genes encoding a third β-AR subtype (β3-AR) has provided a structural basis for the existence of the atypical β-ARs (Emorine et al., 1989; Granneman et al., 1991; Muzzin et al., 1991; Nahmias et al., 1991). The human β3-AR is 50% homologous to the human β1-AR and β2- AR (Nahmias et al., 1991). In contrast to β1 and β2 adrenoceptors, the human and mouse β3-ARs contain several exons and introns (Spronsen et al., 1993; Bensaid et al., 1993; Granneman et al., 1993). β3-ARs have an amino acid sequence which suggests the presence of seven transmembrane spanning regions with an extracellular terminus (Granneman et al., 1993; Strosberg, 1993; Emorine et al., 1992). Different β-AR subtypes as well as β3-AR can activate the same effector and utilize the same physiological agonists. They all couple to stimulatory form of G-protein and in the presence of agonist transduce a signal that results in increased intracellular cAMP. It has been identified that there are several regions of the β1-, β2- and β3-AR which provide for antagonist or agonist subtype-specific binding (Frielle et al. 1988; Liggett and Raymond, 1993).

Unlike β1- and β2-ARs, β3-ARs are resistant to desensitization. It is interesting that β3-AR lacks most of the potential phosphorylation sites for β-adrenoceptor kinase and protein kinase A (Carpéné et al., 1993). There are only 3 serine in the comparable region of the β3-AR which are not in a favourable site for phosphorylation that make the receptor resistant to desensitization (Carpéné et al., 1993; Hausdorff et al., 1990a), whereas the β2-AR has a C terminus containing 11

serine and threonine residues representing potential sites for β-AR kinase phosphorylation, which mediate rapid agonist-induced desensitization,

The β3-AR also lacks an apparently critical 10-amino acid sequence motifs in the cytoplasmic tail which has been implicated as playing a role in β2-AR sequestration (Hausdorff et al. 1991). Thus, the regulation of β3-AR differs with that in β1- and β2-ARs because of the lack of the molecular features for agonist-induced regulation which have been established with β1- and β2-AR. Different subtypes of adrenoceptors may have evolved to respond to different needs for agonist regulation. The physiological importance of β3-ARs probably lies in their capacity to mediate sustained stimulation of brown adipocytes by NA.

AGONISTS FOR SUBTYPES OF β-ARs

Non-selective agonists for β-ARs

NA secreted by sympathetic nerves and adrenaline secreted by adrenal glands are the natural ligands for activation of lipolysis and thermogenesis in animals and humans by acting through β-ARs in both white and BAT. Several other compounds such as isoprenaline, salbutamol and fenoterol were also found to be effective on β-ARs to stimulate metabolic rate. However, the non-selectivity of these compounds makes them unusable for treatment of obesity, because of their unwanted effects on β1- and β2-ARs to increase atrial rate and to induce smooth muscle relaxation. Therefore, most investigations are now focused on finding a potential selective β3-AR

agonist.

Selective agonists for β3-ARs

To support the existence of β3-ARs and to find a potential β3-AR agonist that might control the development of body fat and be useful in treatment of obesity, a series of different drugs has been developed (Champigny et al., 1991; Lönnqvist et al., 1993; Arch et al., 1984; Bloom et al., 1992; Largis et al., 1994). Selective β3-adrenoceptor agonists are potentially useful in treatment of obesity because they could increase energy expenditure with few β1- and β2-adrenergic side effects. Most of them increase thermogenesis, decrease body weight, and improve insulin-resistance in obese animals (Bloom et al., 1992; Revelli et al., 1992; Himms-Hagen et al., 1994; Largis et al., 1994; Meier et al., 1984; Yoshida et al., 1994). The selectivity for β3-adrenoceptors could make them potential agents for treatment of obesity and diabetes in animal models of NIDDM.

Many pharmaceutical companies have developed β3-AR agonists (Table 1). Amongst these compounds, phenethanolamines were found to be very potent agonists for stimulation of lipolysis. For instance, BRL 35,135 and its active metabolite, BRL 37,344 (Figure 2A) show the greatest selectivity (Arch, 1989). These compounds have less effect on atrial rate mediated by β1-ARs or tracheal relaxation mediated by β2-ARs. However, the major problem in treatment of human obesity with β3-AR agonists, so far, has been their stimulatory effect on other β-receptors resulting in

TABLE 1

A list of β3-adrenergic agonists introduced by pharmaceutical companies

Pharmaceutical company	β3-adrenergic agonists
SmithKline Beecham	BRL 37,344, BRL 35,135
Hoffmann-La Roche	Ro 16-8714, Ro 40-2148
Zeneca (ICI)	ZD 7114
Ciba-Geigy	CGP 12177
Lilly	LY 104119, LY 79771
Sanofi-Midi	SR 58611A
American Cyanamid	CL 316,243
(Wyeth-Ayerst	BTA 243)
Merck	L-742,791
Pfizer	
Bristol-Myers Squibb	

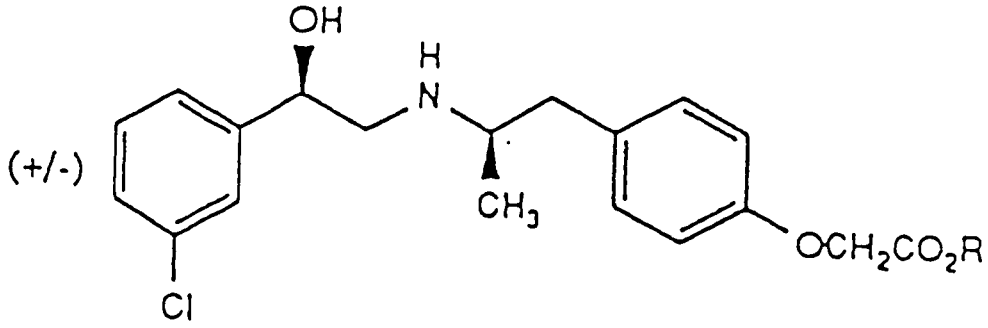
(Revelli et al., 1992; Meier et al., 1989; Blin et al., 1994; Muzzin et al., 1994; Hollenga et al., 1990).

FIGURE 2

The structure of selective β3-adrenergic agonists

Phenethanolamines as β3-adrenergic agonist

A) BRL 35,135 (HBr salt) and its active metabolite, BRL 37,344.

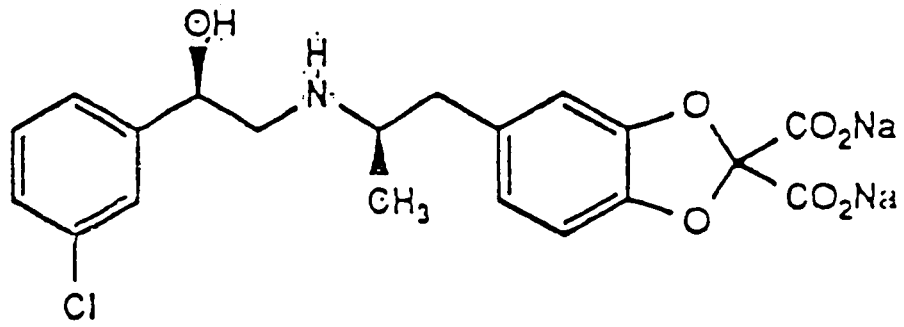


R= CH_3; BRL 35,135 (HBr salt)

R= H; BRL 37,344

B) CL 316,243 is a benzodioxole-containing phenethanolamine and highly selective for β3-ARs with poor affinity to β1- and β2-ARs.

CL 316,243



Bloom, J.D. et al., J. Med. Chem. 35: 3081-3084, 1992

muscle tremor mediated by β2-AR and increased heart output mediated by β1-AR (Bloom et al., 1992). Another problem, realized only more recently, is their relative lack of efficacy for the human β3-AR, compared with their potent effects on rodent β3-ARs (Arch and Wilson, 1996).

The novel β3-AR agonist CL 316,243

The selective β3-AR agonist chosen for study in the work reported in this thesis is a new compound named CL 316,243, disodium [(R,R)-5-[2-[[2-(-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate] (Figure 2B) which is a benzodioxole-containing phenethanolamine and a highly potent β3-adrenergic agonist (Bloom et al., 1992). CL 316,243 is almost totally selective for β3-adrenoceptors and is an extremely poor β1- and β2-AR agonist (Bloom et al.,1992; Dolan et al., 1994). This compound not only possesses anti-obesity effects (Himms-Hagen et al., 1994), but also has anti-diabetic effects and consequently may be useful for treating obesity as well as NIDDM in obese animals (Largis et al., 1994; Yoshida et al., 1994; Umekawa et al., 1997).

ROLE OF β3-ARS IN OBESITY AND INSULIN RESISTANCE

Mutated β3-ARs in obese humans

Studies on β3-adrenoceptors in visceral adipose tissue of obese Pima Indians (Walston et al., 1995), obese Finns (Wilden et al., 1995), and morbidly obese French

subjects (Clément et al., 1995) have indicated that a mutation of the β3-AR is associated with the early onset of NIDDM, a tendency of a low metabolic rate and clinical features of the insulin resistance syndrome including increased blood pressure and high serum concentrations of insulin, glucose, and lipid together with increased capacity to gain weight. This mutation is a replacement of a single tryptophan by arginine (Trp64Arg). These results indicate not only that the Trp64Arg mutation was a candidate for a genetic alteration leading to obesity, but also that the adrenergic system is important for the development of insulin resistance and other metabolic complications of obesity. However, more recent studies have indicated a doubtful significance of mutated β3-ARs (Trp64Arg) in obesity and insulin resistance (Elbein et al., 1996; Oksanen et al., 1996; Hinney et al., 1997; Fujisawa et al., 1997; Gagnon et al., 1996). In these investigations, mutation of Trp64Arg in β3-ARs was not associated with insulin resistance and NIDDM (Elbein et al., 1996; Fujisawa et al., 1997), with regulation of body weight in younger subjects (Hinney et al., 1997; Oksanen et al., 1996), or with body fat including abdominal visceral fat, resting metabolic rate and changes in body weight in Quebec family study (Gagnon et al., 1996). Therefore, it is suggested that Trp64Arg mutation of β3-ARs is not of universal importance for controlling insulin resistance susceptibility and NIDDM and hypertension.

Insulin resistance in obesity

In mammals, insulin is the principal hormone that controls blood glucose. insulin acts by stimulating glucose influx and metabolism in muscle and adipocytes and inhibiting gluconeogenesis by the liver. Insulin action is mediated through the insulin receptor, a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity. The level of tyrosine kinase activity is affected by the serum concentration of insulin and appears to mediate the insulin response through tyrosine phosphorylation of the receptor itself and of substrates like insulin receptor substrate-1 (IRS-1). Insulin resistance, a cause of NIDDM, is a state in which target cells no longer respond to ordinary levels of circulating insulin (White and Kahn, 1994). There are several reports that insulin resistance and abnormal glucose homeostasis are strong correlates of obesity in both animal models and humans (Spiegelman et al., 1993; Hotamisligil et al., 1993, 1995; Hofmann et al., 1994). Obesity is the most common metabolic disorder that affects more than 30% of the adult population in North America and is a major risk factor for NIDDM; more than 80% of NIDDM patients in the USA are obese (Spiegelman et al., 1993).

Role of TNF-α in insulin resistance.

Some other studies have shown that TNF-α, a potent cytokine primarily produced from macrophages and adipocytes (Pennica et al., 1984), mediates insulin resistance in cultured cells as well as in the whole organism (Hotamisligil and

Spiegelman, 1994; Hotamisligil et al., 1995).The expression of TNF-α mRNA in the adipose tissue of obese humans and genetically obese fa/fa rats is significantly elevated and this elevation is strongly correlated with the level of hyperinsulinemia and insulin resistance (Hotamisligil et al., 1995). Administration of TNF-α into humans at certain doses induces a hyperglycaemic state without alteration in insulin levels, suggestive of insulin resistance (Van Der Pol et al., 1991). Furthermore, a constant infusion of TNF-α into rats causes a marked insulin resistance in peripheral tissue and liver (Lang et al., 1992). Neutralization of circulating TNF-α with soluble human TNF-receptor-IgG fusion protein improves the insulin resistance of the fa/fa rat (Hotamisligil et al., 1993). Treatment of obese KK mice with pioglitazone, an insulin sensitizing agent, improves their insulin resistance and reduces the expression of the TNF-α in their WAT (Hofmann et al., 1994). However, in a recently published paper, López-Soriano et al.(1997) have reported that TNF-α is not involved in the lipid metabolism disorders observed in the obese Zucker rats. Any role for β3-ARs in TNF-α expression is unknown.

Role of leptin in regulation of body fat

Leptin is the protein product of the ob gene. Expression of ob gene in adipose tissue and its secretion is part of the system that regulates the body fat stores. Leptin acts on central nervous system to increase energy expenditure and to decrease food intake by inhibiting appetite (Halaas et al., 1995). Murine leptin is a 16 kD protein

containing 167 amino acid residues. Effect of leptin on central nervous system is mediated by leptin receptors. Any mutation of these receptors will result in a decrease in leptin sensitivity, as observed in db/db mice (Chen et al., 1996). It has also been shown that the long intracellular domain form of leptin receptors is crucial for initiating intracellular signal transduction, while leptin receptors in diabetic mice contain a short intracellular domain which make them unable to mediate the effect of leptin on brain (Chen et al., 1996).

More recent reports have shown that the activation of β3-ARs is able to suppress the expression of leptin protein in mice (Mantzoros et al., 1996; Trayhurn et al., 1996; Gettys et al. 1996; Giacobino 1996).

CHAPTER II

OBJECTIVES

The general long-term objective of the research was to elucidate mechanisms by which β3-AR agonists could improve obesity and insulin resistance. The novel β3-AR agonists, CL 316,243, was chosen because it was already known to be highly effective in rats (Himms-Hagen et al., 1994).

The initial objective was to study the way in which chronic stimulation with a β3-AR agonist might increase insulin-sensitivity of BAT in an animal known to be insulin-resistant, the guinea pig. Choice of the guinea pig had the advantage that it was already known that brown adipocytes could be isolated in a state of low thermogenic capacity when the animal is warm-acclimated and high thermogenic capacity when the animal is cold-acclimated, and that BAT becomes a major site of glucose utilization in the in the cold-acclimated state (Himms-Hagen et al., 1995). Unfortunately, guinea pig brown adipocytes were not responsive to the β3-AR agonists, although they were responsive, as expected, to the endogenous ligand, NA. This part of the project, done in collaboration, did yield interesting results which have been published (Himms-Hagen et al., 1993, 1995). My part of study appears as chapter IV in this thesis.

To pursue the long-term objective, subsequent studies were done with rats which we already knew possessed β3-ARs in their BAT:

1. The effect of CL 316,243 on diet induced obesity (DIO) in rats. The objective was to find out whether a long-term infusion of CL could reverse already established obesity in rats eating a high-fat diet. This has been published (Ghorbani et al., 1997). In this study, discussed in chapter V part A, CL reversed obesity by making white adipocytes shrink without reducing their number.

2. The effect of CL 316,243 on mature white adipocytes in genetically obese rats with hyperplastic obesity. The principal objective was to find out whether the excessive number of white adipocytes could be made to disappear by the CL-treatment. Results of this study are in chapter V part B and have been published (Ghorbani and Himms-Hagen, 1997a).

3. The effect of CL 316,243 on the expression of TNF-α. While the experiments outlined in 1. and 2. were in progress the first reports were published about the linkage of TNF-α expression and occurrence of obesity and type II diabetes. Thus, we continued our study with the next experiment to find out whether CL 316,243 suppressed TNF-α expression in WAT. This work is described in chapter V part C of this thesis.

4. When the results from our previous objectives were being collected and calculated in 1995, leptin, a product of the ob gene was identified and the effect of

leptin on body weight gain and food intake was shown in different animal models of obesity. Since expression of leptin in WAT of genetically obese rats and diet-induced obesity in rats eating a high-fat diet was known to be high, we studied the effect of CL on serum leptin level in rats with diet- or aging-associated obesity and in Zucker rats with genetic obesity. This work is described in chapter V part D of this thesis and has been submitted for publication (Ghorbani and Himms-Hagen, 1997b).

CHAPTER III

MATERIALS AND METHODS

1- ANIMALS

Female guinea pigs, male Sprague-Dawley rats and genetically obese Zucker (fa/fa) rats and their lean littermates (Fa/-) were obtained from Charles River Canada.

Guinea pigs arrived either at the age of 8 weeks or at advanced stage of pregnancy. They were housed in plastic cages with wood-chips as bedding at 24°C with free access to food (guinea pig chow).

Thirteen-week-old Sprague-Dawley rats were weighed and housed individually in hanging wire cages and placed at room temperature (24°C) with free access to food and water. Two weeks later, they were separated into two groups, one with access to chow and the other with access to high-fat diet (HF). Rats were weighed twice a week and food intake was measured once a week.

Zucker rats were purchased at the age of 13 weeks. They were kept individually in plastic cages with wood-chips at room temperature (24°C) with free access to food (chow) and water until the age of 35 weeks. Then, they were weighed and the food intake was measured twice a week.

The light cycle in all experiments was 12 hours light/12 hours dark with lights on at 06:00 A.M.

2- FOOD

Animals had free access to water and food, either rat chow (Agway RMH 4020) contained 14.5 % energy from fat, 24% energy from protein and 61.5% energy from carbohydrate, or high-fat diet (4.81 kcal/g). The high-fat diet was obtained from Teklad premier, Wayne Laboratory animal diets, and was composed of 27.5 % casein, 15.37 % sucrose, 14.5 % corn starch, 29.37 % partially hydrogenated vegetable oil, 6.25 % cellulose, 4.374 % mineral mix, 0.5 % calcium carbonate, 1.25 % vitamin mix, 0.63 % choline bitartrate, 0.25 % DL-methionine and 0.006 % ethoxyquin. The food quotient of the HF diet was 0.795. (TD 85418, 54 % of energy from partially hydrogenated vegetable oil).

Guinea pigs had free access to water and guinea pig chow.

3. PROCEDURES INVOLVING ANIMALS

A. Subcutaneous infusion of CL 316,243

A.a) Preparation of the Alzet mini-osmotic pump

The Alzet mini-osmotic pump model 2002 was used to deliver solutions continuously for 14 days. The mean pumping rate for each pump was .49 µl per hour of either saline (0.9%) or CL 316,243 (CL) (1 mg/ kg body weight per day). All ALZET pumps were filled completely with saline or CL and incubated in sterile saline at 37°C for 4 hours, based on the manufacturer's recommendation. For filling the Alzet osmotic pumps, the empty pumps with their flow moderators were first weighed, then the flow moderator was removed and the pump was filled with a 3 ml

syringe and a blunt-tipped, 25-gauge filling needle. The pumps were filled carefully while trying not to introduce air bubbles into the reservoir. The flow moderators were inserted and the pumps were weighed again to verify that enough volume of solution was introduced into the pump.

A.b) Implantation of the Alzet mini-osmotic pumps

The mini-osmotic pumps were implanted subcutaneously in both Sprague-Dawley and Zucker rats under halothane anaesthesia. For subcutaneous placement, the back area of the rat (close to hip) was shaved and scrubbed with Betadine and 70% alcohol and a small incision (max. 2 cm) was made in the skin along the vertebral column. Using a haemostat, a small pocket was formed under the skin, simply by spreading apart the subcutaneous connective tissue. The pump was then inserted into the pocket with flow moderator pointing away from the incision. For prevention of the pump moving, a single suture was made to attach the pumps to connective tissue and the skin incision was then closed with 2-3 wound clips. After two weeks, the pumps were replaced with new ones to have a full 4-week-treatment.

B. Measurement of resting energy expenditure

On day 12 after implantation of pumps, resting metabolic rate was measured using an oxygen analyser (Beckman Industrial Oxygen Analyser Model 755). The rat was placed in a temperature controlled chamber at 24°C and airflow was regulated

with a Brooks thermal mass flowmeter (Brooks Instrument Division, Emerson Electric, Hatfield, PA).Oxygen concentration in the air flowing through the chamber was calculated using a computerized program (Ma and Foster, 1986b).

4) TISSUE SAMPLE PREPARATIONS FROM GUINEA PIGS AND RATS

Animals in each experiment were sacrificed by decapitation. Blood was collected in conical centrifuge tubes placed on ice and centrifuged at 5000 rpm for 20 minutes. The blood serum was then transferred into microfuge tubes and stored at −80°C for appropriate assays. Interscapular BAT (IBAT) and perirenal BAT (PBAT) were dissected and placed in ice cold isolation medium (0.25 M sucrose, 0.2 mM dipotassium EDTA, and 1.0 mM HEPES, in distilled water, pH 7.2) and cleaned from adherent muscles and vasculatures. Small pieces of IBAT and PBAT (2 or 5 mm in diameter) were fixed in either glutaraldehyde or formalin for histology and the remainder of the tissue was homogenized in isolation medium using a Polytron (model PCU-2-110, Brinkman Rexdale, Ontario instrument) with setting at 4. Retroperitoneal and epididymal WAT were also dissected, fixed and homogenized in the same fashion as BAT. Small samples of WAT (150-200 mg) were also used for osmium fixation method as described later.

Preparation of rat and guinea pig BAT mitochondria

Some proportion of homogenate prepared for each sample was placed in a 50 ml tube and centrifuged at 3000 RPM for 10 min. The supernatant was filtered through cheese cloth to get rid of the fat and added to the pellet and centrifuged at 10000 RPM for 14 min. The supernatant was discarded and the pellet was resuspended in 42 ml isolation medium and centrifuged at 10000 RPM for 14 min. The supernatant was discarded and the pellet was resuspended in microcentrifuge tubes with isolation medium and centrifuged again at 10000 RPM for 14 min. The supernatant was carefully discarded without disturbing the pellet. The pellet was then dried and resuspended in 180-300 µl isolation medium and kept at -80°C.

5) HISTOLOGICAL PROCEDURES

A. Light microscopy of thick sections

A.a) Paraffin embedded tissues

RWAT fragments were fixed in 10% buffered formalin. After embedding the tissue in paraffin, thick sections (7 to 10 μm) were cut, deparaffinized and rehydrated as follows: sections were left in xylene for 5 min (twice), then washed twice in 100% ethanol for 3 min, once in 95% ethanol for 3 min, once in 70% ethanol for 3 min, and once in water for 5 min.. Then the sections were stained with haematoxylin and eosin (6 min incubation in filtered haematoxylin solution (Sigma Cat # GHS-2-16), rinsed in water and differentiated in acid-alcohol (30% alcohol+ 1% HCl) for 3 sec., washed

in lithium carbonate (1 g/100 ml distilled H2O) until the section was blue, washed in running tap water for 10 min, placed in alcoholic eosin solution (Sigma Cat. # HT110-1-16) for 2 min, dehydrated once in 95% alcohol for 2 min, twice in 100% alcohol for 2 min, and once in xylol for 5 min, then mounted in Permount (Bancroft 1975; Lillie & Fulmer 1976).

A.b) Immunohistochemistry

Deparaffinization and rehydration of sections were exactly the same as described before. Sections were incubated with the primary antibody to hamster UCP diluted with TCT (1:100) (Tris buffer, 0.6% Carrageenan and 0.3% Triton X-100) overnight at 4°C. (Antibody for UCP was raised in rabbits using purified hamster UCP and stored in glycerol 1:1 at -20). The next day, sections were washed twice in TBS (Tris buffered saline) (0.1 M Tris and 0.14 M NaCl) for 7 min and incubated with a secondary antibody for 30 min (donkey anti-rabbit Ig, biotinylated whole antibody) diluted in TCT buffer (1:50)). Sections were washed in TBS twice for 7 min and neutralized for 10 min in 3% hydrogen peroxide diluted with TBS. Sections were washed again in TBS for 7 min and then were incubated with streptavidin-horseradish peroxidase conjugate diluted with TCT (1:50) for 30 min at room temperature. They were washed in TBS twice for 7 min, then incubated with diaminobenzidene (DAB) (200 μg/100 ml TBS + 700 μl 10% NiCl2 + 10 μl hydrogen peroxide) for 12 min in the dark. Sections were then dehydrated in alcohol, cleared

in xylol and mounted in Permount (Brandtzaeg 1982).

A routine microscope (Zeiss Axioskop 20) for transmitted light and incident-light fluorescence microscopy equipped with a microscope camera (mc 80) was used. The fluorescent emission was generated by a Short Arc Mercury Lamp HBO.

The magnification number in both fluorescence and light microscopy was 400x (objective 40 x OPTvar 1.25 x objective of camera 16).

B. Confocal Microscopy

Immunofluorescence using a double staining method

After deparaffinization and rehydration, sections were rinsed in PBS (phosphate buffered saline) (0.092% K_2HPO_4, 0.02% KH_2PO_4, 0.8% NaCl in distilled water, pH 7.4) for 2 min, then were incubated with primary antibody to UCP for 40 min at room temperature. Then they were washed 3 times and incubated with secondary antibody , donkey anti rabbit Ig, (1:100), for 30 min at room temperature. Sections were washed 3 times and incubated with fluorescein-5-isothiocyanate (FITC) (1:30) for 40 min. Sections were rinsed again in PBS and incubated with bis-benzamide (Hoechst 33258, Sigma Cat. # B-2883) (1:3000) for 2 min. Then they were rinsed in PBS and mounted in permount. The double stained slides were then used for making confocal micrographs as described below.

For the confocal micrographs we used a confocal laser beam scanning microscope (Leica, Germany) with the ability of rotating samples. The depth of the

sample at the point of focal plan illumination was 0.25 to 0.5 μm. The magnification number used for confocal microscopy was also 400x.

C. Light microscopy of semi-thin sections (Epon mixture embedded tissues)

Small pieces of freshly cut tissue (1-2 mm^3) were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight on a rotator. The tissues were washed 3-4 times with 0.1 M cacodylate buffer (pH 7.4). The tissues were then post-fixed with 2% osmium tetroxide for 2 hours on a shaking platform at room temperature in the fumehood. Then, they were washed again with 0.1 M cacodylate buffer with 2 changes. After the last wash, samples were dehydrated with graded ethanol (50%, 65%, 80%, 95%, 100% for 10-15 minutes each), and then incubated in propylene oxide at room temperature (2 times for 10-15 minutes each). They were then incubated in a mixture of 1 part Epon mixture [49.3% Jembed resin, 29.6% dodecenyl succinic anhydride, 19.7% nadic methyl anhydride (JBS, Cat. # JBS-032) 1.4% DMP-30 (tri(dimethylaminomethyl phenol)] and 1 part propylene oxide for one hour on a shaking platform. This step was followed by two incubations with undiluted Epon mixture for at least 1 hour each. The tissue samples were then placed in the tips of the wells in embedding molds containing freshly made Epon mixture and incubated in an oven under vacuum at 45°C overnight. Next, the blocks were trimmed using safety razor blades and sectioned with glass knives. Sections (0.5 to 1 μm) were placed on a drop of double

distilled water on a glass microscope slide and heated to let the water evaporate. Then, they were stained with a mixture of 1% methylene blue, 1% borax (sodium tetraborate), and 1% azure II on a hot plate for 4-5 seconds, then washed, mounted in and viewed under a microscope. The magnification number for semi-thin sections was 2000x using the objective 100x with immersion oil.

6- PROCEDURE TO MEASURE RESPIRATION OF ISOLATED BROWN ADIPOCYTES

Oxygen consumption of brown adipocytes of adult guinea pigs, newborn guinea pigs and rats was measured using a Clark Oxygen Electrode. This is an instrument employing an electrolytic (cathodic) reduction of O_2 under polarographic conditions. A voltage of 0.6 V is applied across two electrodes, one a platinum wire "working electrode" and other an Ag/AgCl reference which is embedded in a plastic body. If the electrodes are enclosed by a thin hydrophobic membrane containing a film of KCl, only O_2 can diffuse through the membrane and the diffusion limited current is directly proportional to the concentration of O_2 outside the membrane and can be measured after amplification. If this electrode probe is immersed in a mixture containing respiring cells, the variations in PO_2 of the contents can be read from the chart which is recording the polarographic diffusion current.

In this procedure, after decapitation of the animal (rats, adult or newborn guinea pigs), interscapular BAT (IBAT) was dissected and immediately transferred

to Krebs-Ringer bicarbonate (KRB) (0.9 % NaCl, 1.15 % KCl, 0.11 M $CaCl_2$, 2.11 % KH_2PO_4, 3.82 % $MgSO_4.7H_2O$, 1.3 % $NaHCO_3$, 0.72 % HEPES, 1.0 % BSA). The tissue was then blotted, weighed, divided into 1.5 g portions and cut and minced finely with scissors. Then, each 1.5 g portion of tissue was placed in a polyethylene scintillation vial containing 5 ml KRB containing 12 mg collagenase for adipocyte isolation (Sigma C-6885) per ml. The vials were, then, gassed with 95% O_2/ 5% CO_2 and incubated at 37°C in a rotatory water bath with shaking for 30 minutes. The vials were removed and shaken up and down energetically 20 times to free the cells from the undigested pieces. At the end of the incubation the free cells were floating. Then, the contents of the vials were filtered through a nylon mesh (Nytex 560, size 250 μm) and washed by flotation using an incubation medium containing bicarbonate-buffered culture medium (Gibco DMEM 380-2320 AG) supplemented with 5.5 mM glucose, 1mM ascorbic acid and 40 mg/ml fatty acid-free bovine serum albumin into a clean 50 ml centrifuge tube to separate the floating cells from undigested tissue. The cells collected in the 50 ml tube were centrifuged at room temperature at 1000 RPM for 1-3 minutes and the infranatant was aspirated with a Pasteur pipet. Then, the remaining cells were resuspended in a few ml incubation medium and allowed to stay at room temperature for 3 minutes and centrifuged again. This step was repeated twice to get rid of the remainder of collagenase in the suspension. After the last wash, the cells were counted under the microscope using a Neubauer improved haematocytometer slide with 0.100 mm depth and the volume of the suspension was adjusted to the

appropriate concentration of cells that was wanted. Then, an appropriate number of cells ($\sim 10^6$ cells) was injected into the chamber containing incubation mixture that had been saturated with continuous gassing with O_2/CO_2 (Rafael et al., 1986). Initially, the basal level of O_2 concentration was recorded by the polarograph before injecting the cells. After injection of the cells into the mixture, the reduction of O_2 content by the respiring cells was monitored by the polarograph and calculated based on the basal oxygen level. After recording the basal oxygen uptake, the β-AR agonist was injected into the incubation medium starting with the low concentration. Five different concentrations of noradrenaline (NA), adrenalin (A), isoproterenol (ISO), CL 316,243 (CL), or BRL 37,344 (BRL) were used. For each concentration, the cells were allowed to respire to reach the maximum rate of oxygen uptake.

7- ASSESSMENT OF ADIPOCYTE SIZE AND NUMBER IN WAT

The fat cell size and number were determined by using the method of Hirsch and Gallian (1968). Unlike other methods, based upon direct microscopic determination of the diameter of fat cells isolated by collagenase and subject to loss of cells damaged by the isolation procedure, this method takes advantage of the spherical shape of fat cells fixed with osmium tetroxide before isolation.

In this method, immediately after sacrificing rats, both epididymal and retroperitoneal fat depots were weighed and cut along the longitudinal axis. Samples (100-300 mg) from the middle sections were weighed and rinsed with chloroform :

methanol (2:1) and the remainder was frozen at -80°C for making homogenates later. Then, samples were incubated with 2 % osmium tetroxide (2 ml) and 0.9 % saline in a scintillation vial for 24 hours with moderate shaking. Osmium tetroxide is highly toxic and carcinogenic and the procedure was done in the fume hood while wearing gloves and mask. After fixation the contents of the vial were washed through 250 μm Nytex nylon mesh with distilled water into a large flask with a large stirring magnet. The shreds were gently rubbed by hand on the Nytex mesh while washing. The trapped white adipocytes were washed with adequate amount of distilled water. Then, the flask contents were passed through a 10 μm Nytex mesh into another flask under vacuum. The cells harvested on the Nytex were transferred into a 50 ml siliconized Corning-ware tube using saline and 2 % Photo Flo solution. The cells were allowed to settle down overnight and as much liquid as possible was removed (leaving 5 ml). The cell suspension was weighed and cells were counted under the microscope using a haematocytometer slide. Then, the total cell number and size were calculated based on the assumption that lipid makes up 90% of total fat depot weight (Newby et al., 1990).

8- ANALYSIS OF CARCASS FAT

The tail and paws of carcasses were removed and the carcasses were autoclaved, minced and homogenized in cold distilled water (1.5-2 L) with a 4 litre Blender (Waring blender, commercial heavy duty). Duplicate samples of homogenate

(10 ml) were placed in Whatman cellulose extraction thimbles (33 mm x 80 mm) and allowed to drain overnight in the cold room. The next day, the thimbles were dried in the oven at 50°C and the fat was extracted using a Soxhlet extraction system and weighed. The total body fat content was then calculated based on the carcass fat plus 90% of the total weight of the other carcass fat depots that had been measured for other purposes (Newby et al., 1990).

9- ASSESSMENT OF TISSUE PROTEIN, CYTOCHROME OXIDASE, UCP, AND DNA CONTENTS

A) Protein assay

The total protein content of BAT and WAT homogenate were estimated using the Lowry method (Lowry et al., 1951) as modified by Schacterle and Pollack (1973). Because a number of substances including sucrose, tris, EDTA and HEPES have been found to interfere with the Lowry protein determination, the protein was precipitated from samples in the isolation media with 12.5 % trichloroacetic acid overnight, then centrifuged at 3500 RPM and the pellet dissolved in 1 ml 0.5 N NaOH and added to 10 ml polypropylene conical tubes, Simultaneously, a series of standards (0, 15, 30, 60, 90, and 120 µg bovine serum albumin (BSA)) was freshly prepared in 1 ml NaOH from a frozen stock solution of 10 mg/ml BSA. Then, 1 ml alkaline copper reagent (10 % Na_2CO_3, 0.1 % potassium tartrate and 0.05 % $CuSO_4.5H_2O$) was added to the tubes containing either samples or standards, and incubated at room temperature for

10 minutes. All tubes were then incubated with 4 ml phenol reagent (BDH, Cat # R01145) at 55°C for 5 minutes and cooled to room temperature in an ice cold water bath. The absorbance was read at 650 nm. The protein concentration was calculated based on the linear standard curve, using a computer programme.

B) Cytochrome oxidase assay (COX):

The cytochrome oxidase activity of both BAT and WAT homogenate was measured using a spectrophotometric method that uses six concentrations of cytochrome c to allow an accurate extrapolation to obtain V_{max}. The original method was modified by Yonetani (1965). In this method, a spectrophotometer is used to measure the rate at which cytochrome oxidase oxidizes its substrate, cytochrome c, by measuring the rate of disappearance of the reduced cytochrome c at an absorbance of 550 nm. In this assay the amount of protein is first assayed in samples of BAT or WAT homogenate (150 µl) (see section A above). For preparation of samples for the assay, for each 10 µg of protein, 1 µl of Lubrol solution (60 mg Lubrol in 20 ml H_2O) was added. The final protein concentration was between 0.25 and 0.8 mg/ml (e.g. 0.65 mg/ml for rat). Phosphate buffer (0.1 M, pH 7.0) was used to obtain the desired concentration of the protein. The homogenate was then mixed well and incubated on ice for 1.5 hours. For the measurement of the absorbance, a Beckman DU-50 spectrophotometer with the capability of loading 6 cuvettes at the same time was used. Prior to starting the measurement, cytochrome c (horse heart, type III, Sigma, Cat.# c-2506) was dissolved in distilled water containing 0.1% ascorbic acid to obtain

the reduced form of cytochrome c and the pH was adjusted to 7 followed by adding water to make the concentration of 202 μM. The 6 cuvettes were then prepared during the incubation of the samples on ice as follows:

	Cyt c (202 μM) (μl)	Phosphate buffer (ml)	Final [Cyt c] (μM)
1	100	2.9	6.7
2	200	2.8	13.4
3	300	2.7	20.1
4	400	2.6	26.8
5	500	2.5	23.5
6	600	2.4	40.2

Then at room temperature, 12 μl of homogenate was added to each cuvette and mixed well and placed in spectrophotometer rapidly (Yonetani, 1965) and absorbance at 550 nm measured. A kinetic soft-pac module was used to process the data using the programme 6:kindata.

C) Rat uncoupling protein radioimmunoassay

The goal of this assay was to quantitate the level of uncoupling protein in homogenates of BAT by using a solid phase radio-immunoassay (RIA). In this assay, 96 flat bottom well microtiter plate (Falcon #3912 Becton Dickinson) were washed in Pierce RBS detergent and rinsed with double distilled water a few times. The plates

were dried by slapping them on paper towels, followed by air drying.

For preparation of samples for assay, the homogenate was initially diluted with PBS and 0.5% Triton-X to make a suspension containing 2.0 mg protein per ml. Then, 50 µl of the diluted homogenates were incubated in microcentrifuge eppendorf tubes at room temperature for 30 minutes. Then 200 µl PBS was added to the samples and mixed and centrifuged for 2 minutes. The infranatants were carefully removed, placed in clean microcentrifuge tubes and stored for no more than one month at -20°C.

For the assay, for each homogenate sample or standard concentration, three wells of a 96-well polyvinylchloride plates were coated overnight with 50 µl of UCP diluted to 20 µg/ml with PBS and 0.02% Triton-X and two wells were treated with 50 µl PBS + 0.02% Triton-X in each well as non-specific binding. The plates were then washed three times with PBS and air dried.

A series of standards of rat UCP were diluted in PBS to give final protein concentration of 0, 5, 20, 40, 65, and 125 ng/µl. Rat tissue homogenate (50 µl containing 2 mg/ml protein), or 50 µl of each UCP standard or PBS + 0.02 % Triton-x was added to each well and plates were incubated in a plastic bag at 37°C for 2 hours. The plates were then washed with PBS + 1% BSA (RIA grade), 3 times and drained for 5 minutes. Then, an antibody diluted with PBS (1:1600), raised in rabbits with hamster UCP, was used to be competed for by either the standards or samples. Again, the plates were placed in a plastic bag kept at 4°C for 24 hours. Then, the plates were

inverted and rinsed 3 times with PBS using a multipipeter pipet and drained for at least 5 minutes.

For detection, [^{125}I]-protein A (30 μCi/ml from ICN Biomedical) with a concentration of approximately 70000 cpm per 50 μl was added to each well. The plates were placed in a plastic bag and incubated at room temperature for 1.5 hours. Then, they were washed 3 times with PBS and drained after the last wash for 10 minutes. The wells were then cut out into plastic tubes and counted with a Beckman #5500 Gamma Counter. The results were later calculated using a computer programme and based on the standard curve and the specific and non-specific binding of the antibody to the UCP protein.

D) DNA assay

Content of DNA in WAT and BAT homogenates was estimated by using a TKO-100 Mini-Fluorometer machine, based on the binding of Hoechst 33258 (H 33258), a fluorescent dye, to DNA. Calf thymus DNA was used as standard. DNA in rat tissue homogenates (5 mg of tissue in homogenate) was hydrolysed in 800 μl AT solution (Ammonium hydroxide +0.2 % Triton X-100) and incubated at 37°C for 10 minutes. Then, 500 μl of sample was taken out and added to 500 μl assay buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.4) and centrifuged at 3000 RPM at 4°C for 30 minutes. The supernatant was then placed on ice and 20 μl added to 2 ml of working dye solution (0.1 μg/ml H33258 in assay buffer) and measured at the

excitation-emission 350-455 respectively. The DNA content was then calculated based on ng/ml of homogenate by a computer programme.

10- ASSESSMENT OF SERUM COMPONENTS

Rat insulin radioimmunoassay (RIA):

Rat serum insulin was measured by RIA using a commercial kit from Linco Research (Cat. # RI-13K). Linco Rat Insulin RIA kit utilizes an antibody made specifically against rat insulin. Sensitivity of 0.1 ng/ml can easily be achieved when using a 100 µl serum sample in an overnight, equilibrium assay (300 µl total volume).

In this method, 200 µl of assay buffer was added to the non-specific binding (NSB) tubes and 100 µl of buffer to reference tubes. Then 100 µl of rat insulin standards (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, ng/ml) and quality controls were added in duplicate. Sample tubes were also prepared in duplicate with 100 µl of serum. Then, 100 µl of ^{125}I-insulin was added to all tubes. The rat insulin antibody (100 µl) was, later, added to all tubes except totals (1-2) and NSB (3-4). All tubes were vortexed and covered and incubated at 4°C overnight. The next day, 1.0 ml of precipitating reagent was added to all tubes which were then vortexed and incubated for 20 minutes at 4°C and centrifuged for 15 minutes at 2000-3000 x g. The supernatant was decanted and the tubes were drained and blotted. Then, they were counted using a Beckman # 5500 Gamma Counter.

For the calculation, a computer programme based on the log of the known

concentration (standards) was used and results were reported as ng insulin/ml serum.

Rat serum glucose measurement

Serum glucose concentration was measured using a One Touch Basic blood glucose monitoring system (Lifescan, Canada Ltd) for quick measurement of blood glucose and expressed as mmol/L.

Rat leptin radioimmunoassay (RIA):

To measure leptin in plasma a Rat Leptin RIA kit (from Linco Research, Inc., Cat. # RI-13K) was used. In this kit, an antibody made specifically against rat leptin was utilized. A sensitivity of 0.1 ng/ml can be achieved by using a 100 µl serum sample in an overnight, equilibrium assay (300 µl total volume).

In this method, 300 µl of assay buffer was added to the non-specific binding (NSB) tubes, 200 µl of buffer to reference tubes, and 100 µl to the rest of tubes. A set of standards was prepared in duplicate based on the following concentrations: 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, and 50.0 ng/ml. The quality controls were also added in duplicates. Sample tubes were prepared in duplicate with 100 µl of sample. In all tubes except totals (1-2) and NSB (3-4), 100 µl of rat leptin antibody was added. All tubes were then vortexed and incubated overnight at room temperature. The next day, 100 µl of ^{125}I-rat leptin was added to all tubes and vortexed and incubated again overnight at room temperature. On the second day, 1 ml of cold precipitating reagent

was added to all tubes, vortexed and incubated 20 minutes at 4°C. Then tubes were centrifuged for 15 minutes at 3000 rpm at 4°C and the supernatant was discarded immediately. The tubes were then drained and blotted counted by using a Beckman #5500 Gamma Counter.

For the calculation, a computer programme based on the log of the known concentration (standards) was used and results were reported as ng leptin/ml rat serum of unknown samples.

11- WESTERN BLOTTING FOR DETECTION OF UCP

Gel electrophoresis was based on the method of Laemmli (1970). Samples, containing 10 μg protein, from WAT or BAT of various rats in different groups were denatured in lysis buffer (2% SDS , 0.1 M Tris-Cl, pH 7.4, 0.04 mM PMSF in isopropanol, 0.02 mM EDTA, 20% glycerol, 10% mercaptoethanol) and incubated in boiling water for 3 minutes. Electrophoresis was performed in a discontinuous gel system with a double vertical slab gel assembly. The stacking gel (upper buffer) contained 3.5% acrylamide and 0.043% bis-acrylamide diluted with 0.1% SDS and 0.4 M Tris, pH 6.8. The separation gel (lower buffer) contained 10% acrylamide and 0.13% bis-acrylamide in 0.5 M Tris and 0.2% SDS, pH 8.8. Samples and standards as well as markers (Bio-Rad low molecular weight proteins) were loaded and the gel was run using the running buffer containing 0.3% Tris base, 1.42% glycine, 0.1% SDS and 100 μM PMSF at room temperature at 180 volts for one hour. After taking

the gel from the glass plate, it was placed in 50 ml transfer buffer (7.2% glycine, 1.5% Tris-base, 20% methanol, 100 μM PMSF) and incubated for at least 30 minutes at room temperature on a shaking platform. Simultaneously, nitrocellulose membrane (NCM), filter papers (Whatman 3mm), and sponge pads were also soaked in transfer buffer. A gel holder cassette including one sponge pad, 2 filter papers, gel, NCM, 2 filter papers, and sponge, was placed in the trans-blot chamber with magnetic stirrer on, with NCM facing the anode. The chamber contained a cooling unit that was filled with water and kept at -20°C. At the time of transfer, the cooling system was placed next to the electrodes. Then, the chamber was filled with transfer buffer and the transfer was performed at 120 Volts (0.110 Amps.) for one hour using a Bio-Rad power pack. After transfer was done, NCM was removed and incubated in a physiological buffered saline (PBS-Tween 20) containing 0,0917% K_2HPO_4, 0.02% KH_2PO_4, 0.8% NaCl, 0.02% KCl, 0.05% Tween-20, pH 7.4, for 30 minutes at room temperature on a shaking platform. Then, the membrane was transferred to another PBS-Tween-20 solution containing the primary antibody (rabbit anti-hamster UCP) at a dilution of 1:10000 for 1 hour at room temperature on a shaking platform. Then, the membrane was washed 3 times for 15 minutes each with PBS containing 0.2% SDS, 0.5% Triton X-100, and 0.5% BSA with 15 minutes incubation intervall. After this washing step, the membrane was incubated with secondary antibody (goat anti-rabbit IgG horse-radish peroxidase conjugate) for one hour at room temperature at the final dilution of 1:10000 in PBS-Tween-20. The blot was washed again with PBS 3

times with 5 minutes incubation between washes. The blot was then incubated in Enhanced Chemiluminescence (ECL) Western blotting detection reagents (Amersham RPN 2109) for one minute, drained and wrapped in Saran Wrap and exposed to Kodak XAR film for 30 to 300 seconds. The film was then developed with an automated machine.

12- NORTHERN BLOTTING FOR ASSESSMENT OF mRNA FOR TNF-α

A. Mouse TNF-α cDNA sub-cloning in bacteria

The mouse cDNA for TNF-α was kindly supplied by Dr. Gökhan Hotamisligil from Dana-Farber Cancer Institute Boston. The plasmid (PUC 9) contained ~1.6 kb mouse TNF-α cDNA insert. The insert was between Pst I and Bam HI sites. The concentration of DNA was about 0.4 μg/μl. The plasmid also contained an ampicillin resistance gene.

The plasmid was diluted to a final concentration of 20 ng/μl and 1-2 μl was added to prepared cells (DH50L cells) in a microcentrifuge tube and incubated for 30 minutes on ice. The tube was then incubated at 42°C for 1.5 hour and after that transferred to an ice bath rapidly for 2 minutes. Then 450 μl of pre-warmed SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 0.05% NaCl and 20 mM glucose in deionized H_2O, pH 7.0) was added to the tube and incubated in 37°C water bath for 1 hour. The tube was transferred again to the ice bath and 100 μl of the content was spread on a Luria-Bertani Medium (LB) plate (containing 1% bacto-

tryptone, 0.5% bacto-yeast extract and 1% NaCl in deionized water, pH 7.0), allowed to dry. The LB plate was then incubated at 37°C overnight. The next day, white colonies were picked up for plasmid isolation and transferred to 5 ml LB in a 10 ml sterile tube and incubated at 37°C with vigorous shaking overnight. Then, the tubes were centrifuged at 2000 RPM for 7 minutes. The supernatant was removed and the tubes were blotted as much as possible. Then, 200 µl of GET solution (solution I) (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA) was added to each tube and vortexed. The content were then transferred to a microfuge tube and 300 µl of solution II (0.2 M NaOH, 0.1% SDS) was added to the tubes and vortexed. Then, 300 µl of solution III (60% potassium acetate, 11.5% glacial acetic acid, 28.5% H_2O) was added to the tubes, vortexed and centrifuged at 12000 RPM for 7 minutes. The supernatant was transferred to new tubes and labelled. Later, 5 µl of RNAase (5 mg/ml) was added to the tubes and incubated at 37°C for 20 minutes. Then, a mixture of phenol and chloroform (1:1) was added to the content of each tube (1:1), vortexed and centrifuged at maximum RPM for 7 minutes. Then, the upper phase was taken and mixed with 600 µl isopropanol, followed by vortexing and incubation at -80°C for 10 minutes. The tubes were then centrifuged at 4°C, at 14000 RPM for 15 minutes. At this point, the pellet could be seen. The supernatant was decanted and the pellet was washed with 500 µl of 70% alcohol and centrifuged at room temperature for 3 minutes. The supernatant was removed and the pellet was dried under vacuum for 5 minutes. Finally, the pellet (plasmid) was dissolved in 50 µl distilled water and

stored at 4°C.

B. Digestion of plasmid and preparation of the probe

The plasmid containing 1.6 kb mouse TNF-α cDNA was digested with restriction enzymes and appropriate buffers in a sterile microfuge tube as follows: 2 µl plasmid DNA, 2 µl 10x incubation buffer for restriction enzymes (Boehringer Mannheim Gmbh, Cat # 1417-99), 0.5 µl Bam HI, 0.5 µl Pst I, and 15 µl distilled water. The tube contents were mixed and incubated at 37°C for at least 2 hours. When the digestion was over, the plasmid was mixed with 5 µl sample loading buffer (SLB) (50% glycerol, 1mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 0.1% of the stock solution of ethidium bromide (10 mg/ml)). and loaded on a 1.2% agarose gel containing ethidium bromide. Simultaneously, 2 µl of a 1 kb DNA Ladder was loaded on a different lane. The gel was run at 82 V and 120 milliampere for 1 hour. then, the 1.6 kb band was cut and stored at 4°C. At that step, the probe was ready to be used.

C. Tissue preparation for northern hybridization

The dissecting instruments (scissors and forceps) were cleaned up and washed out with 70% ethanol followed by washing with diethylpyrocarbonate (DEPC, Sigma Cat# D5758) treated water. DEPC is a strong inhibitor of RNAases (Sambrook et al., 1989). To prepare DEPC-treated water, 0.1% DEPC was vigorously mixed in distilled

water and autoclaved for an hour. DEPC is a suspected carcinogen, therefore, should be handled with caution while wearing gloves and using fume hood. All glasswares and plasticwares used in all steps of this assay were also washed with DEPC-treated water.

Immediately after decapitation of rats, samples (1-2 g) of RWAT were carefully dissected, using sterile scissors and forceps while wearing gloves, and transferred to sterile plastic tubes which were then inserted into liquid nitrogen and kept at -80°C until processed later.

D. Extraction and isolation of RNA

RNA isolation was performed using TRI-reagent, a product of Molecular Research Center Inc., based on a developed method by Chomczynski and Sacchi (1987).

TRI-reagent is a complete and ready to use reagent for isolation of total RNA or for the simultaneous isolation of RNA, DNA and proteins from samples of human, animal, plant, yeast, bacterial and viral origin. The composition of TRI-reagent includes phenol and guanidine thiocyanate in a mono-phase solution.

In this method, samples of tissue (100 mg) were homogenized and lysed in 1 ml TRI-reagent in sterile microfuge tubes using a mini-homogenizer (Polytron). The homogenate were stored at room temperature for 5 minutes to allow the complete dissociation of nucleoprotein complexes. Next, the content of each tube was

supplemented with 0.2 ml chloroform, capped tightly and shaken vigorously for 15 seconds. The resulting mixture was then kept at room temperature for 15 minutes and centrifuged at 12000 g and 4°C for 15 minutes. Following centrifugation, the mixture separated into three phases, a lower red phase, a phenol-chloroform interphase, and colourless upper aqueous phase. RNA remains exclusively in the aqueous phase and the volume of this phase is usually about 60% of the volume of TRI-reagent used for homogenation.

For RNA precipitation, the aqueous phase was transferred to a new eppendorf tube, followed by addition of 0.5 ml of isopropanol, mixed very well and kept at room temperature for 10 minutes, then centrifuged at 12000 g and 4°C for 10 minutes. RNA precipitate forms a gel-like pellet on the side and bottom of the tube. The supernatant was then removed and the RNA pellet was washed once with 1 ml 75% ethanol followed by vortexing and centrifugation at 12000 g. The supernatant was then removed and the RNA pellet was dried under vacuum. Next, the RNA was dissolved in appropriate volume (50 µl) of DEPC-treated water and incubated at 60°C for 10 minutes and used immediately or stored at -80°C.

F. Electrophoresis of RNA, blotting and hybridization

The electrophoresis gel was prepared by dissolving the appropriate amount of agarose in water (1.2% agarose), by heating in a microwave or on a hot plate. Then, 35 ml of gel (cooled down to 60°C) was mixed with 11 ml of 5x concentration gel-

running buffer [(0.1 M MOPS (3-(N-morpholinopropanesulfonic acid) pH 7.0, 40 mM sodium acetate, and 5 mM EDTA pH 8.0 in DEPC-treated water)] and 10 ml formaldehyde. The gel was mixed gently and allowed to cast at least for one hour. RNA samples were prepared during casting the gel by mixing the following volumes in a microfuge tube: RNA sample (>20 μg) 7 μl, gel-running buffer 3.0 μl, formaldehyde 5 μl, and formamide 15 μl. Then, they were incubated at 65°C for 15 minutes followed by chilling them on ice. Before loading the RNA samples, 2 μl of sterile DEPC-treated SLB.

The gel was submerged in 1x gel-running buffer and pre-run for 5 minutes at 5 V/cm. Then, the samples were loaded into the lanes of the gel and run at 4 V/cm for 2 hours until the separation of the two different dyes at least by 1 inch. The gel was then cut and photographed using a transparent ruler for alignment, and transferred to a nylon membrane.

The gel containing the samples was rinsed in DEPC-treated water a few times and placed on gel-transfer system, consisting of a glass baking dish as a buffer reservoir, a support stand for the gel, and a filter paper (3 mm Whatman filter paper). The reservoir dish was filled up almost to the to of the support with 20x SSC (3M NaCl, 0.3 M Na_3 citrate. $2H_2O$, pH 7.0). The gel was placed on the support and centred on the wet 3 mm filter paper in an inverted position with no air bubbles between the 3 mm paper and the gel. The gel was then surrounded with parafilm as a barrier to avoid direct flowing of buffer from the reservoir to paper towel placed

on the top of the gel. A nylon membrane (Amersham Hybond-H^+ membrane Cat # RPN132N), cut to exactly the same size the gel and pre-soaked in 20x SSC, was then placed on the top of the gel with no air bubbles. Then, two pre-wet pieces of 3 mm paper were placed on the membrane followed by a stack of paper towels (5-8 cm high). A glass plate was then placed on the top of the stack and weighed down with a 750-g weight. The gel was transferred overnight and the next day, all paper towels and filter papers were removed and the membrane was marked by either cutting the corner or writing with a pencil and placed on a 3 mm filter paper. The membrane was air dried at room temperature and baked at 80°C for 2 hours in the oven to immobilize the nucleic acids after transfer and to fix RNA to the nylon membrane. The membrane was then ready for hybridization.

The membrane was first pre-hybridized in a pre-hybridization solution [(5X SSC, 5X Denhardt`s solution (0.01% Ficoll, 0.01% polyvinylpyrrolidone, 0.01% BSA (Pentax Fraction V) in water), and 0.5% SDS)] containing 2% denatured salmon-sperm DNA by heating to 100°C for 5 minutes and incubated for one hour in a hybridization chamber at 65°C.

During the pre-hybridization, the probe (TNF-α DNA probe) was diluted to a concentration of 20 ng in 45 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) buffer, and denatured at 100°C for 5 minutes. The probe was then labelled with Redivue [α-^{32}P]dCTP with a specific activity of 3000 Ci/mmol using the rediprime DNA labelling system (Amersham RPN1633.1634) as follows: 45 µl denatured DNA

sample (TNF-α probe) was centrifuged briefly to bring the contents to the bottom of the tube and added to the labelling mix, containing a buffered solution of dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random primers (9 mers) (Amersham RPN1633/1634), and mixed by gently flicking the tube until the blue colour was evenly distributed. Then 5 µl of Redivue [α-^{32}P] dCTP was added to the mixture of the tube and mixed by gently pipetting up and down 4 to 5 times and centrifuged to bring the labelled mix to the bottom of the tube and incubated at 37°C for 20 minutes. After incubation, the reaction was stopped by adding 5 µl of 0.2 M EDTA.

To separate incorporated radioactive DNA from unincorporated ^{32}P-labelled nucleotides a Pharmacia NICK Column (Pharmacia Biotech, Cat # 52-2076-00), containing sephadex G-50 DNA Grade in distilled water and 0.15% Kathon CG as preservative, was used and the purified sample was denatured at 100°C for 5 minutes, then cooled on ice and added to the pre-hybridization solution containing the membrane and incubated overnight at 65°C.

Following hybridization, the membrane was washed in 2x SSC, 0.1% SDS at room temperature for 10 minutes twice, which followed by another wash with 1X SSC, 0.1% SDS at 65°C for 15 minutes. The membrane was then removed and wrapped in Saran-wrap and exposed to a Kodak Bio-Max autoradiograph film for 2-7 days. In some cases the membranes were hybridized in hybridization solution containing formamide at a final concentration of 50%. In those cases, the incubation

temperature was 42°C.

As a control for the loading and integrity of the RNA, the blots were re-hybridized with the 18S ribosomal RNA (considered as a housekeeping gene) probe in the same manner as TNF-α probe.

For quantitation, a densitometer equipped with a computer programme was used to measure the level of TNF-α mRNA.

CHAPTER IV

THERMOGENIC RESPONSE OF GUINEA PIG ADIPOCYTES TO NA AND TO β3-AR AGONISTS

Preface

Cold-acclimation of rats is known to increase insulin-responsiveness of BAT and to stimulate the capacity of glucose transport into BAT in vivo (Vallerand et al., 1990). However, it has been shown that brown adipocytes isolated from BAT of cold-acclimated rats do not respond to NA in spite of their marked thermogenic response in vivo (Nedergaard et al., 1993). In contrast, in cold-acclimated guinea pigs, isolated brown adipocytes show substantial increase in capacity for a thermogenic response to NA (Rafael et al., 1986).

As stated in chapter II, the initial objective was to study the responsiveness of glucose transport to insulin, to NA, and to β3-AR agonists in BAT of an animal known to be insulin-resistant. Since guinea pig seemed to be suitable for this study, it was initially chosen to assess the number, functional state and the role of β3-ARs in BAT cells. These animals were chosen because it was known that BAT cells could be isolated from cold-acclimated guinea pigs with a very high thermogenic capacity and warm-acclimated guinea pigs with a very low thermogenic capacity and it was hypothesized that insulin-sensitivity would be improved by cold-acclimation.

The results of this study showed that no β3-adrenergic agonist used, such as CL 316,243, BRL 37344, was able to stimulate oxygen uptake in BAT cells from cold-acclimated or new born guinea pigs. However, noradrenaline (NA), adrenaline (A) and isoproterenol (ISO) had a marked thermogenic effect on these cells. In contrast, in a comparative study in warm-acclimated rat BAT cells, CL 316,243 was even more potent than NA in stimulating oxygen uptake. Results led to the conclusion that guinea pigs lack β3-ARs in their BAT. Although these findings were disappointing because the stated proposal was based on the role of β3-ARs in BAT and WAT cells, the results were interesting and noteworthy, suggesting that guinea pigs can be a natural model for β3-ARs knockout (Himms-Hagen et al., 1995).

Therefore , I describe the results of this experiment in the current chapter, although it may not follow the objectives of the topic of this thesis.

Introduction

BAT cells from cold-adapted guinea pigs show a greater response to NA compared to cells from animals acclimated to room temperature (Rafael et al., 1986). In most mammalian adipose tissues, the induction of lipolysis by catecholamines is mediated by β-AR subtypes such as β1- and β3-ARs. During cold-acclimation there is a marked increase in cell number, mitochondrial content of each adipocyte, and the ability of each mitochondrion to respond to NA. The increase of thermogenesis per mitochondrion is not because of an increase of respiratory chain, since cytochrome

c oxidase remains unchanged. Instead it is due to a marked increase in the uncoupling protein per mitochondrion (Rial and Nicholls, 1984).

In this experiment, we prepared and incubated brown adipocytes from guinea pigs during the stages of cold adaptation and warm adaptation, and from new born guinea pigs.

The objective of this study was to assess whether NA (NA) and β3-AR agonists such as BRL and CL 316,243 could increase oxygen uptake in brown adipocytes of warm-acclimated guinea pigs, cold-acclimated guinea pigs and newborn guinea pigs.

Material and Methods

Female Dunkin-Hartley guinea pigs were obtained at 3 weeks of age from Charles River Laboratories, St.. Constant, Quebec. They were housed individually at 28°C, with free access to guinea pig chow and water. After one week, some guinea pigs were acclimated to 4°C for up to 4 weeks while other remained at 28°C during the same period. Pregnant guinea pigs were also obtained at advanced stage of gestation from the same company mentioned above. They were housed at room temperature with free access to food and water. The new born guinea pigs were used at the ages of 1, 2, or 3 days after delivery.

In all cases, BAT was isolated and transferred into Krebs-Ringer buffer (see chapter III) and brown adipocytes were isolated by collagenase digestion of

interscapular BAT and washed by flotation using a bicarbonate-buffered culture medium (Gibco DMEM 380-2320 AG) supplemented with 5.5 mM glucose, 1 mM ascorbic acid and 40 mg/ml fatty acid-free bovine serum albumin and equilibrated with 95% oxygen, 5% carbon dioxide, pH 7.4. Oxygen uptake of cells was measured polarographically (YSI Oxygen monitor) in the same medium.

UCP was measured in BAT homogenates by solid phase radioimmunoassay using antibody to purified hamster UCP and [^{125}I]protein A for detection (see chapter III).

As β-AR agonists, we used L-noradrenaline-D-bitartrate (Arterenol)(Sigma A 9512), L-isoproterenol-D-bitartrate (Sigma I2760), L-epinephrine-D-bitartrate (Sigma E4375), CL 316,243 (American Cyanamid Co), and BRL 37,344 (SmithKline Beecham).

Results

UCP content of interscapular BAT was markedly increased in cold acclimated guinea pigs (Figure 3). Since UCP is a specific marker for BAT activity, the increase of UCP content confirms that cold-acclimation has a stimulatory effect on BAT and activates the tissue by increasing UCP concentration in mitochondria to generate heat to fight against cold exposure. In another experiment we demonstrated the substantial

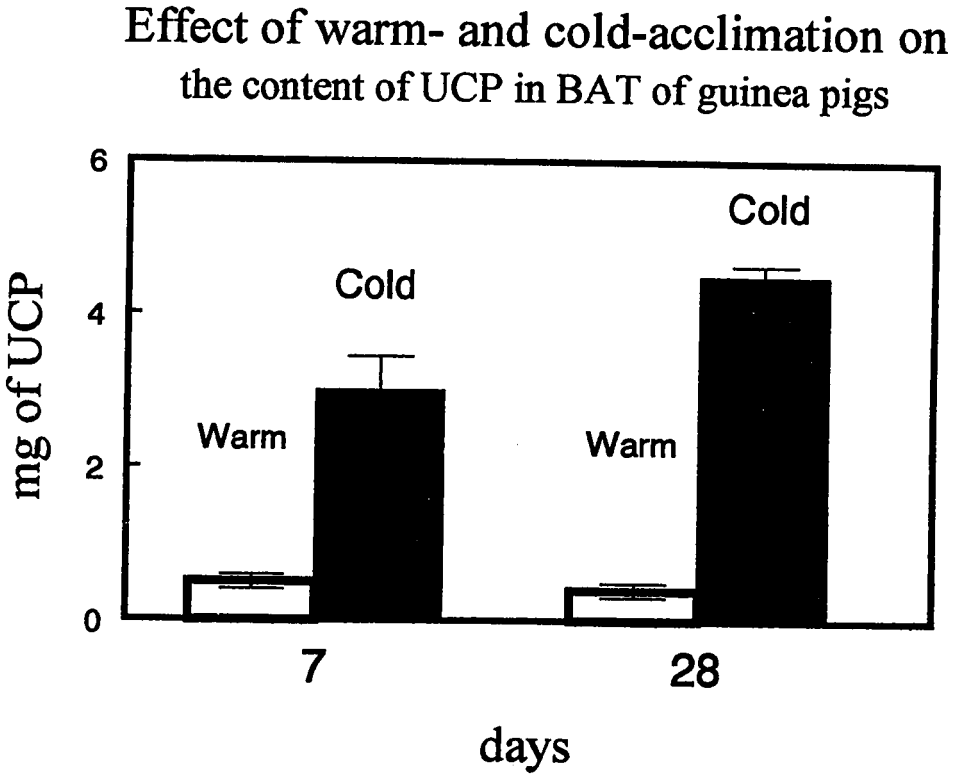


FIGURE 3. **Total UCP of interscapular BAT of warm-acclimated guinea pigs and cold-acclimated (7 to 28 days) guinea pigs. Cold acclimation induced a significant increase in UCP content in both times ($P < 0.001$). The increase is based on the mitochondrial concentration of UCP in µg per mg mitochondrial protein. At 7 days, it was 224 ± 35.6 for cold *vs* 24.5 ± 4.3 for warm ($P < 0.001$). At 28 days, it was 150.0 ± 19.9 for cold *vs* 15.4 ± 1.2 for warm (P , 0.001). Total mg protein changed slightly at 7 days, 84.5 ± 10.46 for cold *vs* 42.0 ± 2.11 for warm (P , 0.001).**

thermogenic response of brown adipocytes isolated from BAT of cold-acclimated but not of warm-acclimated guinea pigs to NA. In warm-acclimated guinea pigs there was no response to NA by brown adipocytes (Table 2), whereas there was a substantial increase in oxygen consumption and thermogenesis in brown adipocytes isolated from cold-adapted guinea pigs (Table 2 and Figure 4). The experiment was continued with adrenaline (A) and isoproterenol (ISO) (Figure 4). Isolated brown adipocytes were then incubated with two selective β3-agonists, BRL 37,344 and CL 316,243 in the same situation and preparations that responded to NA. Surprisingly, there was no response to these β3-AR selective compounds (Figure 5).

Thinking that there might have been a loss of β3-ARs during the isolation of brown fat cells, in another experiment, intact conscious cold-acclimated guinea pigs received a subcutaneous injection containing CL 316,243 and their oxygen uptake measured. Results showed that injection of 10 mg CL per kg body weight had no effect on oxygen uptake (Figure 6). In contrast, injection of NA or cold exposure increased oxygen uptake in guinea pigs by 87% and 108% respectively (Figure 6).

Since brown adipocytes of warm-acclimated guinea pigs had no thermogenic response to NA, due to very low level of UCP, it was not feasible to assess their response to β3-AR agonists. A parallel experiment was performed in collaboration with Dr. N. Begin-Heick of this department by measuring the stimulation of adenylate cyclase activity in crude membranes isolated from BAT of both warm- and cold-acclimated guinea pigs in response to various β-adrenergic agonists. Results showed

TABLE 2

Relative potencies (EC_{50}) of different β-adrenergic agonists in stimulating the respiration of brown adipocytes isolated from BAT of guinea pigs

		warm-acclimated guinea pig	cold-acclimated guinea pig
Agonists		dose response (EC_{50})	dose response (EC_{50})
Isoproterenol		No response	5.0×10^{-7}*
Adrenaline		No response	2.0×10^{-6}*
NA		No response	1.5×10^{-6}*
CL 316,243	β3-agonists	No response	No response
BRL 26810		No response	No response

Iso > A = NA are considered as β1-adrenergic agonists.
*** Concentration of agonists in M**

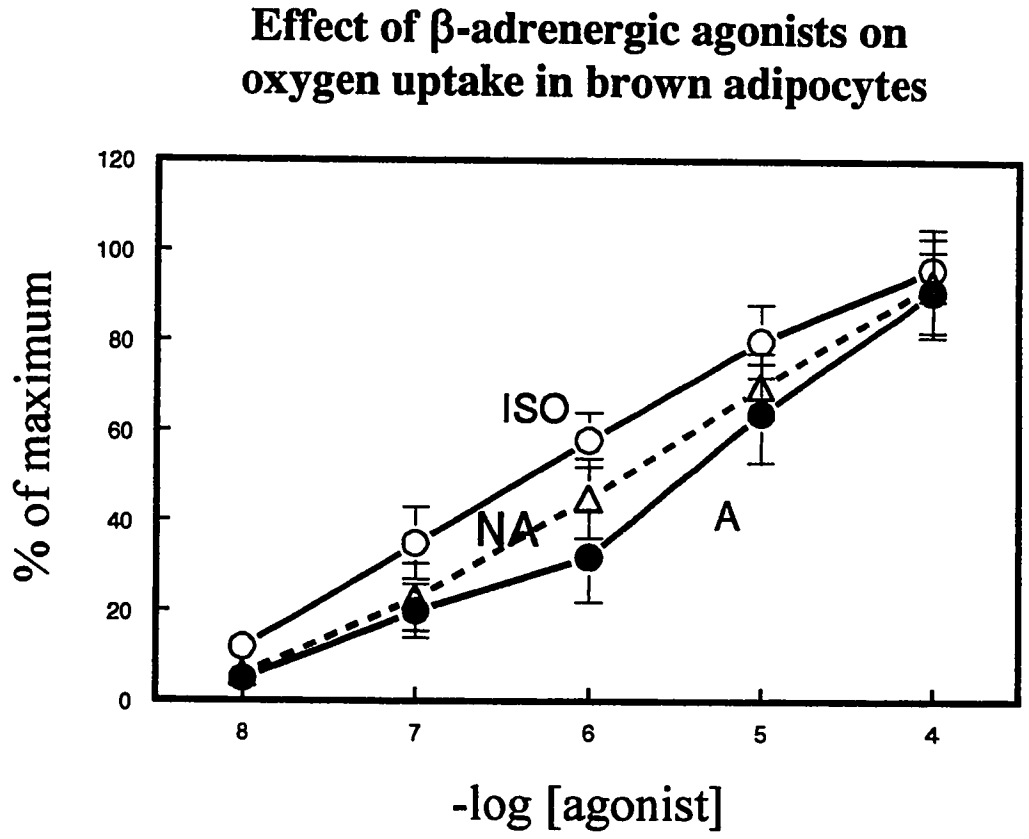


FIGURE 4. Thermogenic effect of adrenergic agonists on brown adipocytes isolated from cold acclimated guinea pigs. Symbols: Isoproterenol (ISO) ○, adrenaline(A) ●, noradrenaline (NA) △. None of these agonists had any effect on isolated brown adipocytes from warm-acclimated guinea pigs. Because of variation in the rate of oxygen consumption from one preparation to another, data are expressed as % of maximum for each agonist in each experiment. Actual maximum rats (in nmol per 10^6 cells, n = 6 cells preparations) were 247 ± 46.8 for ISO, 212 ± 34.0 for A, and 191 ± 43.7 for NA. Calculated EC_{50} was 5.0×10^{-7} M for ISO, 2.0×10^{-6} M for A, and 1.5×10^{-6} for NA.

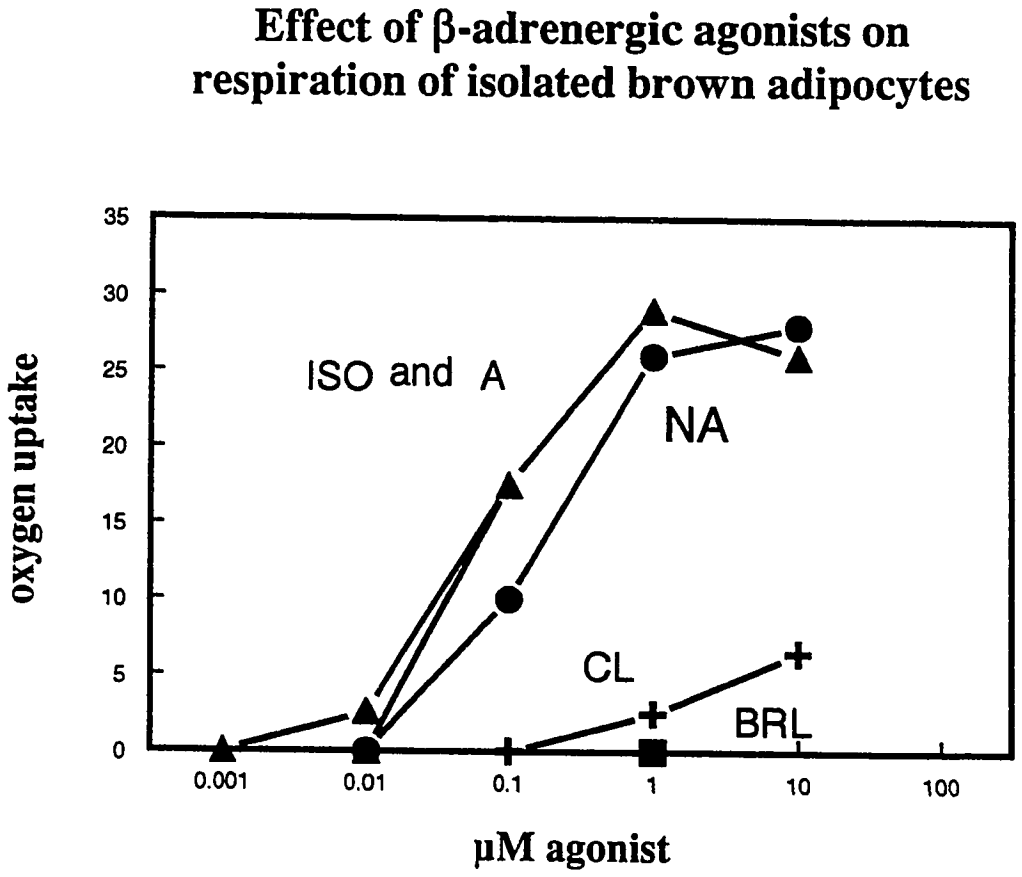


FIGURE 5. Effect of β-adrenergic agonists on respiration of isolated brown adipocytes from cold-acclimated guinea pigs. Symbols: isoproterenol (ISO) and adrenaline (A) ▲, noradrenaline (NA) ●, CL 316,243 (CL) +, BRL 28,410 (BRL) ■, Oxygen uptake is in nmol per 10^6 cells per minute.

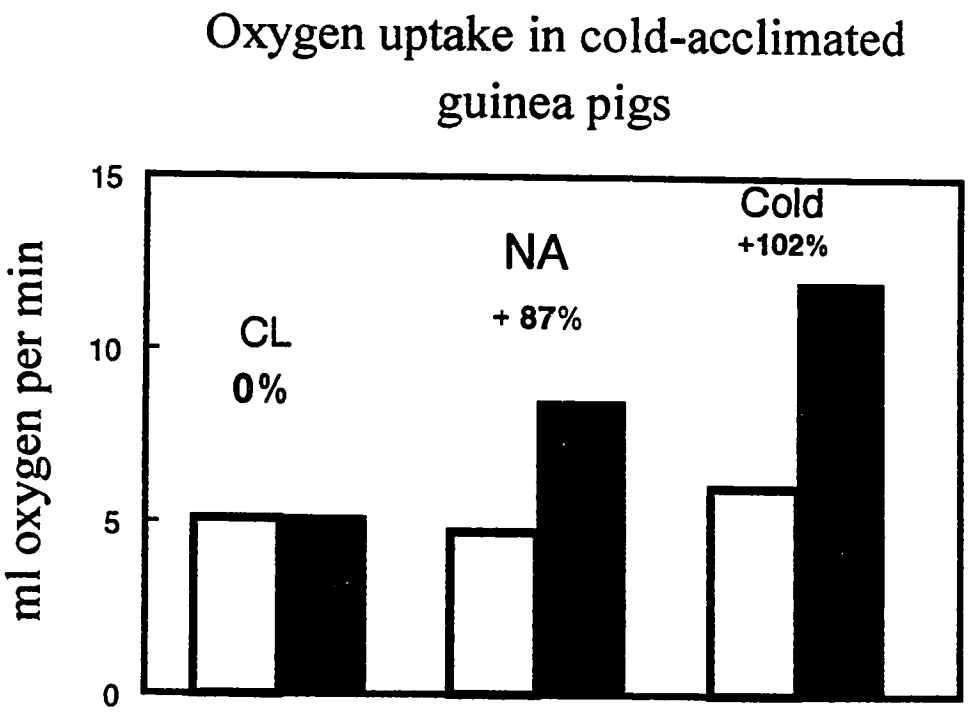


FIGURE 6. Effect of cold-acclimation and β-adrenergic agonists on energy expenditure of conscious cold-acclimated guinea pigs. Animals were either exposed to cold, infused with CL 316,243 (CL) or infused with noradrenaline (NA). Open bars show the oxygen uptake before injection or cold exposure and solid bars show the oxygen uptake after injection or cold exposure.

that adenylate cyclase in BAT membrane from either cold- or warm-acclimated guinea pigs was not stimulated by any β3-AR agonists, including CL 316,243. Whereas it was stimulated by ISO, A, NA and salbutamol in membranes of both warm- and cold-acclimated guinea pigs (Himms-Hagen et al., 1995). Because β3-ARs might have been present in young guinea pigs and lost with aging, as occurs in other precocial mammals such as bovine (Carpéné et al., 1994), we also studied the effects of NA and of CL 316,243 on brown adipocytes isolated from BAT of newborn guinea pigs. β3-AR agonists also failed to stimulate oxygen uptake in BAT cells from cold acclimated or new born guinea pigs, while NA had a marked effect on increasing oxygen uptake as well as stimulation of adenylate cyclase activity in membrane of these cells. In contrast, in a comparative study in rat BAT cells, the capability of CL 316,243 was even more potent than NA in stimulating of oxygen uptake (Figure 7). Therefore, it is suggested that the stimulation of thermogenesis by ISO, A, and NA in both warm- and cold-acclimated guinea pigs is mediated by β1-ARs and not by β3-receptors, and these animals do not express β3-AR in their WAT and BAT.

Discussion

Present results show that chronic exposure to cold increases mitochondrial uncoupling protein synthesis in BAT of guinea pigs and induces thermogenesis in isolated BAT cells. Our data also confirmed the remarkable increase in thermogenic

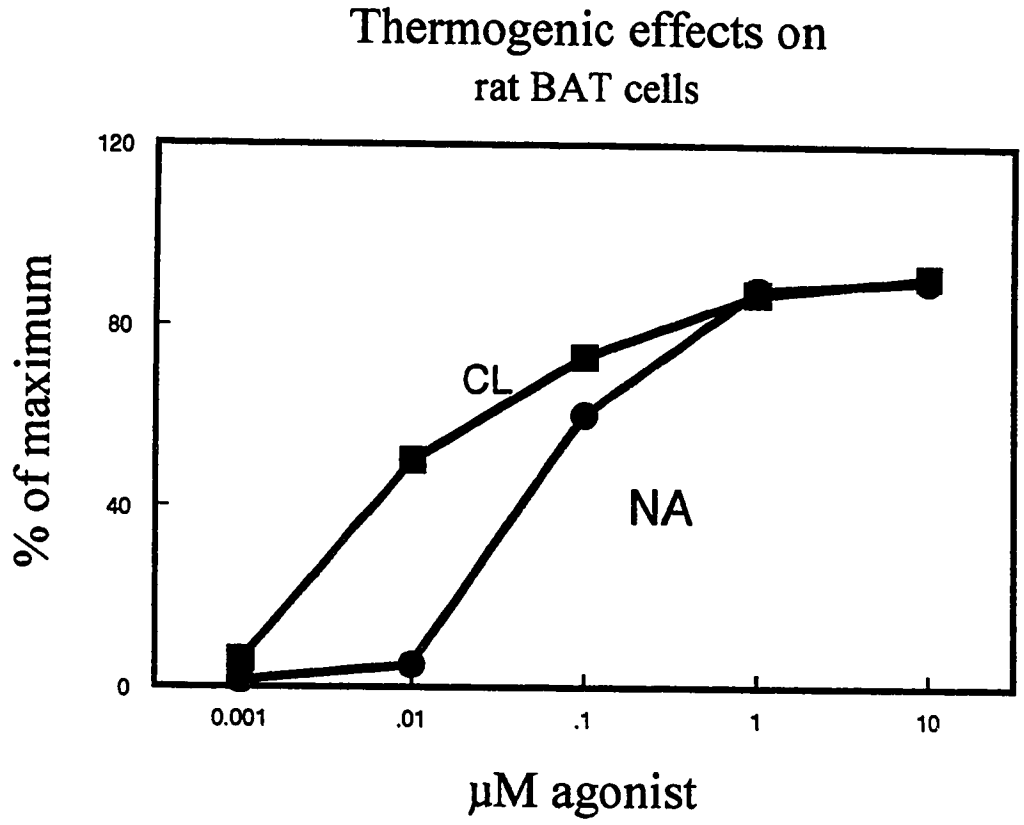


FIGURE 7. **Effect of β-adrenergic agonists on oxygen uptake of brown adipocytes isolated from rats. Symbols, noradrenaline (NA) ●, CL 316,243 (CL) ■.**

responsiveness to NA of brown fat cells isolated from BAT of cold-acclimated guinea pigs (Rafael et al., 1986). Since , NA is able to stimulate lipolysis in brown fat cells of both warm- and cold-acclimated guinea pigs (Cunningham et al., 1986; Pond and Mattocks, 1991) and they exhibit no responses to all the β3-agonists tested, it is possible to conclude that the β-adrenergic responsiveness of guinea pig fat cells essentially involves β1- and β2-ARs and not β3-receptors.

It is also known that β3-adrenergic agonists are inactive on lipolysis in WAT from guinea pigs (Lafontan et al., 1992), in contrast to their lipolytic effect on rat WAT cells. Differences in the adrenergic regulation of lipolysis have also reported between rat and guinea pigs (Carpéné et al., 1994).

However, there is evidence of existence of β3-AR mediating relaxation of various segments of the gastro-intestinal tract of guinea pigs (Bond and Vanhoutte, 1992).

Since, β3-ARs are present in intestinal tissue and absent in adipose tissues of guinea pigs, it is concluded that the regulation of β3-ARs expression is different in guinea pigs and rodents, in which they are present in both adipose tissues and intestine. The guinea pig thus differs from rodents by the absence of β3-adrenergic effects in its adipose tissue and thus resembles the human and bovine species (Himms-Hagen et al.,1995; Strosberg and Pietri-Rouxel, 1996). More recent studies on cloning and sequencing of the guinea pig β3-AR gene have revealed a slightly higher amino acid sequence similarity with the human than with the rodent β3-ARs

(Atgié et al., 1996). β3-ARs in guinea pig ileum have the pharmacological characteristics of human β3-ARs (Emorine et al., 1989). It is now known that the pharmacological characteristics of human β3-ARs differs from that in rodents (Emorine et al., 1994; Strosberg and Pietri-Rouxel, 1996). It is noteworthy that the guinea pig is not a rodent, thus, it is not surprising that it does not possess rodent-type β3-ARs.

Since the objective of this thesis was to assess the role of β3-ARs. in control of BAT function, the guinea pig proved to be an unsuitable model for this purpose. We therefore continued to study the effects of stimulation of β3-ARs in a different species, the rat.

CHAPTER V

REVERSAL OF OBESITY IN RATS BY TREATMENT WITH CL 316,243

PART A: THE EFFECT OF CL 316,243 ON DIET-INDUCED OBESITY (DIO) IN RATS: HYPERTROPHY OF BROWN ADIPOCYTES IN BROWN AND WHITE ADIPOSE TISSUES AND REVERSAL OF DIET-INDUCED OBESITY

Background

It has been shown that β3-ARs are present on the cell surface of both WAT and BAT and can serve as a target for the treatment of obesity (Himms-Hagen and Danforth, 1996). Stimulation of these receptors increases lipid mobilization in WAT and increases energy expenditure in BAT, resulting a reduction of lipid stores in both tissues.

Previous work from this laboratory demonstrated that chronic treatment with CL 316,243 (CL), a new and highly selective β3-AR agonist (Bloom et al., 1992; Dolan et al., 1994; Largis et al., 1994), increased thermogenesis and caused appearance of multilocular adipocytes in WAT and retarded development of obesity in young Sprague-Dawley rats eating a high-fat diet (Himms-Hagen et al., 1994). This

retardation was associated with an increase in metabolic rate, but no change in food intake, and with mitochondrial proliferation in BAT, with a large increase in the level of UCP. The DNA content of two WAT depots was lower in the CL-treated rats but, because no pretreatment group of rats was studied, it was not clear whether the treatment had simply prevented the normal increase in cellularity with age or had made cells disappear. It was also not clear whether the lower DNA level was due to fewer white adipocytes, to fewer cells other than adipocytes, or to both of these. In addition, multilocular adipocytes appeared among the unilocular white adipocytes in WAT of CL-treated rats, but the identity of these cells was not established.

Objective

The first objective of the experiment was to find out whether treatment with CL could reverse diet-induced obesity (DIO) that had already established in older rats by feeding them a high-fat diet, during a period in which they continued to eat the high-fat diet. The second objective was to explore more fully the cellular changes in WAT, in particular the possibilities that the multilocular cells that appeared in WAT might be brown adipocytes expressing UCP and that the treatment might result in disappearance of mature white adipocytes. In order to distinguish between drug-induced prevention of aging-associated accretion of white adipocytes and drug-induced disappearance of mature white adipocytes a pretreatment group of rats was studied.

Other studies have demonstrated an atypical occurrence of UCP in certain WAT depots previously thought to contain only white adipocytes and an increase in UCP content in response to cold-adaptation (Casteilla et al., 1994; Loncar , 1991; P. Young et al., 1984) or to β-adrenergic stimulation (Cousin et al., 1992, 1993a). Based upon the previous observation of multilocular cells in WAT of CL 316,243-treated rats (Himms-Hagen et al., 1994), it seemed likely that CL 316,243 might likewise induce the appearance of UCP in brown adipocytes in one or more typical WAT depots. The definition of a brown adipocyte used here is of a cell that expresses UCP (Himms-Hagen and Ricquier, 1997). We looked for brown adipocytes in WAT depots, using immunohistochemical detection of UCP.

Material and Methods

Eighty male Sprague-Dawley rats were purchased at 13 weeks of age and housed at 24°C in hanging wire mesh cages with free access to food (Agway R-M-H 4020 chow) and water. After one week, food intake and body weights were measured weekly for the next four weeks. Rats were then separated into three groups of equal ranges of weights and mean weights. Design of the experiment is illustrated in Figure 8. Immediately after separation, one group of 8 rats was killed as time zero. The other two groups either continued to eat chow (n = 28) or were switched to a high-fat diet (Teklad 85418) (n = 44) for four weeks. Rats susceptible to DIO were selected as the

3-MONTH OLD MALE RATS WERE TREATED WITH CL 316,243 FOR 4 WEEKS.

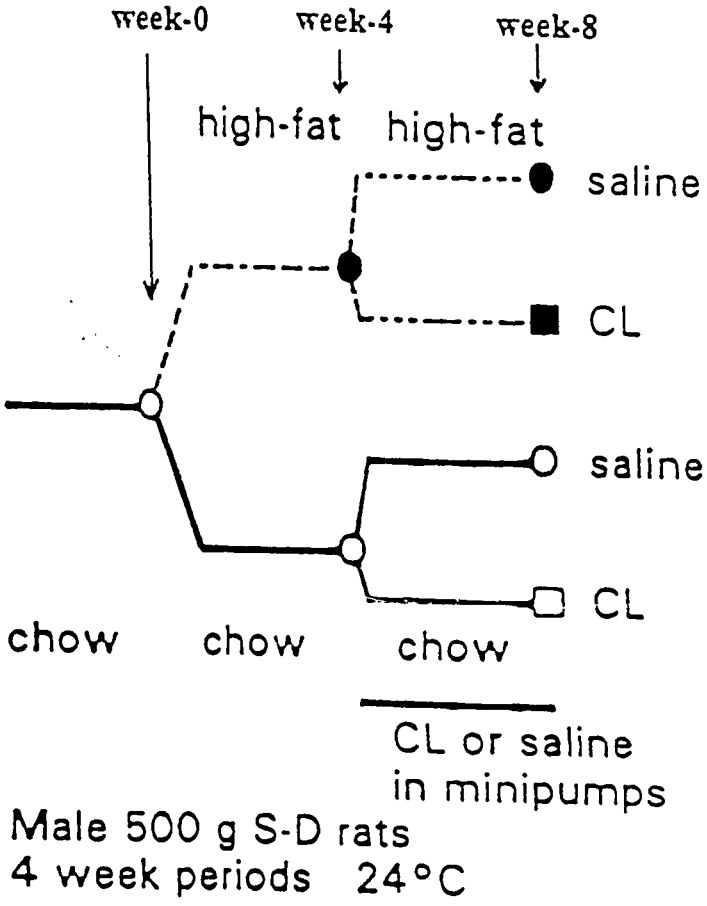


FIGURE 8. The experimental design for treatment of diet-induced obesity in Sprague-Dawley rats.

28 most rapid weight gainers from this last group. Remaining rats eating the high-fat diet (n = 16) were not studied any further. Each of the high-fat diet group or chow group was then separated into three subgroups. One subgroup of 8 from each diet group was killed and two other subgroups of 10 from each diet group were implanted with mini-osmotic pumps (model 2002), infusing either saline or CL for 2 weeks. After 2 weeks pumps were replaced with new ones, infusing CL or saline for another 2 weeks. Then, eight rats out of ten from each group were killed and appropriate tissues (retroperitoneal WAT, epididymal WAT, interscapular BAT, and perirenal BAT) were dissected, cleaned, weighed and homogenized. Samples of homogenates were frozen immediately in liquid nitrogen and stored at -80°C. In all cases after sacrificing rats, blood was collected in conical centrifuge tubes, centrifuged and the serum transferred into microfuge tubes and kept at -80°C for further assays. Resting metabolic rate was measured the week before killing the animals. All other assays such as protein, DNA, cytochrome oxidase, and UCP assay were performed as described in chapter III. The remaining two rats from each group of ten were later killed for histology as described in chapter III.

Statistical analysis

Results are presented as means ± SEM. Statistical analysis used Instat software to do ANOVA followed by Student-Newman-Keuls post hoc test. Significant differences are based on $P < 0.05$.

Results

Energy balance

The fat content of two major abdominal WAT deposits (retroperitoneal WAT and epididymal WAT) (Figure 9A) and other carcass fat (Figure 9B) were significantly greater in rats eating a high-fat diet than in rats eating chow. However, body weights of rats eating chow or a high-fat diet were not significantly different (Table 3). The increase in abdominal fat content observed at 4 weeks was very large in rats eating a high-fat diet compare to those eating chow. This increase was reversed to the level in chow-fed rats after 4 weeks of treatment with CL (Figure 9A). CL-treated chow-fed rats also had less fat in their abdominal depots than did saline-treated chow-fed rats, but the difference was not as marked as that in the rats eating the high-fat diet (Figure 9A). Other carcass fat continued to increase in the rats eating the high-fat diet during the 8 week period of feeding. However, after infusion of CL, this increase stopped without any decrease in the size (Figure 9B). Only a small increase occurred in this fat compartment in the chow-fed rats during the 8-week period and CL had no effect on it.

Energy intake in the rats eating the high-fat diet was the same as in rats eating chow and it was not altered by CL-treatment (Figure 10A). Resting metabolic rate of

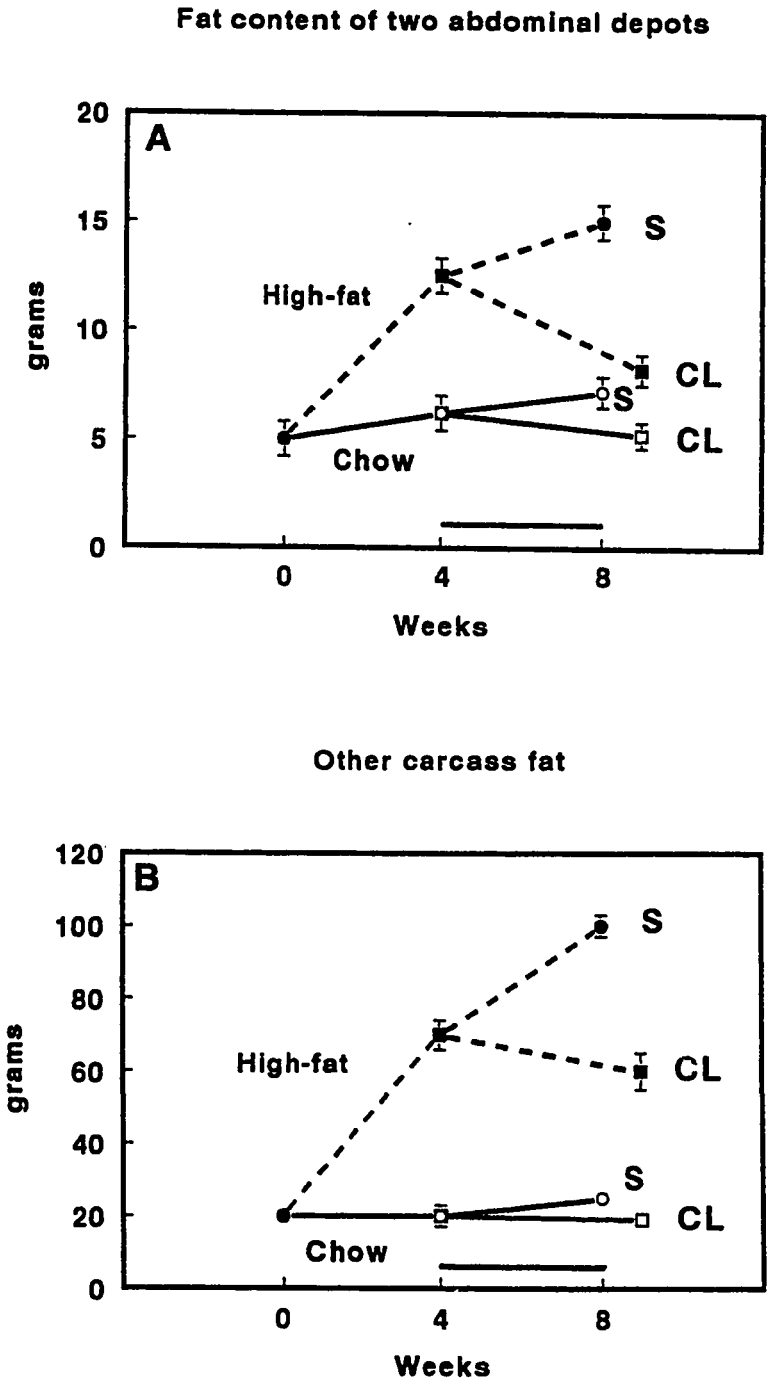


FIGURE 9. Body fat content in high-fat diet and chow-fed rats. All values are means ± SEM (n=8).

A) Total fat content of two major abdominal depots (RWAT and EWAT). Closed symbols and dotted lines are DIO rats which ate high-fat diet from week 0 to week 8 (High-fat). Open symbols and solid lines are control rats (Chow). Rats were treated with saline (S) or CL 316,243 (CL) between 4 and 8 weeks. Effect of high-fat diet at 4 week, $P < 0.001$. CL-DIO *vs* S-DIO at 8 weeks, $P < 0.001$. CL *vs* saline at 8 weeks for chow-fed rats, $P < 0.05$, CL-chow rats at 8 weeks *vs* chow rats at 4 weeks, N.S.

B) Carcass fat content. Symbols and lines are as in A. Effect of high-fat diet at 4 weeks, $P < 0.001$. CL *vs* saline at 8 weeks, $P < 0.001$; CL-DIO rats at 8 weeks *vs* DIO rats at 4 weeks, N.S. S-DIO rats at 8 weeks *vs* DIO rats at 4 weeks, $P < 0.001$.

Table 3

Body weights and weights of retroperitoneal WAT

	Chow-fed			High-fat diet	
	Week 0	week 4	week 8	week 4	week 8
Body weights (g)					
pretreatment	502 ±7.9	556 ± 18.9		605 ± 7.3	
saline			588 ± 17.3		623 ± 9.9
CL			580 ±17.3		600 ± 9.1
Weights of RWAT(g)					
pretreatment	2.48 ± 0.28	3.00 ± 0.53		7.92 ± 0.57‡	
saline			4.82 ±0.59		9.31 ± 0.46‡
CL			2.45 ± 0.35 *		4.76 ± 0.54*†‡

Values are means ±SEM for weights all groups of rats at the time they were killed. Rats were treated between week 4 and 8. Weight of RWAT in CL-treated rats was significantly lower than that in saline-treated rats in both group of rats. Symbols indicate: * significant effect of CL ($P < 0.05$) compared with saline -treated rats eating same diet; † significant effect of CL ($P < 0.05$) compared with pretreated state at week 4; ‡ significant effect of diet ($P < 0.05$) compared with same time and treatment group.

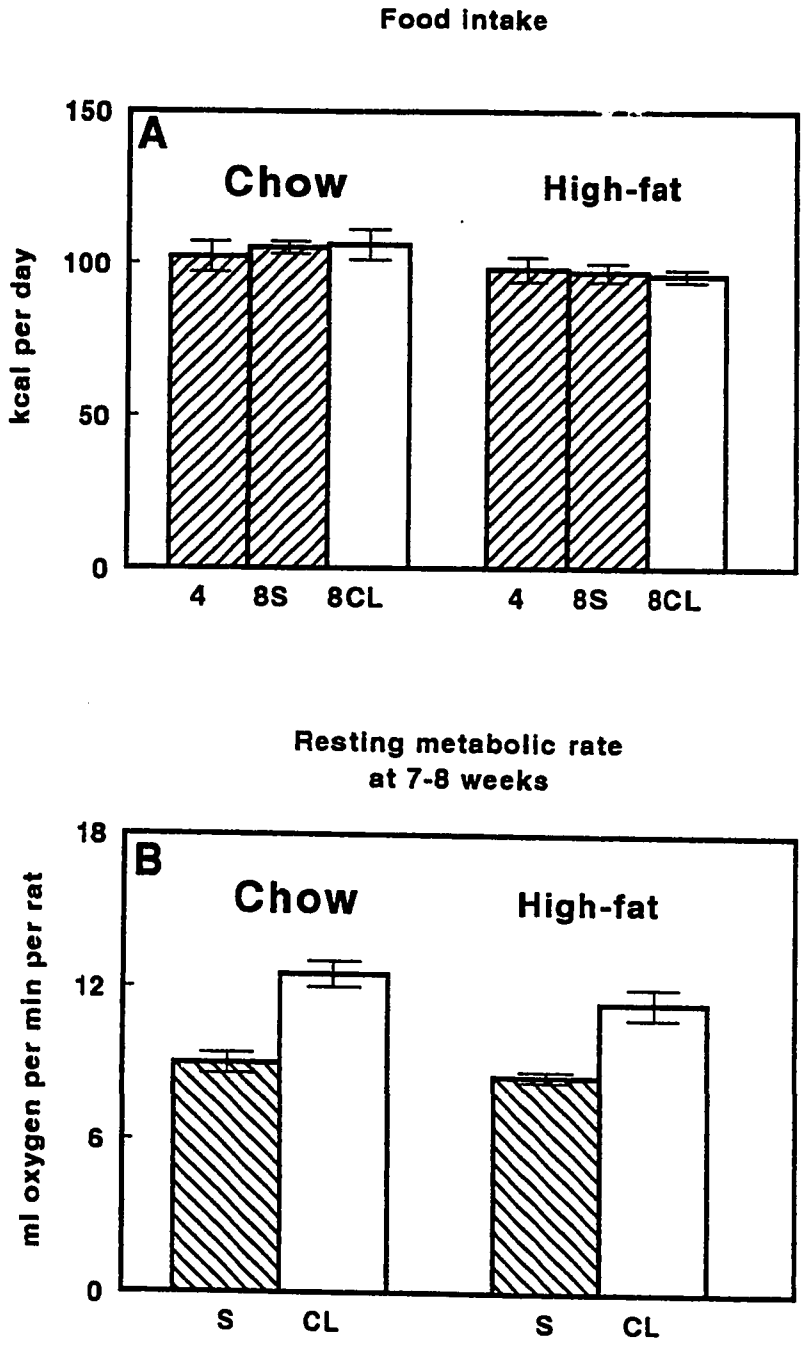


FIGURE 10. Energy intake and resting metabolic rates. All values are means ± SEM (n=8). A) Energy intake during the first 4 weeks of diet (bar labelled 4) and during the subsequent 4 weeks of treatment with saline (bar labelled 8S) or CL (bar labelled 8CL) in chow-fed rats (Chow) and DIO rats (High-fat). There are no significant differences.
B) Resting metabolic rate measured during weeks 7-8. It is significantly higher ($P < 0.001$) in CL treated rats for both diet-treatments. Rectal temperatures of the rats were 37.70 ± 0.093 (S-Chow), 38.00 ± 0.195 (CL-Chow), 37.73 ± 0.180 (S-High-fat), 37.96 ± 0.150 (CL-High-fat). There are no significance in rectal temperature.

rats treated with CL was substantially increased in both chow and high-fat diet by more than 44% (Figure 10B). Rectal temperatures remained unchanged by the CL-treatment in both groups (legend to figure 10B).

Effect on WAT

Although both retroperitoneal and epididymal WAT depots were studied, only data for retroperitoneal WAT (RWAT) are presented, because changes in epididymal WAT were similar although of lesser magnitude. CL-treatment decreased the elevated weight of RWAT induced by the high-fat diet (Table 3). Treatment with CL also prevented the aging-associated increase in RWAT weight in the chow-fed rats but did not decrease it from that seen before the treatment (Table 3). Fat cell size showed a marked increase during the development of obesity in the high-fat diet (Figure 11A). This increase in adipocyte size was decreased by the treatment with CL (Figure 11A; 12B and D). However, the number of mature white adipocytes was not significantly altered by the high-fat diet and CL-treatment did not alter this number (Figure 11B). Despite the unchanged number of mature white adipocytes, DNA content of RWAT increased progressively with age, especially marked in the rats eating the high-fat diet (Figure 13A); CL reversed the increased in DNA content induced by high-fat diet feeding at 4 week of treatment (Figure 13A) and prevented the aging-associated increase in chow-fed rats. In contrast to DNA content, the

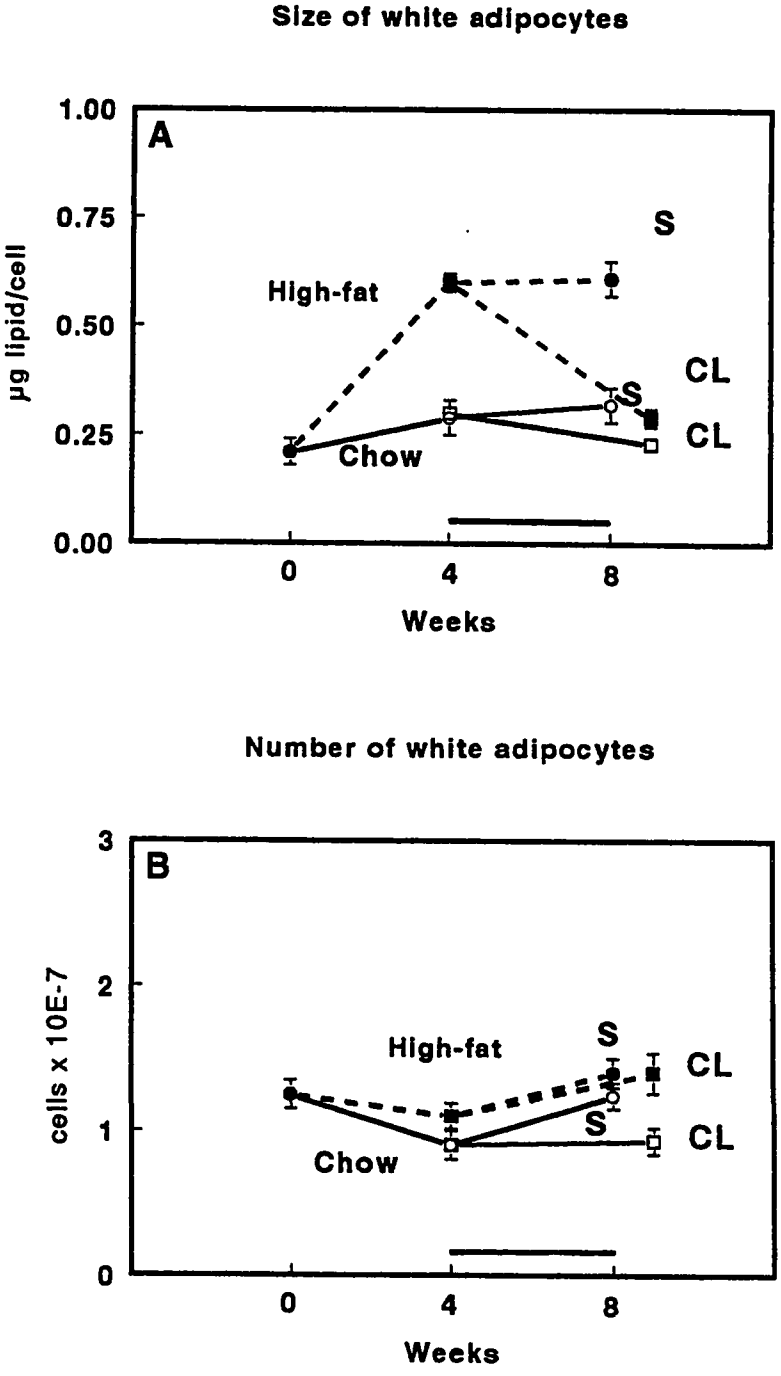


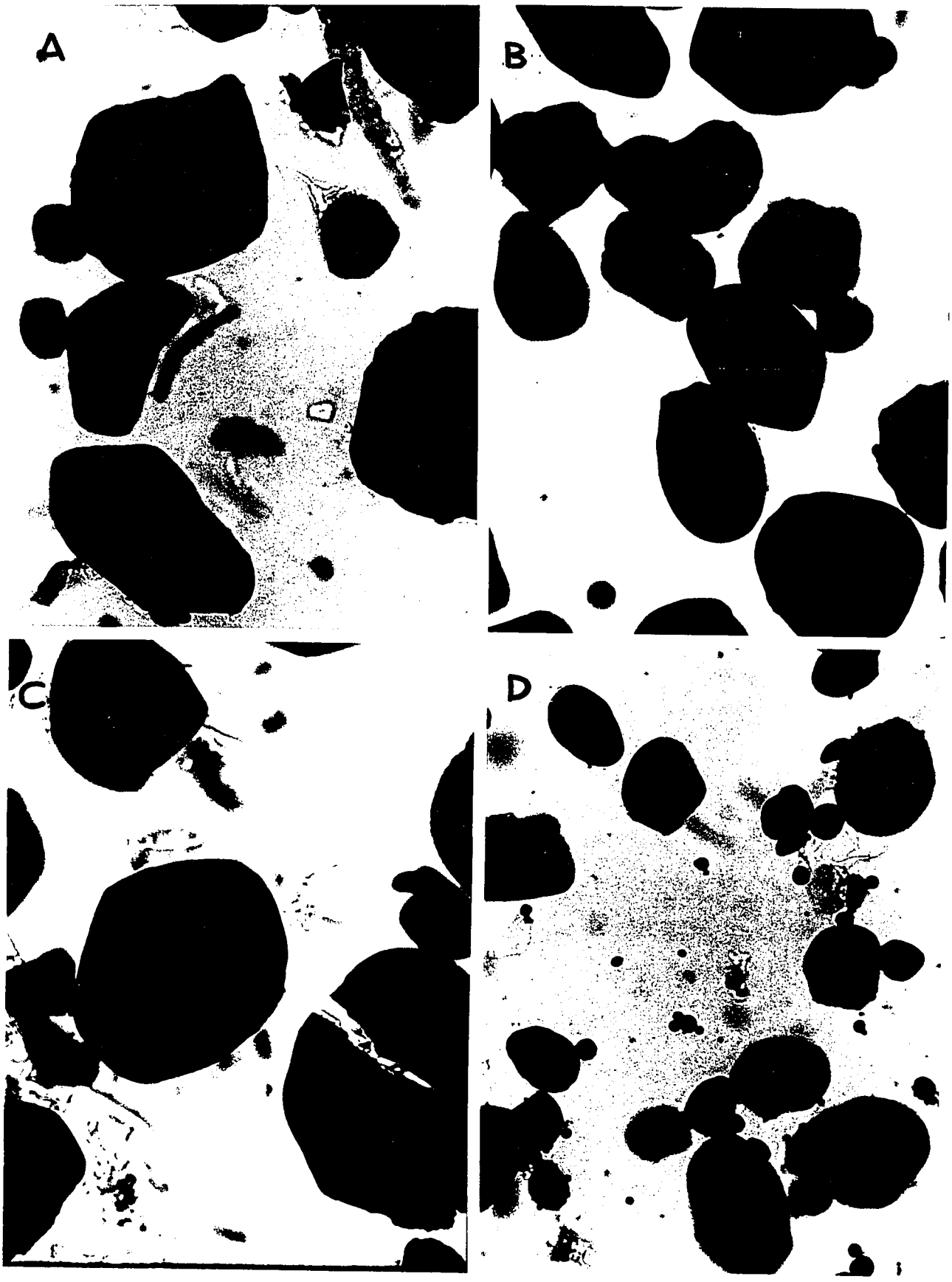
FIGURE 11. Size and number of white adipocytes in RWAT as assessed by the osmium fixation method (Hirsch and Gallian 1968). All values are means ± SEM (n = 8). Symbols, lines and labels as in figure 9A.

A) The size of adipocytes. DIO rats at 4 weeks *vs* chow rats at 4 weeks. $P < 0.001$. CL-DIO rats at 8 weeks *vs* S-DIO rats at 8 weeks, $P < 0.001$, and *vs* DIO rats at 4 weeks, $P < 0.001$. S-DIO rats at 8 weeks *vs* DIO rats at 4 weeks, N.S.

S-chow rats at 8 weeks *vs* chow rats at 4 weeks. N.S.

B) Total adipocytes number. The Y axis shows total number of cells multiplied by 10^{-7}. There are no significant differences.

FIGURE 12. **Micrographs of white adipocytes from RWAT fixed by the osmium fixation method (Hirsch and Gallian 1968) in saline (S) and CL-treated rats eating a chow or high-fat diet. (A) S-Chow, (B) CL-Chow, (C) S-high-fat, (D) CL-high-fat. Magnification x 200. Note the adipocytes in CL-treated rats are smaller than those in saline-treated rats.**


A
B
C
D

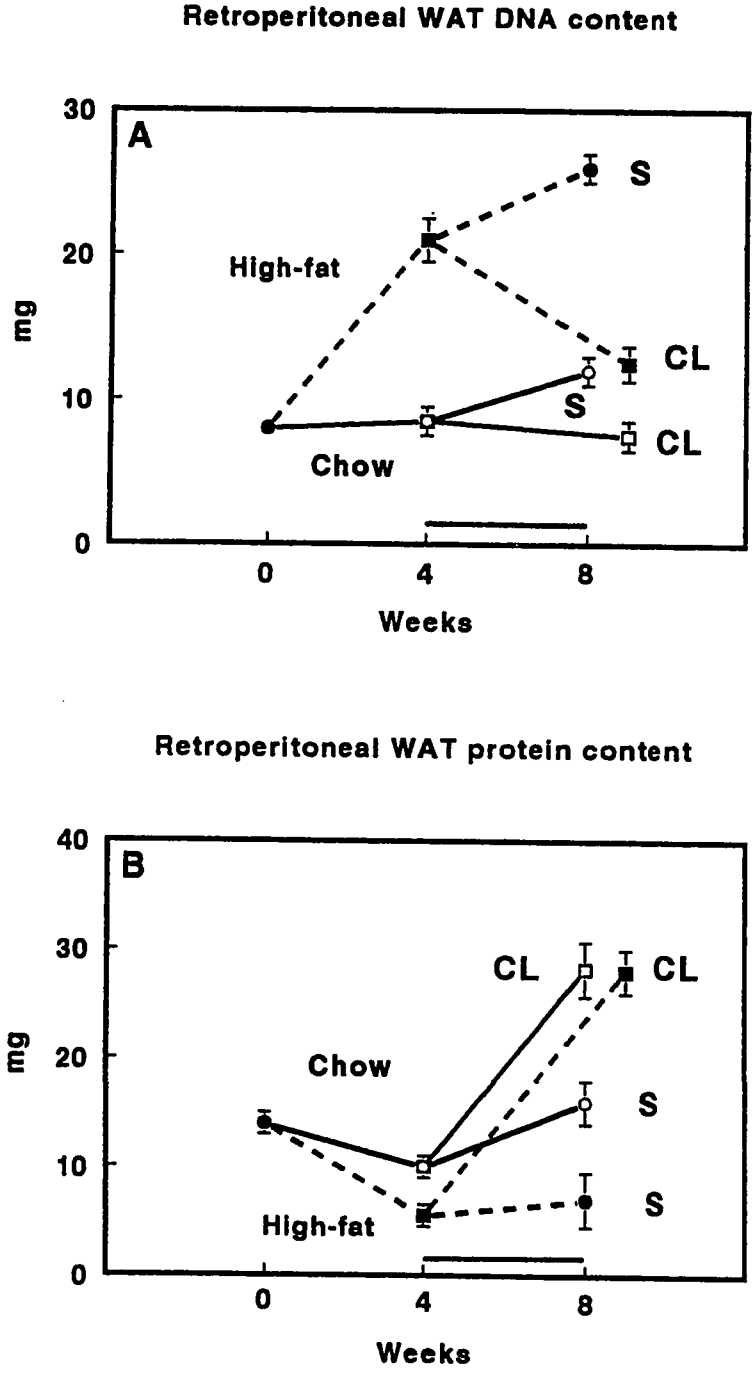


FIGURE 13. DNA and protein contents of RWAT. All values are mean ± SEM (n = 8). Symbols, lines and labels as in Figure 9A.
A) DNA content of saline and CL-treated rats. DIO rats at 4 weeks *vs* chow at 4 weeks, $P < 0.001$. CL-DIO at 8 weeks *vs* S-DIO at 8 weeks. $P < 0.001$ and *vs* DIO at 4 weeks, $P < 0.001$. S-DIO at 8 weeks *vs* DIO at 4 weeks, N.S. CL-chow at 8 weeks *vs* S-chow at 8 weeks, $P < 0.05$ and *vs* chow at 4 weeks, N.S.
B) Total protein content. CL-DIO at 8 weeks *vs* S-DIO at 8 weeks, $P < 0.001$ and *vs* DIO at 4 weeks, $P < 0.001$. CL-chow at 8 weeks *vs* S-chow at 8 weeks. $P < 0.001$ and *vs* chow at 4 weeks. $P < 0.001$.

protein content of RWAT was not altered by diet but was increased markedly in both high-fat diet and chow-fed rats in response to CL-treatment (Figure 13B).

Histology of WAT

Interpretation of the CL-induced changes in DNA content and weight (both decreased), mature white adipocyte number (unchanged) and protein content (increased) required histological and immunohistochemical assessment of the tissue to assess its cellular composition. Typical appearance of numerous large unilocular white adipocytes in RWAT of saline-treated rats was used as a control to compare with the changes in RWAT of CL-treated rats. In RWAT of saline-treated rats, white adipocytes were apparently the predominant cell type in the tissue; in the high-fat diet rats, adipocytes appeared to be larger (Figure 14A and C). In CL-treated rats, white adipocytes were markedly smaller than those in saline-treated rats eating the same diet (Figure 14B and D). Furthermore, appearance of abundant densely stained multilocular and much smaller cells than white adipocytes in the tissue was apparent (Figure 14B and D). For further investigation, the presence of UCP in the multilocular cells that appeared in RWAT of CL-treated rats was detected by using immunohistochemistry (Figure 15B and D). UCP was not detected at all in RWAT of the saline-treated rats (Figure 15A and C). Higher power light microscopy of semi-thin stained sections showed the multilocular cells to have densely-stained cytoplasm,

FIGURE 14. Histology of RWAT. Haematoxylin and eosin-stained 10 μm sections of RWAT of S-chow (A), CL-chow (B), S-high-fat (C), and CL-high-fat (D). Magnification x 400. The appearance of the large number of very small cell identified as multilocular cells in CL-treated rats is apparent (Figures B and D).

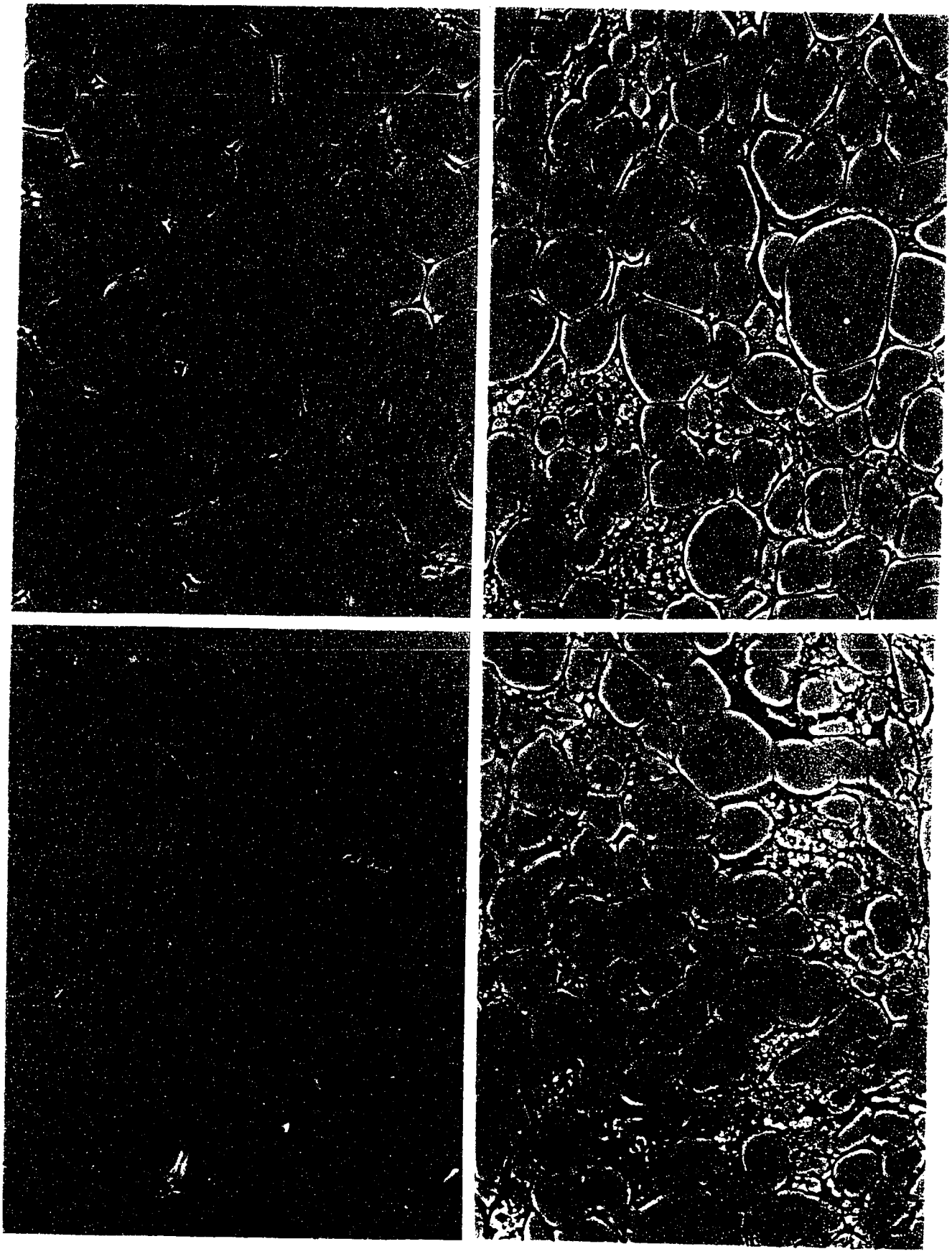
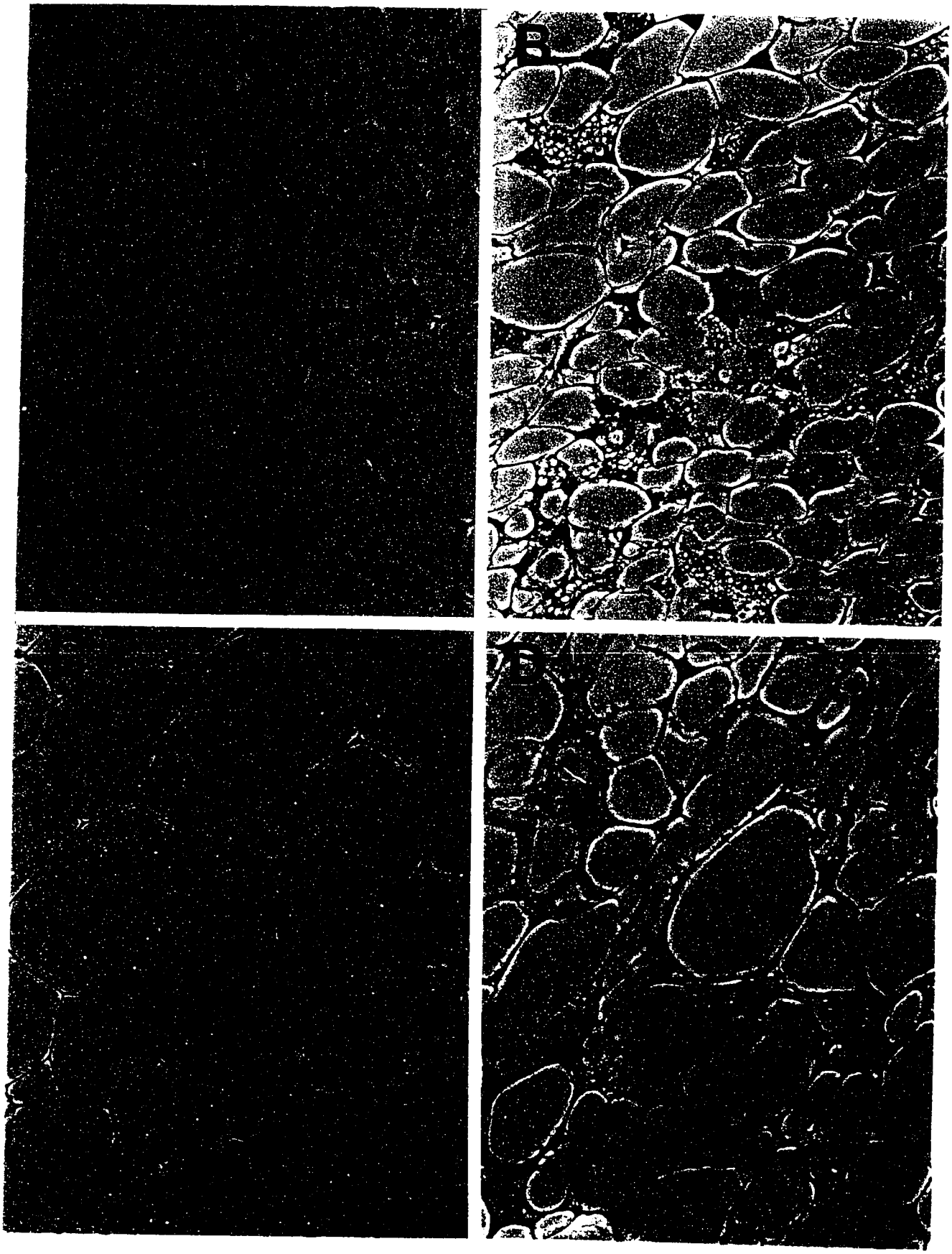


FIGURE 15. Immunohistochemical detection of UCP in RWAT. Thick sections (10 μm) of RWAT were immunodetected with the UCP antibody. Black stains are UCP immunoreactions. (A) S-chow, (B) CL-chow, (C) S-high-fat, (D) CL-high-fat. Magnification x 400. Multilocular cells in RWAT of CL-treated rats contain UCP and therefore are defined as brown adipocytes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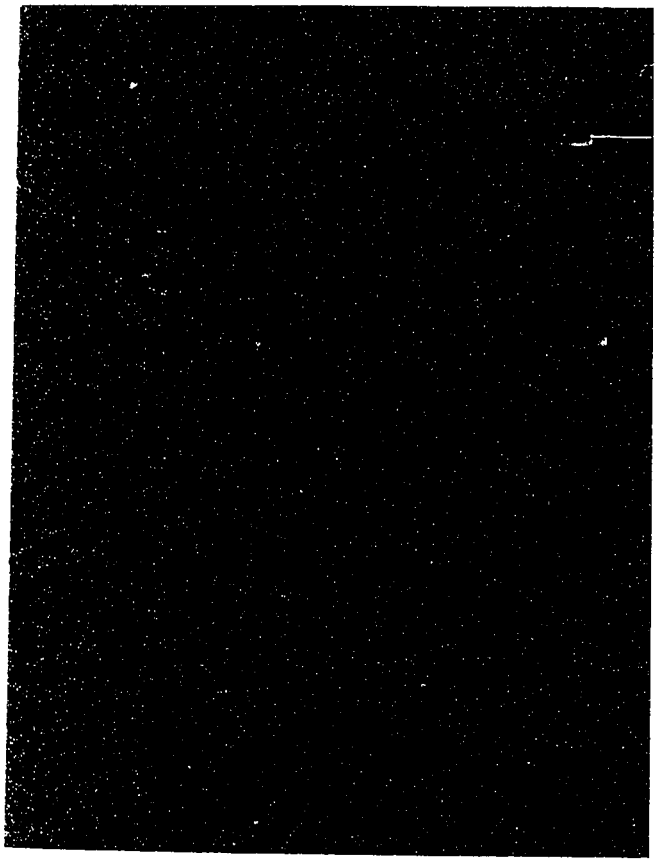
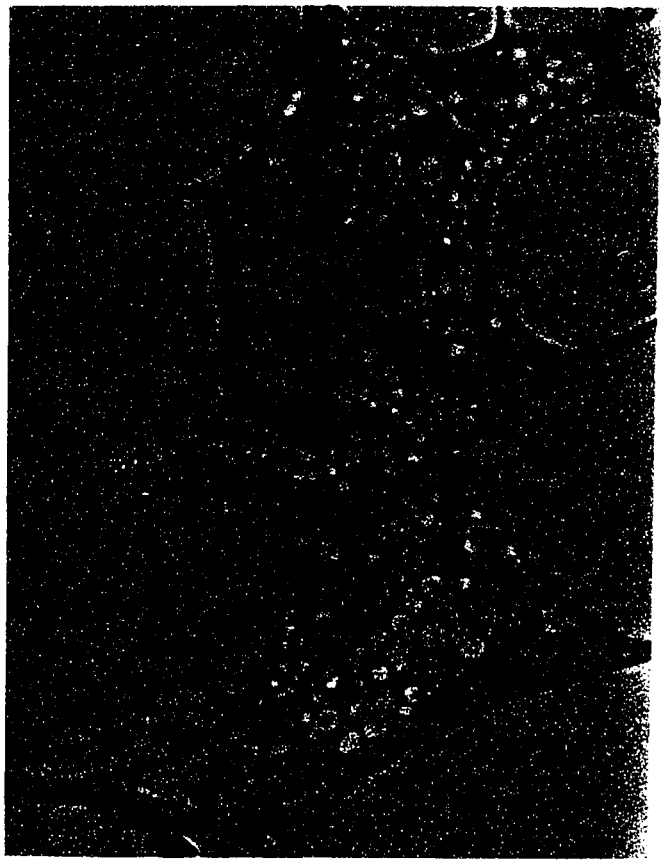
numerous very small lipid droplets and large pale nuclei with a prominent nucleolus, all characteristic of brown adipocytes (Figure 16B and C). Clustering of multilocular cells between mature white adipocytes and their association with numerous capillaries was another characteristic of the tissue under influence of CL. Multilocular cells were not seen in sections of WAT of saline-treated rats (Figure 16A and C). CL-treatment induced the appearance of a 32 kDa protein, identified as UCP, as assessed by Western blotting. The effect was somewhat variable, but more marked in the rats eating the high-fat diet (Figure 17).

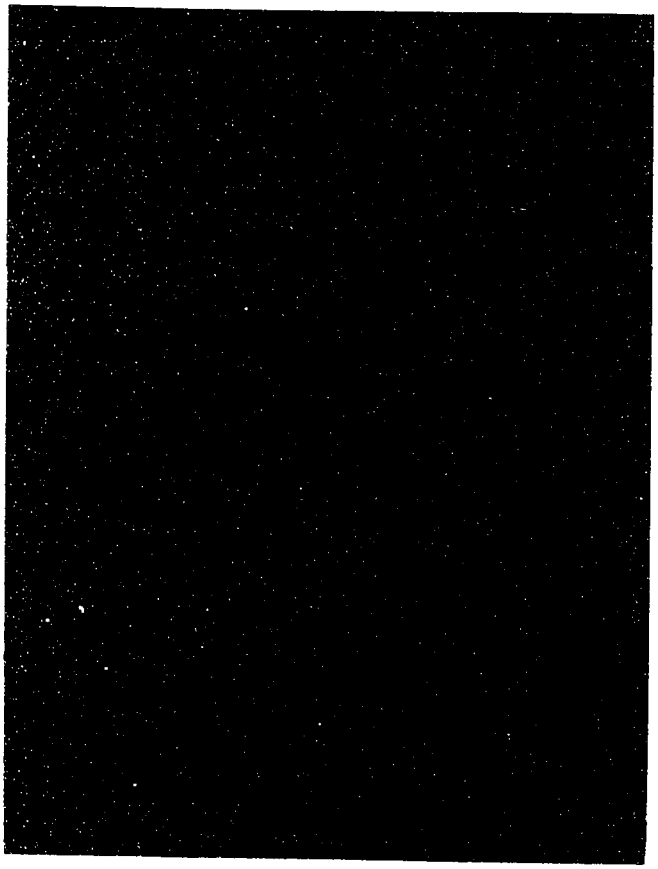
Effect on BAT

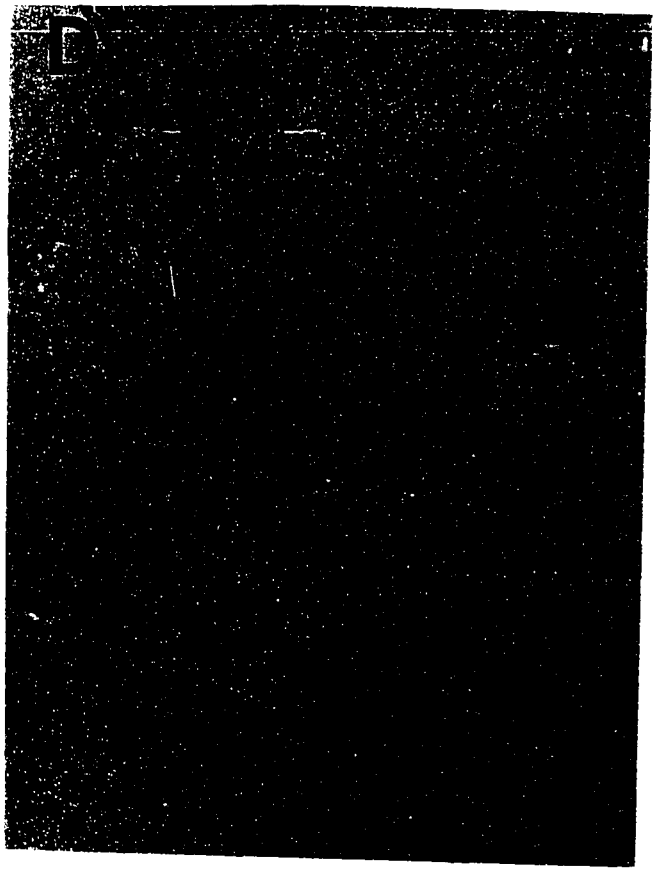
Although both interscapular and perirenal BAT depots were studied, data for interscapular BAT (IBAT) only are present in this section, because changes in perirenal BAT were similar to those in IBAT. In IBAT, there was a substantial increase in total protein and UCP contents induced by CL-treatment. These increases were similar in rats eating chow and in rats eating the high-fat diet (Figure 18A and B). Cytochrome oxidase activity also increased by 4-10 fold, indicating a marked proliferation of mitochondria during the CL-treatment (Figure 19). Despite the hypertrophy of BAT, DNA content of IBAT remained unchanged (Figure 20A), and the weight of BAT was not increased by the drug treatment (Figure 20B).

FIGURE 16. Semi-thin sections of retroperitoneal WAT of S- or CL-treated rats. Sections (0.5 µm) were stained with methylene blue and azur blue II. (A) S-chow, (B) CL-chow, (C) S-high-fat, (D) CL-high-fat. Magnification x 2000. The appearance of multilocular cells with a large palely-stained nuclei and a dense nucleolus (all characteristics of brown adipocytes) associated with numerous capillaries is observed in WAT of CL-treated rats (Figures B and D).









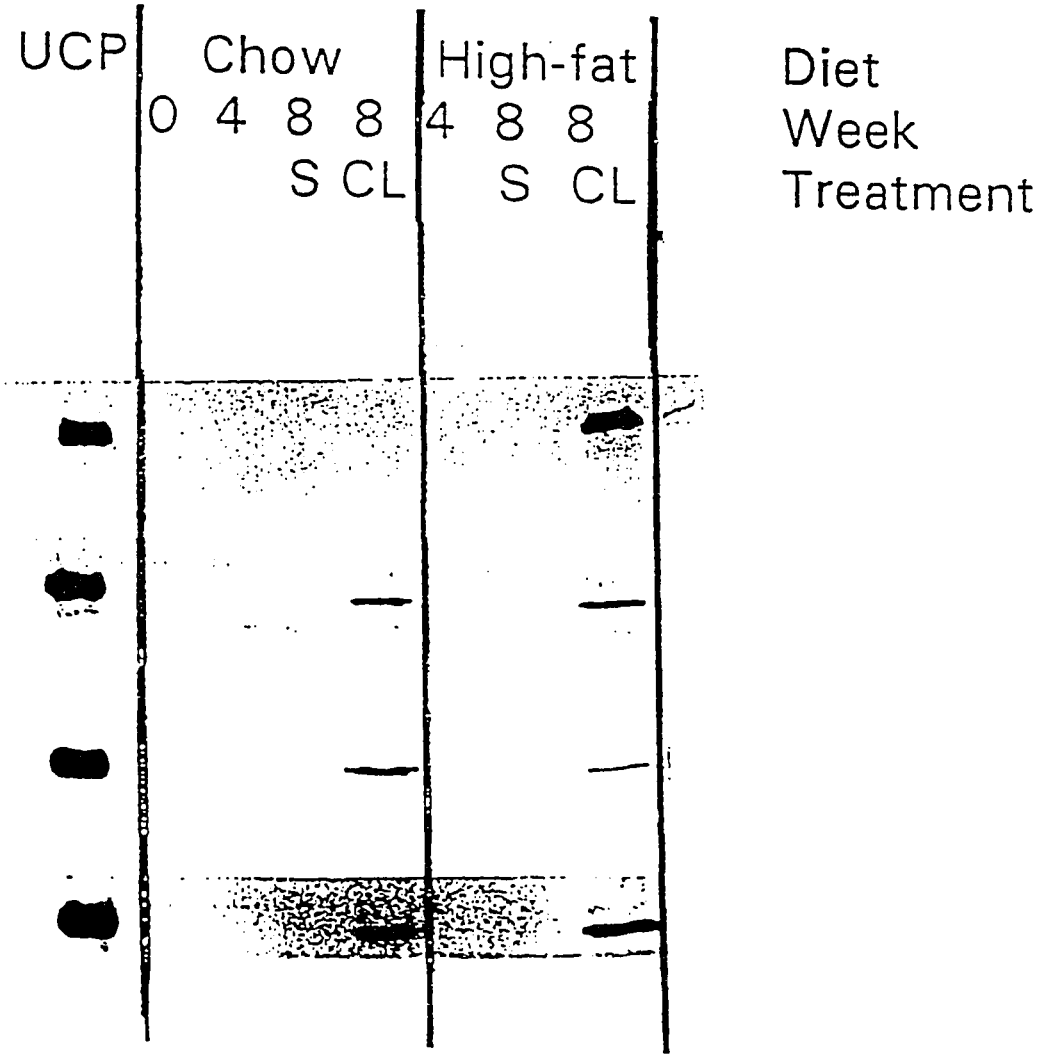


FIGURE 17. Western blots of UCP in RWAT (10 µg protein). Four rats from each group of 8 are shown . Chow-fed rats from 0, 4 and 8 weeks, high-fat diet rats from 4 and 8 weeks. Rats were either saline- (s) or CL-treated (CL). UCP appeared after CL-treatment, particularly in WAT of rats eating the high-fat diet.

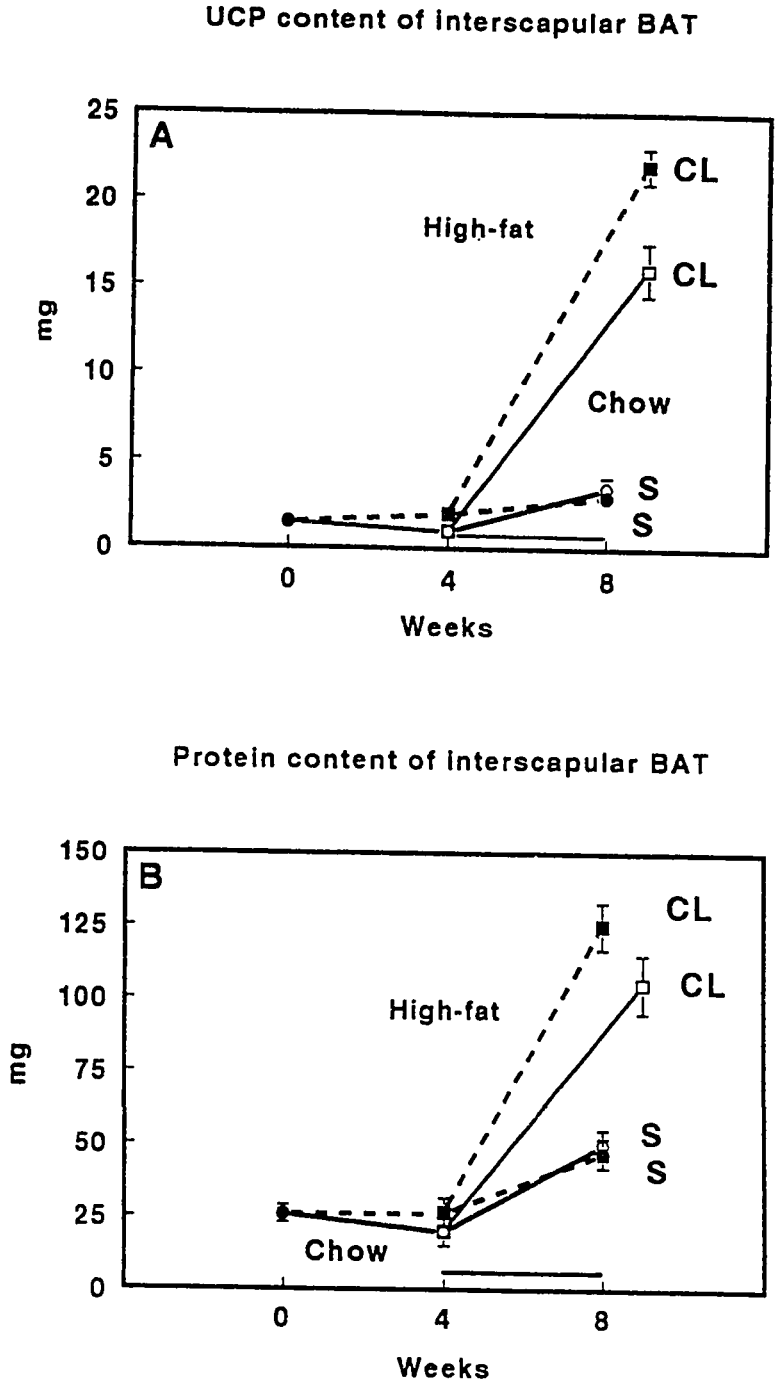


FIGURE 18. UCP and protein content of interscapular BAT. Values are means ± SEM (n = 8). Symbols and lines and labels as in figure 9A.
A) UCP content of interscapular BAT. CL-treatment significantly increased UCP content in both chow and high-fat diet, ($P < 0.001$).
B) Total protein content of interscapular BAT. The protein content also increased by treatment CL in both diets, ($P < 0.001$).

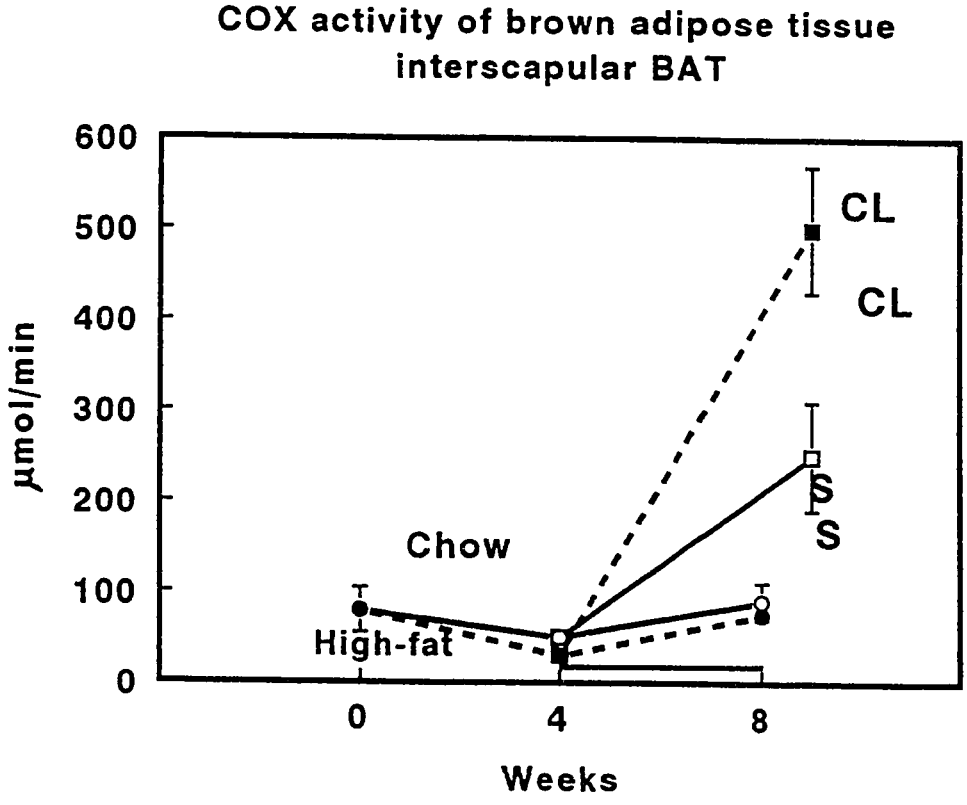
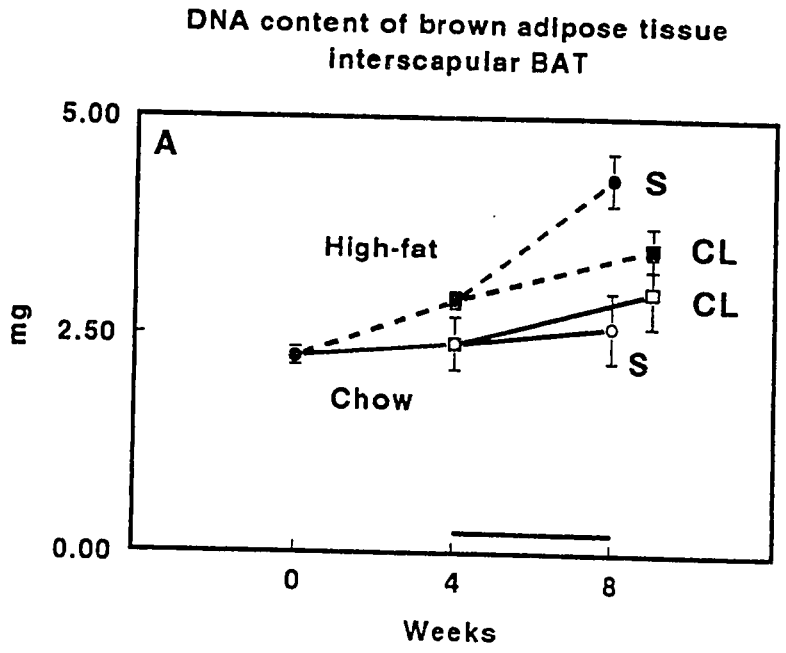


FIGURE 19. Cytochrome oxidase content of interscapular BAT. Values are means ± SEM (n = 8). Symbols and lines and labels as in figure 9A. CL-treatment induced a very large increase in cytochrome oxidase content of brown adipose tissue in both chow and high-fat diet rats, ($P < 0.001$).



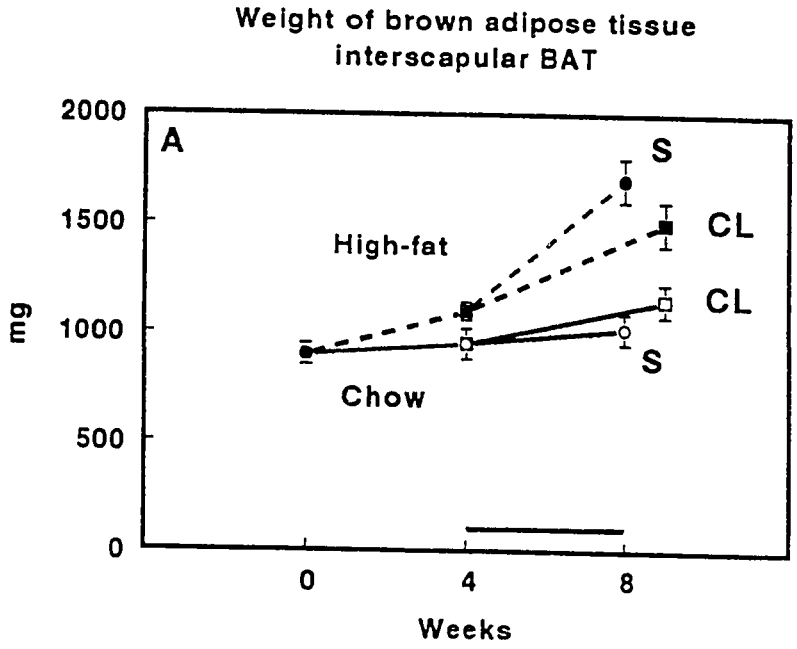


FIGURE 20. DNA content and weight of interscapular BAT. Symbols and lines and labels as in figure 9A.
A) CL-treatment did not alter DNA content of interscapular BAT, perhaps retarded the aging-associated in rats eating the high-fat diet.
B) The wet weight of IBAT was not altered by CL-treatment.

Discussion

Results of this experiment show that CL-treatment is able to reverse established diet-induced obesity in rats that continue to eat a high-fat diet during the treatment. The DIO brought about an increase in total body fat, both intra-abdominal and other carcass fat, without any increase in energy intake which shows the high metabolic efficiency in the processing of a high-fat diet. The enlarged adipose depots contained large adipocytes but there was no increase in adipocyte number. The DIO was a hypertrophic obesity. Treatment with CL induced a negative energy balance associated with increased thermogenesis, but without any change in energy intake. The loss of fat was primarily from the intra-abdominal WAT depots with no reduction in other carcass fat. The enlarged size of the adipocytes was returned to normal by treatment with CL. The drug had less effect on the smaller cells in the chow-fed control rats. On the other hand, CL-treatment did not induce any change in the number of white adipocytes in either the high-fat diet or the chow-fed rats, despite reversal of the obesity in the DIO rats.

BAT, a probable site for the increase of energy expenditure, was also influenced by CL-treatment. The substantial increase in energy expenditure induced by CL-treatment is associated with hypertrophy of brown adipocytes. Treatment with CL induced a very marked increase in UCP content of IBAT (by 10-fold) and in cytochrome oxidase activity by 10-fold, evidence for a marked proliferation of IBAT mitochondria. CL-treatment did not have any effect on DNA content of BAT,

probably because the precursors interstitial cells possess only β1-ARs (Bronnikov et al., 1992). Hyperplastic growth of BAT that occurs during cold exposure is mediated by the effect of NA released from sympathetic nerve on β1-ARs (Géloën et al., 1992); since CL is a selective β3-adrenergic agonist, it is consistent not to observe hyperplasia of BAT in CL-treated rats. One report of an increase in DNA content of BAT in response to β3-AR stimulation (Arbeeny et al., 1995) used a different and less selective β3-AR agonist, BRL 35135, acknowledged to possess both β1- and β2-adrenergic agonist properties (Arbeeny et al., 1995).

In WAT, the appearance of abundant multilocular brown adipocytes that expressed UCP was accompanied by a large increase in total protein content which was probably due to the development of brown adipocytes in WAT. The size of white adipocytes decreased whereas the number of mature white adipocytes remained unchanged. Since, the high-fat diet feeding did not increase the number of white adipocytes as assessed by the Hirsch and Gallian osmium fixation method, the increase in total cell number, as assessed from DNA content, implies that this increase was associated with other cell types rather than white adipocytes, and these other cell types disappeared under the influence of the CL-treatment. Appearance of multilocular cells in WAT is suggested to be a remodelling phenomenon under the influence of CL-treatment. The origin of the multilocular, UCP-expressing cells which appeared in RWAT of CL-treated rats is still unknown. It will be further discussed in the general discussion at the end of this thesis.

PART B: DOES CL 316,243 TREATMENT MAKE MATURE ADIPOCYTES DISAPPEAR IN AN OBESE ANIMAL WITH HYPERPLASTIC OBESITY?

Background

In the previous studies, the new β3-AR agonist, CL 316,243, increased thermogenesis and prevented DIO in young rats (Himms-Hagen et al., 1994) and reversed established DIO in older rats eating a high-fat diet (see chapter V part A). White adipocytes in CL-treated rats showed a significant decrease of the content of lipid compared to saline-treated rats. However, there was no change in the number of mature white adipocytes in drug-treated rats. Abundant multilocular cells that appeared in WAT were identified as brown adipocytes. CL-treatment also induced the disappearance of non-adipocytes which appeared during feeding with the high-fat diet. The question arises whether CL-treatment could have any effect on the number of mature white adipocytes in hyperplastic obesity. Unfortunately, the DIO was a hypertrophic obesity and the effect of the drug on hyperplastic obesity could not be assessed in this model.

Objective

The objective of this study was to determine whether mature white adipocytes

present in increased numbers in hyperplastic obesity can be made to disappear by treatment with CL 316,243. For this purpose, the genetically obese Zucker fa/fa rats were chosen and the design of the experiment was similar to that of previous experiment except that the rats were allowed to eat chow until they reached the age of 30 weeks which is known to be age that hyperplastic obesity is marked in fa/fa rats (Cleary et al., 1979).

An additional objective was to explore more fully the extensive remodelling of WAT that occurred under the influence of CL using additional histological techniques.

Methods

Animals

Twenty four lean and twenty four genetically obese fa/fa Zucker rats were purchased from Charles River, Canada at 11-12 weeks of age. They were housed at 24°C in plastic cages with wood chips and free access to chow (Agway R-M-H 4020 chow, 14.5% energy from fat) and water until they reached 30 weeks of age. At this time, each genotype was separated into three groups of 8 and one of these groups of each genotype was killed (week 0). The other two groups of 8 from each genotype were then implanted with osmotic mini-pumps (Alzet 2002, Alza, Palo Alto CA) under halothane anaesthesia and received 0.49 µl per hour containing either saline or CL 316,243 (1 mg per kg body weight per day) for the next 2-3 weeks. Design of the

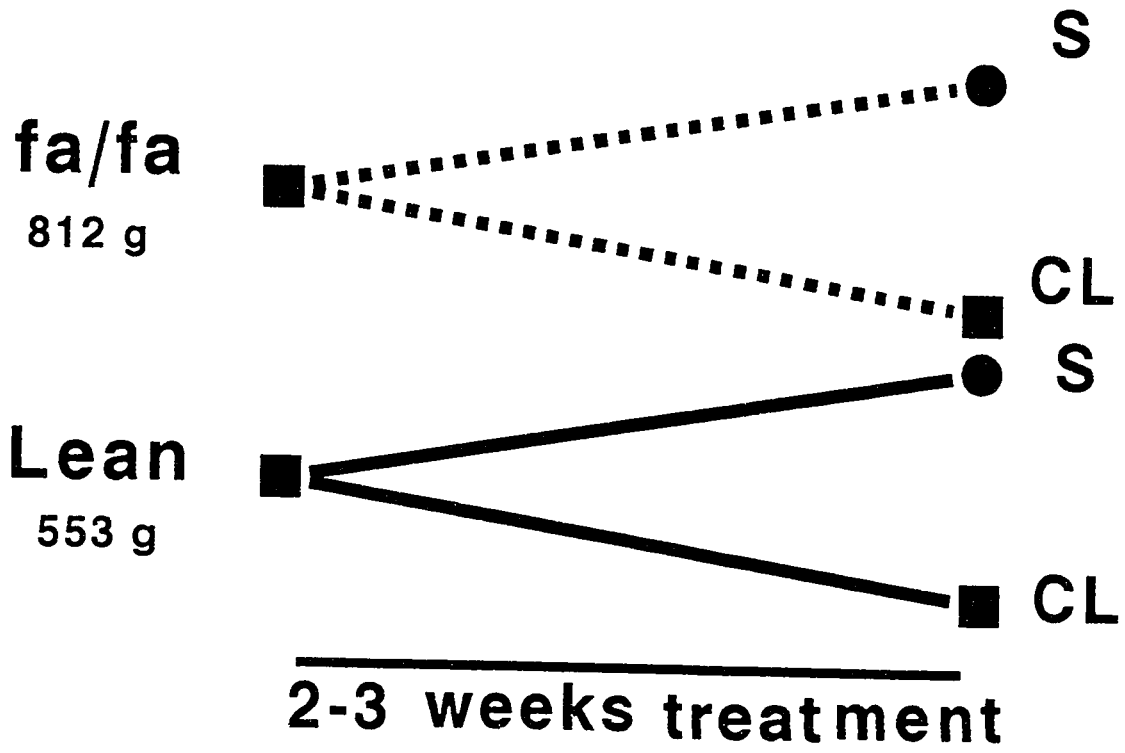
experiment is illustrated in Figure 21. Pumps were replaced after 2 weeks. Body weights and food intakes were measured weekly. Resting metabolic rates were measured during the second or third week as in the previous experiment. Some CL treated fa/fa rats died before the end of the second week. The remainder of this group plus the saline-treated fa/fa rats were therefore studied immediately. The CL-treated and saline treated lean rats were studied as soon as possible thereafter.

Rats were sacrificed by decapitation. Blood was collected in conical centrifuge tubes, centrifuged and the plasma was transferred into microfuge tubes and kept at -80°C. Interscapular and perirenal BAT depots were removed and placed in isolation medium on ice (Cui and Himms-Hagen, 1992). They were then dissected free of adhering muscle, vasculature and WAT, weighed, homogenized and kept at -80°C. Retroperitoneal WAT and epididymal WAT depots were also removed, weighed and homogenized. Small portions (150-200 mg) were used for osmium fixation for counting and sizing of mature adipocytes (Hirsch and Gallian, 1968). Some portions of all tissues mentioned above were fixed in either formalin or glutaraldehyde for histology and immunohistochemistry. The remaining carcass had tail and paws removed and was frozen for later carcass analysis as described in chapter III.

All biochemical assays such as DNA, protein, cytochrome oxidase, UCP, and radioimmunoassays as well as resting metabolic rate, histology and immunohistochemistry were performed as described in chapter III.

Treatment of lean and obese fa/fa rats with CL 316,243

2-3 weeks treatment, 1 mg/kg/day



8 month old, chow, 24°C

FIGURE 21. Design of experiment for the treatment of lean and obese (fa/fa) Zucker rats with CL 316,243. See text for more details.

Statistical analysis

Results are presented as means ± SEM. Statistical analysis used Instat software to do ANOVA followed by Student-Newman-Keuls post hoc test. Significance are based on $P < 0.05$.

Results

The weight of fa/fa rats was highly variable, much more than that of the lean rats. Since the variation of body weight in fa/fa rats was very high, the decrease in mean body weight of these rats induced by the CL-treatment did not reach statistical significance (Table 4). However, the fa/fa rats which survived lost an average of 62 ± 19.8 grams in two weeks and the fa/fa rats which did not survive for 2 weeks lost an average of 35.5 ± 3.8 grams in the first week of treatment. CL-treatment reduced abdominal fat content in both fa/fa and lean rats (Figure 22A). The increase of other carcass fat content stopped in fa/fa rats but no reduction occurred (Figure 22B). There was a marked decrease in food intake induced by the CL-treatment in the hyperphagic fa/fa rats but not in the lean rats (Figure 23A). Resting metabolic rate was increased by 69% in the fa/fa rats and by 39% in the lean rats (Figure 23B). Rectal temperatures remained unchanged by the CL-treatment (legend to Figure 23B). Both resting metabolic rate and energy intake were higher in the saline-treated fa/fa rats compared with the lean rats (Figure 23A and B).

Table 4

Body weights and cellularity of retroperitoneal WAT (RWAT)

	Lean		Obese (fa/fa)	
	Week 0	week 2-3	Week 0	week 2-3
Body weights (g)				
pretreatment	553 ± 20.6 (8)		812 ± 52.0 ‡ (6)	
saline		558 ± 13.9 (8)		820 ± 26.7 ‡ (8)
CL		513 ± 12.4 (8)		743 ± 39.3 ‡ (4)
Weights of RWAT(g)				
pretreatment	6.04 ± 0.61		48.9 ± 6.57 ‡	
saline		5.21 ± 0.65		51.84 ± 6.38 ‡
CL		2.23±0.23*†		31.9 ± 4.13 *†
Number of cells in RWAT(from DNA) x 10-9				
pretreatment	1.8 ± 0.18		14.0 ± 2.0 ‡	
saline		1.6 ± 0.22		16. ± 2.0 ‡
CL		0.7 ± 0.08 *†		9.8 ± 1.1 *‡
Number of adipocytes in RWAT (osmium method) x 10^{-7}				
pretreatment	1.0 ± 0.16		7.3 ± 1.45 ‡	
saline		1.2 ± 0.16		9.4 ± 1.08‡
CL		1.6 ± 0.23		6.3 ± 0.13‡

Values are means ±SEM for the numbers of rats shown in parentheses. All rats ate cho Treatment with saline or CL was between week 0 and weeks 2-3. Symbols indicate: significant effect of CL, compared with saline-treated rats of same genotype; † significan effect of CL compared with pretreated state at week 0; ‡ significant effect of genotyp compared with same time and treatment group.

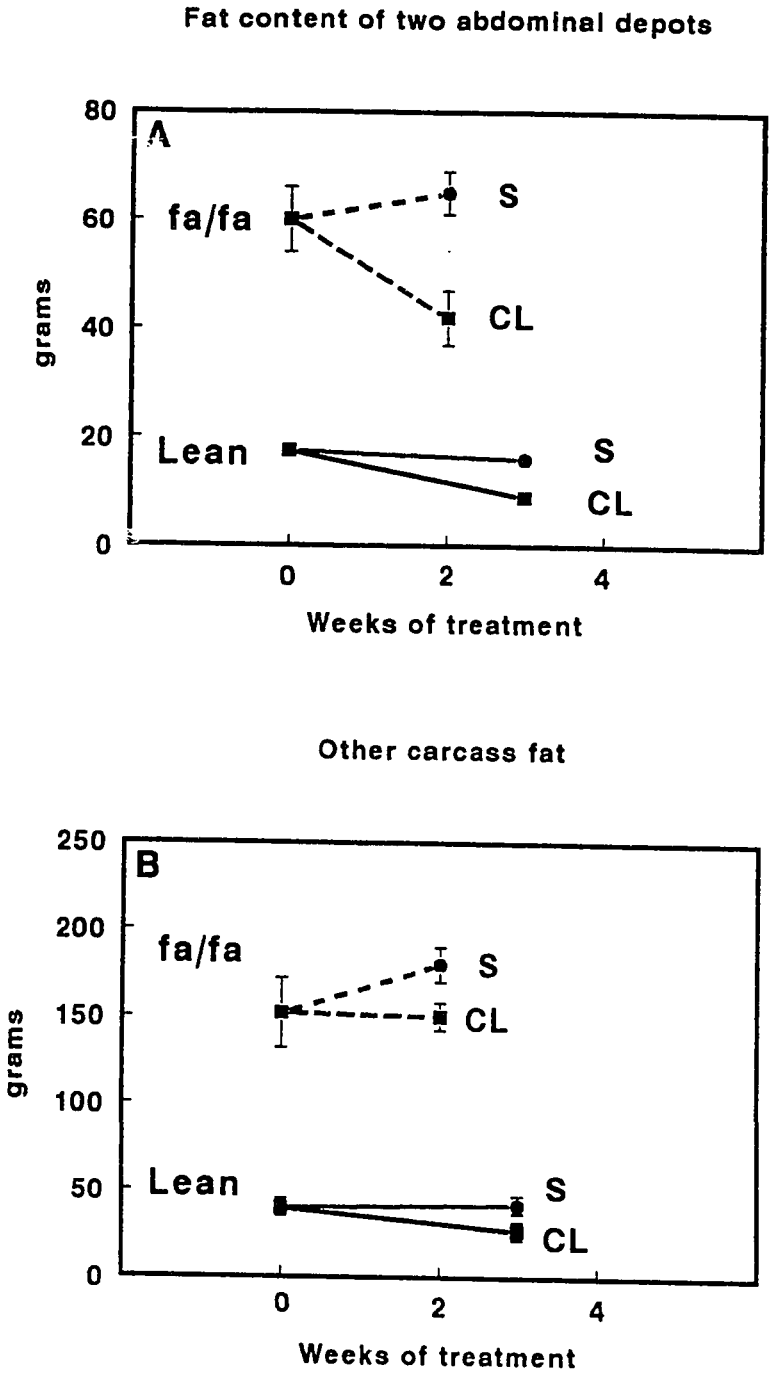


FIGURE 22. Body fat in lean and fa/fa rats.

A) Fat content of two major abdominal depots (epididymal and retroperitoneal). CL-treated rats are indicated by solid circles; all other groups are indicated by solid squares. Dotted lines link values for fa/fa rats; solid lines link values for lean rats. All rats ate chow. CL-treatment significantly decreased abdominal fat content in fa/fa rats (CL -fa/fa *vs* S-fa/fa at 2 weeks, $P < 0.05$, and *vs* fa/fa at 0 weeks, $P < 0.01$). In lean rats, CL-lean *vs* S-lean at 3 weeks, $P < 0.001$, and *vs* lean at 0 weeks, $P < 0.001$, were significant.

B) Other carcass fat. Symbols, lines and labels as in Panel A. CL-fa/fa at 4 weeks *vs* S-fa/fa at 4 weeks, $P < 0.05$ and *vs* fa/fa at 0 weeks, N.S. S-fa/fa at 4 weeks *vs* fa/fa at 0 weeks. $P < 0.05$. CL-lean at 3 weeks *vs* S-lean at 3 weeks, $P < 0.01$ and *vs* lean at 0 weeks $P < 0.05$. S-lean at 3 weeks *vs* lean at 0 weeks, N.S.

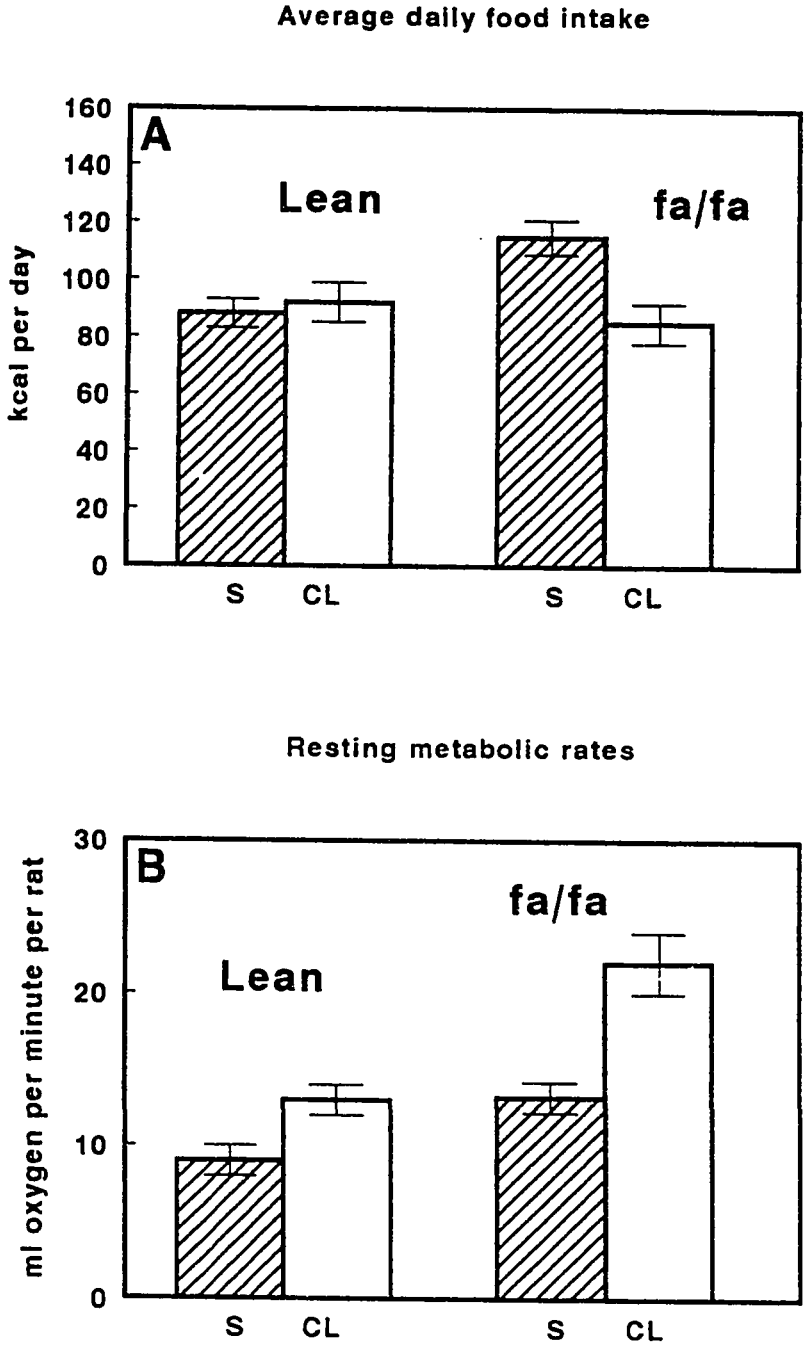


FIGURE 23. Food intake and resting metabolic rates.

A) Food intake was measured during the treatment for saline (S) and CL (CL) treated lean and fa/fa rats. S-fa/fa higher than S-lean, $P < 0.001$. CL-treatment reduced food intake in fa/fa rats ($P < 0.001$) but had no effect on food in lean rats.

B) Resting metabolic rate was measured during weeks 7-8. CL-treatment significantly increased resting metabolic rate in both genotypes ($P < 0.001$). S-fa/fa metabolic rate is higher than that for S-lean ($P < 0.05$). It is also significantly higher in CL-fa/fa rats than in CL-lean rats ($P < 0.001$). Rectal temperature of the rats were 37.7 ± 0.20 (S-lean), 37.9 ± 0.15 (CL-lean), 37.6 ± 0.16 (S-fa/fa), 37.9 ± 0.21 (CL-fa/fa). There was no significance in rectal temperature.

WAT depots

In this experiment both retroperitoneal and epididymal WAT depots were studied. Since changes in epididymal WAT were similar to retroperitoneal WAT (RWAT), data are reported only for RWAT. Wet weight of RWAT was almost 10 times higher in fa/fa rats than in lean rats and was reduced by 35% by the CL-treatment (Table 4). Weight of RWAT was also reduced by the CL-treatment in the lean rats (Table 4). The average size of white adipocytes in the fa/fa rats was normal and adipocyte size was reduced by the CL-treatment in both fa/fa and lean rats (Figure 24A). The number of mature white adipocytes was about 7 times greater in the fa/fa rats than in the lean rats (Figure 24B). CL-treatment did not reduce number of white adipocytes. However, it seemed that the treatment with CL stopped any increase of adipocyte numbers associated with age in fa/fa rats (Figure 24B). DNA content of RWAT was much greater in the fa/fa rats than in lean rats and was reduced by CL-treatment in both fa/fa and lean rats (Figure 25A). Protein content of RWAT was substantially increased by the CL-treatment in both lean and fa/fa rats (Figure 25B). Western blotting showed that RWAT of CL-treated rats contained a 32 kDa protein, identified as UCP, more abundant in the lean rats than in the fa/fa rats (Figure 26).

The proportion of mature white adipocytes (as assessed by the osmium fixation method) to total cell number (as assessed by DNA content) was lower than that has

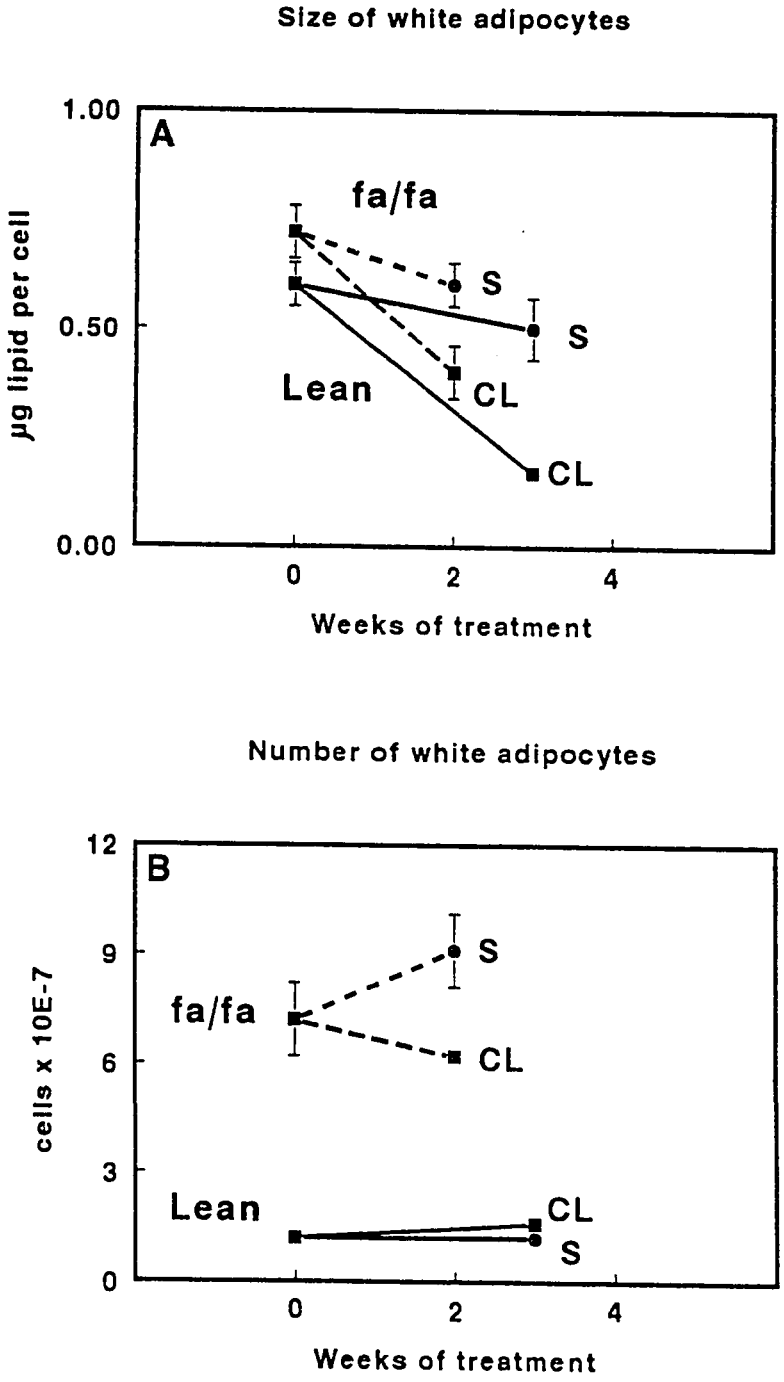


FIGURE 24. Size and number of white adipocytes in RWAT as assessed by the osmium fixation method (Hirsch and Gallian 1968). All values are means ± SEM (n = 8). Symbols, lines and labels as in figure 22A.

A) The size of adipocytes. CL-fa/fa at 2 weeks *vs* at 2 weeks, N.S., *vs* fa/fa at 0 weeks, $P < 0.05$. CL-lean rats at 3 weeks *vs* S-lean rats at 3 weeks, $P < 0.001$, and *vs* lean rats at 0 weeks, $P < 0.001$.

B) Total number of white adipocytes. There are no significant differences for fa/fa rats except the total number of cells was greater ($P < 0.001$) than in lean rats.

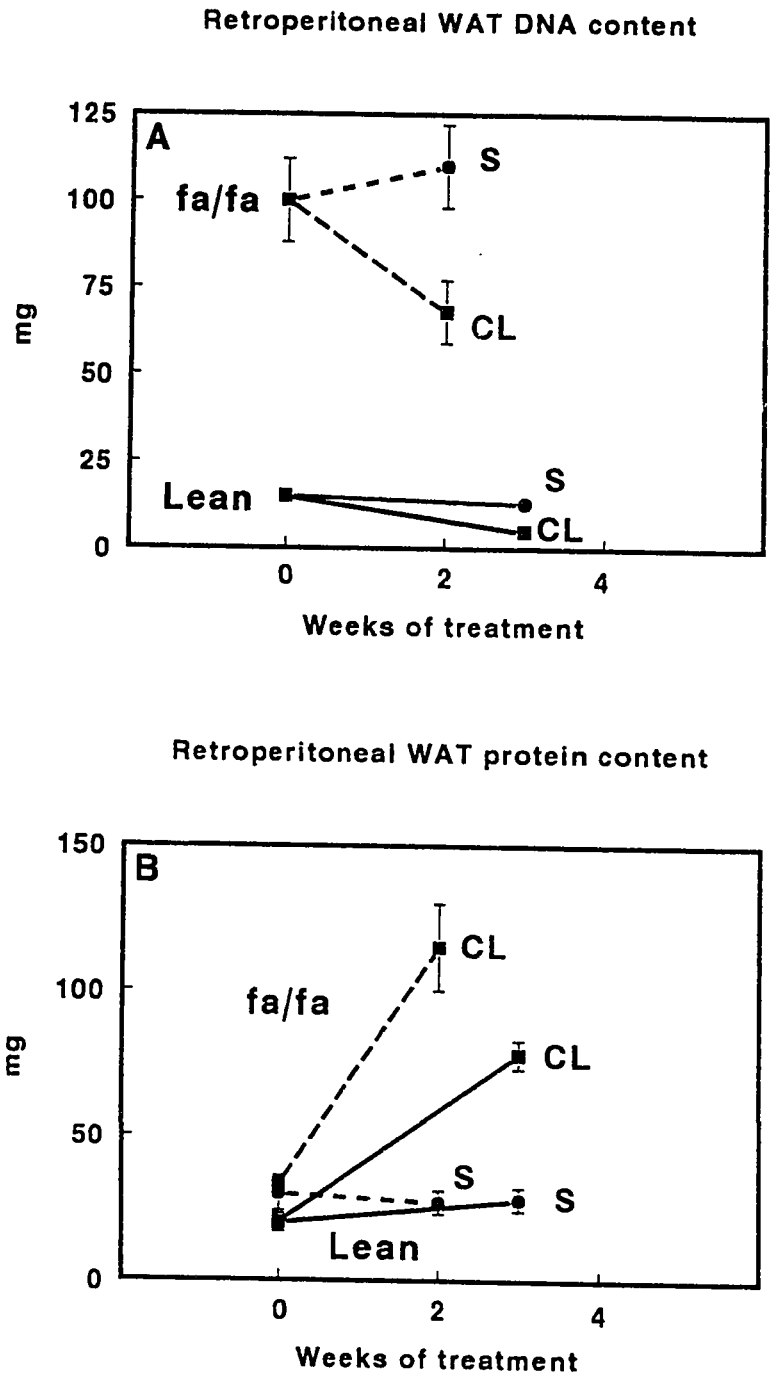


FIGURE 25. DNA and protein contents of RWAT. All values are means ± SEM. Symbols, lines and labels as in figure 22A.

A) DNA content is higher in fa/fa rats than in lean rats ($P < 0.001$). CL-fa/fa at 2 weeks *vs* S-fa/fa at 2 weeks, $P < 0.05$ and *vs* fa/fa at 0 weeks, $P < 0.05$. CL-lean rats at 3 weeks *vs* S-lean rats at 3 weeks, $P < 0.01$ and *vs* lean rats at 0 weeks, $P < 0.001$.

B) Total protein content increased in CL-fa/fa at 2 weeks *vs* S-fa/fa at 2 weeks, $P < 0.001$ and *vs* fa/fa at 0 weeks, $P < 0.001$. CL-lean rats at 3 weeks *vs* S-lean rats at 3 weeks, $P < 0.001$ and *vs* lean rats at 0 weeks, $P < 0.001$.

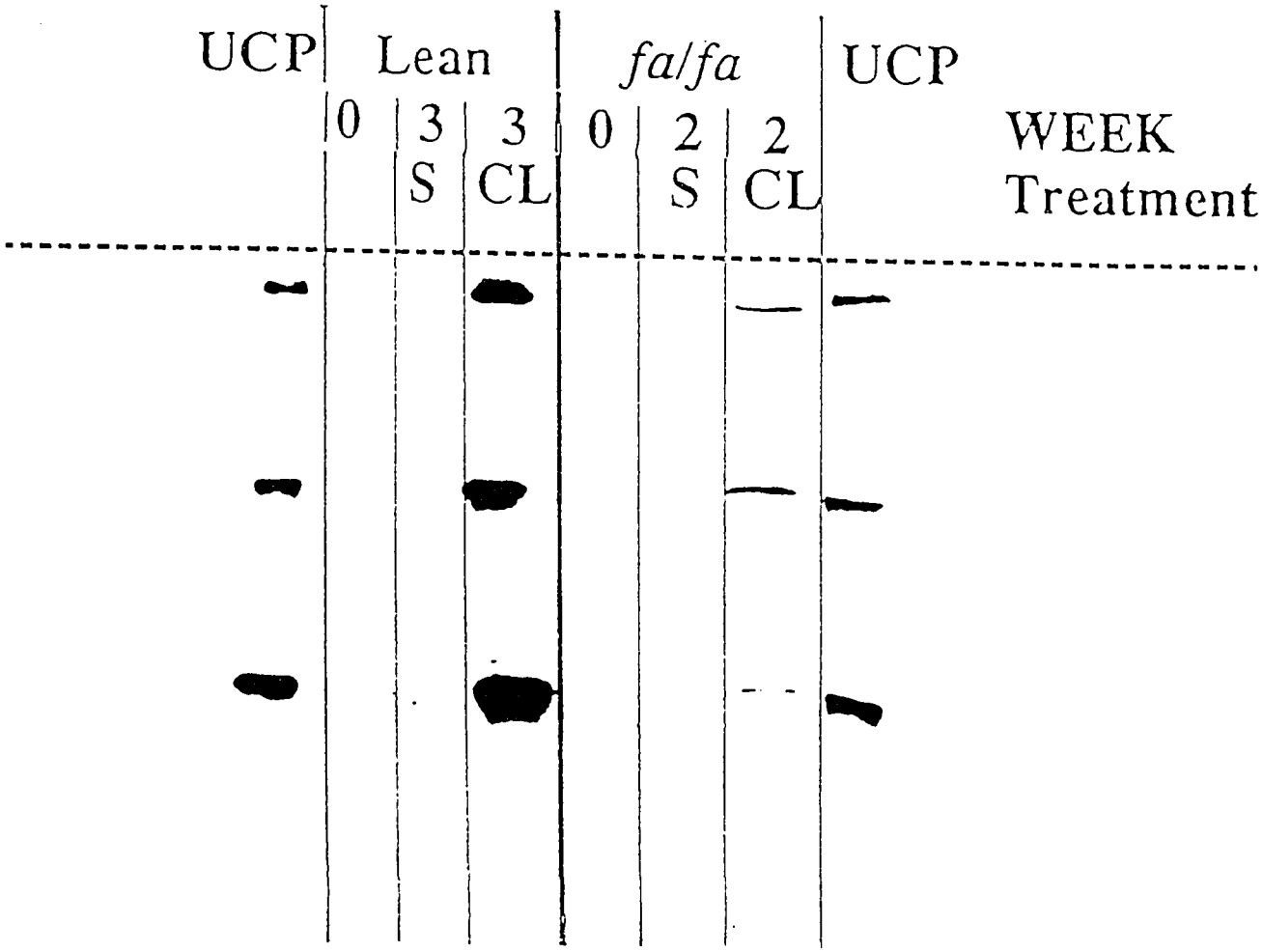


FIGURE 26. Western blots of UCP in RWAT (10 µg protein). Three rats from each group are shown. Rats were treated with either saline (S) or CL (CL). CL-treatment induced the appearance of UCP in WAT, particularly in lean rats.

previously been described (0.6 to 2-3%) (Table 4).

Histology of WAT

Numerous typical large unilocular white adipocytes apparently the predominant predominant cell type in RWAT in both lean and fa/fa rats were seen by routine histology (Figure 27A and C). Histology of RWAT of CL-treated rats showed unilocular white adipocytes that were smaller than those in saline-treated rats and also abundant more densely stained multilocular cells which were smaller than white adipocytes (Figure 27B and D). The presence of UCP in these multilocular cells was demonstrated by using an immunohistochemistry method (Figure 28B and D). UCP was not seen in unilocular white adipocytes (Figure 28A and C) in saline-treated rats. In addition, to demonstrate the presence of brown adipocytes containing UCP in WAT of CL-treated rats, confocal immunofluorescence microscopy was used. Confocal micrographs showed the appearance of abundant multilocular cells with numerous small lipid droplets and expressing UCP in CL-treated lean and fa/fa rats (Figure 29B and D). A faint immunofluorescence was seen in the cytoplasm of unilocular adipocytes of both saline-treated and CL-treated rats (Figure 29A and C). A further study on semi-thin sections with higher magnification of light microscopy showed the multilocular cells contained densely-stained cytoplasm and numerous very small lipid droplets and pale nuclei with a prominent nucleolus, all characteristic

FIGURE 27. Histology of RWAT. Haematoxylin and eosin-stained 10 μm sections of RWAT of (A) S-lean, (B) CL-lean, (C) S-fa/fa and CL-fa/fa rats. Magnification x 400. Note the large number of very small cells, many multilocular, in CL-treated rats (B and D) and the apparent predominant unilocular white adipocytes in saline-treated rats (A and C).

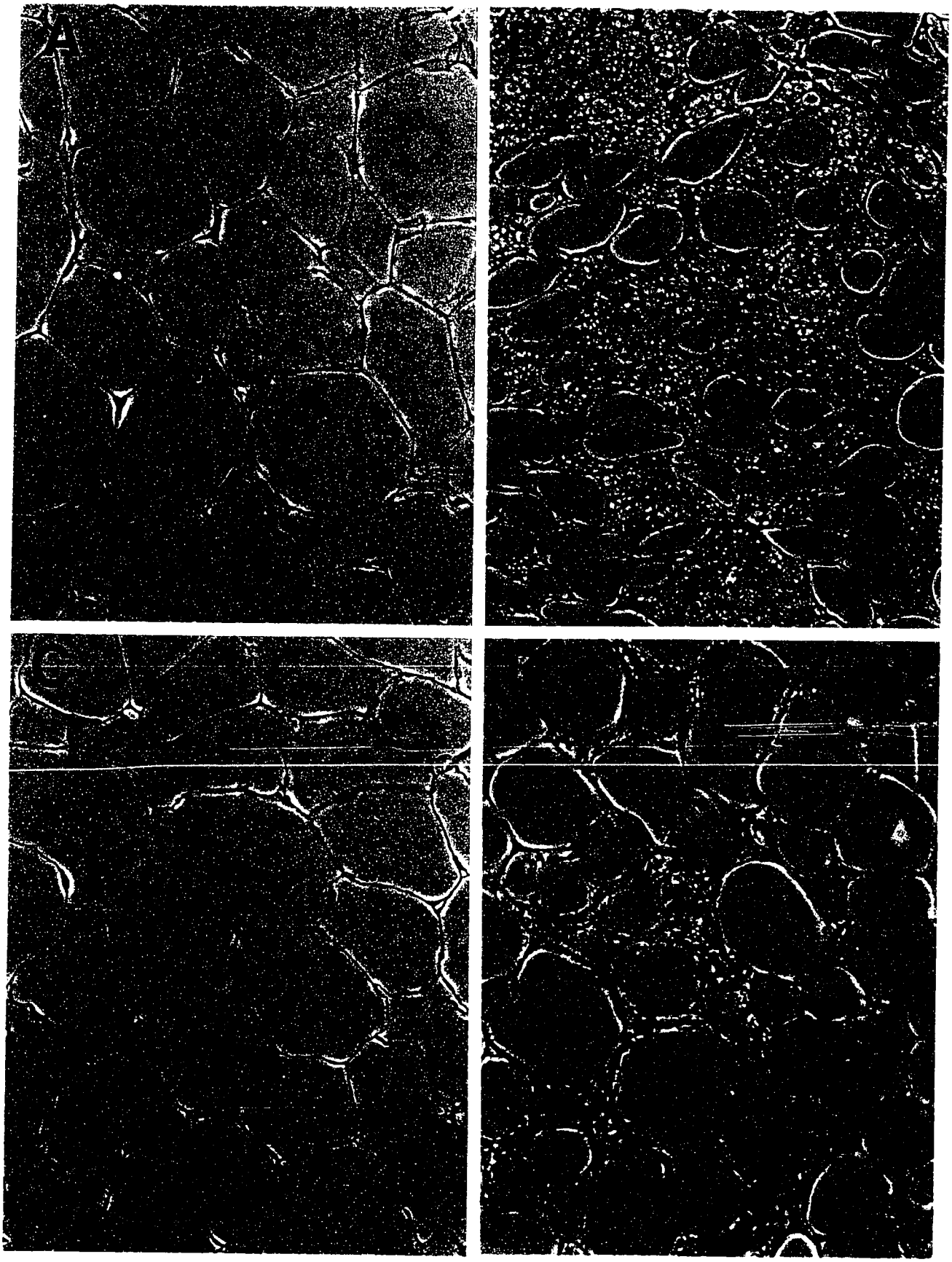
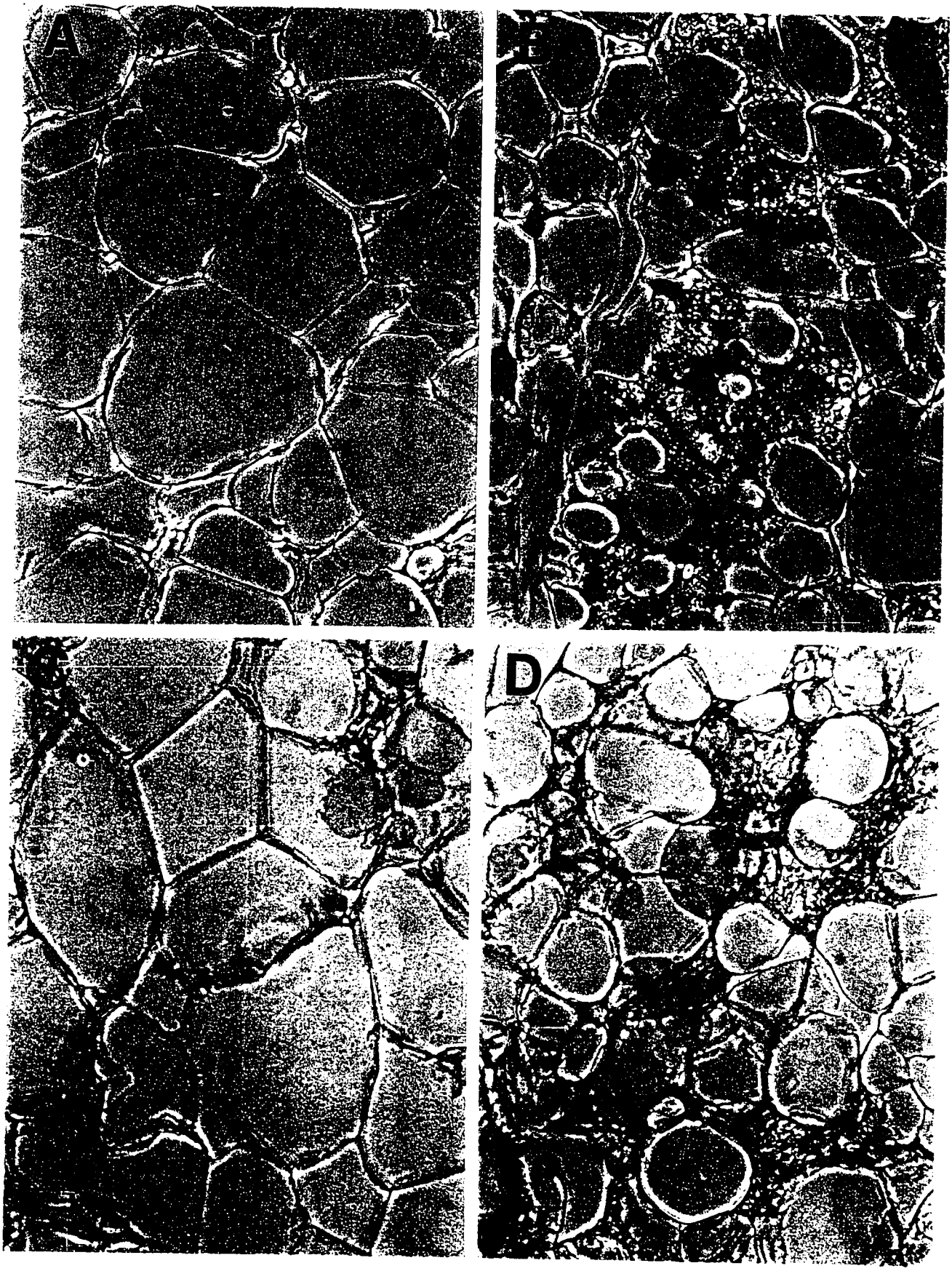

A

FIGURE 28. **Immunohistochemical detection of UCP in RWAT. UCP in 10 μm sections of RWAT.**
(A) S-lean, (B) CL-lean, (C) S-fa/fa and CL-fa/fa rats. Magnification x 400. Multilocular cells in CL-treated rats contain UCP (black stain), hence are defined as brown adipocytes. They are less apparent in the fa/fa rat (D) than in the lean rat (B) but note that the WAT of the fa/fa rat weight more than five times as much as the WAT of the lean rat (Figure 29).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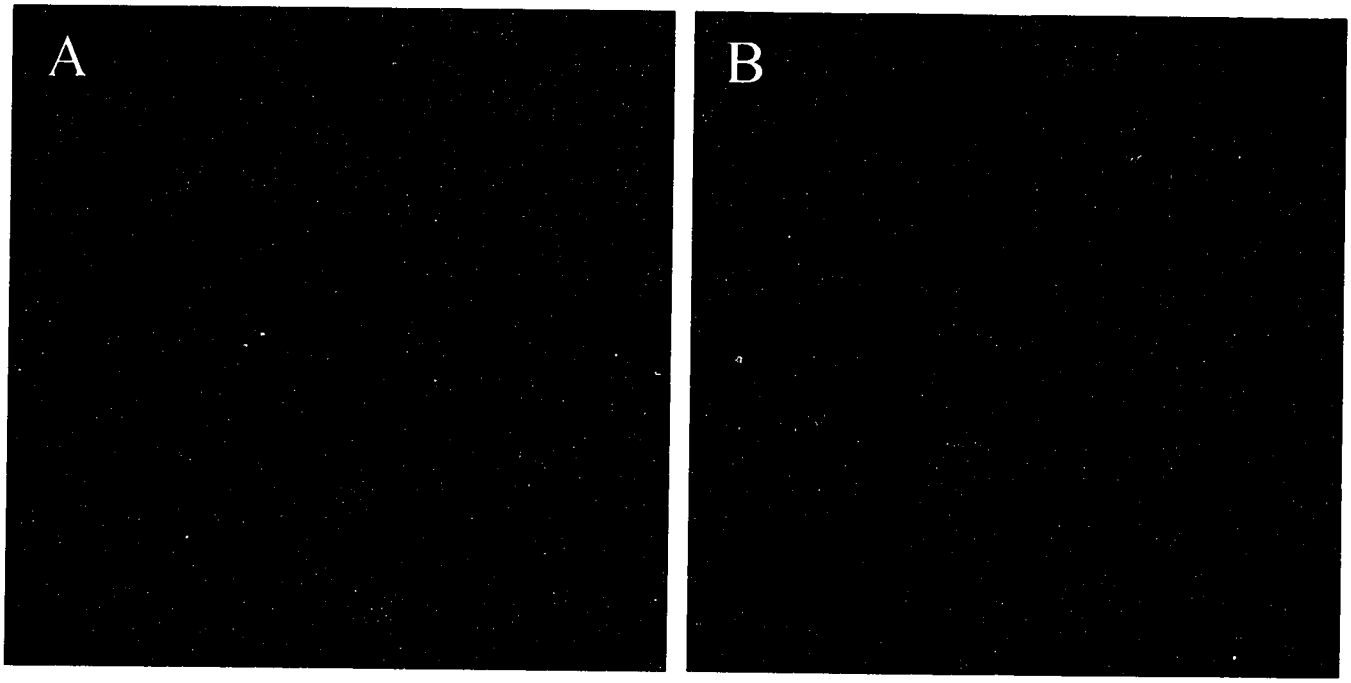
D

FIGURE 29. Confocal micrographs of immunofluorescence detection of UCP in RWAT of lean and fa/fa rats treated with S or CL.

Very thick sections (25-30 µm) were double stained with FITC and propidium iodide and immunodetected by a polyclonal antibody to UCP. The section were viewed by a laser beam passing through 0.5 µm section. Graphs are RWAT sections of (A) S-lean, (B) CL-lean, (C) S-fa/fa, (D) CL-fa/fa rats. Magnification x 800. In the CL-treated lean and fa/fa rats (B and D) apparent multilocular cells with numerous small lipid droplets (shown in black) and abundant immunolabelled UCP stained with FITC (green stain) are documented as brown adipocytes. The nuclei of both brown adipocytes and other cells are visible in red (propidium iodide-stain).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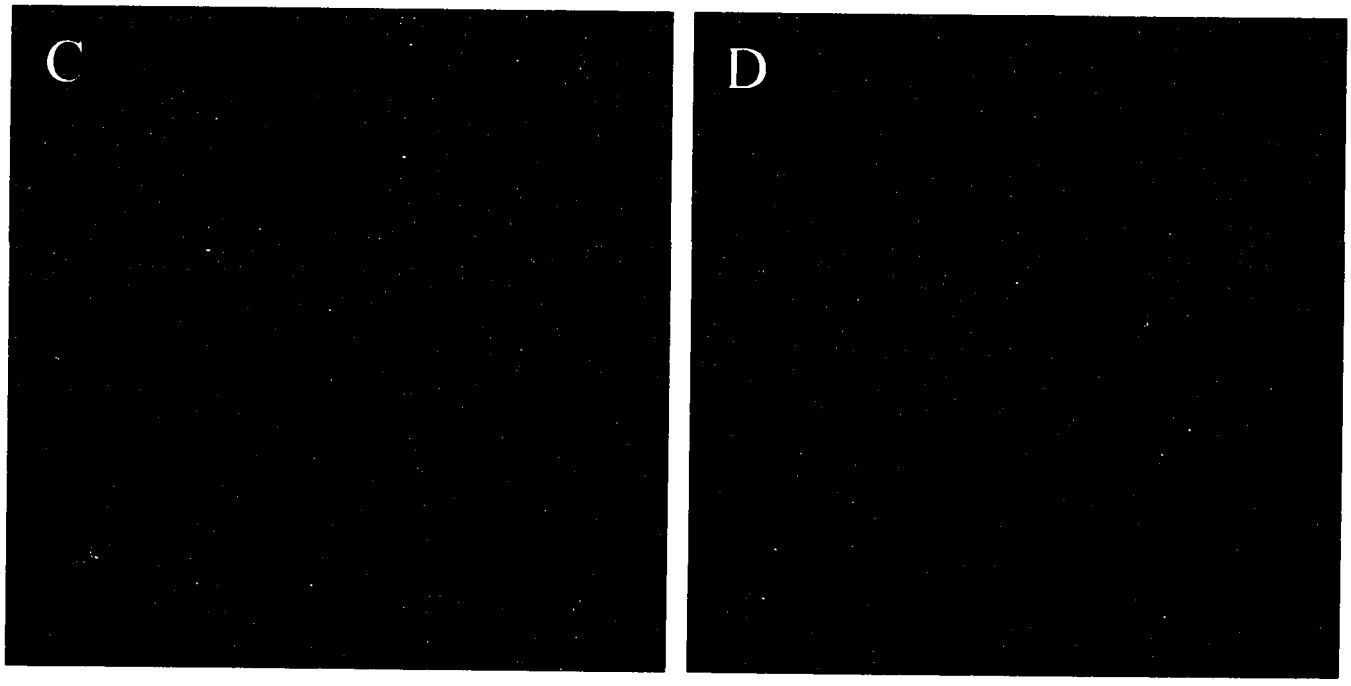
C
D

of brown adipocytes (Figure 30B and D). In RWAT of CL-treated fa/fa rats some mature white adipocytes were surrounded by multilocular cells (Figure 30D). To confirm the low contribution of mature white adipocytes to other cells, 10 μm sections were stained with propidium iodide and viewed by fluorescence microscopy. The appearance of these sections suggests the presence of abundant non-adipocyte nuclei in RWAT (Figure 31A, B, C, and D). The high proportion of non-adipocyte nuclei is more obvious in RWAT of the CL-treated rats, probably because the size of the adipocytes has decreased markedly so that the section contains far more of the non-adipocyte nuclei.

BAT

Both interscapular and perirenal BAT were studied. However, data are presented only for interscapular BAT (IBAT), since the changes in perirenal BAT similar to those in IBAT.

Total protein content and UCP content of BAT were 50% lower in fa/fa rats than in lean rats (Figure 32A and B). Both UCP and total protein content of IBAT markedly increased after treatment with CL in both lean and fa/fa rats (Figure 32A and B). DNA content of IBAT was not altered by CL-treatment in any group (data not shown).

FIGURE 30. Semi-thin sections of RWAT of S- or CL-treated rats. Semi-thin sections (0.5 µm) of RWAT of (A) S-lean, (B) CL-lean, (C) S-fa/fa, and (D) CL-fa/fa were stained with methylene blue and azur blue II. Magnification x 2000. In the CL-treated lean rat the multilocular nature and large palely-stained nuclei with a dense nucleolus characterize these cells further as typical brown adipocytes. Also visible are endothelial cells associated capillaries and triangular-shape interstitial cells. In the fa/fa rat, multilocular cells with very small lipid droplets surrounded unilocular white adipocytes (D).

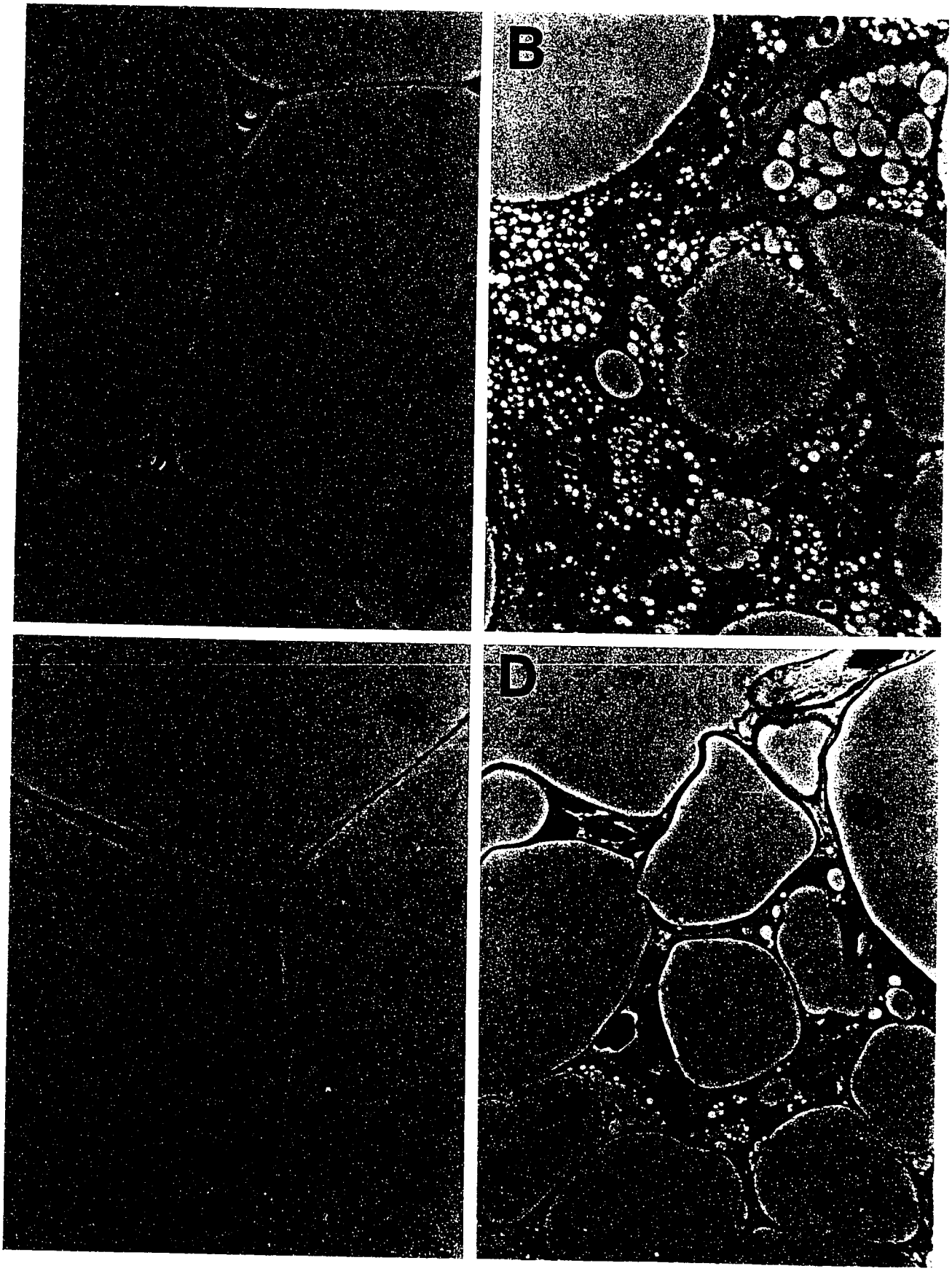
B
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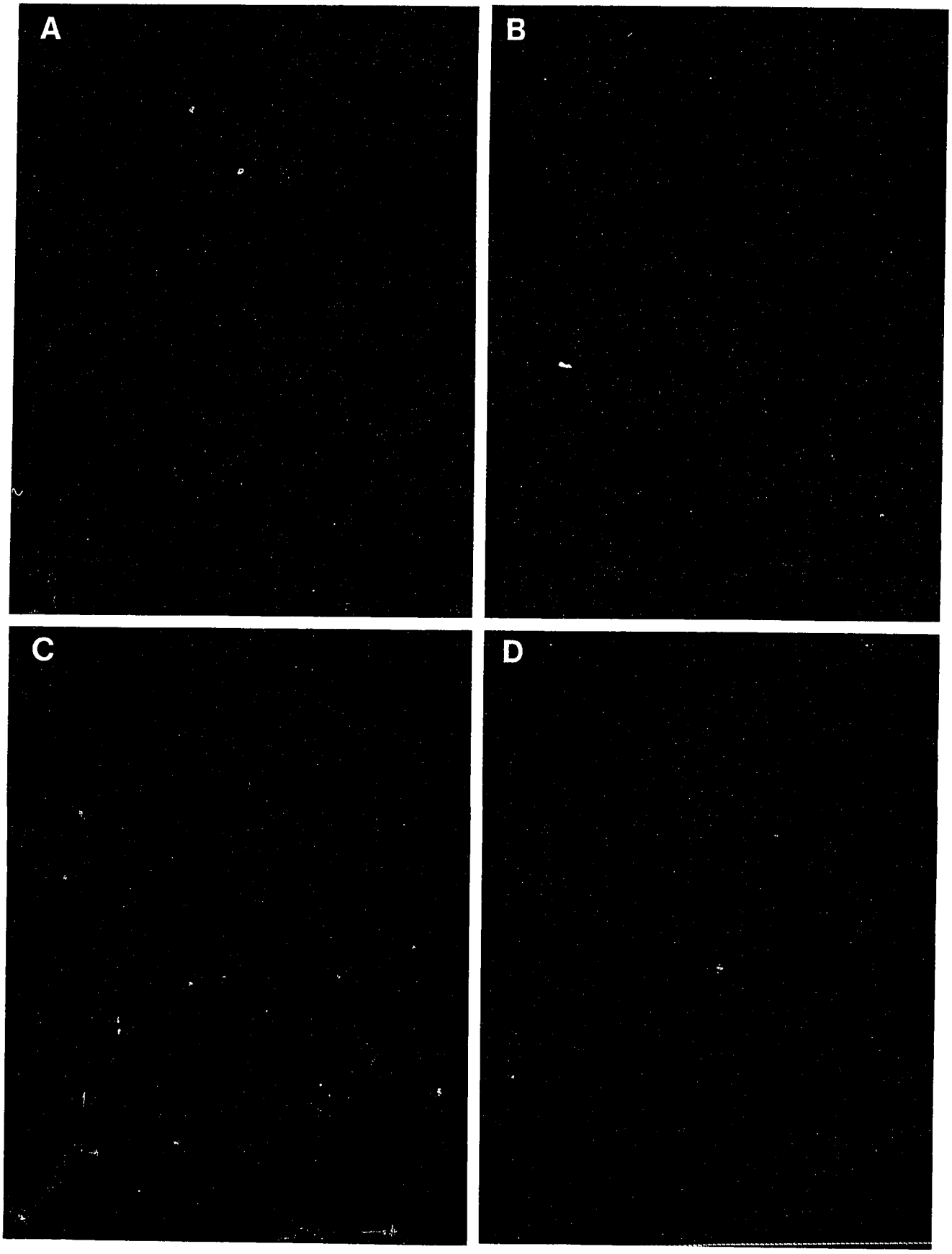
FIGURE 31. Fluorescence micrograph of propidium iodide-stained RWAT. Graph shows 10 μm sections of propidium iodide-stained sections of RWAT of (A) S-lean, (B) CL-lean, (C) S-fa/fa, (D) CL-fa/fa rats. Magnification x 400. The small white spheres are nuclei. White adipocytes appear as large black spheres. Since these are 10 μm sections and the average adipocyte diameter is 80-120 μm, relatively few of the nuclei can be present in adipocytes. The non-adipocyte nuclei are clearly seen in saline-treated rats (A and C). They become much more apparent in CL-treated rats (B and D), probably due to the shrinkage of the white adipocytes in these drug-treated animals. Indeed, they can barely be distinguished from each other in these fairly thick sections. These sections provide visible confirmation that white adipocytes are not the predominant cell type in WAT, when considered as proportion of total cells present (see table 4).


A
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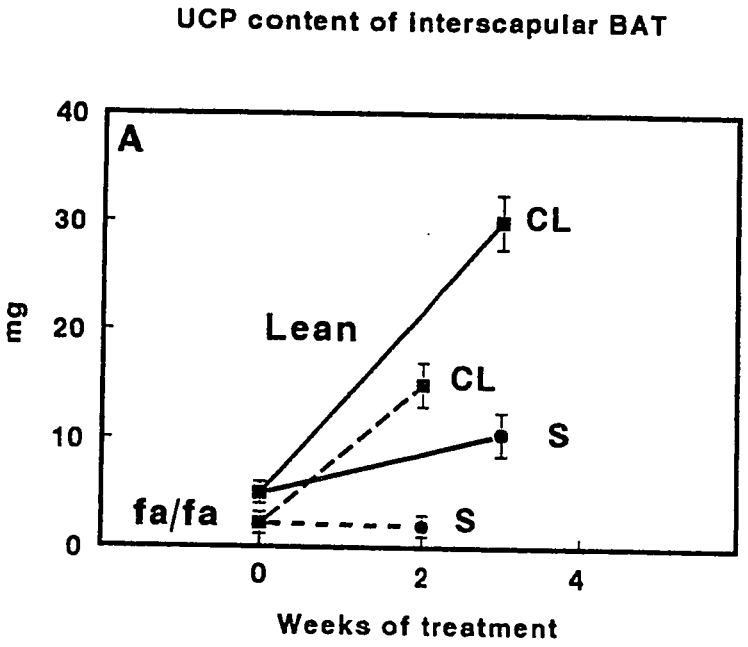


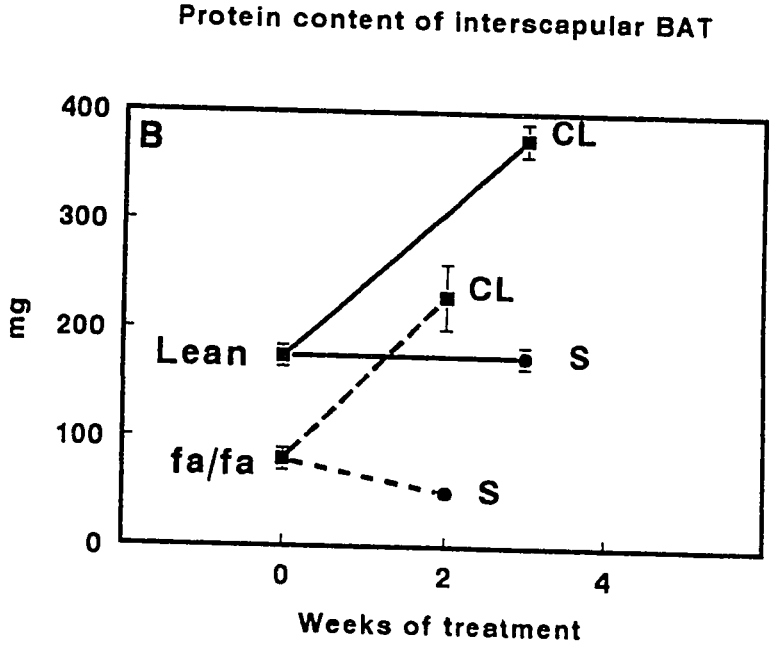


FIGURE 32. UCP and protein content of interscapular BAT. Symbols, lines and labels as in Figure 22A.

A) Panel A shows UCP content. CL-treatment significantly increased UCP in both group of rats during 2 or 3 weeks treatment, $P < 0.001$ for both genotypes. fa/fa rats at 0 weeks *vs* lean rats at 0 weeks, $P < 0.005$, and at 2-3 weeks, $P < 0.001$.

B) Shows total protein content. CL-treatment significantly increased protein content in both group of rats during 2 or 3 weeks treatment, $P < 0.001$ for both genotypes. fa/fa rats 0 weeks *vs* lean at 0 weeks, $P < 0.001$, and at 2-3 weeks, $P < 0.001$.

Discussion

In this experiment, the fa/fa rats seemed to be much more responsive to CL 316,243-treatment than the lean rats. They showed a very marked increase in energy expenditure of 69%, and a suppression of the hyperphagia to a normal level during the treatment with CL. In contrast, treatment did not have any effect on food intake in lean rats and induced a smaller increase in energy expenditure than in fa/fa rats.

CL-treatment also induced a marked decrease in white adipocyte size in both fa/fa and lean rats (Figure 24A). In genetically obese fa/fa rats the hyperplastic obesity is obvious and compared to the lean rats the number of white adipocytes is much higher. CL-treatment, however, did not have any effect on the increased number of adipocytes (Figure 24B). It was unable to reverse the hyperplasia of white adipocytes. Appearance of UCP in multilocular cells in what has been traditionally regarded as WAT was another result of CL-treatment. Their appearance was accompanied by a large increase in total protein content of the WAT, presumably present mainly in mitochondria in these cells and occurring despite a decrease in the total number of cells (as assessed by the DNA content), an unchanged number of smaller mature white adipocytes and a decrease in tissue weight. The nature of the immunofluorescence detected by UCP antiserum in unilocular white adipocytes is discussed further in chapter VI.

BAT

An increase in energy expenditure induced by a β3-AR agonist is likely to be occurring in brown adipocytes. The substantial increase in energy expenditure induced by the CL-treatment was associated with alterations in two distinct populations of brown adipocytes. First, there was expansion of the mitochondrial compartment in brown adipocytes in BAT. In our previous study we showed that there was no change in the relative proportions of different cell types in BAT of CL-treated rats (Himms-Hagen et al., 1994).

The DNA content of the BAT was not changed by CL-treatment, as also seen in our previous study (Himms-Hagen et al., 1994, and chapter V part A), probably because the precursor interstitial cells possess only β1-ARs (Bronnikov et al., 1992) so that the mitogenic effect of NA, seen for example during cold-acclimation (Géloën et al., 1990), does not occur with selective β3-adrenergic stimulation.

WAT

Comparison of the number of mature white adipocytes as assessed by the osmium-fixation method with the total number of cells in WAT as assessed from DNA content suggests that the proportion of white adipocytes in retroperitoneal WAT is lower than generally thought. Such a low proportion white adipocytes has also been reported by other investigators (Cleary et al., 1979). The appearance of abundant stained nuclei in the 10 μm sections stained with propidium iodide strengthens this

conclusion (Figure 31). The average diameter of white adipocytes is 80-120 μm which means that relatively few of their nuclei could be present in a 10 μm section; most of the nuclei seen in Figure 31 must be non-adipocyte nuclei.

It is interesting to notice that the cells which disappeared with CL-treatment were not mature white adipocytes, because the number of these cells was unchanged. It is also noticeable that the very marked elevation in total cell number in WAT in the obese fa/fa rats was reduced by CL-treatment. The reduction of total number of cells was associated with the appearance of brown adipocytes in the tissue while the number of white adipocytes remained unchanged. Other reports have also demonstrated a decrease in DNA content of WAT in long-term food deprived rats (Miller et al., 1983) and in streptozocin-diabetic rats (Géloën et al., 1989a). The decrease in DNA content in the food deprived rats was not accompanied by any loss of white adipocytes (Miller et al., 1983). A possible explanation for the nature of the cells which disappeared from WAT of both genetically obese (fa/fa) rats and DIO rats after treatment with CL is offered here. They are suggested to be vascular cells, recruited to support the increased circulation of blood in the expanded mass of the WAT. Under the CL-treatment, white adipocytes start shrinking and the mass decreases which results in a lower need for substrates and energy. It has been shown that WAT has a very rich blood circulation and the angiogenic properties of WAT during the development of obesity and growth of adipose tissue have long been recognized (Silverman et al., 1988). Secretion of at least one angiogenic substance or

precursor is known to be increased in obesity. Thus, angiotensinogen production is increased in obesity and is under nutritional regulation (Cassis, 1994; Frederich et al., 1992). While there is some information about the role of angiogenesis in altered blood flow in WAT in obesity (Crandall et al., 1994) mechanisms involved in the disappearance of the expanded vasculature of WAT when its fat content has been reduced appear to be unknown.

PART C: THE EFFECT OF CL 316,243 ON THE EXPRESSION OF TUMOUR NECROSIS FACTOR (TNF-α):

Background

TNF-α is a cytokine expressed by both macrophages and adipocytes. TNF-α is known to induce insulin resistance associated with hyperinsulinemia by impairment of insulin action on peripheral glucose uptake and hepatic glucose output (Lang et al., 1992). This impairment of insulin action is suggested to occur because of phosphorylation of insulin receptors and IRS-1 by TNF-α (Hotamisligil et al., 1994b).

In the previous studies we demonstrated that treatment with CL 316,243, a highly selective agonist for β3-ARs partially reversed obesity in fa/fa Zucker rats. Other studies have previously shown that CL-treatment improves insulin-resistance in KKA mice (Yoshida et al., 1994; Largis et al., 1994).

Objective

Since the role of TNF-α in inducing insulin-resistance in obese animals is now well-known (Hotamisligil et al., 1993, 1994a; Spiegelman and Hotamisligil, 1993) we hypothesised that CL-induced improvement in insulin-resistance might be associated with the suppression of TNF-α mRNA expression in adipose tissue. To test this hypothesis, two groups of rats (fa/fa and lean Zucker rats) treated with either CL

316,243 or saline (the same rats which were used in the previous experiment) (Chapter V part B) were used to demonstrate the effect of CL 316,243 on the expression of TNF-α mRNA as well as blood glucose and serum insulin levels.

Results

Before treatment, serum insulin levels were significantly higher in obese and insulin-resistant fa/fa rats than in lean rats (Figure 33A). Treatment with CL significantly decreased the serum insulin level in these animals (Figure 33A). Blood glucose level was also slightly higher in obese animals than lean rats (Figure 33B). After treatment with CL, the level of blood glucose was significantly decreased in fa/fa rats (Figure 33B). These results showed that treatment with CL 316,243 improved insulin-resistance in fa/fa rats, allowing normoglycemia at a normal concentration of insulin in the blood. The average fat content of RWAT and EWAT depots was higher in fa/fa rats than in lean control animals (Figure 22A and Table 4). CL 316,243-treatment decreased the fat content of these abdominal depots in both lean and fa/fa rats. The level of TNF-α mRNA expression in RWAT was measured in both groups of animals. The expression of TNF-α mRNA in fa/fa rats was not significantly different with the lean rats. However, treatment with CL 316,243 significantly decreased the expression of TNF-α in WAT of obese Zucker rats (Figure 34A and B).

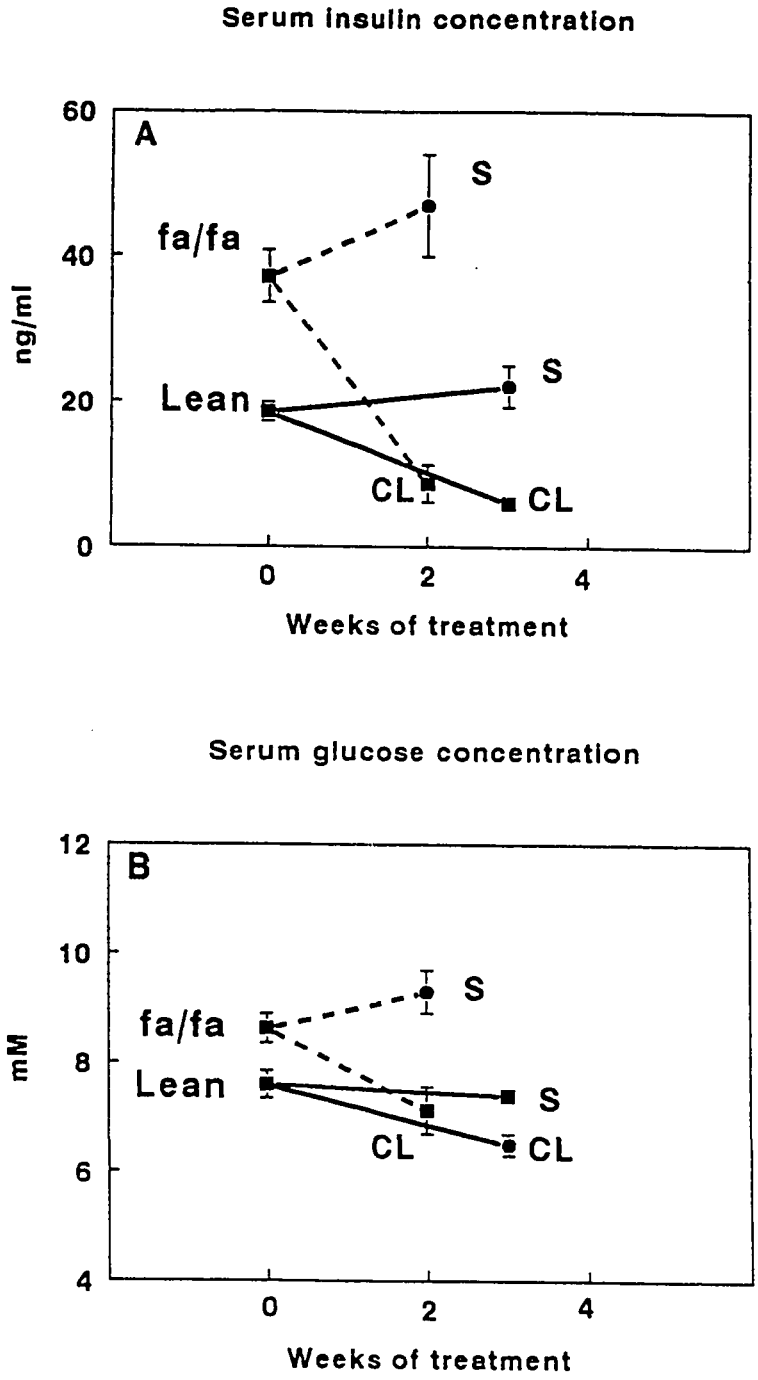


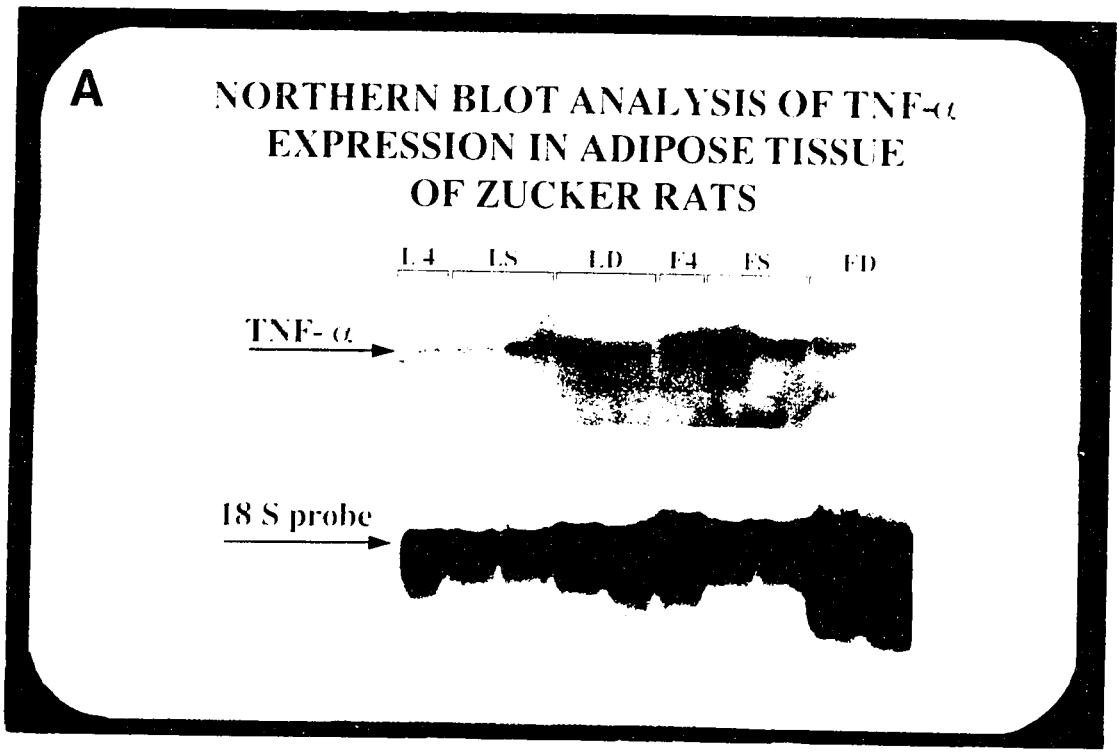
FIGURE 33. Serum glucose and insulin concentration. Symbols, lines and labels as in Figure 22A.

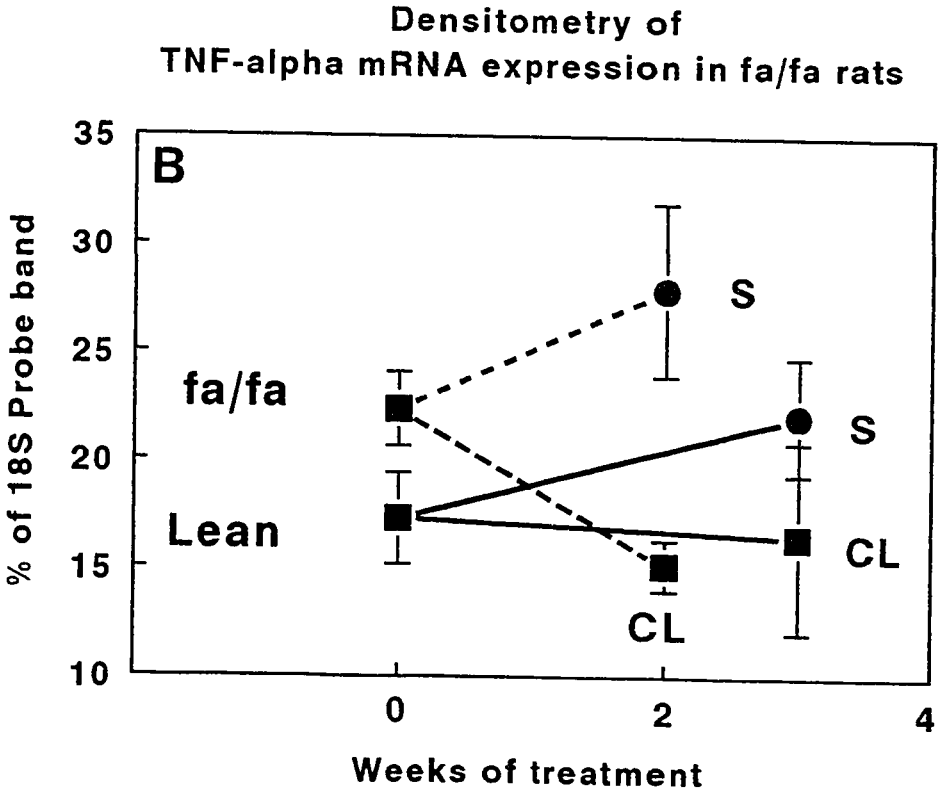
A) shows serum insulin concentration. CL-fa/fa at 2 weeks *vs* S-fa/fa at 2 weeks and fa/fa at 0 weeks, $P < 0.001$. CL-lean at 3 weeks *vs* S-lean at 3 weeks and *vs* lean at 0 weeks, $P < 0.05$. Insulin is higher in fa/fa rats than in lean rats at 0 weeks and in S-fa/fa than in S-lean at 2-3 weeks, $P < 0.001$. There is no significant difference between CL-fa/fa and CL-lean rats at 2-3 weeks.

B) shows serum glucose concentration. CL-fa/fa at 2 weeks *vs* S-fa/fa at 2 weeks, $P < 0.001$, and *vs* fa/fa at 0 weeks, $P < 0.05$. CL-lean at 3 weeks *vs* S-lean at 3 weeks and *vs* lean at 0 weeks, N.S. Glucose is higher in fa/fa than lean at 0 weeks, $P < 0.05$, and in S-fa/fa than in S-lean at 2-3 weeks, $P < 0.001$. There is no significant difference between CL-fa/fa and CL-lean at 2-3 weeks.

FIGURE 34. Northern blot analysis of the expression of TNF-α mRNA in the RWAT of lean and fa/fa rats.

A) shows northern hybridizations of TNF-α mRNA and 18S ribosomal RNA (as control) . Total RNA from retroperitoneal WAT was extracted by Chomczynski method (Chomczynski 1993). Total RNA (~20 µg) was denatured and separated on formaldehyde gel. Lane 1, lean-pretreatment; lanes 2 and 3, S-lean; lanes 4 and 5, CL-lean; lane 6, fa/fa-pretreatment; lanes 7 and 8, S-fa/fa and lanes 9 and 10, CL-fa/fa rats. The 18S ribosomal probe is shown as a control for the loading and integrity of the RNA. B) Densitometry of the TNF-α mRNA bands as compared to 18S bands in the same lane. All values are means ± SEM. Symbols, lines and labels as in figure 22A. CL-fa/fa at 2 weeks *vs* S-fa/fa at 2 weeks, $P < 0.001$. CL-fa/fa at 2 weeks *vs* S-lean at 3 weeks, $P < 0.05$. CL-lean at 3 weeks *vs* saline-fa/fa at 2 weeks, $P < 0.001$. CL-lean at 3 weeks *vs* S-lean at 3 weeks and *vs* S-lean at week 0, N.S. There is no significant difference between CL-fa/fa and CL-lean rats at 2-3 weeks.


A
NORTHERN BLOT ANALYSIS OF TNF-α
EXPRESSION IN ADIPOSE TISSUE
OF ZUCKER RATS
L4
LS
LD
F4
FS
FD
TNF-α
18 S probe


Densitometry of
TNF-alpha mRNA expression in fa/fa rats
B
% of 18S Probe band
35
30
25
20
15
10
fa/fa
Lean
S
S
CL
CL
0
2
4
Weeks of treatment

Discussion

Elevated TNF-α mRNA expression in WAT usually correlates with massive obesity and insulin resistance (Hotamisligil et al., 1993; Spiegelman et al., 1993; Hofmann et al., 1994). Elevated TNF-α mRNA expression has been shown in ob/ob, tub/tub, and KKA mice , and Zucker fa/fa rats (Hotamisligil et al., 1993), as well as transgenic model of obesity/insulin resistance created by ablation of BAT via a bacterial toxin gene driven by the UCP promoter (Lowell et al., 1994; Hotamisligil et al., 1994b). It is possible that adipose tissue is able to generate mediators that influence the activity of insulin in various target tissues (Spiegelman, 1993).

In this study we show that treatment of fatty Zucker rats with CL 316,243 improves both obesity and insulin resistance. In this experiment we have demonstrated the correlation of expression of TNF-α in RWAT and insulin-resistance in genetically obese fa/fa rats, and that improvement of NIDDM in these animals by treatment with CL 316,243 is linked with the suppression of TNF-α mRNA expression in WAT. These results confirm the association between TNF-α expression and the insulin-resistant condition in rats as shown by other investigators. Taken together, our finding indicated that the hyperglycaemia and hyperinsulinemia induced by insulin-resistance correlated positively with the expression of TNF-α mRNA in an abdominal WAT depot. Others have shown that chronic infusion of CL in lean and obese diabetic rats improves glucose tolerance and insulin resistance in obese-diabetic rats associated with a marked increase in glucose uptake in BAT and

WAT (D'Allaire et al., 1996). Thus CL not only possesses anti-obesity effects, but also has anti-diabetic effects and consequently may be useful for treating obesity as well as NIDDM in obese animals.

PART D: EFFECT OF CL 316,243 ON SERUM LEPTIN LEVEL IN RATS WITH DIET- OR AGING-ASSOCIATED OBESITY AND IN ZUCKER RATS WITH GENETIC (fa/fa) OBESITY.

Background

As it was mentioned in the general objective, the current section (part D) is a complementary experiment to measure the serum leptin concentration of rats in the experiments described in part A and part B of this chapter, already completed before leptin was discovered.

In previous sections (chapter V part A and B) chronic treatment with CL 316,243 was shown to have an anti-obesity effect associated with shrinking of white adipocytes and with reversal of the hyperphagia of fa/fa rats. No effect of CL-treatment was observed on normal food intake in Sprague-Dawley rats or in older lean Zucker rats. In both experiments, the increase in energy expenditure induced by CL was not compensated for by any increase in energy intake.

It is known that treatment with a β3-AR agonist suppresses leptin expression in WAT and decreases the concentration of leptin in blood (Gettys et al., 1996; Giacobino, 1996; Mantzoros et al., 1996; Collins and Surwit, 1996; Trayhurn et al., 1996). The failure of energy intake to rise to meet expenditure must therefore be independent of any decrease in leptin concentration (Mantzoros et al., 1996), which

would itself be expected to increase intake. It seems likely that the extra heat production induced by CL was responsible for suppressing energy intake (Himms-Hagen, 1995).

Objective

The objective of the present study was to assess the effect of chronic β3-AR stimulation by CL on leptin concentration in blood of obese and lean rats. The animals, procedures and other data are described in previous sections (chapter V part A and B).

Results and discussion

The serum leptin concentration in DIO rats was more than 3 times that in lean rats and was reduced to normal by the CL-treatment (Figure 35A). Serum leptin level decreased in obese fa/fa Zucker rats was 3 times higher than that in old lean Zucker rats and almost 10 times higher than that in younger lean Sprague-Dawley rats (Figure 35A and B). The elevated level in fa/fa rats was not reduced by the CL-treatment whereas the elevated serum leptin concentration in the old lean Zucker rats was reduced by treatment. In DIO rats, the number of adipocytes remained unchanged during the period of eating a high-fat diet, whereas the size of adipocytes increased significantly to the level of that in fa/fa rats by high-fat diet feeding (Table 5). In

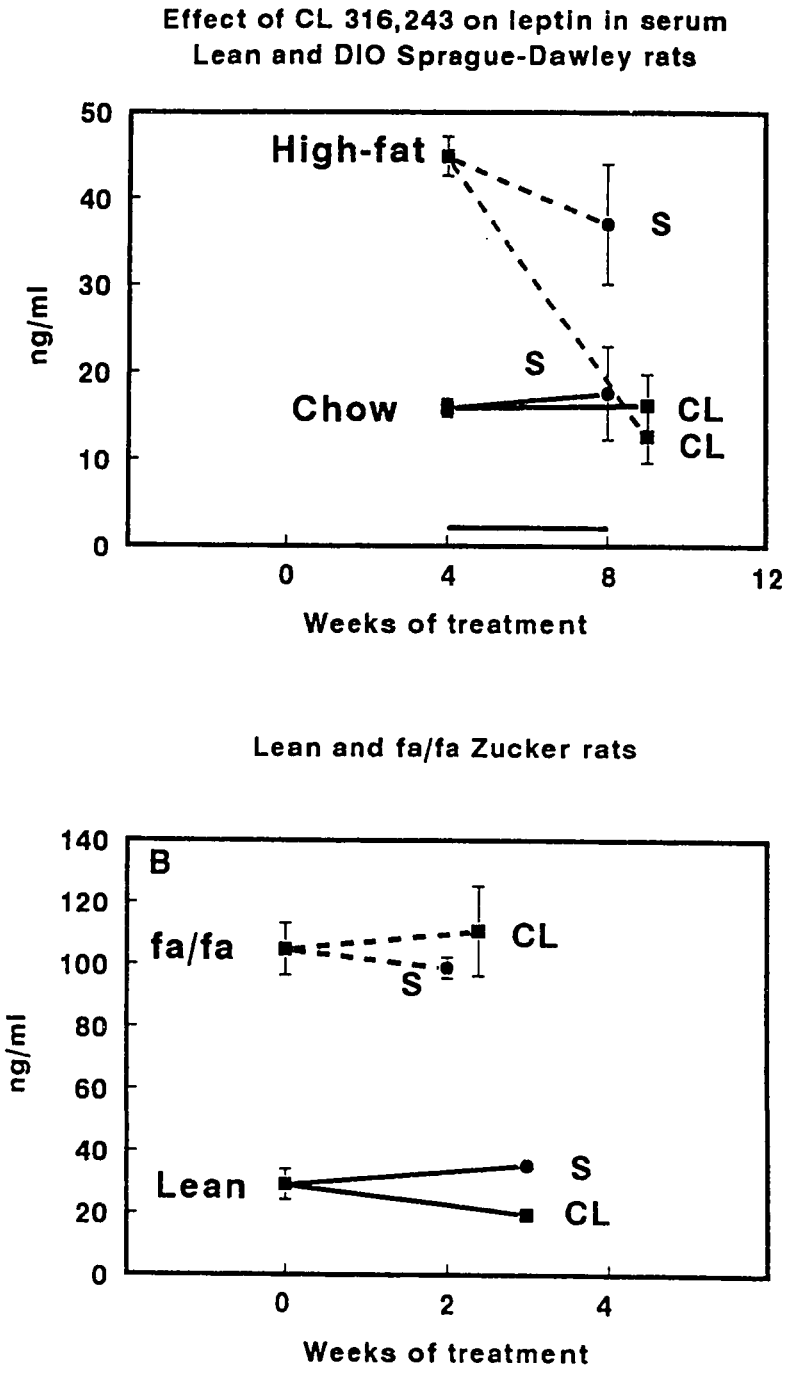


FIGURE 35. Effect of S- or CL-treatment on serum leptin concentration. All values are means ± SEM. Symbols, lines and labels as in figure 22A.
A) Serum leptin in DIO rats. Leptin concentration is higher in high-fat diet rats than in chow-fed rats, $P < 0.001$. CL-high-fat *vs* S-high-fat and *vs* high-fat, $P < 0.001$. CL-chow *vs* S-chow and *vs* chow, N.S.
B) Serum leptin in fa/fa rats. Leptin concentration is higher in fa/fa rats than in lean rats ($P < 0.001$). CL-lean at 2 weeks *vs* S-lean at 2 weeks, and *vs* lean at 0 week, $P < 0.001$. CL-fa/fa at 2 weeks *vs* S-fa/fa at 2 weeks and *vs* fa/fa at 0 weeks, N.S.

Table 5

Summarized data for adipocyte size and number for both DIO and genetically obese, fa/fa, rats

	Cell number	Cell size (μg lipid per cell)
DIO rats		
S-chow	$1.3 \times 10^7 \pm 0.13 \times 10^7$	0.35 ± 0.043
CL-chow	$1.0 \times 10^7 \pm 0.1 \times 10^7$	$0.22^* \pm 0.016$
S-high-fat	$1.4 \times 10^7 \pm 0.15 \times 10^7$	$0.63^{\S} \pm 0.06$
CL-high-fat	$1.5 \times 10^7 \pm 0.24 \times 10^7$	$0.30^* \pm 0.013$
Genetically obese (fa/fa) rats		
S-lean	$1.2 \times 10^7 \pm 0.15 \times 10^7$	0.52 ± 0.04
CL-lean	$1.6 \times 10^7 \pm 0.23 \times 10^7$	$0.15^* \pm 0.01$
S-fa/fa	$9.4 \times 10^{7\dagger} \pm 1.3 \times 10^7$	0.56 ± 0.05
CL-fa/fa	$6.3 \times 10^{7\ddagger} \pm 0.13 \times 10^7$	$0.42^* \pm 0.025$

Values are means ±SEM (n=8 except CL-fa/fa for which the number is 4). Symbols indicate: * significant effect of CL, compared with saline-treated rats of same genotype or diet; † significant difference compared with S-high-fat in DIO group and with S-lean in the same group; ‡ significant difference compared with S-lean in the same group. § significant difference compared with S-chow in the same group. Table 5 compare data from Figure 11 and Figure 24 to allow direct comparison of control lean rats in two experiments.

Parallel to the increase of adipocyte size of DIO rats, serum leptin level was also increased (Figure 35A). The larger size of the adipocytes in the old lean Zucker rats also correlated with a higher concentration of serum leptin. There was no difference between the size of adipocytes in lean and fa/fa Zucker rats, presumably because of aging-associated hypertrophy of adipocytes in lean Zucker rats. However, the adipocyte number was more than 7-fold greater in fa/fa rats as compared with lean rats (Table 5). In parallel to the greater adipocyte number, the serum leptin also was 3-fold greater (Figure 35B).

CL-treatment induced a significant decrease of adipocyte size in both high-fat diet and young chow-fed rats as it was accompanied by a marked decrease in serum leptin level in high-fat diet rats, while there was no significant difference in CL-treated chow-fed rats as compared with saline-treated rats in the same group (Figure 35A). CL-treatment also decreased adipocyte size and reduced serum leptin concentration in lean old Zucker rats. In genetically obese rats, despite the reduction in adipocyte size (Table 5), CL-treatment did not have any effect on serum leptin level in fa/fa rats (Figure 35B). In spite of the unchanged serum leptin level in fa/fa rats, CL-treatment decreased the food intake of these hyperphagic animals (Figure 23A).

Others have shown that β3-AR stimulation suppresses the expression of leptin in WAT in normal rats (Collins and Surwit, 1996; Trayhurn et al., 1996; Gettys et al., 1996; Giacobino et al., 1996; Mantzoros et al., 1996). The decrease in serum leptin

concentration seen in the present study was associated with the shrinking of white adipocytes in two abdominal depots in young DIO rats (Figure 35A) and old lean Zucker rats (Figure 35B) treated with CL. The inability of CL-treatment to reduce serum leptin level in fa/fa rats could be due to leptin production by other fat depots, such as extra-abdominal, mainly subcutaneous, WAT which remain enlarged in CL-treated fa/fa rats (Figure 22). It has been shown that the elevated leptin mRNA level in subcutaneous (inguinal) WAT of fa/fa rats is not suppressed when hyperinsulinemia is normalized by 3 days of fasting (Cusin et al., 1995). Another possible suggestion for the lack of change in leptin concentration in CL-treated fa/fa rats is based on the defect in all forms of leptin receptors in these animals (Chua et al., 1996; Phillips et al., 1996). Since leptin is rapidly concentrated in the kidney and excreted in urine (Van Heek et al., 1996; Cumin et al., 1996), and leptin receptors are expressed in kidney (Hoggard et al., 1997; Wang et al., 1996), including a form known to be involved in leptin transport (Wu-Peng et al., 1997), it may be that leptin excretion is impaired in the fa/fa rat. This impairment might contribute to maintenance of the very high level of leptin in blood despite the reversal of both obesity and diabetes in CL-treated fa/fa rats.

Results showed a dissociation of changes in leptin levels and changes in food intake. Thus, leptin level decreased in both DIO rats and old lean rats when they were CL-treated but food intake did not change in either. If leptin acts as a satiety factor, we might have expected a compensatory increase in food intake in these animals to

balance their increased energy expenditure. Moreover, leptin level did not decrease in CL-treated fa/fa rats while their elevated food intake was decreased by the treatment. The genetically obese, fa/fa, rat, presumed to be hyperphagic because of lack of central response to its own elevated leptin levels (Chua et al., 1996; Cousin et al., 1996; Moinat et al., 1995). One may raise this question that why there is a marked decrease in food intake in fa/fa Zucker rats treated with CL without any alteration in leptin level.

It is likely that the CL-treatment increases thermogenesis by BAT, thus leading to a decrease in food intake due to the large increase in heat production in the fa/fa rats (Figure 23B). On the other hand, in lean Zucker rats and in DIO rats, the serum leptin was reduced significantly by the CL-treatment while there was no effect on food intake. A plausible explanation for this phenomenon is that the tendency to increase food intake because of reduction of leptin production is inhibited by the increase of heat production by BAT (Himms-Hagen 1996). Therefore, the overall effect would be unaltered food intake, as was seen in these animals, and prevention of any compensatory increase to meet the increased energy expenditure.

CHAPTER VI

DISCUSSION

A. GENERAL DISCUSSION AND CONCLUSION

(a) Summary of major findings

Treatment with CL of rats with diet-induced obesity or genetic obesity is here shown to reverse the obesity, bringing about shrinking of mature white adipocytes but no reduction in number of these cells, even when their number is elevated, as in the fa/fa rat.

Reversal of obesity was associated with a large increase in energy expenditure but no change in food intake, except in the hyperphagic fa/fa rat in which intake was reduced to a normal level. The increase in energy expenditure was associated with hypertrophy of BAT and also, remarkably, with appearance of brown adipocytes in WAT. Extensive remodelling of WAT appeared to be induced by the treatment.

Improvement of the insulin-resistant state of the fa/fa rat was associated with reduced expression of TNF-α in WAT; TNF-α is a putative mediator of insulin resistance in obesity.

The level of leptin in serum was correlated with the degree of obesity, being low in young lean rats, somewhat elevated in moderately obese rats with DIO or aging-associated obesity and markedly elevated in the grossly obese fa/fa rats. Drug-treatment reduced leptin level only in the moderately obese rats. There was no

correlation between leptin level or change in leptin level and food intake. These finding have recently been published (Ghorbani and Himms-Hagen, 1997a, b; Ghorbani et al., 1997).

These major findings are further discussed in relation to more recent findings in the literature in parts (b) and (c) below. Possible directions for further research are outlined in part B of this chapter.

(b) Reversal of obesity and insulin-resistance

Treatment of rats with CL increased thermogenesis, growth of BAT, and shrinking of white adipocytes through acting on β3-ARs. Although fa/fa rats have down-regulated β3-ARs in their BAT (Muzzin et al., 1991; Onai et al., 1995) and appear to regulate these receptors differently from lean rats (Onai et al., 1995) and although treatment with a β3-AR agonist itself down-regulates β3-ARs (Revelli et al., 1992), fa/fa rats exhibit a very marked response to the action of the β3-AR agonist, CL 316,243, on energy balance. Presumably enough β3-ARs remained functional to allow this response to occur.

Improvement of insulin resistance associated with reduced TNF-α expression is consistent with TNF-α as a mediator of the resistant state. The site of improved glucose utilization is probably brown adipocytes, since CL-treatment is known to increase the level of Glut4 in BAT and WAT, but not muscle (Umekawa et al., 1997). BAT is known to be important site of glucose utilization when sympathetic

stimulation is high (Assimacopoulos-Jeannet et al., 1992; Vallerand et al., 1990; Marette et al., 1991).

CL-treatment induced an increase in energy expenditure and growth of BAT expected from previous studies (Himms-Hagen et al., 1994) and resulted in a negative energy balance without any change in energy intake, except in hyperphagic fa/fa rats in which the negative energy balance was associated with both reduced energy intake and increased energy expenditure. This was associated with loss of fat particularly from intraabdominal depots in both DIO and genetically obese fa/fa rats. Others have recently shown similar effects of CL-treatment on energy expenditure and food intake in obese mice (Nagase et al., 1996) and Otsuka Long-Evans Tokoshima fatty rats (Umekawa et al., 1997).

Feeding rats with a high-fat diet induces an increase in leptin level as the white adipocytes grow (Masuzaki et al., 1995; Moinat et al., 1995). CL-treatment in DIO rats is expected to decrease the elevated leptin level (Mantzoros et al., 1996), but at the same time to increase heat production by BAT (Himms-Hagen 1995). The overall effect would be unaltered food intake, as was seen in DIO rats in chapter V part A. However, genetically obese, fa/fa, rats are presumed to be hyperphagic because of the lack of central response to their elevated leptin levels (Moinat et al., 1995; Cusin et al., 1995; Chua et al., 1996), whereas the thermogenic effect of CL still occurs. Thus, the overall effect would be a reduction in the elevated food intake due to increased heat production as was seen in experiment B of chapter V.

(c) Remodelling of WAT

In WAT, DIO induced hypertrophy (increased size) of white adipocytes and the enlarged size of white adipocytes was returned to normal by the CL-treatment. CL had however less effect on the smaller adipocytes in the chow-fed control rats. In older lean and fa/fa rats eating only chow, the average size of white adipocytes was as large as in the DIO rats and CL-treatment induced a marked decrease in cell size in both fa/fa and lean rats.

CL-treatment did not alter the number of mature white adipocytes in either the fa/fa rats with hyperplastic obesity or DIO rats with hypertrophic obesity. It has been shown that in streptozocin-diabetic rats (Géloën et al., 1989a) some adipocytes disappeared. However, it seems likely the adipocytes which remained were so small as to be indistinguishable from interstitial cells by light microscopy, therefore were not counted in the histological procedure used.

DIO induced appearance of some non-adipocyte cells which then disappeared after treatment with CL. The cells which appeared, then disappeared, were not mature white adipocytes, because the number of these cells were unchanged. The reduction occurred at a time when brown adipocytes were actually appearing in the tissue and white adipocytes remaining unchanged in number. A similar reduction in non-adipocyte cells was seen in CL-treated fa/fa rats. A decrease in DNA content of WAT has also been shown in long-term food deprived rats (Miller et al., 1983) and in

streptozotocin-diabetic rats (Géloën et al., 1989a). In food-deprived rats, as in the present experiment, the decrease in DNA content was not accompanied by any loss of white adipocytes (Miller et al., 1983). It is suggested that the cells which disappeared are vascular cells which are recruited to support the increased blood circulation in the expanded WAT during the period of feeding and lost under influence of the CL-treatment.

The substantial increase in energy expenditure induced by the CL-treatment was associated with alterations in two distinct populations of brown adipocytes. First, the expansion of the mitochondrial compartment in brown adipocytes in BAT (Himms-Hagen et al., 1994) which is associated with a 10-fold increase UCP content in brown adipocytes as was seen in these experiments. Second, the appearance of abundant multilocular brown adipocytes that expressed UCP in retroperitoneal WAT. The appearance of these brown adipocytes was accompanied by a large increase in total protein content of the WAT, presumably present mainly in mitochondria of these cells and occurring despite a decrease in the number of non adipocyte cells, an unchanged number of smaller mature white adipocytes and a decrease in tissue weight. The overall increase in energy expenditure induced by CL thus presumably occurred in two distinct populations of thermogenically component brown adipocytes, those in BAT and those induced to appear in WAT by the drug treatment.

The origin of the multilocular, UCP-expressing cells which appeared in RWAT of CL-treated rats remains unknown. Their expression of UCP defines them as brown

adipocytes (Himms-Hagen and Ricquier, 1996). It is unlikely they arose by conversion of unilocular white adipocytes to brown adipocytes because the number of white adipocytes did not change. Since the DNA content of the tissue actually decreased at the time these cells appeared it seems improbable that brown adipocyte precursor cells had proliferated in response to the stimulus, although we cannot exclude the possibility that proliferation of one cell type was more than balanced by disappearance of another cell type. It also seems unlikely that CL promoted proliferation and differentiation of precursor interstitial cells because these would be expected to possess β1- rather than β3-ARs. We suggest therefore that very small precursors to brown adipocytes, perhaps brown preadipocytes or protoadipocytes (Géloën et al., 1990) are present in WAT depots and are induced to differentiate into mature brown adipocytes by sustained β3-adrenergic stimulation, undergoing both marked mitochondrial proliferation and lipogenesis, thereby acquiring triacylglycerol stores. These precursors to brown adipocytes should always be present in WAT, but are very small, with a low content of mitochondria and UCP and lipogenic enzymes, and not readily seen in routine histological sections. Perhaps they lack the rich sympathetic and sensory innervation characteristics of brown adipocytes in BAT depots. We suggest that these precursor cells are already committed to the brown adipocyte lineage and possess β3-ARs but are in an inactive state because of lack of suitable stimulation. Thus, they are induced to differentiate and hypertrophy only by high concentrations of β3-AR agonists. Others have described dormant or masked

brown adipocytes in certain WAT depots (Cousin et al., 1992; Casteilla et al., 1994; Loncar, 1991; P. Young et al., 1984). Moreover, precursors of brown adipocytes are present in the stromal-vascular fraction of WAT (at least in the Siberian hamster) and mature into brown adipocytes during culture, when they constitute 10-15% of the total adipocytes formed (Klaus et al., 1995). Our hypothesis differs from the three hypotheses advanced by Casteilla and colleagues (1994) to take into account the apparent presence of β3-ARs on the cells (brown adipocyte precursor interstitial cells in BAT do not possess these receptors) and the lack of measurable change in the number of white adipocytes (the brown adipocytes which appeared could not have been derived from cells with the characteristics of white adipocytes).

Another possible explanation for the origin of the brown adipocytes in WAT is that uncommitted precursor cells are available that are stimulated to differentiate and become committed to the brown adipocyte lineage by a product released locally from the intensely stimulated white adipocytes surrounding them. Activation by free fatty acids (FFA) (or a specific FFA or product thereof such as a prostaglandin) of a member of the PPARγ family of nuclear hormone receptors and promotion of adipocyte differentiation is a possibility (Forman et al., 1995; Kliewer et al., 1995). PPARγ is strongly expressed in adipose tissue (Tontonoz et al., 1995). While forced expression of PPARγ can induce adipose differentiation in cell lines that normally would not follow this route (Tontonoz et al., 1995) its possible role in determining whether a cell will follow a brown or white adipocyte lineage is unknown.

The level of expression of the β3-AR in adult human adipose tissues is controversial (Himms-Hagen and Danforth, 1996), although there is no doubt that it is expressed in newborn human BAT (Deng et al., 1996). However, precursor cells from adult human adipose tissues can differentiate in culture and can be induced to express UCP by β3-AR agonists, including CL 316,243, that is, they differentiate into brown adipocytes (Champigny and Ricquier, 1996). Therefore, it is possible that use of β3-AR agonists in adult obese humans might increase energy expenditure by inducing the appearance of brown adipocytes in adipose tissues which could contribute to the reversal of obesity in humans.

B. DIRECTIONS FOR FURTHER RESEARCH

The results described in this thesis raise several unanswered questions which should be the subject of further research.

1. The recent discovery of UCP2 (Fleury et al., 1997; Gimeno et al., 1997) and of UCP3 (Vidal-Puig et al., 1997; Boss et al., 1997) raises the possibility that the antiserum to UCP used in the study probably reacts with UCP1, UCP2, and UCP3, either because they share homologies recognized by a single antibody or because the preparation of antiserum used a preparation of UCP1 that was contaminated with other UCP homologues known so far (all are present in BAT and have very similar properties). Therefore, further investigations are required to distinguish between the

three form of UCP under the influence of CL-treatment by using Northern-blot analysis of mRNA levels of these proteins using the probes now available.

It has recently been reported that UCP1 is not expressed in any liver cells while the reactivity of UCP antiserum to the recently cloned UCP2 is detected only in Küpffer cells, cells which express UCP2 mRNA, in the rat liver tissue (Larrouy et al., 1997). We have confirmed that our antiserum reacts with UCP2 in Küpffer cells in liver (results not shown).

Recent evidence in literature supports the presence of UCP1 mRNA in RWAT of cold-acclimated rats (Cousin et al., 1992) but not in inguinal WAT (Cousin et al., 1992, 1996). CL-treatment has been shown to increase UCP1 mRNA in RWAT and inguinal WAT of rats and mice (Nagase et al., 1996; Umekawa et al., 1997; Collins et al., 1996). UCP2 mRNA is expressed in many tissues including WAT, BAT, and skeletal muscles. Cold-acclimation and CL-treatment do not alter the expression of UCP2 in these tissues (Fleury et al., 1997). UCP3 is mainly expressed in skeletal muscle and BAT (Vidal-Puig et al., 1997; Boss et al., 1997) and is not altered by cold-acclimation in muscle (Boss et al., 1997). The effect of CL or other β-AR agonists on the expression of UCP3 is still unknown. It seems likely that the UCP present in RWAT of CL-treated rats was UCP1.

The marked immunoreaction (green) seen in multilocular cells in RWAT of CL-treated rats in immunofluorescence study in this thesis (Figures 29B and D) is therefore suggested to be due to the appearance of UCP1. The little trace of

immunoreactivity in unilocular white adipocytes in RWAT of saline treated rats is probably due to the existence of UCP2 in these cells (Figures 29A and C). Since it has been shown that cold-acclimation and CL-treatment do not alter the level of UCP2 mRNA in WAT of mice (Fleury et al., 1997) but increase UCP1 mRNA in WAT (Umekawa et al., 1997; Nagase et al., 1996, Cousin et al., 1996), it is concluded that the immunoreaction observed in multilocular adipocytes in RWAT of CL-treated lean and fa/fa rats is UCP1. We cannot yet exclude that it might be UCP3.

2. The origin of brown adipocytes which appear in WAT is another interesting subject to be investigated in future. Cell culture of different cell types including preadipocyte, fibroblast, and endothelial cells might provide information regarding the variation of β-adrenoceptors by western blotting (requires specific antibody for each receptor) and/or northern blot analysis (requires specific probe for each receptor). Since β-adrenergic receptors seem to be the essential regulatory elements for cell activation and probably cell differentiation as well as lipolysis and thermogenesis, incubation of each cell line with a β1-, β2-, or β3-AR agonist would give some idea of the cellular changes due to the stimulation by different agonists. Although newly described proteins are becoming hot topics for consideration in treatment of obesity and increase in energy expenditure, their regulation seems to be controlled by β-ARs.

Another possible way to trace cells which appear during the treatment could

be a time course study using histological procedures and studying the ultrastructure of the cells as they developed.

3. It still remains to be examined whether use of drugs selective for human β3-ARs are able to improve obesity and insulin resistance in humans. So far, there is disappointing evidence that agonists which are effective in rodents do not act on the human β3-ARs. Future experiment should use new compounds with good selectivity and efficacy at the human β3-AR.

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Professional experience: **(May 1992-Sep. 1997):** Graduate student in the PhD program in the department of Biochemistry at the University of Ottawa, supervised by Dr. Himms-Hagen. The topic of my thesis

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(Jan. 1993-Sep. 1997): Lab demonstrator for 3rd year metabolism course (BCH 3046-2) and for 2nd year introductory Biochemistry (BCH 2936).

(Oct. 1990-Jan. 1992): Fish diseases and health manager at the Iranian fisheries cooperative company in Tehran. I was involved in fish diseases diagnosis and treatment as well as controlling the water quality in cold and warm water fish cultivating and rearing farms.

(Feb. 1987-Oct. 1990): Vet practitioner at the animal husbandry farms. I was involved in diagnosis and treatment of internal diseases in cattle, sheep, dogs and cats. As a part of my job I have experience in large and small animal surgery including caesarean, rumenotomy, foreign object removal from the stomach, and castration.

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Publications:

1- **Ghorbani M.**, and J. Himms-Hagen. Appearance of brown adipose tissue during CL 316,243-induced reversal of genetic obesity and diabetes in Zucker fa/fa rats. Int. J. Obesity 21: 465-475,1997.

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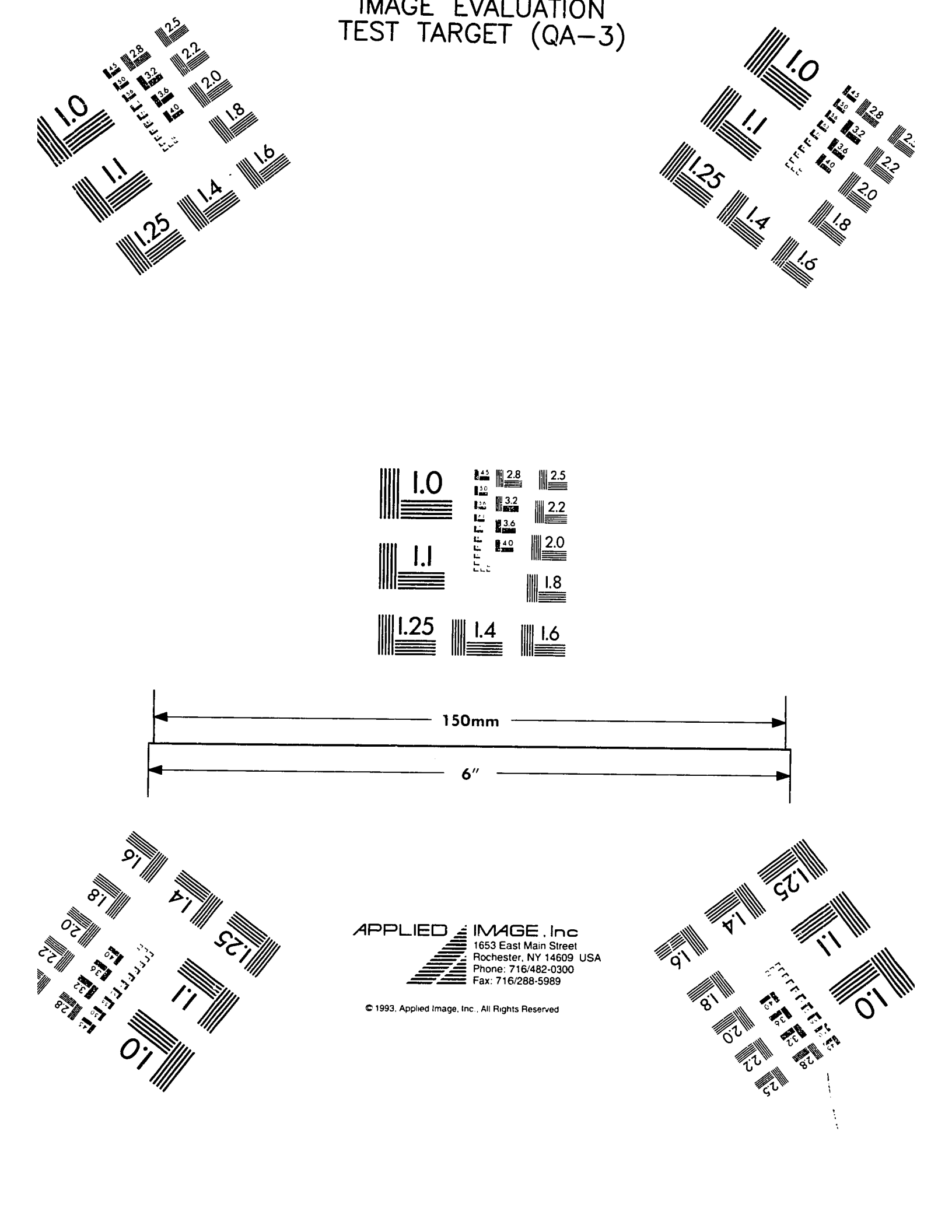
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