NOREPINEPHRINE DEPENDENT INHIBITION OF PROTEOLYSIS IN BROWN ADIPOCYTES

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ABSTRACT

Brown adipose tissue (BAT) is specialized for heat production. Brown adipocytes contain an uncoupling protein (UCP-1) in the mitochondria, which uncouples oxidative phosphorylation and produces heat. Thermogenesis in BAT is under the control of sympathetic nervous system. In addition, BAT growth and atrophy occurs in response to changes in the sympathetic activity. So, the focus of this research was to evaluate the mechanism by which norepinephrine (NE) affects proteolysis in brown fat cells differentiated in culture. An inhibitory effect of NE on proteolysis and ATP level was observed in mature brown adipocytes but not in pre-adipocytes and 3T3-L_1 adipocytes. The inhibitory effect of NE was not affected by the amount of UCP1 in mature brown adipocytes. To see if NE's action on proteolysis in mature brown adipocytes was linked to the onset of thermogenesis via activation of UCP-1 and decreasing energy level of the cell, the effect of NE on proteolysis and ATP was compared with that of fatty acids known to activate UCP-1 directly. Unlike in pre-adipocytes and 3T3-L_1 adipocytes, both proteolysis and cell ATP level were decreased in mature brown adipocytes. Furthermore, bromopalmitate, a non-metabolisable fatty acid, which is known to activate UCP-1, reduced proteolysis and ATP by a greater extent than NE. There was a linear relationship between reduction in cell ATP and protein degradation. So, NE may inhibit proteolysis in mature brown adipocytes by decreasing the cell ATP level, and thus may play a significant role in affecting the cell content of protein under certain physiological and pathological conditions, known to activate thermogenesis and stimulate BAT growth.

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LIST OF ABBREVIATIONS

AA, ascorbic acid;

AC, adenylyl cyclase;

ADP, adenosine 5'-diphosphate;

AMP, adenosine 5'-monophosphate;

ANOVA, analysis of variance;

ATP, adenosine 5'-triphosphate;

BAT, brown adipose tissue;

BSA, bovine serum albumin;

cAMP, cyclic adenosine 5'-triphosphate;

CEBP,CCAAT/enhancer-binding protein;

COX, cytochrome oxidase;

CPM, count per minute;

CPT, carnitine palmitoyl transferase;

CRE, cAMP response element;

CREB, cAMP response element binding protein;

DMEM, Dulbecco's modified eagle medium;

DNA, deoxyribonucleic acid;

ECL, enhanced chemiluminescence luminol;

EDTA, ethylenediamineacetic acid;

FADH, reduced flavin adenine dinucleotide;

FAs, fatty acids;

FCCP, carbonyl cyanide p-trifluoromethoxy phenyl hydrazone;

FFAs, free fatty acids;

GDP, guanosine 5'-diphosphate;

Gs, stimulatory GTP-binding protein;

GTP, guanosine 5'-triphosphate;

HEPES, 4-(2-hydroxyethyl)-1-Piperazine ethane sulphonic acid;

HRP, horse radish peroxidase;

HSL, hormone-sensitive lipase;

IgG, immunoglobulin G;

kDa, kiloDalton;

LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one;

mRNA, messenger ribonucleic acid;

NADH, reduced nicotinamide adenine dinucleotide;

NE, norepinephrine;

PBS, phosphate-buffered saline;

PKA, protein kinase A;

PPAR, peroxisome proliferator-activated receptor;

SDS, sodium dodecyl sulfate;

SNS, sympathetic nervous system;

TCA, trichloroacetic acid;

TTBS, Tween 20 tris-buffered saline;

T3, 3,5,3'-triiodothyronine;

UCP, uncoupling protein;

WAT, white adipose tissue;

1.0 INTRODUCTION

1.1 BROWN AND WHITE ADIPOSE TISSUES

In mammals, there are two types of adipose tissues with some anatomical/structural and vastly different physiological functions: white adipose tissue (WAT) and brown adipose tissue (BAT) (Daikoku *et al* 2000). The amount of BAT differs between small and large mammals. While the tissue is especially abundant in small mammals (such as mice and rats), in larger mammals including dogs, cows, and primates, distinct deposits of the tissue are present at birth, but become relatively sparse during later development (Lowell and Spiegelman, 2000; Palou *et al* 1998). Humans are not an exception (Surwit *et al* 1998). Brown fat depots are well-developed only in newborns. In adult humans, brown fat is almost absent and white fat depots constitute most of the adipose tissue. However, some brown adipocytes still remain interspersed within white adipose depots (Oya *et al* 1997; Danforth and Himms-Hagen, 1997; Villa *et al* 2000).

1.1.1 ANATOMICAL LOCATION AND GENERAL STRUCTURE OF BAT AND WAT

Depending on the species, BAT is restricted to specific areas mainly in the interscapular and auxiliary regions with minor accumulations near the thymus gland and great vessels of the thorax and abdomen (Tanaka *et al* 1997). WAT is also located in several sites including subcutaneous, perivascular, perigonadal, intermuscular, peritoneal, and perirenal (Villa *et al* 2000).

The cell population in BAT consists of 40% mature brown adipocytes, 10% pre-adipocytes, interstitial stem cells and mast cells, and the remaining 50% include endothelial cells forming the capillaries (D'Allaire *et al* 1995). A mature brown adipocyte is a terminally differentiated cell that no longer divides. It is characterized by many small spherical, oval or polygonal fat droplets (multilocular) with slightly eccenteric (not centrally placed) nucleus. In addition to that, it contains a large number of mitochondria packed with dense cristae that are rich in a protein called uncoupling protein 1 (UCP1) (Palou *et al* 1998; Morroni *et al* 1995). In pre-adipocytes, on the other hand, multilocular fat droplets and large number of mitochondria-containing UCP1 are absent. They contain some small fat droplets as well as a small number of mitochondria with a few cristae. These cells also contain a very large nucleus compared to the rest of the cell (Nedergaard *et al* 1993). Pre-adipocytes are cells that have the capacity to become mature brown adipocytes during the process of tissue recruitment (tissue growth).

In contrast to brown fat, cell types that can be found in WAT include white adipocytes, macrophages, endothelial cells, fibroblasts, and mast cells (DeMartinis *et al* 1987). White adipocytes contain a spherical to oval shaped, large and centrally placed intracellular lipid droplet (unilocular). The nucleus is usually located peripherally as a flattened or pressed structure (Tanaka *et al* 1997). Unlike mature brown fat cells, white adipocytes contain a few mitochondria with sparse cristae and no UCP1 (Enerback *et al* 1997; Kozak *et al* 1994).

BAT and WAT are mainly under the influence of sympathetic nervous system (SNS). However, sympathetic innervation is dense in BAT and much reduced

in WAT (Bamshad *et al* 1999; Morrison *et al* 1999). Similarly, the vascular supply to BAT is extremely rich compared to being sparse in WAT.

1.1.2 MITOCHONDRIA IN BAT AND WAT

The two types of adipose tissues, BAT and WAT, have different functions. The major function of white adipocytes is to serve as a storage depot for excess metabolic energy. Their stores of triglycerides can be released during periods of net caloric insufficiencies as fatty acids (FAs). On the other hand, BAT contains specialized adipocytes, which also store triglycerides but use them as an energy source dedicated to heat production (Rohlfs *et al* 1995; Tanaka *et al* 1997).

The mechanism of heat production in brown adipocytes is based on the function of a unique mitochondrial protein UCP1 (Kopecky *et al* 1995). UCP1 is a dimer protein of 32 kDa, each monomer consisting of 306 amino acids, located in the inner mitochondrial membrane (Palou *et al* 1998). The protein contains six transmembrane helices with both the C- and N-termini protruding to the cytosolic side (Klingenberg, 1999).

In mitochondria of all tissues such as WAT, an electrochemical proton gradient is produced by passing electrons through the respiratory chain and expelling protons from the matrix to intermembrane space, upon the oxidation of reduced equivalents such as reduced nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$). This process is followed by two events: (1) the transfer of electrons to O_2 at the end of the respiratory chain to form water (O_2 consumption); and (2) the re-entry of protons, through ATP-synthase, for the

synthesis of adenosine 5'-triphosphate (ATP) from adenosine 5'-diphosphate (ADP) (Figure 1.1 A). In BAT mitochondria, however, UCP1 dissipates the proton gradient across the inner mitochondrial membrane as heat instead of ATP (Nedergaard *et al* 1999; Monemdjou *et al* 1999; Matthias *et al* 1999, Figure 1.1 B). Therefore, whereas the respiratory activity is mainly coupled to the phosphorylation of ADP and ATP synthesis (coupled oxidative phosphorylation), oxidative phosphorylation is uncoupled in brown adipocytes. In this case, the high respiratory rate is not accompanied by ATP production but by heat production (Denjean *et al* 1999; Villa *et al* 2000).

The proton transport activity of UCP1 is regulated by FAs as activators (Section 1.3.1.2), and purine nucleotides [ATP, ADP, guanosine 5'-triphosphate (GTP), and guanosine 5'-diphosphate (GDP)] as inhibitors (Echtay *et al* 1998). The binding capacity of UCP1 for nucleotides was identified to be one nucleotide per UCP1 dimer (Klingenberg and Huang, 1999). Brown fat mitochondria from wild-type mice normally exhibit very low membrane potentials. Upon addition of GDP, however, membrane potential immediately increased. In contrast, mitochondria from UCP1-ablated mice did not show any effect of GDP on the membrane potential (Matthias *et al* 1999). Consequently, UCP1 has been proposed to be the only protein in BAT mitochondria, which binds to and is inhibited by GDP (Matthias *et al* 1999; Nedergaard *et al* 1999). Binding of GDP by UCP1 was a key in identifying and purifying this protein (Klingenberg and Huang, 1999).

Finally, in addition to the presence of UCP1 in BAT, there are other differences between the mitochondria of brown and white adipocytes, which help

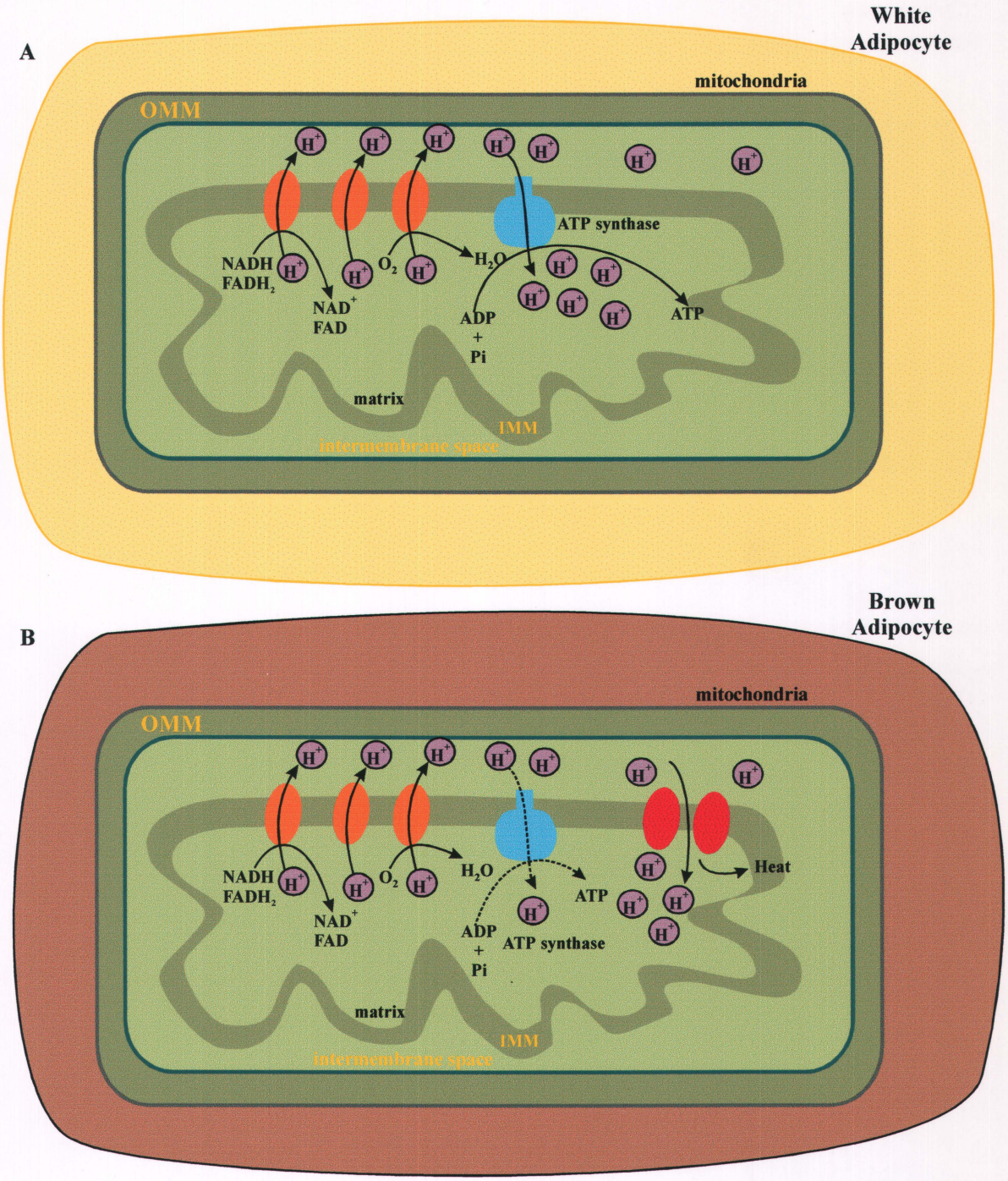


Figure 1.1 Mitochondria of white and brown adipocytes. (A) oxidation of substrates is coupled to phosphorylation of ADP and ATP synthesis. (B) oxidation of substrates is uncoupled from synthesis of ATP and accompanied by heat generation.
OMM = outer mitochondrial membrane
IMM = inner mitochondrial membrane

brown adipocytes to produce heat. The fuel for thermogenesis comes from the oxidation of FAs derived from triglyceride stores in cytosol. There is a high capacity for carnitine-mediated transport of FAs into BAT mitochondria where β-oxidation occurs, driving FAs toward oxidation and thermogenesis. The situation is opposite in WAT; a relatively low capacity for carnitine-mediated transport of FAs in mitochondria directs the compounds toward lipogenesis (Kopecky *et al* 1996). Another important difference is that brown fat mitochondria, unlike white fat, have a high content of enzyme components of the respiratory chain such as cytochrome oxidase (COX), and a low content of ATP-synthase (Brand *et al* 1999; Skulachev, 1999). Thus, BAT mitochondria are specially adapted for production of heat rather than ATP.

1.2 IMPORTANCE OF BAT FOR MAINTENANCE OF BODY TEMPERATURE AND ENERGY BALANCE

Brown fat thermogenesis is activated in response to changes in environmental temperature or energy balance. BAT heat production is important in protecting animals from hypothermia in cold environment, for re-warming during arousal from hibernation, or in preventing obesity (Shima *et al* 1994; Harper and Himms-Hagen, 2001). There are several lines of evidence to support the importance of UCP1, and thus of BAT in thermoregulation and energy balance. Lowell *et al* (1993) and Melnyk *et al* (1997) used the brown fat specific regulatory elements of the gene for UCP1 to drive expression of diphtheria toxin A-chain (UCP-DTA) in the brown adipocytes of transgenic mice. The toxin killed brown adipocytes and ablated BAT. Surprisingly, two lines of transgenic mice were created, both could not regulate their body temperature upon cold exposure and became obese upon feeding high fat diet. However, two lines of transgenic mice differed with respect to the status of BAT ablation over time. Whereas the first line had persistent BAT deficiency, the second transgenic line had near complete regeneration of BAT by age 8 weeks. Interestingly, while the second line of transgenic mice were obese at 16 days of age, total body lipid stores returned to normal by 8 weeks. Regeneration of BAT accompanied by reversal of obesity strongly suggests that BAT is involved in regulation of the energy balance, and that obesity in these mice was a consequence of BAT deficiency. In another study, Enerback *et al* (1997) (reviewed by Harper and Himms-Hagen, 2001) inactivated the UCP1 gene, and the UCP1 knockout mice were not able to maintain their body temperature in a cold environment (5°C). However, the UCP1 knockout

mice did not become obese upon feeding a high fat diet. This discrepancy between BAT-ablated mice and UCP1-ablated mice in the susceptibility for diet-induced obesity was explained by the high expression of another putative uncoupling protein, UCP2, which may have compensated for the absence of UCP1 (Boss *et al* 1997). There are several UCP1 homologues, UCP2 and UCP3 (Fleury *et al* 1997), and it was shown that UCP2 expression, for example, in BAT increases 14-fold in UCP1-knockout mice (Monemdjou *et al* 1999).

Kopecky *et al* (1996) and Stefl *et al* (1998) introduced UCP1 into white adipocytes, and thus showed its importance in thermoregulation and energy balance. By use of the fat-specific promoter of the adipocyte lipid-binding protein aP2 gene, a transgenic mouse expressed UCP1 not only in BAT but also in WAT. Mice with the aP2-UCP1 transgene were partially resistant toward fat accumulation and obesity compared to their non-transgenic control littermates. It was suggested that energy dissipation by UCP1 in mitochondria of white and not brown fat tissue affected energy balance and determined the phenotype of the transgenic mice, as endogenous UCP1 in brown fat was down regulated. However, these mice were cold sensitive, and it was concluded that cold-induced thermogenesis in brown fat can not be substituted by other tissues and UCP1 expressed in WAT is not enough for protection against cold.

Prior to these most recent studies with transgenic animals, several studies suggested an important role of BAT in thermoregulation and energy balance. Cold exposure or feeding palatable high fat/carbohydrate diets (cafeteria diet) in rats and mice induced not only BAT thermogenesis, but also its recruitment (LeBlanc and

Labrie, 1997; Ricquier *et al* 2000; Lowell and Spiegelman, 2000; Hamann *et al* 1996; Surwit *et al* 1998). During the process of recruitment, pre-adipocytes undergo proliferation and differentiation. During proliferation, they go through several rounds of mitosis as shown by 6-8 times increase in the BAT content of deoxyribonucleic acid (DNA) (Geloen *et al* 1992; Bukowiecki *et al* 1982). The pre-adipocytes differentiate and acquire the characteristics of mature brown adipocytes as described in Section 1.1.1. Mature brown adipocytes also undergo some changes which include increases in total amount of cell proteins and mitochondrial proteins such as glycolytic, lipogenic, lipolytic, respiratory chain enzymes, and specially increase in expression of UCP1 (Cousin *et al* 1996; Morroni *et al* 1995; Denjean *et al* 1999; Rippe *et al* 2000; Ribeiro *et al* 2000). All these changes are responsible for a large increase in the tissue thermogenic capacity (Emilsson *et al* 1998; Guardiola-Diaz *et al* 1999).

In addition to the above, it was demonstrated that the composition of fat in diet is also an important factor for stimulation of BAT thermogenic activity and recruitment (Nedergaard *et al* 1983). Recruitment state of BAT and its capacity for heat production is augmented by a diet that has a normal fat content, but in which the fat is enriched in polyunsaturated FAs such as n-3 unsaturated FAs (Matsuo *et al* 1995; Sadurskis *et al* 1995; Oudart *et al* 1997). Additionally, it has been suggested that it is not the fat content of diet that is the only important factor in diet-induced BAT activity and recruitment, but rather the protein content of the diet. Diets low in proteins are able to stimulate BAT activity and growth (Sadurskis *et al* 1995).

1.3 CONTROL OF BAT ACTIVITY AND THERMOGENIC CAPACITY BY SYMPATHETIC NERVES

BAT receives a dense innervation by SNS. The two lobes of interscapular BAT are each innervated by 5 intercostal nerves which directly innervate each brown adipocyte, as well as a nerve that innervates the thoracodorsal blood vessels (Bamshad *et al* 1999; Morrison *et al* 1999; Geloen *et al* 1992). Release of the neurotransmitter norepinephrine (NE) from SNS is increased in BAT during cold exposure and changes in dietary intake (Schwartz *et al* 1983; Young *et al* 1982). In contrast, NE release from SNS neurons decreases during conditions of fasting, deacclimation (transfer of cold-acclimated animals to a thermoneutral environment in which animals do not need to produce heat in order to maintain their body temperature), and lactation (Young *et al* 1982; Yoshida *et al* 1983; young and Landsberg, 1977; Sakaguchi *et al* 1988; Reichling *et al* 1987; Trayhurn and Jennings, 1988; Villarroya *et al* 1987). NE is thought to play the major role in the regulation, not only of acute switching on and off of thermogenesis, but also of the tissue growth in response to chronic stimulation. Cessation of SNS activity is associated with disuse and BAT atrophy (Park and Himms-Hagen, 1988; Nedergaard *et al* 1995; Atgie *et al* 1997; Rothwell and Stock, 1984, Section 1.4).

1.3.1. ADRENERGIC CONTROL OF BAT THERMOGENESIS

Several studies have shown *in vitro* and *in vivo* that NE administration or sympathetic nerve stimulation results in increased O_2 consumption and heat production (Rothwell and Stock, 1981; Young *et al* 1982; Matthias *et al* 2000). The

importance of NE for BAT thermogenesis also became evident using dopamine β-hydroxylase knockout mice, the enzyme responsible for NE synthesis. Dopamine β-hydroxylase knockout mice have atrophied BAT and does not maintain body temperature in cold environments (Thomas and Palmiter, 1997). In addition, studies using adrenergic agonists and antagonists demonstrated not only the importance of NE in tissue thermogenesis but also the type of adrenergic receptor involved. Zhao *et al* (1994, 1998) pointed out that β_3-adrenergic receptor agonist (BRL-37344) was as effective as NE in stimulating thermogenesis, whereas β_1- and β_2-adrenergic receptor antagonists had no effect. Therefore, in brown fat cells, it is mainly through β_3-receptor that β-adrenergically mediated thermogenesis is stimulated. However, there is also a role for α_1-adrenoceptors in potentiating the thermogenic response by β_3-receptors (Zhao *et al* 1997).

NE released from the sympathetic nerve endings interacts with β_3-adrenergic receptors on brown fat cells (Himms-Hagen, 1989; Cannon *et al* 1996; Atgie *et al* 1997). Upon NE binding to β_3-adrenergic receptors, there is activation of adenylyl cyclase (AC), via stimulatory GTP binding proteins (Gs), leading to the conversion of ATP to cyclic adenosine monophosphate (cAMP) (Cannon *et al* 1996; Nedergaard *et al* 1995, Figure 1.2). Increased cAMP activates protein kinase A (PKA) which phosphorylates hormone-sensitive lipase (HSL). Activation of HSL leads to the release of free fatty acids (FFAs) from the triglyceride stores (Himms-Hagen, 1989; Cannon *et al* 1996; reviewed by Rial and Gonzalez-Barroso, 2001). FFAs are then transported to the mitochondria and play two major roles in the

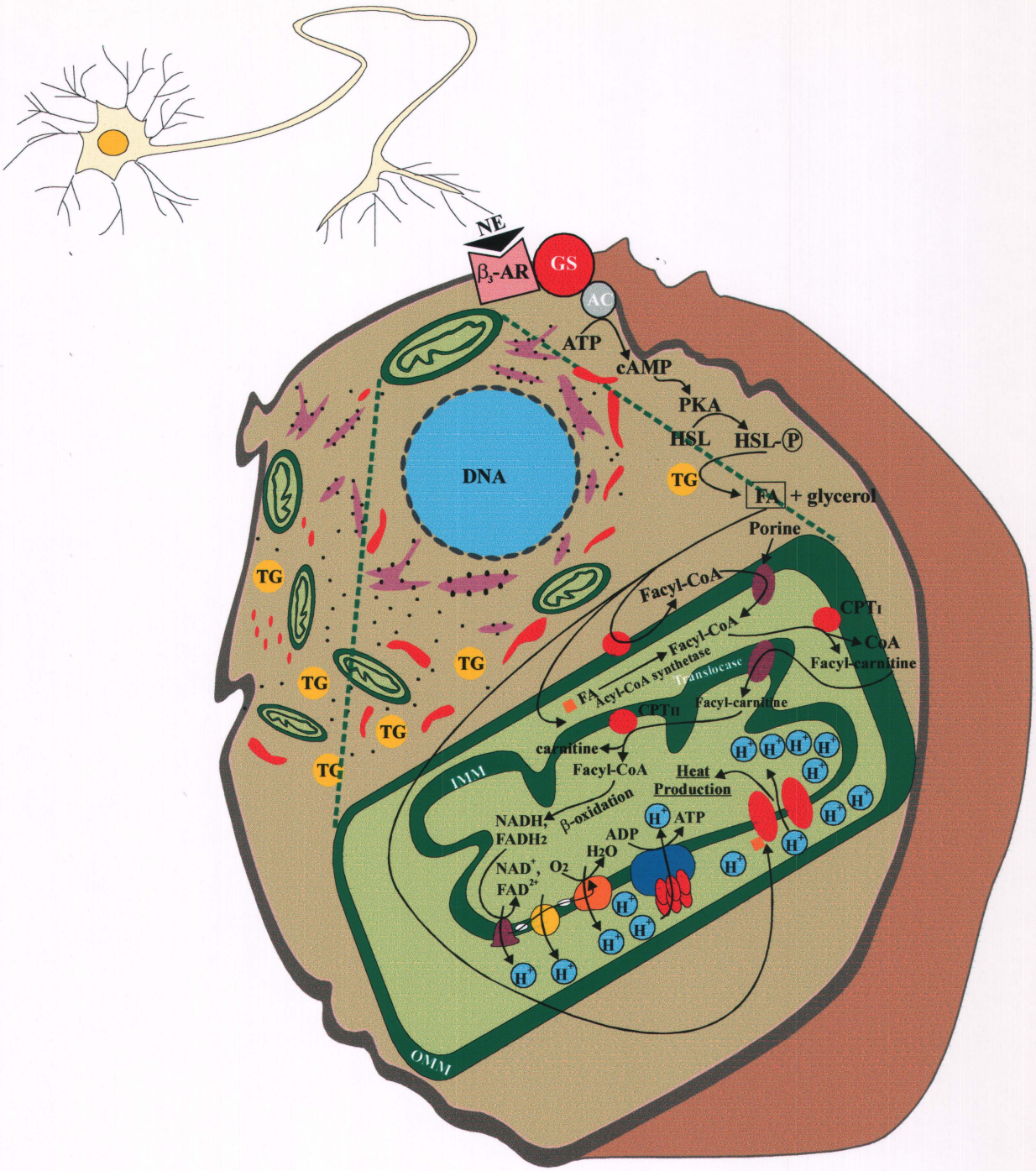


Figure 1.2 Mechanism of heat production by brown adipocytes. Upon activation of UCP1 by fatty acids released by NE-induced lipolysis, protons extruded by electron respiratory chain re-enter the mitochondrial matrix through UCP1. This cycle generates heat instead of ATP.
FA = Fatty Acid
TG = Triglyceride Store
β_3-AR = β_3-adrenergic receptor

induction and support of thermogenesis (reviewed by Rial and Gonzalez-Barroso, 2001, Figure 1.2).

1.3.1.1 FATTY ACIDS AS ENERGY SUBSTRATES FOR β-OXIDATION

FFAs play a key role in thermogenesis as an energy substrate for mitochondrial β-oxidation. FAs in the intermembrane space are converted to acyl-CoA derivatives by acyl-CoA synthetase (reviewed by Rial and Gonzalez-Barroso, 2001, Figure 1.2). It should be noted that acyl-CoA synthetase is also located on the cytosolic side of outer mitochondrial membrane, therefore some fatty acyl-CoAs may also be formed on the outer mitochondrial membrane, and then enter the intermembrane space. Fatty acyl-CoAs are then converted to fatty acyl-carnitines by the enzyme carnitine palmitoyl transferase-I (CPT-I), located on the matrix side of the outer mitochondrial membrane. Fatty acyl-carnitines are then transported into the mitochondrial matrix by a translocase. In the mitochondrial matrix, fatty acyl-carnitines are converted back to acyl-CoA derivatives by carnitine palmitoyl transferase-II (CPT-II), and are served as substrates for β-oxidation (reviewed by Rial and Gonzalez-Barroso, 2001; Jezek *et al* 1998). β-oxidation of FAs produces NADH and $FADH_2$, which are the principal sources of electrons for mitochondrial respiration (Figure 1.2). Thus, defects in one of the proteins associated with β-oxidation of FAs affect BAT thermogenesis and render mice cold sensitive (Guerra *et al* 1998). For example, a mutation in the gene which encodes short chain acyl-CoA dehydrogenase enzyme, an enzyme involved in β-oxidation which catalyzes the initial dehydrogenation of straight-chain FAs in mitochondria, renders the mice unable to

maintain their body temperature when exposed to cold, indicating that intact FA oxidation is essential for BAT thermogenesis (Guerra *et al* 1998). In addition, other studies have shown that agents which interfere with import of FAs into the mitochondria or with β-oxidation affect BAT respiration, and thus thermogenesis (Bukowiecki *et al* 1981). The potent inhibitor of mitochondrial CPT, 2-tetradecylglycidic acid (McN-3802), or a specific inhibitor of FA oxidation (e.g. methylpalmonitrate) rapidly abolished respiration stimulated by NE. The conclusion is that long chain FAs from NE-dependent stimulation of lipolysis are the principal substrates oxidized by brown adipocytes (Bukowiecki *et al* 1981). However, there is additional role for FFAs in brown adipocytes.

1.3.1.2 FATTY ACIDS AS ACTIVATORS OF UCP1

FFAs are the physiological activators of UCP1 in brown adipocytes (Figure 1.2). Several studies have shown that FFAs increase membrane proton conductance, lower the membrane potential, and stimulate respiration and thermogenesis by direct activation of UCP1 (Locke and Nicholls, 1981; Locke *et al* 1982; and Bukowiecki *et al* 1981).

Bukowiecki *et al* (1981) were first to demonstrate that FAs increase respiration in isolated brown fat cells. The capacity of FAs for stimulating respiration was critically dependent upon the length of their carbon chain. Palmitic (16:0), oleic (18:1), and linoleic (18:2) acids were nearly equivalent in stimulating respiration, while hexanoic acid did not elicit a respiratory response, and octanoic acid was approximately one-half as efficient as palmitic, oleic, and linoleic acids. Locke *et al*

(1982) measured the time course of changes in mitochondrial membrane potential, and O_2 consumption during infusion of palmitic acid in brown adipocytes. Upon addition of palmitic acid, there was a significant decrease in mitochondrial membrane potential, and an immediate increase in O_2 consumption. On the termination of infusion, all changes were rapidly reversed. The decrease in mitochondrial membrane potential upon palmitic acid infusion was attributed to proton permeability change of the inner mitochondrial membrane (Locke *et al* 1982). Direct estimation of mitochondrial membrane proton permeability change upon palmitic acid addition confirmed that proton conductance was largely increased which parallel to the decrease in mitochondrial membrane potential. In contrast to brown fat mitochondria, such findings could not be observed in the liver mitochondria, which do not contain UCP1. Therefore, these results clearly suggested that FAs function not only as fuel, but also to uncouple mitochondrial respiration.

The increased proton conductance found when FAs interact with brown fat mitochondria appeared to be the consequence of a direct interaction with UCP1, rather than a non-specific action in the membrane. Rial *et al* (1983) suggested that if FAs interact with UCP1, the increment in proton permeability should be sensitive to inhibitors of UCP1 function, such as GDP. Alternatively, if FAs act in a non-specific manner, their effects on proton permeability should be similar to that of an artificial proton ionophore. Addition of carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) increased mitochondrial proton permeability and respiration, which was not affected by the presence of GDP. Unlike FCCP, low concentrations of palmitic acid increased the membrane permeability to protons and the effects were reduced as the

GDP concentration was increased. Thus, GDP was able to inhibit the proton conductance induced by FFAs. These results suggested that FAs act at a locus at or near UCP1.

In addition, the uncoupling effect of FAs was influenced by the amount of UCP1 in the mitochondria. Rial *et al* (1983), Locke *et al* (1982), and Cunningham *et al* (1986) prepared the mitochondria from BAT of guinea pigs adapted to either cold or warm temperatures. The BAT mitochondria of cold-adapted animals possessed an increased amount of UCP1, and were very sensitive to the uncoupling effect of FAs. Mitochondria from warm-adapted animals, however, were 9-fold less sensitive to FAs and resembled liver mitochondria. Therefore, the presence of UCP1 was necessary for the expression of a high sensitivity to FA uncoupling in BAT.

This was also confirmed by the expression of UCP1 in reconstituted systems. Murdza-Inglis *et al* (1991) and Arechaga *et al* (1993) expressed UCP1 in yeast *Saccharomyces cerevisiae* mitochondria, and then compared the FA sensitivity of these mitochondria with that of control mitochondria. Control mitochondria showed a very low sensitivity to FAs, whereas yeast mitochondria with ectopically expressed UCP1 showed a very high FA sensitivity comparable to that of brown fat mitochondria. Similarly, Strieleman *et al* (1985), Klingenberg and Winkler (1985), and Winkler and Klingenberg (1992) reconstituted UCP1 into liposomes, and examined the effects of added palmitic acid on the proton conducting activity. Proton conductance by the reconstituted UCP1 was specifically activated by palmitic acid, and the increase in proton conducting activity was dependent on the chain length.

Although there was strong evidence that FAs interact directly with UCP1, no FA binding site had been detected by that time. Thereafter, several studies have been done by the Jezek's group to find such a binding site on UCP1 (Jezek and Freisleben, 1994; Jezek *et al* 1995; Jezek *et al* 1996). They synthesized a photoreactive analogue of dodecanoic acid, 12-(4-azido-2-nitrophenylamino) dodecanoic acid (AzDA), and mentioned that if binding sites for FAs exist on UCP1, one should be able to identify them using photoaffinity labeling. Indeed, FA binding sites were shown to exist on UCP1. This was also confirmed by Ruzicka *et al* (1996).

Finally, construction of UCP1-ablated mice opened a new opportunity to re-examine the importance of UCP1 in FA-induced uncoupling in brown fat cells and mitochondria. Nedergaard *et al* (2001), using UCP1-ablated mice, confirmed that FA-stimulated respiration in brown fat cells is UCP1-dependent. Addition of FAs to brown fat cells stimulated respiration, but not in cells from UCP1-ablated mice. Hofmann's group (2001) also showed that brown fat mitochondria from UCP1-ablated mice do not respond to FAs. Overall, the conclusion is that FAs induce uncoupling of BAT mitochondria, and this effect is dependent on UCP1.

1.3.2. ADRENERGIC CONTROL OF BAT RECRUITMENT

Chronic stimulation of BAT, as with cold exposure or changes in diet, increases tissue mass, cell number, total mitochondrial protein content, as well as UCP1 content of the mitochondria. These changes induced by cold or diet can be mimicked by chronic NE infusion, and suppressed by sympathectomy (removal of the sympathetic nerves) (Mory *et al* 1982, 1984; Ricquier *et al* 1983; Park and Himms-Hagen, 1988; Bianco *et al* 1988). Furthermore, NE infusion in rats in which BAT is surgically denervated, stimulates tissue growth and restores the thermogenic capacity in the denervated tissue to that of the innervated tissue (Geloen *et al* 1992; Mory *et al* 1980; Geloen *et al* 1988; Rehnmark *et al* 1989). The effects of NE can be induced in cells in culture. Addition of NE to brown fat cells in primary culture causes cell proliferation, increases the cell mitochondrial content, as well as mitochondrial UCP1 messenger ribonucleic acid (mRNA) and protein level (Rehnmark *et al* 1989; Herron *et al* 1990; Kopecky *et al* 1990; Houstek *et al* 1990; Puigserver *et al* 1992; Bronnikov *et al* 1992; Yamashita *et al* 1995; Cannon and Nedergaard, 1996; Foellmi-Adams *et al* 1996). The conclusion is that NE functions as a mitogen (induces cell proliferation), and is required for the trophic response of BAT to stimulation by cold or diet.

The use of adrenergic agonists and antagonists confirmed the importance of NE in the process of recruitment, and also allowed determination of the type of adrenergic receptors involved in the process. CL-316243 and BRL 35135 (β_3-adrenergic receptor agonists) infusion in rats caused a marked growth of interscapular BAT, but the growth pattern differed somewhat from that induced by cold exposure.

CL-316243 treatment caused an increase in BAT protein content as well as 3-4 folds increase in UCP1 and COX content. However, there was no cell proliferation, as indicated by the lack of increase in DNA content. Thus, there is involvement of β_3-adrenergic receptors in differentiation. This is in agreement with the observation that propranolol (mainly β_1/β_2 antagonist, in high concentrations is also a β_3-antagonist) failed to inhibit NE-induced differentiation *in vivo* or *in vitro* (Bronnikov *et al* 1992, 1999). Although CL-316243 failed to induce cell proliferation of pre-adipocytes, β_1-adrenergic receptor agonists were effective in the stimulation of proliferation of pre-adipocytes in tissue cultures (Bronnikov *et al* 1992; Rehnmark *et al* 1990; Champigny *et al* 1992; Yoshitomi *et al* 1998; Emilsson *et al* 1998), and the effects were inhibited by low concentrations of propranolol (Nedergaard *et al* 1993).

1.3.2.1 NE-SIGNALING PATHWAY RESPONSIBLE FOR PROLIFERATION OF BROWN PRE-ADIPOCYTES

NE directs proliferation of brown pre-adipocytes. Very little is known about the molecular mediation of the mitogenic effect of NE. NE interacts with β_1-adrenergic receptors on the surface of pre-adipocytes resulting in an increase in cAMP production (D'Allaire *et al* 1995; Granneman, 1995, Figure 1.3). The events downstream of cAMP activation have not been fully characterized.

In many cell types, proliferative processes are regulated or accompanied by an increased expression of the proto-oncogene c-myc (Nedergaard *et al* 1995).

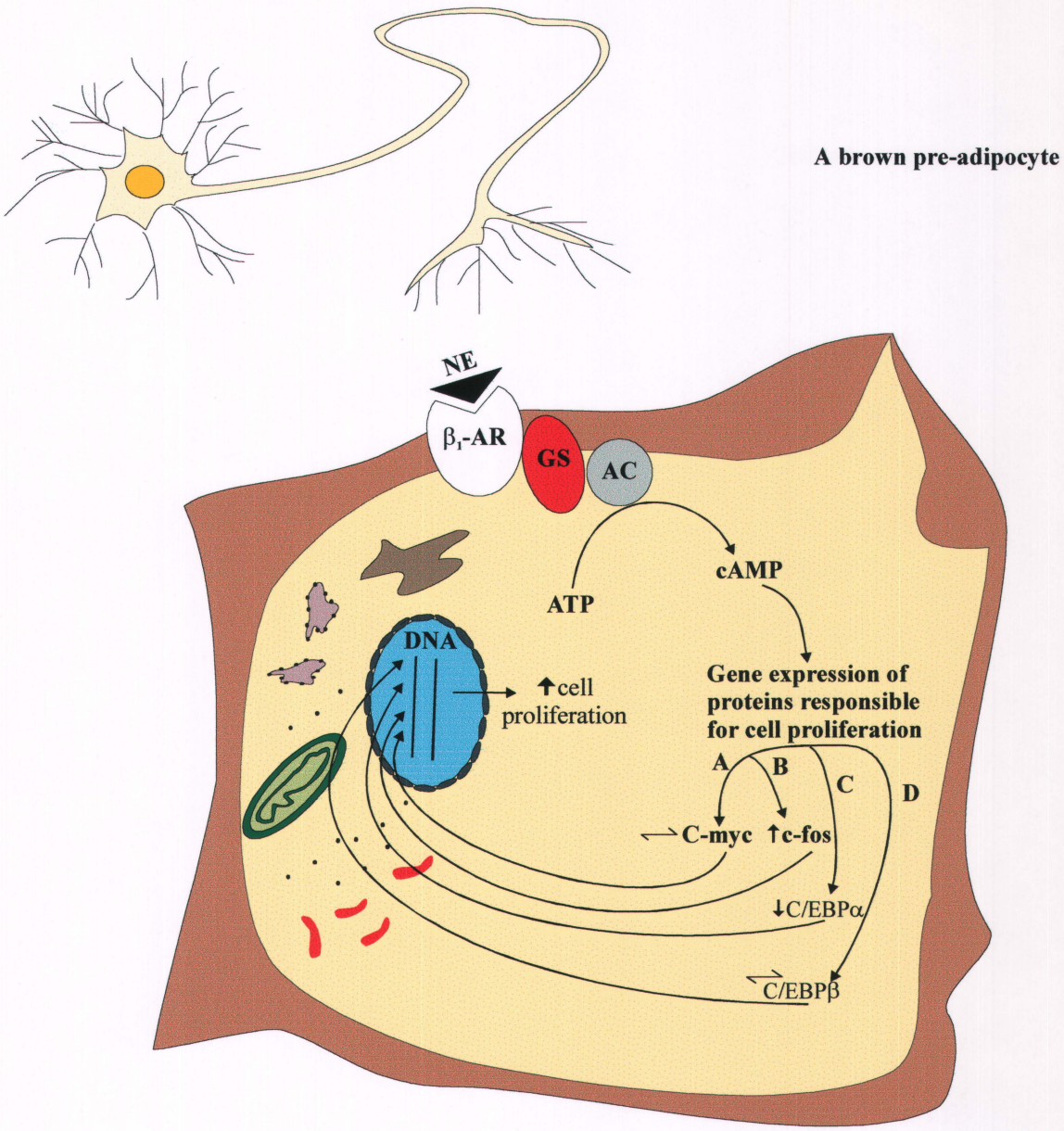


Figure 1.3 NE-signaling pathway which leads to proliferation of brown pre-adipocytes. Upon NE binding to β_1-adrenergic receptor on the cell surface, the expression level of some transcription factors involved in cell proliferation may change.
β_1-AR = β_1-adrenergic receptor

This is not the case for adrenergic activation of proliferation in brown pre-adipocytes. Neither *in vivo* during cold exposure, nor in cell cultures, is the NE-stimulation of proliferation accompanied by an increase in c-myc expression (Cannon and Nedergaard, 1996; Cannon *et al* 1996, Figure 1.3). However, the proto-oncogene c-fos expression is elevated under these conditions (Thonberg *et al* 1994; Cannon *et al* 1996). An association to NE-induced cell proliferation was also found for the transcription factor CCAAT/enhancer-binding protein α (C/EBPα). In the same cell stages that respond proliferatively to NE-stimulation, NE caused a decrease in the expression of C/EBPα (Rehnmark *et al* 1993; Cannon *et al* 1996). This effect was specific in that the transcription factor C/EBPβ did not show this NE-induced repression (Cannon *et al* 1996, Figure 1.3). It has also been suggested that acceleration of cell division would require increased capacities of enzymes directly involved in DNA synthesis, such as ribonucleotide reductase (Nedergaard *et al* 1995), and there are positive effects of adrenergic stimulation on the expression of the enzyme (Nedergaard *et al* 1995).

1.3.2.2 NE-SIGNALING PATHWAY RESPONSIBLE FOR DIFFERENTIATION OF BROWN ADIPOCYTES

Once pre-adipocytes in culture reach confluence, they start acquiring the brown adipocyte phenotype. First, there is down-regulation of β_1-adrenergic receptors and appearance of the β_3-adrenergic receptors (Bronnikov *et al* 1999). The cells do differentiate in the absence of NE, but acquire great thermogenic capacity in its

presence. NE binds to β_3-adrenergic receptors on the plasma membrane and stimulates expression of UCP1 (Rehnmark *et al* 1990; Bronnikov *et al* 1999; Nedergaard *et al* 1995, Figure 1.4). The most likely explanation for this effect is that PKA, when stimulated by increased cAMP levels, phosphorylates a transcription factor called cAMP response element binding protein (CREB) (Nedergaard *et al* 1995). There are cAMP response elements (CRE) identified on the promoter region of UCP1 gene and presumably phosphorylated CREB interacts with CRE that results in increased UCP1 expression (Kopecky *et al* 1990; Kozak *et al* 1994; Nedergaard *et al* 1995, Figure 1.4).

Other transcription factors may also be involved in the NE-induced UCP1 gene expression. The transcription factor peroxisomal proliferator-activated receptor α (PPARα) may be activated by endogenous FAs released by NE stimulation of lipolysis (Kliewer *et al* 1994). Recently, Barbera *et al* (2001), using deletion and mutation analysis, have identified a PPARα response element in the upstream enhancer region of the rat UCP1 gene (Figure 1.4). Another isoform of PPARα is PPARγ_2, which has also been suggested to be a pivotal transcription factor for adipocyte differentiation. This is reflected by its increased expression during maturation of brown adipocytes in culture (Tontonoz *et al* 1994). Another transcription factor, C/EPBα, has been proposed as a determinative factor in brown adipocyte differentiation, and its gene expression is also increased by NE in brown fat cells (Nedergaard *et al* 1995). Transgenic mice with a targeted disruption of the c/ebpα gene have severely reduced mass of BAT as well as a reduced expression of

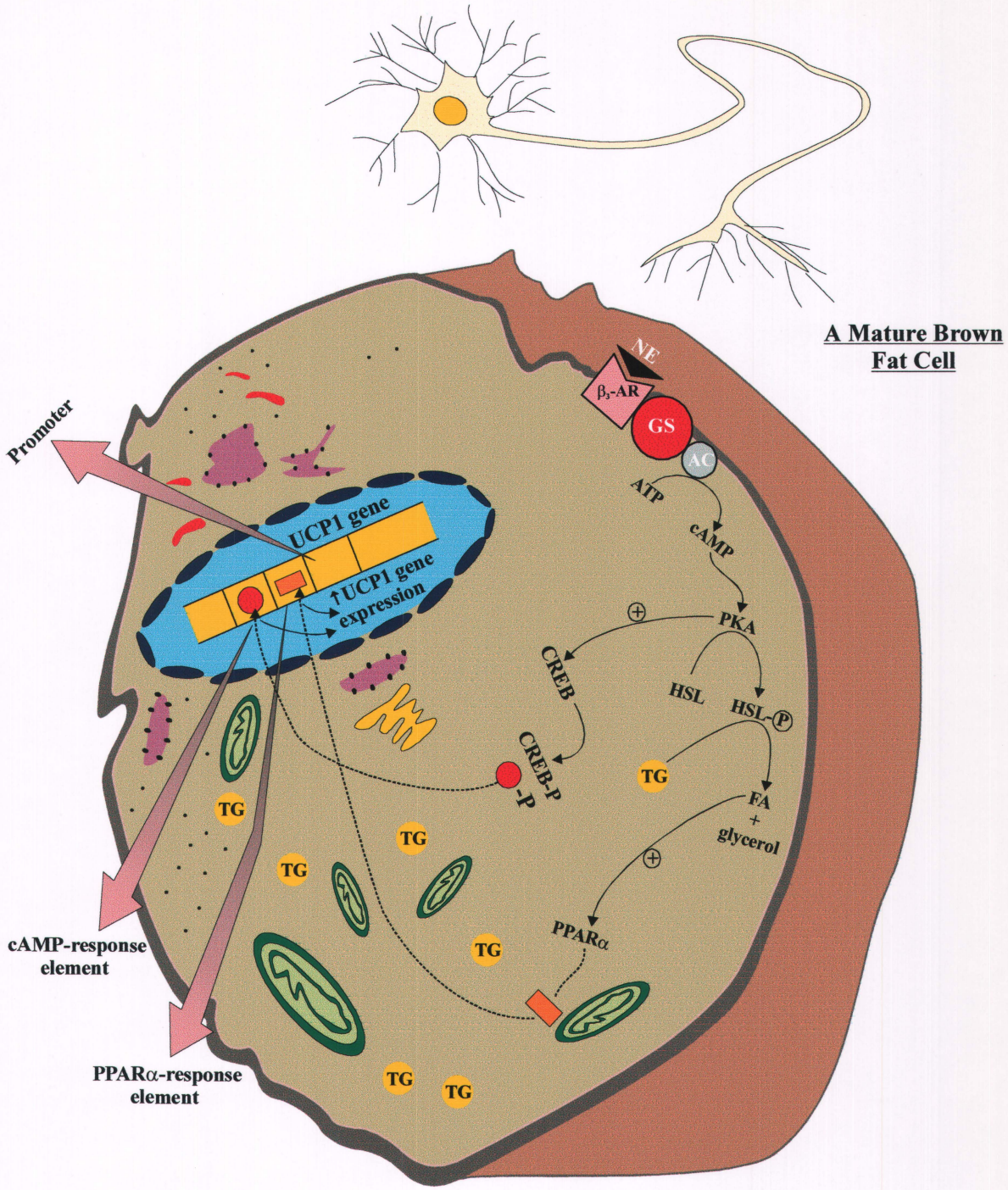


Figure 1.4 NE-signaling pathway which leads to differentiation of brown adipocytes. Several transcription factors are involved in the process of differentiation, the level of which or their activities are regulated by NE released from sympathetic nerves adjacent to brown adipocytes.
TG = Triglyceride Store
β_3-AR = β_3-adrenergic receptor

UCP1, suggesting that C/EBPα is essential for adipocyte differentiation *in vivo* (Wang *et al* 1995).

Another most important aspect of brown adipocyte differentiation is accumulation of multiple fat droplets (Himms-Hagen, 1989). Several studies evaluated what controls the expression of enzymes responsible for lipolysis and lipogenesis, such as glyceraldehyde 3-phosphate dehydrogenase (Sirover, 1996;Cao *et al* 1991; Manchado *et al* 1994; Wang *et al* 1995; Yeh *et al* 1995; Barroso *et al* 1999). Two transcription factors, C/EBPβ and C/EBPγ, appeared to play dominant roles in this respect (Tanaka *et al* 1997). Mice lacking C/EBPβ and/or γ died at the early neonatal stage (Tanaka *et al* 1997). Unlike wild-type animals, brown adipocytes from gene disrupted mice, 20 hours after birth, did not accumulate fat droplets.

1.4 BAT ATROPHY IN RESPONSE TO SUPPRESSION OF SYMPATHETIC ACTIVITY

There are a number of conditions such as fasting (Desautels, 1985; Rothwell *et al* 1984; Trayhurn and Jennings, 1988), deacclimation (Desautels *et al* 1986; Desautels and Dulos, 1990), and lactation (Trayhurn *et al* 1982), when heat production is no longer needed and/or energy should be conserved. In these cases, there is reduction in sympathetic activity to BAT (Young *et al* 1982; Yoshida *et al* 1983; young and Landsberg, 1977; Sakaguchi *et al* 1988; Reichling *et al* 1987; Trayhurn and Jennings, 1988; Villarroya *et al* 1987), and thus decrease in BAT thermogenic activity and capacity (BAT atrophy) (Desautels, 1985; Desautels *et al* 1986; Desautels *et al* 1990; Desautels and Dulos, 1988; Trayhurn and Jennings, 1988; Agius and Williamson, 1980; Williamson, 1980). In mice, there is also marked tissue atrophy during denervation (Desautels and Dulos, 1990). BAT atrophy is generally characterized by loss of tissue cellularity (DNA content), tissue total protein content, tissue mitochondrial protein content, and mitochondrial content of UCP1 (Desautels and Dulos, 1988; Muralidhara and Desautels, 1994; Desautels, 1985; Trayhurn *et al* 1987; Desautels *et al* 1986; Trayhurn and Jennings, 1988; Trayhurn, 1989). Whereas loss of tissue cellularity is observed during deacclimation of mice (Desautels, 1985), it is not seen under conditions of fasting, denervation, or lactation (Desautels and Dulos, 1990; Desautels, 1985; Desautels and Dulos, 1988).

1.4.1 INTRACELLULAR PROTEOLYSIS IN BAT AND ITS IMPORTANCE

Changes in a tissue protein mass always depend on the balance between rates of protein synthesis and degradation (Blommaart *et al* 1997). BAT protein loss during conditions of BAT atrophy is associated with a fall in protein synthesis. For instance, a marked loss of UCP1 mRNA precedes the loss of UCP1 from the mitochondria during re-acclimation of cold-exposed animals to warm environments (Patel *et al* 1987; Reichling *et al* 1987), and fasting (Champigny and Ricquier, 1990). Increase in intracellular proteolytic activity is also likely an important contributing factor to brown fat protein loss, particularly in cases when the tissue cellularity is unchanged such as during fasting, lactation, and denervation (Desautels and Dulos, 1990; Desautels, 1985). Brown fat has a large capacity for lysosomal proteolysis as suggested by specific activities of lysosomal proteases in BAT homogenates larger than in many other organs (Desautels *et al* 1990). Lysosomes are the organelles of intracellular protein degradation (Dunn, 1994; Seglen and Bohley, 1992). Containing 15-20 proteolytic enzymes such as cathepsins B, H, L, and D as well as numerous other hydrolases, lysosomes are well equipped to degrade the variety of proteins (e.g. membrane proteins, and proteins with long half-lives) and other macromolecules which are continually transported to these organelles from other regions of the cell (Seglen and Bohley, 1992). The bulk of material is delivered to lysosomes by the process known as autophagy, which involves three main steps (Schellens and Meijer, 1991): (a) sequestration, during which cytoplasmic components are surrounded by a membrane that is probably derived from the endoplasmic reticulum. Sequestration results in the formation of an autophagosome; (b) fusion, during which

autophagosomes acquire proteolytic enzymes by fusion with lysosomes, resulting in formation of autolysosomes; and (c) digestion, which is the enzymatic degradation of sequestered material inside the autolysosomes (Schellens and Meijer, 1991). The bulk loss of BAT proteins and mitochondrial proteins during a 24-hour fast in mice likely results from activation of the lysosomal proteolytic pathway (Desautels *et al* 1990). When mice acclimated at 4°C were fasted overnight, which caused a marked increase in protein loss, increases in specific activities of cathepsins D and L were observed. Selective increases in cathepsins D and L activities during accelerated BAT protein loss are consistent with the major role in intracellular protein degradation generally ascribed to these enzymes (Beynon and Bond, 1986). In the brown fat of 24-hour fasted mice kept at 21°C there was no change in the specific activities of the lysosomal proteases. Nevertheless, the lysosomal proteolytic pathway of protein degradation was clearly involved (Desautels *et al* 1990), as injections of chloroquine and leupeptin caused a marked reduction in the tissue protein loss (Desautels *et al* 1990). Chloroquine is an acidotropic agent that raises the lysosomal pH and is also an inhibitor of cathepsin B (DeDuve, 1983). Leupeptin strongly inhibits cathepsins B, and L, but is a weak inhibitor of cathepsin H (Barrett and Kirschke, 1981).

However, the above findings concerning the importance of lysosomal pathway in BAT protein loss during fasting, do not exclude the contribution of the other proteolytic systems in BAT such as that within mitochondria (Desautels *et al* 1990). This is emphasized by the observation that reductions of UCP1 content in isolated mitochondria during BAT atrophy lag behind the loss of mitochondrial proteins from the tissue and are not accompanied with corresponding reductions of

other proteins associated with the mitochondrial inner membrane, such as the adenine nucleotide transporter or succinate dehydrogenase (Desautels, 1985; Desautes *et al* 1986; Trayhurn and Jennings, 1988). Brown fat mitochondria possess soluble and membrane-associated proteases that may be involved in the more selective changes in the organelle protein composition during BAT atrophy (Desautels, 1992). Accordingly, Desautels and Dulos (1994) found that there is a protease within BAT mitochondrial membranes that may be involved in the breakdown of UCP1. Proteolytic activity within mitochondria may have many other functions (Desautels and Dulos, 1993). It is likely involved in the processing of precursor proteins imported into the organelle, in the removal of proteins with abnormal conformations (as may arise by mutations, error in synthesis, improper assembly into multimeric enzyme complexes, or post-translational damages), in the regulation of enzyme levels in response to changes in metabolic requirements, and in the general turnover of the proteins that constitute the organelles (Desautels, 1986; Desautels and Dulos, 1993).

1.4.2 ADRENERGIC CONTROL OF PROTEOLYSIS IN BAT

NE affects the amount of proteins in a brown fat cell. Change in the cell content of protein results from alterations in the balance between rates of synthesis and degradation (Blommaart *et al* 1997). NE stimulates protein synthesis (Waldbillig and Desautels, 1992) and inhibits protein degradation (Desautels and Heal, 1999). In brown adipocytes differentiated in culture, without serum or hormonal additions, approximately 2% of cell proteins are degraded per hour (Desautels and Heal, 1999). Addition of NE reduced protein degradation significantly (Desautels and Heal, 1999).

The inhibition of proteolysis by NE was observed only in fully differentiated brown fat cells, suggesting there is a requirement for a late event in the differentiation of brown fat cells (Desautels and Heal, 1999).

The effect of NE was dominantly due to inhibition of autophagy. Addition of inhibitors of sequestration step of autophagy; 3-methyl adenine, wortmannin, and 2-(4- Morpholinyl)-8- Phenyl- 4H-1- Benzopyran-4- one (LY294002), significantly reduced proteolysis almost to the same extent as NE (Desautels and Heal, 1999). Thus, NE and inhibitors of autophagic sequestration were likely affecting the same pathway (Desautels and Heal, 1999). However, this is not to say that reduction of autophagy is the only way by which NE inhibits proteolysis. When NE was added together with inhibitors of autophagy or lysosomal function, proteolysis was always slightly less than from addition of inhibitors alone (Desautels and Heal, 1999). This difference did not reach statistical significance, but suggested that NE may be affecting other proteolytic pathways within brown adipocytes, in addition to autophagy (Desautels and Heal, 1999).

The mechanism by which NE inhibits proteolysis in differentiated brown adipocytes involves the binding of NE to β_3-adrenergic receptor on the cell surface of mature brown adipocytes and a subsequent increase in intracellular cAMP level (Desautels and Heal, 1999). The concentration of propranolol required to inhibit even partially the effect of NE on proteolysis was much higher than required to inhibit β_1- or β_2-adrenergic receptor-mediated event (Desautels and Heal, 1999). This points to a β_3-receptor-mediated effect. In addition, the order of potency of adrenergic

agonists in reducing proteolysis (BRL-37344>isoproterenol>dobutamide) was consistent with an important role of the β_3-adrenoceptors. It was the same order of potency for stimulation of the lipolytic and thermogenic response in brown adipocytes. BRL-37344 is a β_3-adrenergic agonist, isopreterenol is a β_1/β_2-adrenergic agonist, and dobutamide is a β_1-adrenergic agonist. Addition of forskolin, an activator of AC, also inhibited proteolysis to the same extent as NE, likely underlying its mechanism of action (Desautels and Heal, 1999).

However, the mechanism responsible for NE inhibitory action on proteolysis in mature brown adipocytes beyond the increase in intracellular cAMP level is not understood. Intracellular cAMP level increases upon NE binding to the β_1-adrenergic receptors in brown pre-adipocytes, as well as upon NE binding to the β_3-adrenergic receptors in mature brown adipocytes (Bronnikov *et al* 1999). Since NE does not have inhibitory effect on proteolysis in brown pre-adipocytes (Desautels and Heal, 1999), it points to the appearance late in differentiation of a protein, perhaps a protein or lipid kinase, to couple β_3-adrenergic receptor function and proteolysis (Desautels and Heal, 1999, Figure 1.5). Changes in protein and lipid phosphorylation are involved in the control of autophagy (Blommaart *et al* 1997). Another possibility is a link between NE-dependent uncoupling of metabolism and proteolysis, since expression of UCP1 is also a late event in differentiation (Desautels and Heal, 1999, Figure 1.5). NE-induced uncoupling of mitochondria may be important to reduce the cell content of ATP. Some studies (Pettersson and Vallin, 1976; LaNoue *et al* 1982), but not all (Waldbillig and Desautels, 1992), have shown a reduction in cell ATP content upon NE addition to brown fat cells isolated by collagenase digestion.

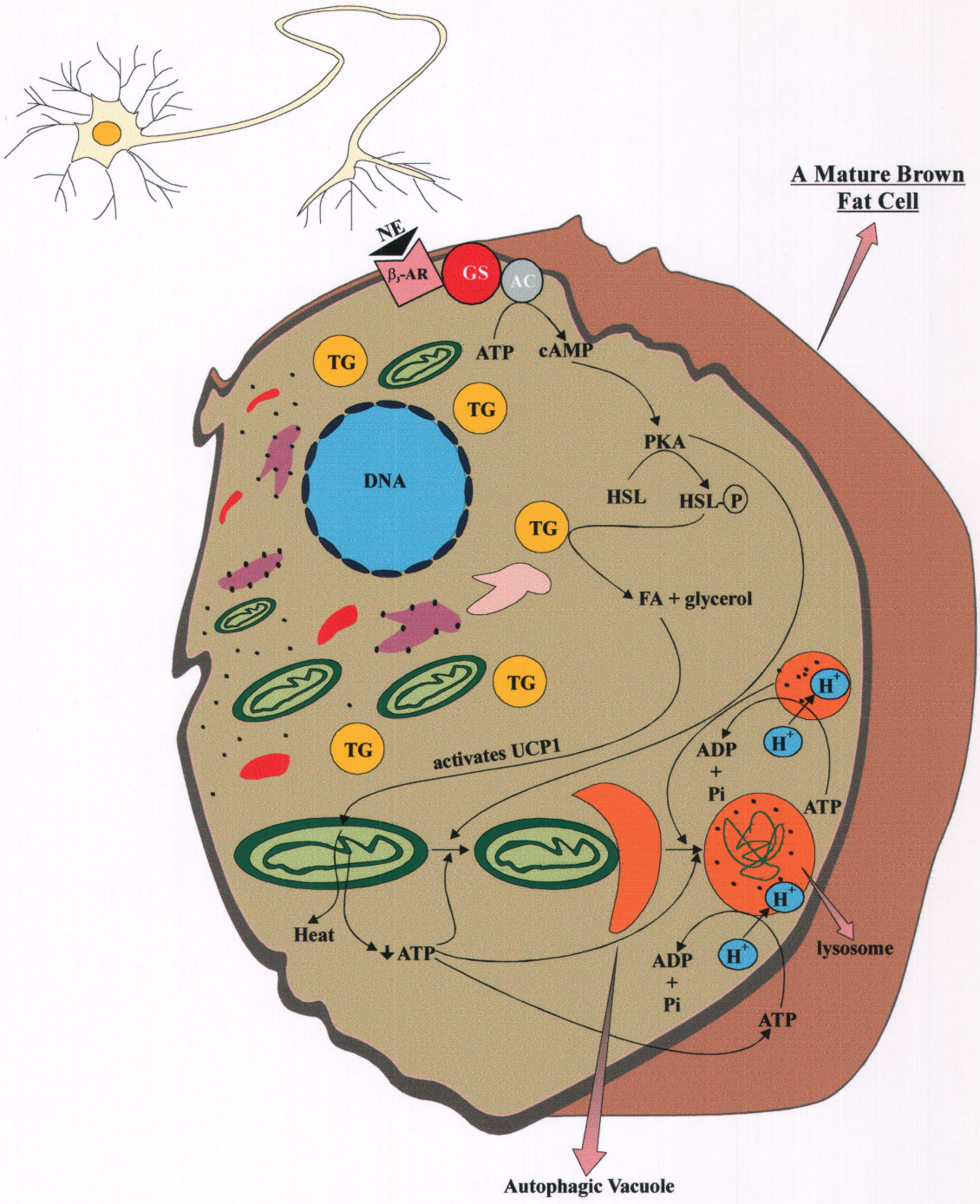


Figure 1.5 Possible mechanisms for inhibition of proteolysis by NE released from sympathetic nerves adjacent to brown adipocytes. Upon binding to β_3-adrenergic receptor on the cell surface, NE may activate a protein or lipid kinase that inhibits proteolysis. Furthermore, NE could inhibit proteolysis by activation of UCP1 and subsequent reduction in ATP level.
β_3-AR = β_3-adrenergic receptor
TG = Triglyceride Store

Autophagy in hepatocytes is susceptible to relatively small changes in ATP (Schellens and Meijer, 1991; Schellens *et al* 1988). ATP is required for each step involved in autophagy: for sequestration, vesicle fusion, and intralysosomal proteolysis (Plomp *et al* 1989, 1987; Schellens and Meijer, 1991). Furthermore, it was demonstrated that there is a linear relationship between intracellular ATP and autophagic proteolysis in hepatocytes (Schellens and Meijer, 1991). In addition to the energy dependence of autophagic lysosomal pathway of protein degradation shown in hepatocytes, there is proteolytic activity within mitochondria that also requires ATP (Langer and Neupert, 1996). BAT mitochondria possess a proteolytic system capable of degrading proteins (Desautels, 1992). This proteolytic activity requires ATP as it is inhibited by addition of atractyloside, an inhibitor of adenine nucleotide transport across the inner membrane, and by addition of arsenate and vanadate, two phosphate analogs known to inhibit ATPase enzymes (Desautels and Dulos, 1993). The requirement of ATP is likely for the activity of ATP-dependent proteases located in the membrane and matrix of the organelle (Desautels, 1992).

OBJECTIVE

The objective of this work is to evaluate the mechanisms by which NE inhibits proteolysis in brown fat cells. The hypothesis is that the inhibitory effect of NE on proteolysis in differentiated brown adipocytes is linked to activation of uncoupled mitochondria and a subsequent reduction in intracellular ATP level.

2.0 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Aprotinin, ATP assay mix (# FL-AAM), ATP dilution buffer (# FL-AAB), ATP releasing reagent (# FL-SAR), Dexamethasone, Dithiothreitol, DMEM, Glycerol, Insulin, Nonidet, NE, Palmitic acid, Paraformaldehyde, Penicillin G, Phenyldiamine, Phenyl methyl-sulfonyl fluoride, Poly-L-lysine, Sodium fluoride, Texas Red-conjugated goat anti-rabbit IgG, Thyroid hormone (T_3), and Triton X-100 were obtained from Sigma Chemical Company.

Bovine serum albumin (BSA), Fetal calf serum (Cellect-Silver), Hepes, Mercaptoethanol-2, Methionine, Oil red O, Pantothenic acid, and Tricine were purchased from ICN Biomedicals, Inc. Biotin, Leupeptin, Pepstatin, and Streptomycin from Calbiochem Company. Coomassie brilliant blue G-250, Sodium dodecyl sulfate, and Tween 20 were obtained from Bio-Rad.

Collagenase was from Worthington Biochemical Corporation (CLS type1), Bromopalmitic acid from Aldrich Chemical Company, $MgSo_4$ and Sodium pyrophosphate from J.T. Baker Chemical Company, Sodium Chloride from EM Science, Citifluor AF1 from Marivak Ltd, and Acs scintillator fluid was from Amersham.

Ascorbic acid, EDTA, and TCA were obtained from BDH Chemicals. Bromophenol Blue and Sodium vanadate were from Fisher Scientific Company.

2.2 CELL CULTURES

2.2.1 CULTURES OF BROWN ADIPOCYTES

Newborn mice (CD1 strain, 3-4 days old) were killed by decapitation and brown adipose tissue was dissected under sterile conditions and placed in a small volume of DMEM (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 33 μM biotin, 17 μM pantothenic acid, 100 μM ascorbic acid, 100 μg/mL streptomycin, 6 μg/mL penicillin G, and 4% (w/v) bovine serum albumin (BSA), previously kept for 30 minutes at 37°C under 95% O_2 and 5% CO_2 atmosphere. Animal protocols were reviewed and approved by the University Committee on Animal Care and Supply. The tissue was cut in small pieces with a sterile scalpel blade on a plastic petri dish and transferred to a 50 mL sterile centrifuge tube containing 6 mL DMEM + BSA as well as 1 mg/mL collagenase (Worthington; CLS type 1). The tissue was incubated for 20 minutes at 37°C under 95% O_2 and 5% CO_2 gas atmosphere with shaking (vortex, 10-15 shakes at setting=4 on vortex mixer) every 2.5 minutes. Digestion mixture was filtered through a sterile nitex mesh and added to 30-40 mL DMEM + BSA. Mature brown fat cells were allowed to float for 20 minutes at room temperature and the pre-adipocyte fraction was spun down at 2500 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 10 mL DMEM + BSA by repeated pipetting, filtered through nitex mesh and the volume brought up to 45 mL with DMEM + BSA. Two mL of cell suspension was added to 35 mm cell culture dishes in sterile cabinet. Cell cultures were kept in a CO_2 incubator (5% CO_2 and 95% air) at 37°C and 100% relative humidity. After 1 or 2 hours, cell suspension was aspirated off and the cells bound to

the culture dishes were washed once with 2 mL DMEM. Culture medium (2 mL) was then added and culture dishes were returned to CO_2 incubator. Culture medium consisted of DMEM with antibiotics, biotin, pantothenic acid, ascorbic acid, and 10% (v/v) fetal calf serum (ICN, Cellect-Silver). It was prepared with sterile water and filtered through sterile nitrocellulose filter prior to use. Culture medium was replaced every second to third day. Once the cell cultures reached confluence (about day 5), 3, 5, 3'-triiodothyronine (T_3, 1 nM) and insulin (50 nM) were added to the culture medium to stimulate cell differentiation. Culture medium (with T_3 and insulin) was replaced every second day until most or all cells acquired brown fat phenotype (about 5 more days). Differentiation of brown adipocytes was followed by the appearance of fat vacuoles detected by light microscopy and of UCP1 detected by immunofluorescent microscopy and Western blotting.

2.2.2 CULTURES OF 3T3-L_1 ADIPOCYTES

3T3-L_1 adipocytes were obtained from Dr. D. Anderson from the Saskatoon Cancer Research Center. They were originally from the American Types Tissue Culture Collection (ATCC, cat # CL-173). Growth and differentiation of 3T3-L_1 cells were carried out as described by Green and Meuth (1974).

3T3-L_1 adipocytes in 35 mm cell culture dishes were allowed to grow in the presence of DMEM and 10% (v/v) fetal calf serum in the CO_2 incubator (5% CO_2, 95% air, 100% relative humidity), exactly the same as brown adipocytes. Culture medium was replaced every second day until cells reached confluence (day 5). Upon confluence, dexamethasone (1μM) was added to the culture medium in addition to T_3

(1nM) and insulin (50 nM) to stimulate cell differentiation. Culture medium (DMEM supplemented with antibiotics, vitamins, 10% (v/v) fetal calf serum, T_3, insulin and dexamethasone) was replaced every second day. Differentiation of 3T3-L_1 adipocytes was followed by the appearance of fat vacuoles detected by light microscopy. UCP1 immunostaining was also done for 3T3-L_1 adipocytes to show the absence of UCP1 expression in these adipocytes.

2.3 LIGHT AND IMMUNOFLUORESCENCE MICROSCOPY

2.3.1 PREPARATION OF COVERSLIPS FOR CELL CULTURES

Coverslips were dropped into a beaker containing soap and hot water for 30 minutes. To remove the soap, beaker was placed under gentle running hot water for 5 minutes. Coverslips were then washed with distilled water and 2x with 95% ethanol, each time for 30 minutes. Coverslips were placed in a sterilizing oven overnight to remove the excess alcohol and to dry the coverslips. Coverslips were added to poly-L-lysine solution (0.001 g in 100 ml sterile distilled water) for 2-24 hours. The coverslips were then dried on sterile paper towel in the biological safety cabinet, placed in the appropriate petri plates (35 mm Falcon culture dishes) and stored at 4°C, until use.

2.3.2 CELL FIXATION AND UCP1 IMMUNOSTAINING

Adipocytes were grown on the coverslips for 4-12 days (4 days for brown pre-adipocytes and 10-12 days for mature brown adipocytes and 3T3-L_1 adipocytes). Cells were washed 3x with phosphate-buffered saline (PBS) and incubated for 30

minutes with paraformaldehyde (4% w/v) in PBS. Cells were then washed 3x with PBS and incubated for 30 minutes in PBS + BSA (1% w/v) + triton X100 (0.1% v/v). Cells were washed in PBS for 3x, and stored in PBS + azide (0.04% w/v) at 4°C.

For UCP1 immunostaining, polyclonal antibodies from rabbit antiserum against UCP1 [1:320 dilution in PBAL (PBS, 1% w/v BSA, and 0.1 M lysine)] was added to the cell monolayers and adipocytes were incubated at 4°C overnight on a shaking platform. Antiserum against UCP1 was prepared in our laboratory (Desautels, 1985). To adsorb contaminating antibodies to other mitochondrial proteins and increase the specificity of the antiserum to UCP1, antiserum against UCP1 was pre-incubated for 2 hours at room temperature with sonicated rat heart mitochondria (Desautels and Dulos, 1994). Heart mitochondria do not have UCP1 but contain all other proteins associated with mitochondria. Cell monolayers were washed 5x in PBAL with the last wash for 20 minutes. The second antibody (Texas Red-conjugated goat anti-rabbit IgG, from Sigma) with 1:320 dilution in PBAL was added to the cell monolayers in the dark at room temperature for 2 hours. Cell monolayers were washed in the dark 5x using PBAL with the last wash for 20 minutes. The coverslips were mounted onto microscope slides using 10 µl Citifluor AF1 (an anti-fading agent) mounting solution (Marivak Ltd) supplemented with 1 mg/mL p-phenyldiamine, and sealed with nail polish. Cell monolayers were examined with a Reich Fluorescent microscope. Kodak Technical Pan Films were used for photography.

2.3.3 OIL RED O STAINING

For staining fat vacuoles, cell monolayers were incubated at room temperature with oil red O dye (4% w/v in PBS) for 30 minutes. Cell monolayers were washed 3x with PBS, and coverslips were mounted onto microscope slides in the same way stated above.

2.4 CELL PROTEIN LABELING AND MEASUREMENTS OF PROTEIN DEGRADATION

2.4.1 PROTEIN LABELING AND ESTIMATION OF SPECIFIC ACTIVITY

Standard cell culture medium was aspirated off and replaced by cell culture medium with reduced methionine content for protein labeling. Cell culture medium for protein labeling was made of 10% (v/v) fetal calf serum, 10% (v/v) DMEM, and 80% (v/v) methionine-free DMEM. Supplementation with vitamins and antibiotics was as in standard DMEM. [^{35}S] methionine was added at 5μCi/mL. Cell cultures were kept in the CO_2 incubator for 72 hours. Reduced methionine content of the culture medium did not affect the apparent differentiation of either 3T3-L_1 cells or brown fat cells.

The specific activity of labeled cell proteins, i.e., the amount of label amino acid incorporated (cpm incorporated) per μg protein in culture dish was estimated at time= 0 hour: After cell collection and disruption by sonication into 1 mL 0.5 N NaOH, 0.85 mL was transferred to a microcentrifuge tube and mixed with 0.15 mL trichloroacetic acid [TCA (100% w/v)] to precipitate cell proteins. After at least 1 hour at 4°C, acid precipitated proteins in aliquots of 0.4 mL (for brown pre-

adipocytes) and 0.1 mL (for mature brown adipocytes and 3T3-L_1 adipocytes) were collected by filtration (whatmann GF/C glass fiber filters), washed 5x with 5 mL TCA (10% w/v) + 5mM methionine (TCA + Met), and counted using 10 mL of Amersham ACS scintillator fluid.

For estimation of protein content, acid precipitated proteins (from 0.5 mL aliquots) were collected by centrifugation, re-solubilized in 0.5 N NaOH and used for protein estimation by the method of Bradford (1976). Specific activity of labeled proteins were then calculated as cpm [^{35}S]/µg protein.

2.4.2 ESTIMATION OF RATES OF PROTEIN DEGRADATION

After 3 days labeling period, cell monolayers were washed 3x with 2 mL of DMEM +2 mM unlabelled methionine and incubated with 2.5 mL of DMEM +2 mM unlabelled methionine and other additions, dependent upon the experiment. At time 0 hour and various times after addition (up to 4 hours), 0.25 mL culture medium was collected into microcentrifuge tubes and mixed with 0.035 mL TCA (100% w/v) and 0.015 mL BSA (5% w/v). After at least 1 hour at 4°C, samples were centrifuged for 5 minutes, and 0.15 mL of acid-soluble supernatant was added to 4 mL of Amersham ACS scintillator fluid and counted. Protein degradation was measured as the release of acid-soluble radioactivity from the cells into the cell culture medium per unit time, after subtraction of residual acid-soluble radioactivity at time= 0 hour.

2.5 CELL PROTEIN CONTENT

The Bio-Rad protein assay by Bradford (1976) was used for protein estimation using BSA as protein standard. Protein content of samples was calculated using a standard curve relating the change in OD600 when dye reagent (Coomassie brilliant blue G-250) reacts with the protein, against the concentration of the protein in the standard. There was a linear relationship between the amount of the protein in the standards (over the range 0-10 μg) and changes in OD600 (R^2= 0.99).

2.6 CELL CONTENT OF ATP

Culture ATP content was measured with Sigma's bioluminescent somatic cell assay kit # FL-ASC. Cell monolayers were washed 3x with PBS and collected in 1mL ice cold Sigma's somatic cell ATP releasing reagent (Sigma # FL-SAR). The samples were diluted 1:100 in ATP assay mix dilution buffer (25 mM tricine, 5mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 100 μg BSA). Twenty μl was added to cuvettes containing 50 μl ATP assay mix (Sigma # FL-AAM, containing luciferine and luciferase), 100 μl cell ATP releasing reagent, and 30 μl ATP assay mix dilution buffer (Sigma # FL-AAB). Light emission in millivolts, which is proportional to the ATP content of the sample, was measured with an LKB 1251 luminometer set at peak mode and room temperature. ATP content of the samples was evaluated using a standard curve attained by plotting the logarithm of the relative light intensity generated by luciferine-luciferase reaction against the logarithm of the concentration of ATP in the standard solutions. There was a linear relationship

between the concentration of ATP in standards (over the range 0-1392 fentomoles) and the relative light intensity generated by luciferine-luciferase reaction (R^2>0.96).

2.7 CELL CONTENT OF UCP1

Cell monolayers were washed 3x with PBS at room temperature. The cells were collected in 1mL ice cold lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM sodium chloride, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM sodium vanadate, 1% v/v Nonidet P40, 0.2 mM phenylmethylsulfonylfluoride, 5 µg/mL each of leupeptin, pepstatin, aprotinin, and stored at -80°C. Samples of whole cell lysates were mixed with an equal volume of 2X sample buffer containing 62.5 mM Tris-base, 4% w/v SDS (sodium dodecyl sulfate), 5% v/v 2-mercaptoethanol, 10% v/v glycerol and Bromophenol Blue, pH 6.8. Aliquots of cell lysates in sample buffer containing 20 µg protein, as well as 5 µl of Bio-Rad pre-stained molecular weight standards were loaded on 15% (w/v) acrylamide: 0.4% (w/v) bis-acrylamide gel. Proteins on the gel were separated depending on their size. Proteins were then electrophoretically transferred from the gel to a nitrocellulose membrane. The membrane was blocked at 4°C overnight in a covered container containing blocking solution [5% (w/v) defatted milk in 0.05% (v/v) TTBS (Tween 20 in tris-buffered saline)]. Membrane was removed from the blocking solution and washed 2x, each time for 5 minutes, in a tray containing TTBS. The membrane was incubated for 1 hour with primary antibody solution (rabbit polyclonal antibody against UCP1) at 1:1000 dilution in 5% (w/v) defatted milk in TTBS. The unbound antibody was removed by washing the membrane 4x in TTBS,

each time for 5 minutes, with gentle agitation. The secondary antibody [horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG, from Bio-Rad] was added at 1:3000 dilution in 5% (w/v) defatted milk in TTBS to the membrane, and membrane was incubated at room temperature for 1 hour. Nitrocellulose membrane was washed 4x, each time for 5 minutes with TTBS, and incubated in chemiluminescence luminol reagent [a commercial ECL kit (Amersham)] for 1 minute and exposed to Kodak film.

2.8 STATISTICAL ANALYSIS

Differences between groups were examined by one-way and two-way ANOVA and Duncan's multiple range tests for comparisons between means. NCSS statistical software (Kaysville, UT) was used for computation. The results were considered statistically significant when $P<0.05$. Results are presented as means ± standard error (S.E.).

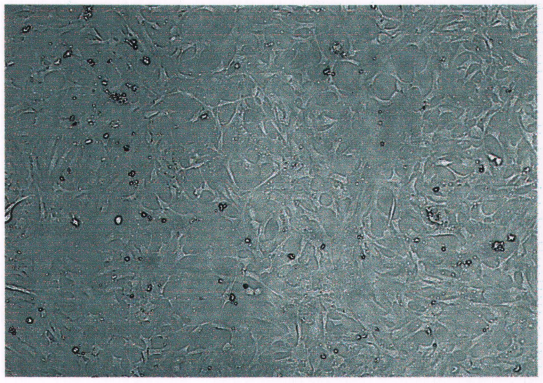
3.0 RESULTS

3.1 DIFFERENTIATION OF BROWN AND 3T3-L_1 ADIPOCYTES IN CULTURE

After isolation from mouse interscapular region, brown pre-adipocytes grew rapidly until confluence (at about day 5) in the presence of DMEM supplemented with fetal calf serum. Upon reaching confluence, brown pre-adipocytes were allowed to differentiate into mature brown adipocytes in the presence of T3 and insulin for 7 days (days 5-12). Immunostaining for UCP1 and staining of fat vacuoles using oil red O dye were done to confirm the differentiation of brown pre-adipocytes to mature cells. Brown pre-adipocytes (day 4 cells) are fibroblast-like adipose cells that are rapidly dividing and do not contain fat droplets (Figure 3.1 A). Mature brown adipocytes (day 10-12 cells), on the other hand, are round cells with several fat droplets of different sizes (Figure 3.2 A). The most important distinction is expression of UCP1. UCP1 immunostaining shows that the protein is present in mature brown adipocytes (Figure 3.2 B) and absent from brown pre-adipocytes (Figure 3.1 B). The antibody against UCP1 is specific so that the pre-immune serum (serum collected from the same animal prior to immunization) in the same concentration as serum against UCP1, does not give any immunostaining in mature brown adipocytes (Figure 3.2 C). Western blots using antibody against UCP1 also confirm the presence of the protein in mature brown fat cells (Figure 3.15).

3T3-L_1 line is a pre-adipose, fibroblast-like cell line that has the ability to accumulate triglyceride fat and obtain the characteristics of the mature white adipose cells. 3T3-L_1 cells were grown to confluence (about day 5) in the presence of DMEM

(A)



(B)

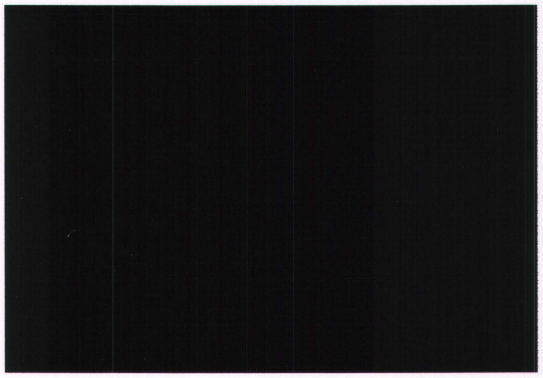


Figure 3.1

Brown pre-adipocytes after 4 days in culture. (A) The fibroblast-like appearance and absence of fat vacuoles are clear. Magnification is 200X. (B) The absence of UCP1 from brown pre-adipocytes is evident by immunofluorescent microscopy using antibody against UCP1.

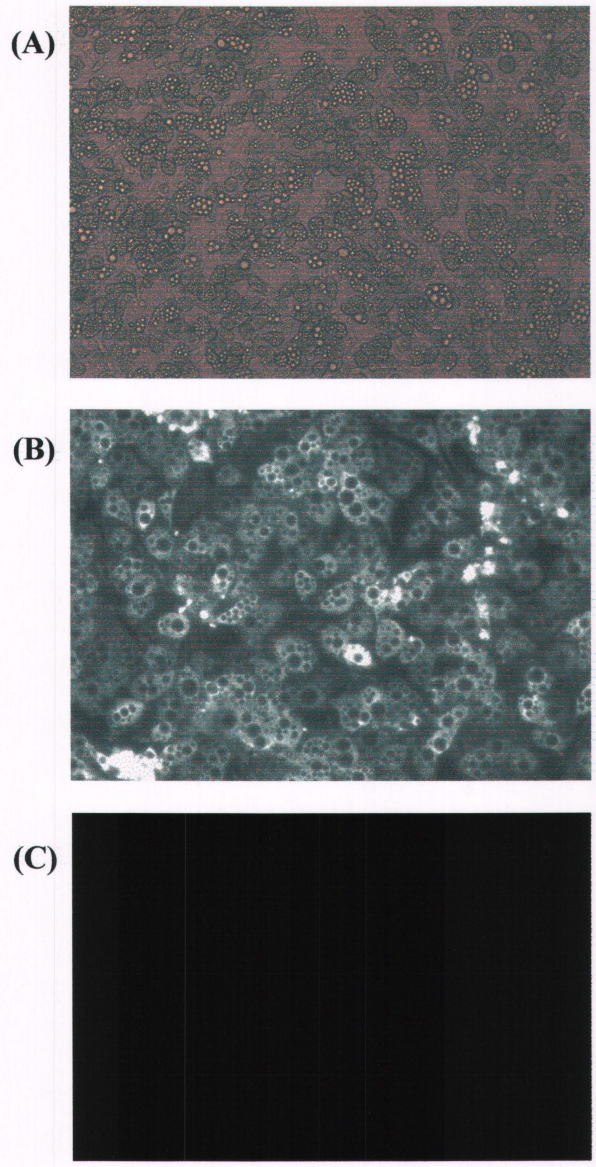


Figure 3.2

Mature brown adipocytes after 10 days in culture. (A) Appearance of multiple small fat vacuoles is evident. Magnification is 200X. (B) Presence of UCP1 in mature brown fat cells is visualized by immunofluorescent microscopy using antibody against UCP1. Magnification is 400X. (C) There is no immunofluorescence using pre-immune serum.

supplemented with fetal calf serum, the same as brown pre-adipocytes. Upon reaching confluence, the conversion of 3T3-L_1 cells from pre-adipose to mature white adipose cells was stimulated by addition of dexamethasone as well as T3 and insulin for 7 days (days 5-12). Staining of fat vacuoles using oil red O was done to follow the differentiation of 3T3-L_1 adipocytes. The same as mature brown fat cells, 3T3-L_1 adipocytes are round cells with several fat droplets of different sizes (Figure 3.3 A). The size of fat droplets, however, appears to differ between mature brown fat cells and 3T3-L_1 adipocytes, being larger in 3T3-L_1 adipocytes (Figures 3.3 A and 3.2 A). Immunostaining for UCP1 reveals the important distinction between mature brown fat cells and 3T3-L_1 adipocytes. In contrast to mature brown fat cells, 3T3-L_1 adipocytes lack UCP1 (Figures 3.3 B and 3.2 B). Western blots using antibody against UCP1 also confirm the absence of the protein in 3T3-L_1 adipocytes (Figure 3.15).

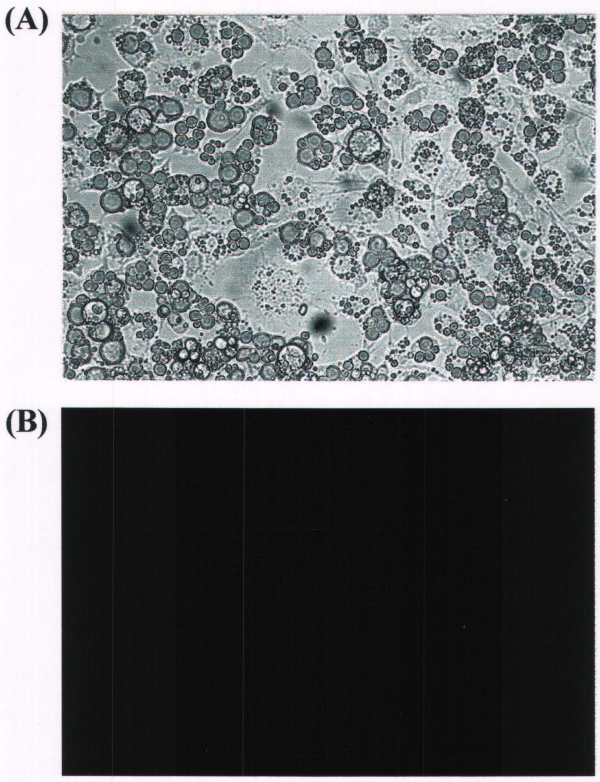


Figure 3.3

3T3-L_1 adipocytes after 10 days in culture. (A) Presence of multiple fat vacuoles with relatively large size is evident. Magnification is 200X. (B) Immunofluorescence of 3T3-L_1 adipocytes using antibody against UCP1, shows the absence of the protein in these cells.

3.2 NE INHIBITS PROTEIN DEGRADATION IN MATURE BROWN ADIPOCYTES BUT NOT IN PRE-ADIPOCYTES OR 3T3-L_1 ADIPOCYTES

BAT atrophy caused by fasting and return of cold-acclimated animals to thermoneutrality is associated with suppression of sympathetic activity to BAT. Moreover, surgical denervation of BAT partially mimics the tissue atrophy. The atrophy is characterized by rapid loss of mitochondrial proteins and slower loss of UCP1 from the mitochondria (Young *et al* 1982; Rothwell *et al* 1984; Trayhurn and Jennings, 1986; Desautels *et al* 1986; Trayhurn *et al* 1982; Desautels and Dulos, 1990; Desautels *et al* 1990). These observations suggested that NE might have an inhibitory influence on protein degradation in BAT. Furthermore, it was recently found that NE inhibits protein degradation in mature brown adipocytes differentiated in culture, via a β_3-adrenergic receptor-mediated increase in cAMP level, possibly contributing to brown fat growth and atrophy (Desautels and Heal, 1999). The inhibition of proteolysis by NE in brown adipocytes was an event dependent on the differentiation-state of the cell; i.e., it could not be seen in pre-adipocytes.

In the present work, the effect of NE on protein degradation was measured as described in Methods. Mature brown adipocytes were incubated with [^{35}S] methionine over 72 hours to uniformly label all cellular proteins. Labeling period and conditions (reduced methionine content of the culture medium) did not change the appearance of adipocytes. After 72 hours of labeling period in the presence of [^{35}S] methionine, 99.1 ± 0.1% total radioactivity in brown adipocytes was present in acid-precipitable form (labeled proteins) and 0.9 ± 0.1% in acid-soluble form (n=3). After extensive washes to remove the excess label amino acid, the amount of

acid-soluble radioactivity outside the cells was negligible. Adipocytes were then incubated for a period of 4-hour in DMEM supplemented with 2 mM unlabeled methionine to prevent re-incorporation of labeled precursors. The time for measurement of protein degradation (4h) was chosen as brown fat cells differentiated in culture had a high rate of protein turnover which was linear over a 6h period (Desautels and Heal, 1999). The proportion of total radioactivity present in brown adipocytes in acid-precipitable form decreased linearly to 82.1 ± 2.1%, while the acid-soluble radioactivity released from the cells into the culture medium increased to 17.8 ± 7%. The proportion of acid-soluble radioactivity within brown fat cells decreased only slightly over 4 hours from 0.9 ± 0.1% to 0.6 ± 0.1%. Therefore, the release of acid-soluble radioactivity from cells into the culture medium over a period of 4 hours, as labeled cell proteins (acid-precipitable) were degraded, was used to calculate rates of protein degradation.

Mature brown adipocytes were incubated with DMEM without serum, supplemented with 2 mM unlabelled methionine, in the presence or absence of NE (1μM). NE was dissolved in 1% ascorbic acid (AA) and equivalent volume of AA solution was added to control cultures. Protein degradation in mature brown adipocytes over a 4 hour-period was linear (R^2=0.97, Figure 3.4). The rate of protein degradation estimated by linear regression was 27.2 ± 4.5 μg labeled proteins degraded/h in the absence of NE (Figure 3.4). In the presence of NE (1 μM), the rate of protein degradation was significantly (P=0.03) decreased to 16.1 ± 5.5 μg labeled proteins degraded/h (Figure 3.4). Calculated as the percent of acid-precipitable radioactivity (labeled proteins at T=0 h) released from the cells as acid-soluble

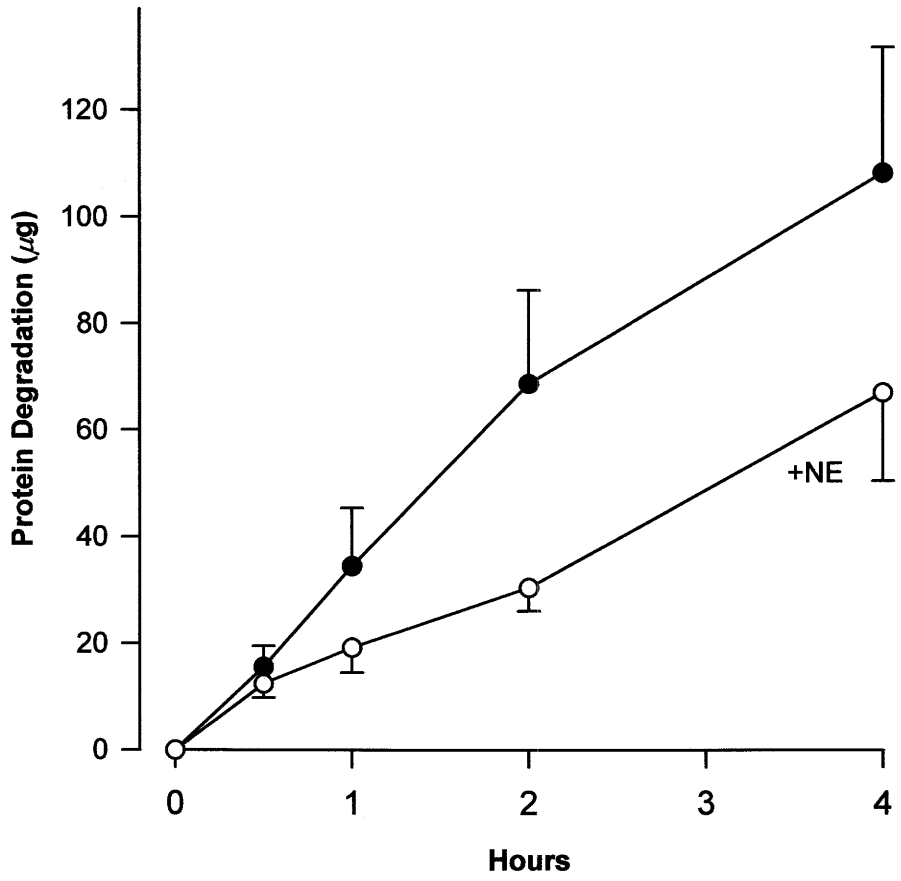


Figure 3.4

NE inhibition of protein degradation in mature brown adipocytes. Protein degradation is expressed as μg of labeled protein released from cells as acid-soluble products into the culture medium over a 4 hour period of incubation with (O) or without (●) NE (1μM). Results are means ± S.E., with n=3 separate cell cultures. Statistical analysis is given in the text.

products over 4 hours, the rate of proteolysis in mature brown adipocytes in the absence of NE represented 3.7 ± 0.9 % of the total labeled cell proteins turning over per hour (Figure 3.5). Addition of 1 μM NE, decreased protein degradation to 2.2 ± 0.5 % of labeled cell proteins turning over per hour (Figure 3.5). Furthermore, the inhibitory effect of NE on protein degradation in mature brown fat cells was within physiological range (0-100 nM). As NE concentration increased, protein degradation was significantly reduced ($P<0.0001$, Figure 3.6).

To evaluate if the inhibitory effect of NE on protein degradation was specific to differentiated brown fat cells, the effects of NE in brown pre-adipocytes, mature brown adipocytes and 3T3-L_1 adipocytes were compared. Inhibition of protein degradation by NE was only observed in differentiated mature brown fat cells, and not in brown pre-adipocytes or differentiated 3T3-L_1 adipocytes (Figure 3.5). The rate of protein degradation in brown pre-adipocytes was slightly higher than in mature brown fat cells or 3T3-L_1 adipocytes. In the absence of NE, the rate of proteolysis in brown pre-adipocytes was 4.2 ± 1.8 %/h, while it was 3.5 ± 0.9 %/h in differentiated 3T3-L_1 adipocytes, a rate similar to that in mature brown fat cells (Figure 3.5). In the presence of 1 μM NE, however, unlike the significant decrease shown in the rate of proteolysis in mature brown fat cells, there was no effect of NE observed on proteolysis in brown pre-adipocytes (4.1 ± 1.6 %/h, $P=0.9$, Figure 3.5) or in differentiated 3T3-L_1 adipocytes (3.3 ± 0.9 %/h, $P=0.8$, Figure 3.5).

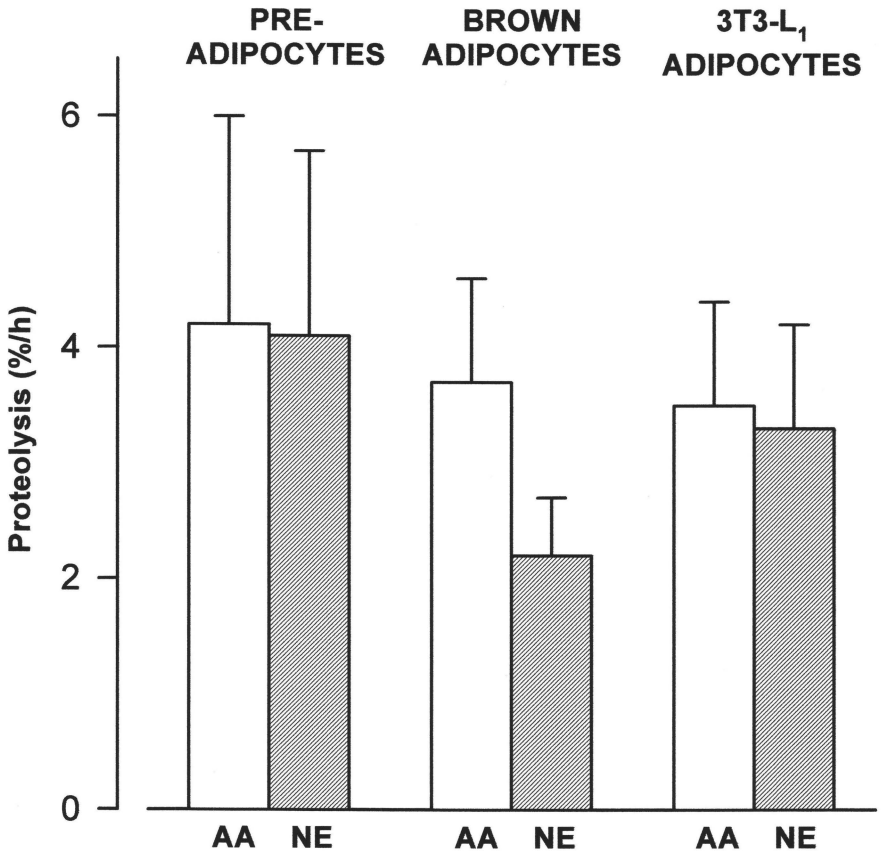


Figure 3.5

Effect of NE (1 μM) on proteolysis in pre-adipocytes, mature brown fat cells and 3T3-L_1 adipocytes. NE was prepared in ascorbic acid (AA) and equivalent volume of AA solution was added to control cultures. Results are expressed as % labeled proteins degraded to acid-soluble products per hour, estimated by linear regression. Results are means ± S.E., with n=3-7 separate cell cultures. Statistical analysis is given in the text.

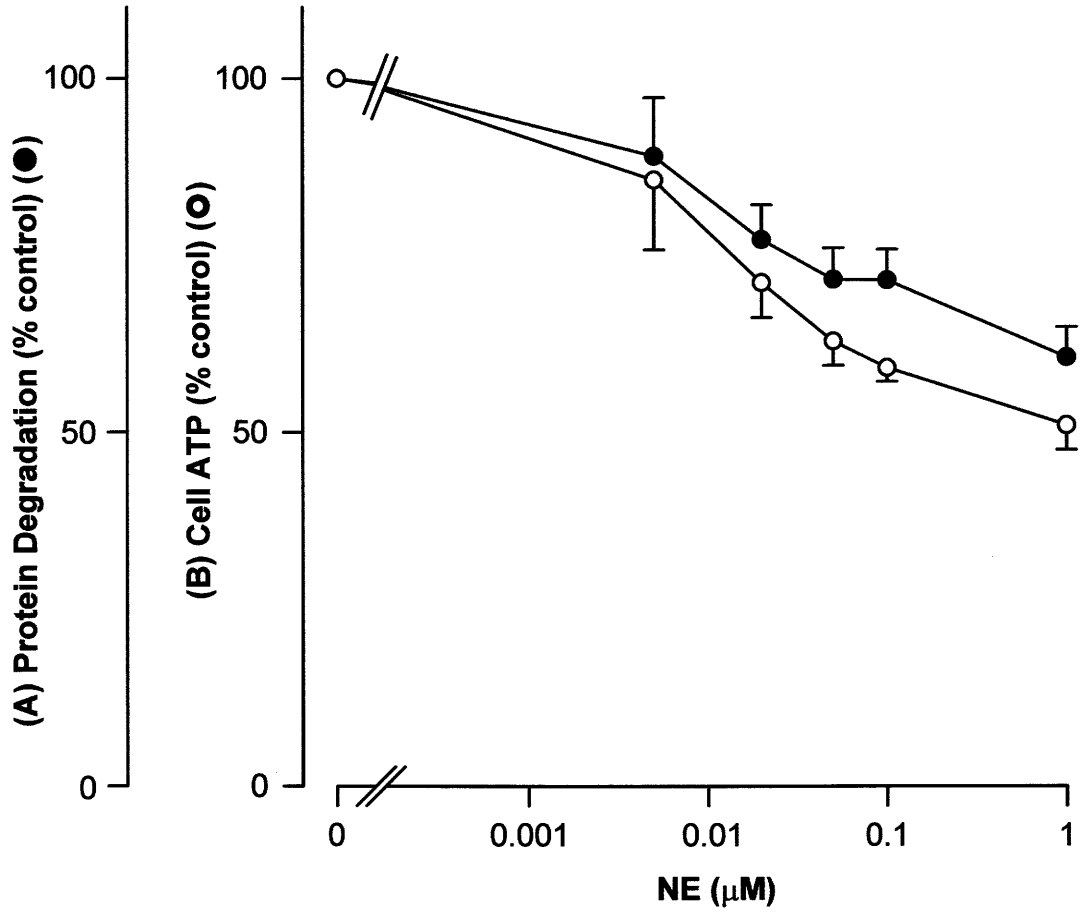


Figure 3.6

Effect of NE concentrations on (A) protein degradation and (B) cell ATP content in mature brown adipocytes. Results are expressed as % control where (A) 100% is 35±8 μg labeled proteins degraded to acid-soluble products in 4 hours in cells incubated with ascorbic acid (AA), and (B) 100% is 5.06±1.1 pmoles ATP/μg protein after 4 hours of incubation with AA. NE was dissolved in 1% w/v AA and equivalent volume of AA was added to cultures that served as control. Results are expressed as means ± S.E., with n=6 separate cell cultures. Statistical analysis is given in the text.

3.3 NE DECREASES ATP CONTENT IN MATURE BROWN ADIPOCYTES BUT NOT IN PRE-ADIPOCYTES OR 3T3-L_1 ADIPOCYTES

Since the inhibitory effect of NE on protein degradation is observed only in mature brown fat cells, it is possible that NE mediates its action via activation of UCP1, which is absent from brown pre-adipocytes and differentiated 3T3-L_1 adipocytes. It was previously shown that brown fat cells isolated from wild-type mice responded to NE with a large increase in thermogenesis, while in brown fat cells isolated from UCP1-ablated mice, NE had lost its ability to stimulate thermogenesis (Nedergaard *et al* 1999). Now the same situation may apply here. NE activates uncoupled mitochondrial respiration, and thus thermogenesis via activating UCP1 in mature brown adipocytes. The increase in heat production may result in a decrease in ATP production, which could affect protein degradation. In isolated rat hepatocytes, maintenance of ATP level is important for autophagic proteolysis (Blommaart *et al* 1997). It was shown that the consecutive steps of autophagy all respond to relatively small changes of intracellular ATP concentration. Furthermore, in mature brown fat cells, inhibition of protein degradation by NE was recently shown to be due at least in part to inhibition of autophagy (Desautels and Heal, 1999). Therefore, the focus of the present research was to evaluate if NE inhibits protein degradation by activation of UCP1 and reduction in intracellular energy level in mature brown fat cells. So, brown pre-adipocytes and 3T3-L_1 adipocytes were used as negative controls for these experiments.

ATP level in mature brown adipocytes was measured following an incubation period of 4 hours in the presence or absence of NE (1μM), and expressed as pmoles ATP/μg protein. In mature brown fat cells, ATP level was 12.2 ± 1.7 pmole/μg protein at time 0 hour and remained constant over 4 hours of incubation (P=0.8), as cell ATP level was 12 ± 1.2 pmole/μg protein after 4 hours (Figure 3.7). However, NE addition (1μM) under same conditions used to measure protein degradation, significantly decreased cellular ATP level (P=0.004) being 6.4 ± 2.3 pmole/μg protein after 4 hours of incubation (Figure 3.7). The effect of NE on reduction of cellular ATP level was rapid, as decrease in the cell ATP content was observed in the first 30 minutes and remained constant afterwards. The effect of NE on ATP content of mature brown fat cells was within physiological range (0-100 nM, Figure 3.6). It is noteworthy that there were corresponding changes in the NE concentration-dependent decreases in protein degradation and cellular ATP level (Figure 3.6).

A decrease in the cellular ATP content was observed only in mature brown fat cells and not in pre-adipocytes or differentiated 3T3-L_1 adipocytes. While the ATP content of mature brown adipocytes exposed to 1μM NE for a period of 4 hours was 53.1 ± 19.4% of the ATP content of cells incubated without NE (P= 0.005, Figure 3.8), similar decrease in the cellular ATP level was not observed in differentiated 3T3-L_1 adipocytes (P=0.3, Figure 3.8). In brown pre-adipocytes, cell ATP level was slightly greater after 4 hours of incubation with 1μM NE (P<0.05, Figure 3.8).

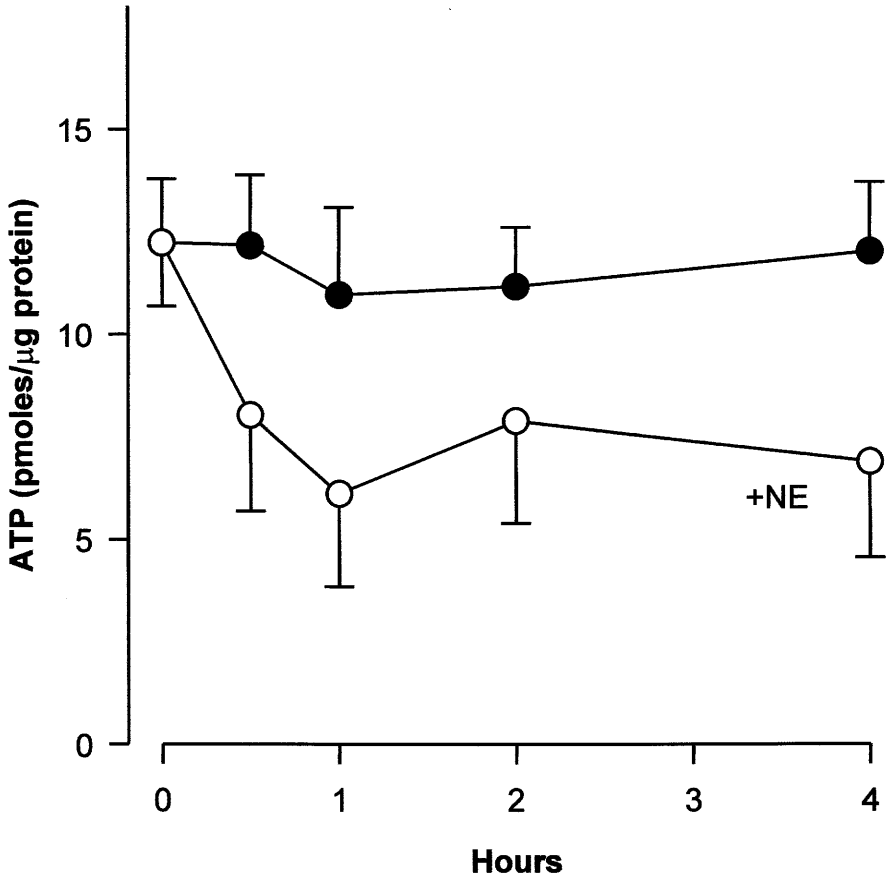


Figure 3.7

Time-course of changes in the ATP content of mature brown fat cells over 4 hours of incubation in the presence (O) or absence (●) of NE (1μM). Values are means ± S.E., with n=4 separate cell cultures. Statistical analysis is given in the text.

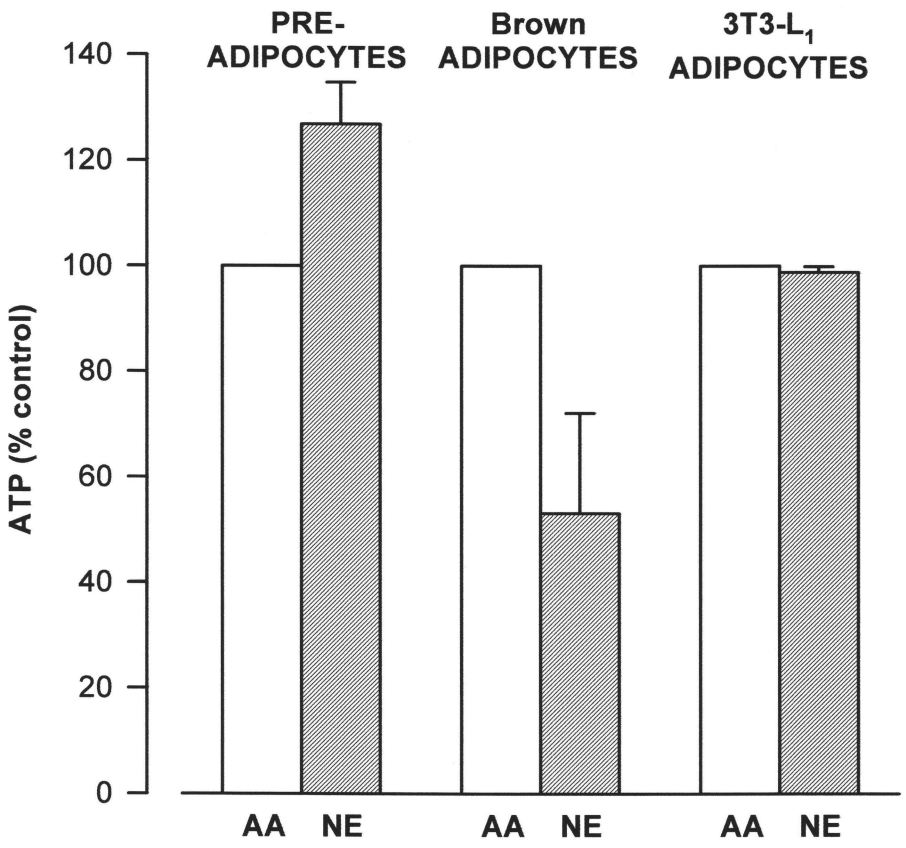


Figure 3.8

Comparison of changes in cell ATP content after 4 hours of incubation with 1 μM NE, in pre-adipocytes, mature brown fat cells and 3T3-L_1 adipocytes relative to cells incubated without NE [ascorbic acid (AA), 100% control]. NE was dissolved in 1% w/v AA and equivalent volume of AA was added to cultures that served as control. Results are means ± S.E., with n=3-7 separate cell cultures. Statistical analysis is given in the text.

3.4 PALMITIC ACID REDUCES PROTEOLYSIS AND CELL ATP LEVEL IN MATURE BROWN ADIPOCYTES BUT NOT IN PRE-ADIPOCYTES OR 3T3-L_1 ADIPOCYTES

FAs, like palmitic acid (16:0), have been known to function as intracellular physiological activators of UCP1 and to increase BAT thermogenesis (Locke and Nicholls, 1981; Locke *et al* 1982; Bukowiecki *et al* 1981; Jezek and Freisleben, 1994; Jezek *et al* 1995; Jezek *et al* 1996). Addition of FAs to isolated brown fat cells stimulates a thermogenic response which is absent in brown fat cells from UCP1-ablated mice (Nedergaard *et al* 1999). Therefore, to further evaluate the hypothesis that NE action on protein degradation in mature brown adipocytes is linked to activation of UCP1 and subsequent reduction in cell ATP content, the effect of palmitic acid was examined. Use of palmitic acid allows to by-pass the functions of NE-activated kinase A, and to evaluate if NE action on protein degradation and cell ATP content is linked to FA-dependent activation of UCP1.

Palmitic acid was dissolved in ethanol (20 mg/ml w/v) and then mixed with BSA (fatty acid free, 78 μM) with increasing molar ratio of palmitic acid:albumin (0, 1:1, 2:1, 4:1, 6:1 and 8:1). Mature brown adipocytes were incubated for a period of 4 hours, either alone or in the presence of 1μM NE to determine if exogenously added FA could mimic the effects of NE on the cell ATP content and proteolysis (Figure 3.9). FFA concentration in μM was calculated from the known affinity constant of albumin for FA and the equation $[FFA](\nu) = m\nu + b + Ce^{k\nu}$ (Richieri *et al* 1993). Parameters m, b, c, and k are known and FFA levels are determined as a function of the ratio (ν) of total FA to albumin (Richieri *et al* 1993).

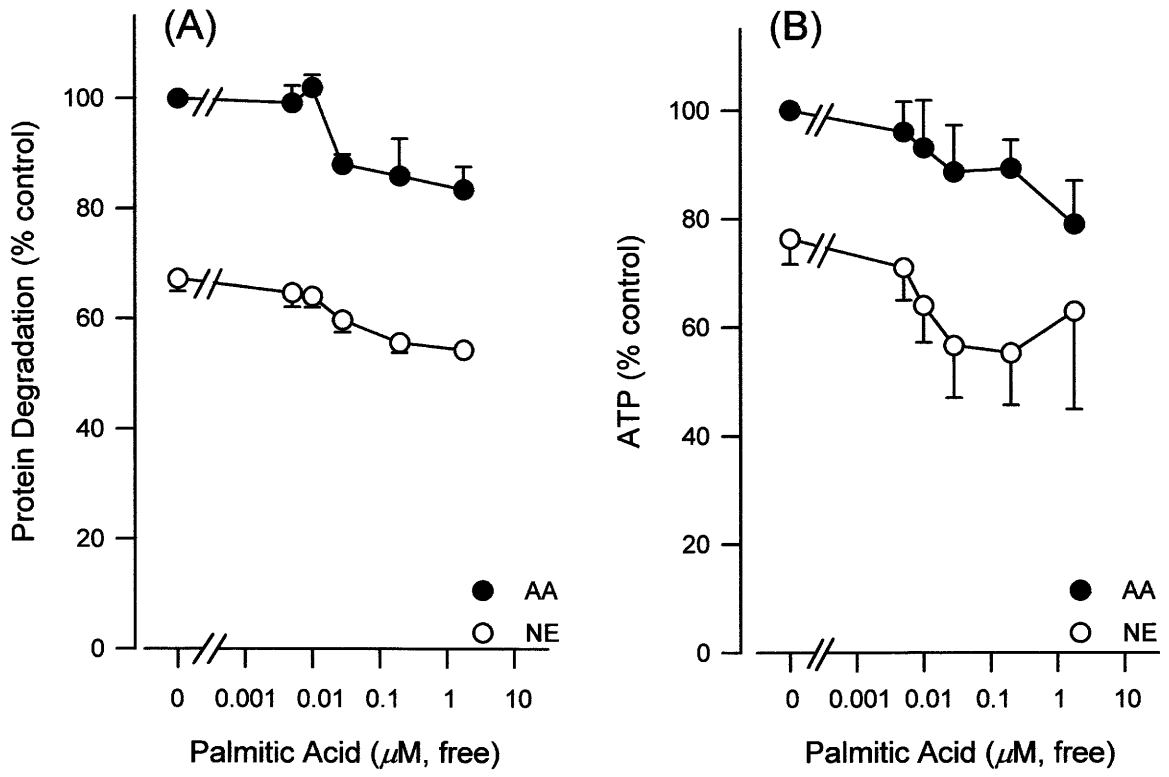


Figure 3.9

Inhibitory effect of palmitic acid on protein degradation (A) and cell ATP level (B) in mature brown adipocytes in the presence or absence of NE (1 μM). Results are expressed as % control where (A) 100% is 27.6±6 μg labeled proteins degraded to acid-soluble products in 4 hours in cells incubated with albumin, and (B) 100% is 7.3±1.9 pmoles ATP/μg protein after 4 hours in cells incubated with albumin. Results are described as means ± S.E., with n=7 separate cell cultures. Statistical analysis is given in the text.

Free palmitic acid concentrations corresponding to different molar ratios of palmitic acid:albumin of 0, 1, 2, 4, 6 and 8 were 0, 0.005, 0.01, 0.04, 0.3 and 2.5 μM, respectively. Addition of albumin and ethanol alone had no effect on either protein degradation or cellular ATP content. In the absence of albumin or ethanol, proteolysis was 35.6 ± 8.9 μg protein degraded to acid-soluble products in 4 hours, while it was 35.4 ± 10.8 and 37.5 ± 8.7 μg protein degraded/4hours in the presence of albumin ($P>0.5$) or ethanol ($P>0.5$) respectively. In the absence of albumin or ethanol, the cell ATP content was 10.1 ± 0.7 pmoles ATP/μg protein, while it was 11.4 ± 1.1 and 10.1 ± 0.9 pmoles ATP/μg protein in the presence of albumin ($P=0.4$) or ethanol ($P>0.5$), respectively.

Increasing palmitic acid concentrations caused significant reductions in protein degradation ($P<0.005$) in mature brown fat cells (Figure 3.9 A). The effect of palmitic acid was not as large as that of NE ($P<0.0001$, Figure 3.9 A). Two-way analysis of variance indicated no interaction between the effects of NE and those of palmitic acid ($P>0.5$). The same concentrations of palmitic acid also reduced cell ATP content but the difference did not reach statistical significance ($P=0.2$, Figure 3.9 B). The effect of palmitic acid was not as large as that of NE ($P<0.0001$) and there was no interaction between the effect of palmitic acid and that of NE ($P>0.5$).

The effect of palmitic acid on protein degradation and cell ATP content was also examined in brown pre-adipocytes (Figure 3.10) and 3T3-L_1 adipocytes (Figure 3.11). Brown pre-adipocytes and 3T3-L_1 adipocytes lack UCP1. Therefore, if NE inhibitory action on protein degradation in mature brown fat cells is linked to UCP1 activation, and subsequent reduction in the cell ATP content, then there should

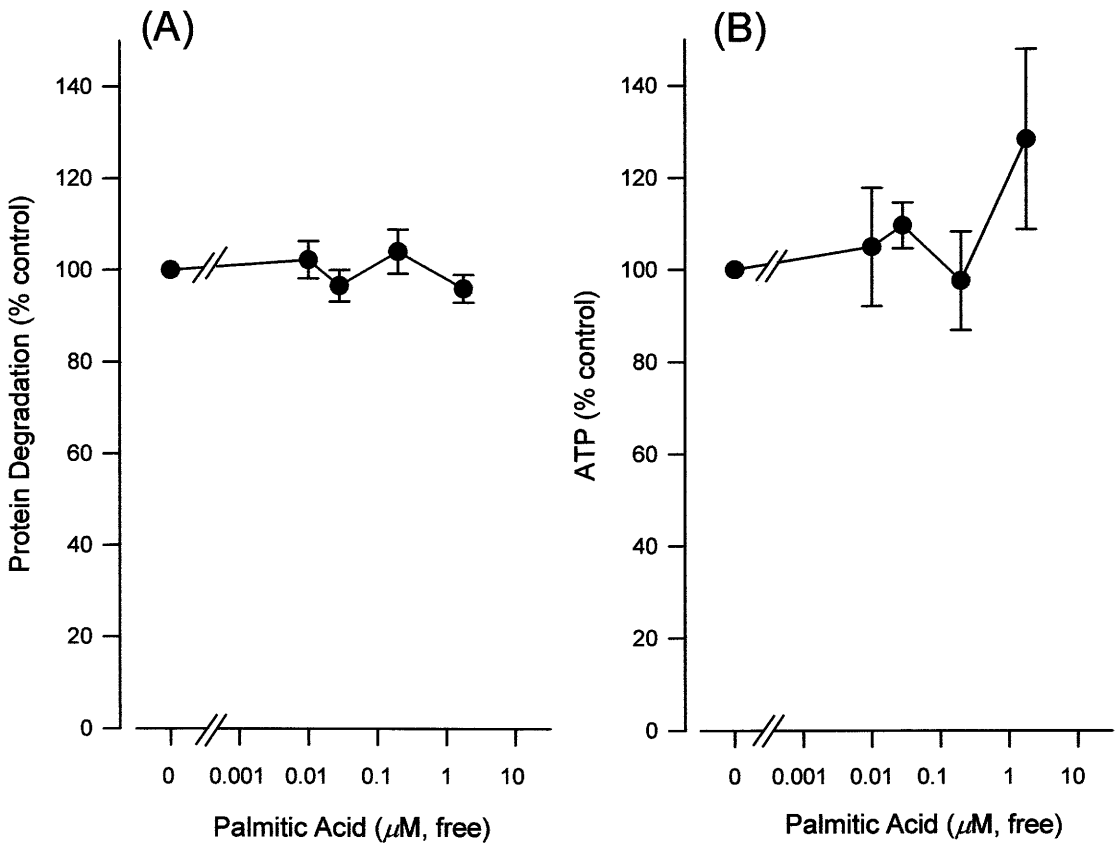


Figure 3.10

Effects of palmitic acid on degradation of cellular proteins (A) and cell ATP (B) in brown pre-adipocytes. Results are expressed as % control where (A) 100% is 18.7±4 μg labeled proteins degraded to acid-soluble products in 4 hours in cells incubated with albumin, and (B) 100% is 9.7±1.1 pmoles ATP/μg protein after 4 hours in cells incubated with albumin. Results are expressed as means ± S.E., with n=3 separate cell cultures. Statistical analysis is given in the text.

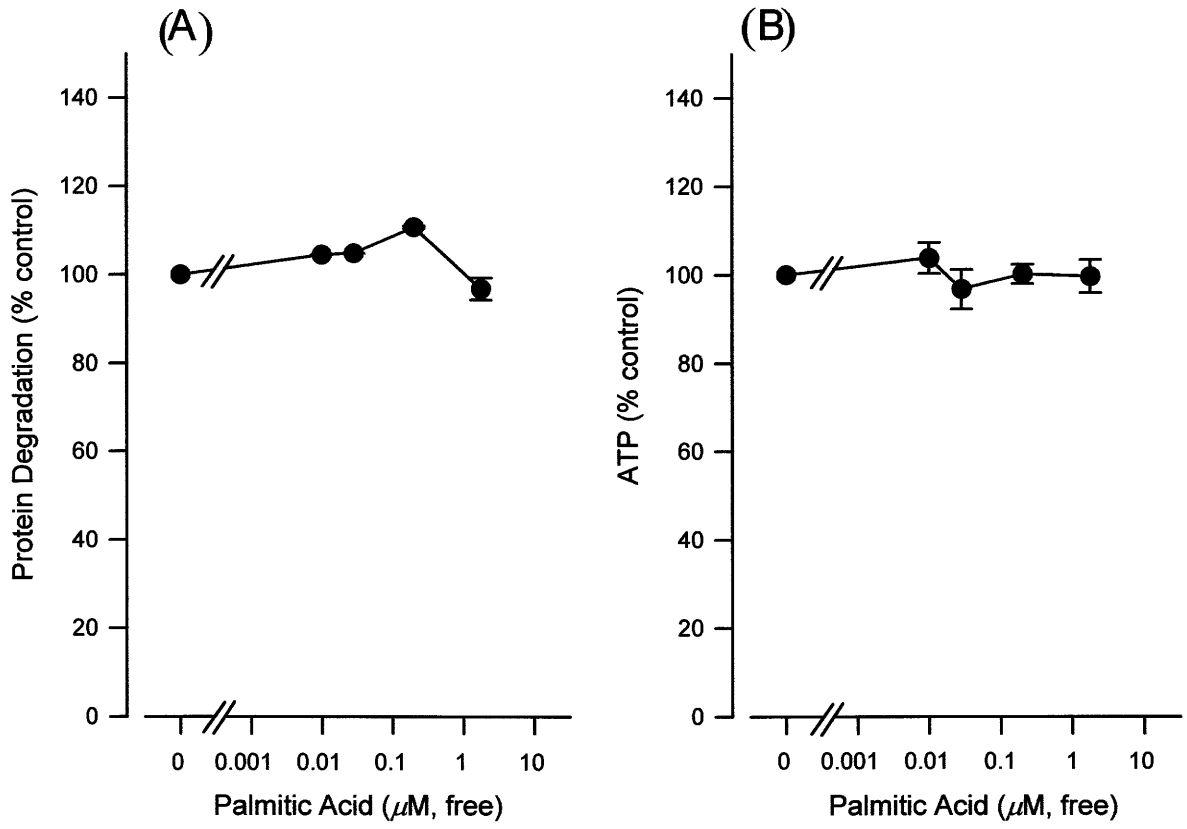


Figure 3.11

Effects of palmitic acid on protein degradation (A) and cell ATP (B) in 3T3-L_1 adipocytes. Results are expressed as % control where (A) 100% is 53.9±14 μg labeled proteins degraded to acid-soluble products in 4 hours in cells incubated with albumin, and (B) 100% is 28.3±2.2 pmoles ATP/μg protein after 4 hours in cells incubated with albumin. Results are expressed as means ± S.E., with n=3 separate cell cultures. Statistical analysis is given in the text.

be no reduction in cell ATP content and thus, no inhibition of proteolysis in brown pre-adipocytes or 3T3-L_1 adipocytes exposed to palmitic acid. Palmitic acid in the same concentrations as in mature brown adipocytes had no effect on protein degradation (P=0.5, Figure 3.10 A) or cell ATP content (P=0.4, Figure 3.10 B) in brown pre-adipocytes. In addition, increasing concentrations of palmitic acid also had no effect on either protein degradation (P=0.5, Figure 3.11 A) or cell ATP content (P=0.5, Figure 3.11 B) in 3T3-L_1 adipocytes.

3.5 COMPARISON OF THE EFFECTS OF DIFFERENT FATTY ACIDS ON PROTEOLYSIS AND ATP CONTENT

The effect of palmitic acid (16:0) on protein degradation and also cell ATP content was smaller than that of NE. Exogenous FAs might not completely mimic the effects of endogenous FAs and exogenously added FAs may not reach intracellular concentration similar to that produced by NE-induced lipolysis. The solubility of FAs can limit their intracellular concentrations. Different long chain FAs have distinct solubilities in aqueous solutions (Richieri *et al* 1993). Likewise, different long chain FAs are not equivalent in terms of their ability to interact with UCP1 and to de-energize mitochondria (Klingenberg and Huang, 1999). Therefore, we evaluated whether other long chain FAs were able to affect cell ATP content and proteolysis in brown adipocytes.

Brown adipocytes were incubated for a period of 4 hours, with increasing molar ratio of FAs:albumin of three long chain FAs with varying chain length and degree of unsaturation, oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4). Protein degradation and cellular ATP content were measured and the effects were compared to those of palmitic acid (Figure 3.12).

Increasing the molar ratio of FA:albumin inhibited protein degradation significantly ($P=0.02$) and also reduced cell ATP content ($P<0.0001$). The concentrations of FFAs in μM corresponding to different molar ratios FA:albumin of 2, 4, 6 and 8 were 0.02, 0.09, 1.38 and 29.19 μM respectively for oleic acid; 0.03, 0.47, 11.10 and 324.27 μM respectively for linoleic acid; and 0.03, 0.54, 8.90 and 146.27 μM respectively for arachidonic acid (Figure 3.12). Oleic acid, linoleic acid as

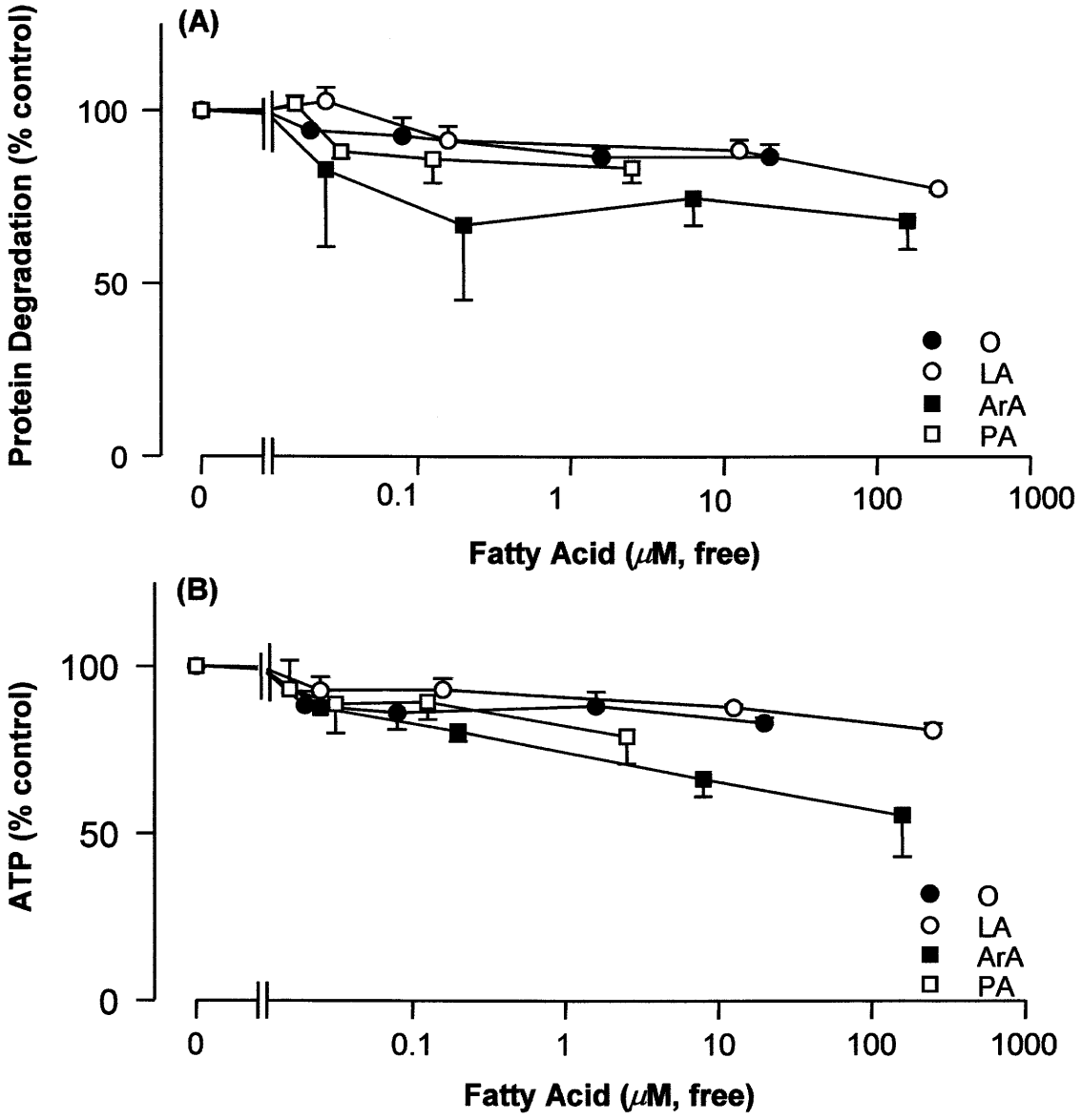


Figure 3.12

Comparison of the effects of Palmitic Acid (PA), Oleic Acid (O), Linoleic Acid (LA) and Arachidonic Acid (ArA) on (A) protein degradation and (B) cell ATP content in brown fat cells. Fatty acids were mixed with albumin in molar ratios of 2:1 to 8:1 and the amount of free fatty acids in μM calculated as described by Richieri *et al* (1993). Results are expressed as % control, where (A) 100% is 22.2±11.5 μg labeled proteins degraded to acid-soluble products in 4 hours in cells incubated with albumin, and (B) 100% is 6.2±0.4 pmoles ATP/μg protein after 4 hours in cells incubated with albumin. Results are expressed as means ± S.E., with n=3 separate cell cultures. Statistical analysis is given in the text.

well as arachidonic acid are much more soluble than palmitic acid. The effects of oleic acid and linoleic acid were similar to those of palmitic acid. However, arachidonic acid was more effective relative to other FAs ($P<0.05$, Figure 3.12 A and B).

3.6 BROMOPALMITIC ACID REDUCES PROTEOLYSIS AND ATP CONTENT TO A GREATER EXTENT THAN PALMITIC ACID

Another factor responsible for limiting increases in the intracellular concentration of exogenously added FAs is what happens to them once they are inside the cell. FAs have two functions in brown adipocytes. One is to activate UCP1, and the other, to be used as substrate for β-oxidation yielding reduced NADH and $FADH_2$, which provide the electrons for the electron transport chain. Therefore, FAs serve both as activators and fuel for thermogenesis. Bromopalmitic acid is a non-hydrolyzable analog of palmitic acid. Thus, although it is able to activate UCP1 (Klingenberg and Huang, 1999), it can not be used as fuel for thermogenesis (Fong *et al* 1997). Compared to palmitic acid, the hypothesis is that bromopalmitic acid may cause a greater reduction in cell ATP and consequently, a larger effect on proteolysis if the two are linked.

The same FA:albumin molar ratios were used with bromopalmitic acid as with palmitic acid. Results are only expressed in molar ratio of FA:albumin, as the affinity constant for interaction of bromopalmitic acid with albumin is not known. Bromopalmitic acid was a good inhibitor of protein degradation (Figure 3.13 A, $P<0.0001$). It also significantly decreased cell ATP content (Figure 3.13 B, $P<0.0001$). At a molar ratio of 6:1, while bromopalmitic acid reduced protein degradation and cell ATP content to 60.8 ± 3% and 43.3 ± 8.7% of control (cells incubated without bromopalmitic acid) respectively, palmitic acid decreased proteolysis to 86 ± 6.7% and cell ATP content to 89.3 ± 5.2% of control (cells incubated without palmitic acid). At the highest molar ratio of bromopalmitic

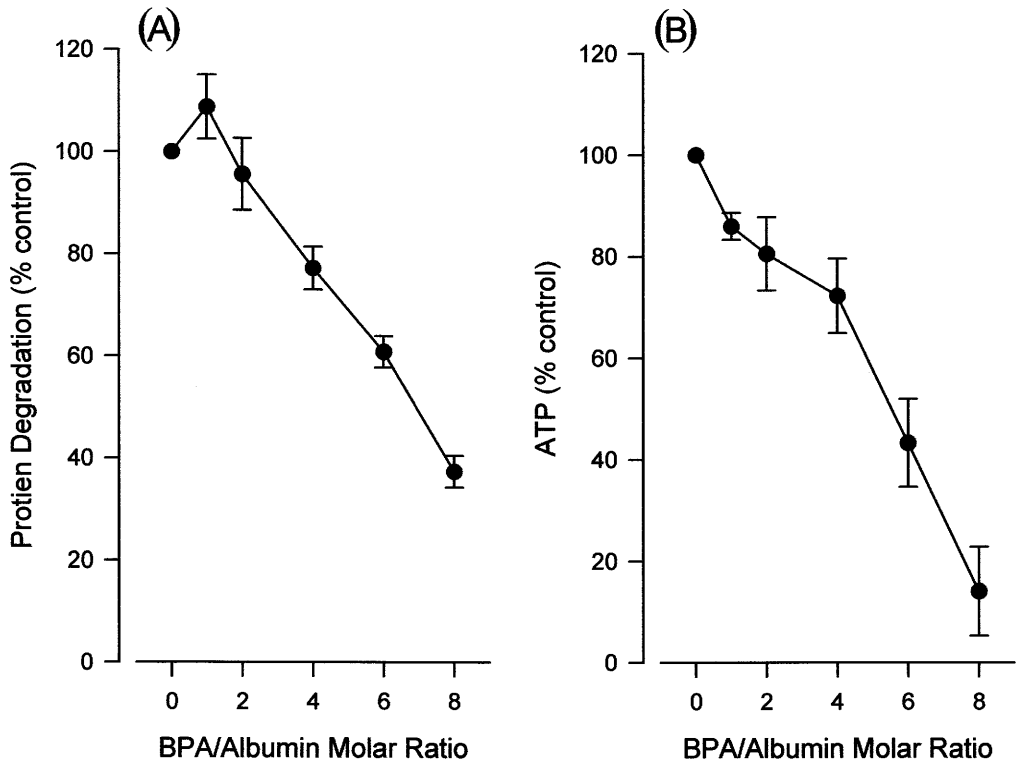


Figure 3.13

Effects of bromoplamitic acid (BPA) on protein degradation (A) or cell ATP level (B) in mature brown adipocytes. Results are expressed as % control where (A) 100% is 21.8±2.8 μg labeled proteins degraded to acid-soluble products in 4 hours in cells incubated with albumin, and (B) 100% is 11.8±1 pmoles ATP/μg protein after 4 hours in cells incubated with albumin. Results are expressed as means ± S.E., with n=6 separate cell cultures. Statistical analysis is given in the text.

acid:albumin (8:1) used, bromopalmitic acid caused almost a complete loss of cell ATP content. BPA was previously shown to be more effective in activating UCP1 (Klingenberg and Huang, 1999). As H^+ transport is generally accepted to be central to the uncoupling activity of UCP1, Klingenberg and Huang (1999) measured the dependence of H^+ transport activation on the structure of fatty acid. The H^+ transport activity was 0.4 μmol (mg protein)$^{-1}$ min^{-1} with PA, and reached 1.81 μmol (mg protein)$^{-1}$ min^{-1} with BPA.

Overall, a linear relationship (R^2=0.58) between changes in protein degradation and changes in cell ATP content was observed when all results from the effects of different concentrations of NE, and different concentrations and types of FAs on protein degradation and ATP level were combined and plotted versus each other (Figure 3.14).

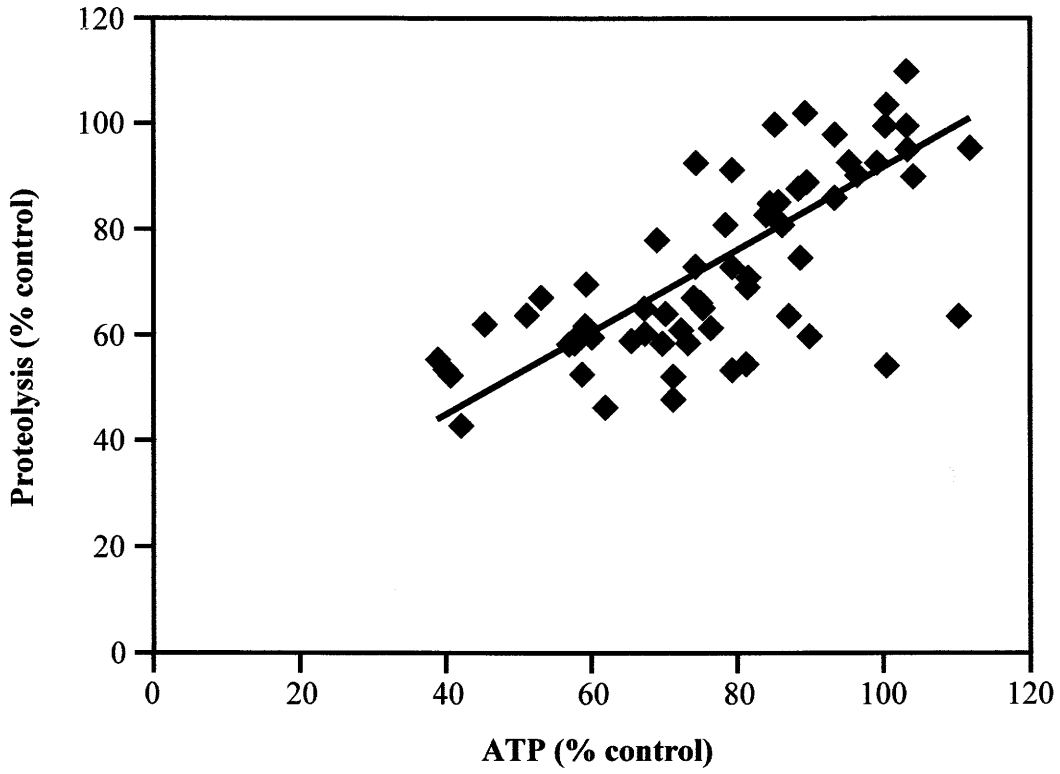


Figure 3.14

Relationship between changes in cell ATP content and protein degradation after 4 hours of exposure of adipocytes to different concentrations of NE, as well as to different concentrations and types of fatty acids. The regression equation is y=0.7459 x +23.353, with R^2=0.58.

3.7 REPEATED EXPOSURE OF BROWN ADIPOCYTES TO NE DOES NOT CHANGE THE CELL RESPONSE TO NE IN TERMS OF REDUCTION IN PROTEOLYSIS OR CELL ATP CONTENT

NE and FAs decrease protein degradation and cellular ATP content in mature brown fat cells (Sections 3.2-3.6). This inhibitory effect was not found in brown pre-adipocytes or 3T3-L_1 adipocytes. The hypothesis was that NE interacts with β_3-adrenoceptor on the cell surface, activates AC, which finally results in the release of FAs within the cell. FAs activate UCP1, and thus decrease the amount of ATP which can normally be produced by coupled respiration. Proteolysis is dependent on ATP, so less ATP, less proteolysis. This led to the next question, namely, "do cells with an increased capacity to respond calorigenically to NE also have an altered response to NE in terms of inhibition of protein degradation? For instance, would a cell from cold-acclimated mice possess the same, higher or lower rate of protein degradation in the presence or absence of NE?

In order to answer the question, NE (10 μM) was added to brown adipocytes daily for 7 days. NE was added daily as it disappears rapidly from culture medium (Waldbillig and Desautels, 1992). Prolonged and repeated exposure of brown adipocytes to NE is a way to up-regulate the cell thermogenic capacity, which may include increases in the cell content of mitochondrial proteins (including UCP1), but also possibly other aspects of brown fat cell functions, such as the cell capacity for glycolysis or lipolysis. Western blots for UCP1 showed a much greater level of UCP1 expression in brown adipocytes repeatedly exposed to NE for 7 days, compared to cells exposed to ascorbic acid for 7 days (Figure 3.15). Furthermore, there was no any

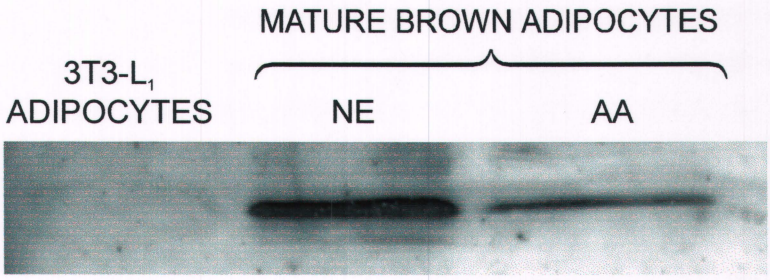


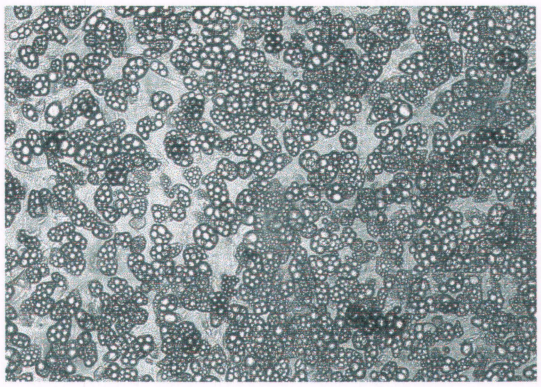
Figure 3.15

Immunoblots for UCP1 in mature brown adipocytes exposed either to 10 μM NE or AA for seven days. Mature brown adipocytes treated with NE for seven days have increased expression of UCP1 compared to AA-treated cells. Differentiated 3T3-L_1 adipocytes do not express UCP1.

obvious morphological abnormalities following prolonged exposure of brown adipocytes to NE for 7 days. Brown adipocytes treated with ascorbic acid for 7 days were round and filled with many small fat droplets (Figure 3.16 A). Morphology of brown adipocytes treated with NE for 7 days was similar (Figure 3.16 B).

Following repeated exposure of brown adipocytes to ascorbic acid or NE for 7 days, cells were incubated for a period of 4 hours in the presence or absence of NE (1 μM) and protein degradation and cell ATP content were measured. Repeated exposure of the cells to NE for 7 days did not alter basal or NE-dependent inhibition of proteolysis (Figure 3.17 A, $P>0.5$). The same is true regarding cellular ATP content. Upon addition of NE, the reduction in cell ATP level seen after 4 hours ($P<0.0001$) were not altered by the prior exposure of the cells to NE (Figure 3.17 B, $P= 0.3$).

(A)



(B)

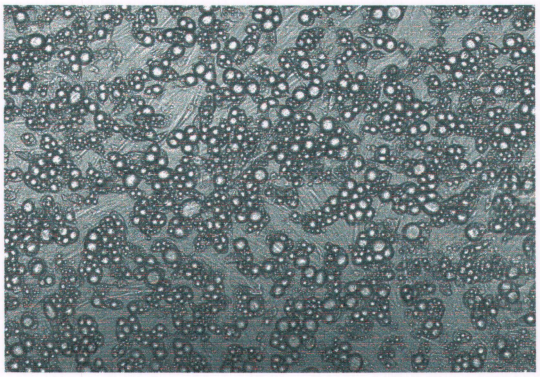


Figure 3.16

Mature brown adipocytes in culture (A) after treatment with ascorbic acid (AA) for seven days or (B) after prolonged and repeated exposure to 10 μM NE for seven days. Presence of multilocular fat droplets is evident in both (A) and (B). Magnification is 200X.

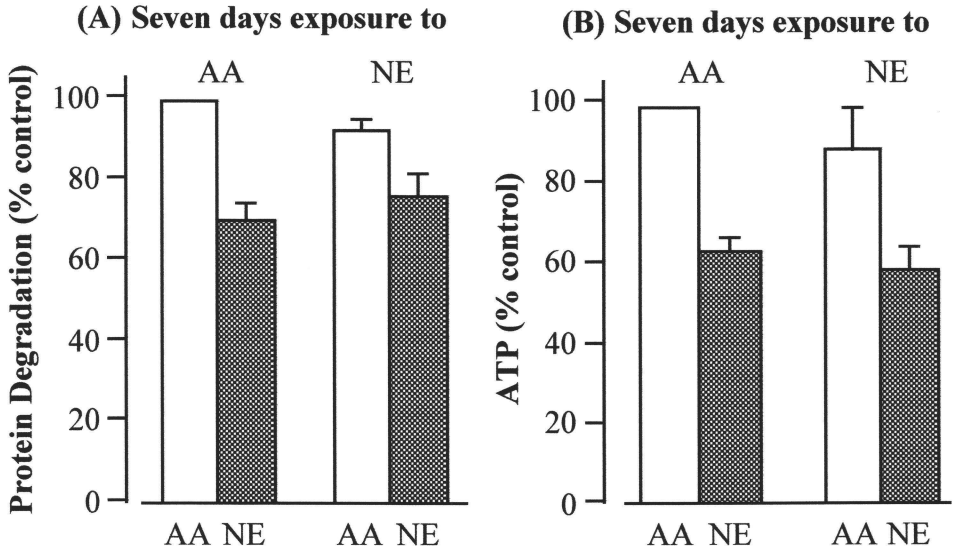


Figure 3.17

Effect of 4h exposure to ascorbic acid (AA) or NE (1μM) on proteolysis (A) and cell ATP content (B) after 7 days treatment with AA or NE. Results are expressed as % control where (A) 100% is 33.4±4.7 μg labeled proteins degraded to acid-soluble products in 4 hours in cells treated with AA for seven days and exposed to AA for four hours, and (B) 100% is 11.1±2 pmoles ATP/μg protein in cells treated with AA for seven days and exposed to AA for 4 hours. Results are described as means ± S.E., with n=7 separate cell cultures. Statistical analysis is given in the text.

4.0 DISCUSSION

4.1 NE INHIBITS PROTEIN DEGRADATION IN BROWN ADIPOCYTES

The thermogenic capacity of BAT varies with the need for thermoregulation and energy balance. It is increased in newborns, in animals exposed to cold environments, and in animals with prolonged intake of calories in excess of requirements (Jacobsson *et al* 1986; Jagus, 1987). Growth of BAT is usually associated with increases in tissue cellularity (tissue cell number), in the cell content of mitochondrial proteins, and in the concentration of UCP1 in the mitochondria (Himms-Hagen, 1984, 1989; Ricquier *et al* 1991). In contrast, there is BAT atrophy in conditions such as fasting, lactation, and return of cold-acclimated animals to a thermoneutral environment (Young *et al* 1982; Rothwell *et al* 1984; Trayhurn and Jennings, 1988). BAT atrophy is generally characterized by loss of cell mitochondrial proteins, and loss of UCP1 from the mitochondria with or without loss of tissue cellularity (Desautels, 1985; Desautels *et al* 1986; Trayhurn and Jennings, 1988).

In rats and mice, increase or decrease in BAT sympathetic activity correlates well with increase or decrease in BAT thermogenic capacity, respectively (Himms-Hagen, 1989). Many reports suggest that NE is an important trophic agent. For instance, NE infusions can mimic the increases in tissue cellularity, in the cell mitochondrial protein content, and UCP1 observed after cold stress or prolonged hyperphagia (Mory *et al* 1984; Geloen *et al* 1988; Ricquier *et al* 1986). Furthermore, surgical denervation and β-adrenergic antagonists cause BAT atrophy (Park and Himms-Hagen, 1988; Denjean *et al* 1999; Jacobsson *et al* 1986) and reduce the cold-

or diet-induced growth of the tissue (Himms-Hagen, 1989; Park and Himms-Hagen, 1988; Rothwell and Stock, 1984).

Change in cell protein content is determined by the balance between rates of protein synthesis and degradation. Waldbillig and Desautels (1992), and Desautels *et al* (1996), using brown fat cells isolated by tissue collagenase digestion, demonstrated that NE significantly increases the incorporation of [^{35}S] methionine in the cellular proteins, mitochondrial proteins, and UCP1, and thus promotes protein synthesis. The role of NE on protein degradation could be equally important. NE directly inhibits protein degradation by 30-40% in brown adipocytes differentiated in culture (Figures 3.4 and 3.6). Protein degradation was measured as the release of [^{35}S] acid-soluble radioactivity from the pre-labeled proteins into the culture medium, as cell proteins are degraded. During a 4-hour incubation of brown adipocytes in the presence of DMEM + 2 mM unlabeled methionine, the amount of [^{35}S] labeled proteins (acid-precipitable) decreased, with a corresponding and simultaneous increase in the amount of acid-soluble radioactivity outside the cells. Since the amount of acid-soluble radioactivity inside the cells did not change, measurement of acid-soluble radioactivity released into the culture medium is an adequate measurement of rate of proteolysis.

The inhibitory effect of NE on proteolysis was not seen in brown pre-adipocytes and 3T3-L_1 adipocytes, both lacking UCP1 (Figure 3.5). Similar results were attained previously, with no effect of NE on proteolysis in brown pre-adipocytes (day 3 and day 5 cultures), some inhibition of proteolysis in day 7 cultures [in keeping with some differentiation of these cells (low expression of

UCP1)], and a significant inhibitory effect in day 10 cultures of mature brown adipocytes (Desautels and Heal, 1999). This is not because NE does not interact with pre-adipocytes and 3T3-L_1 cells. NE increases rate of proliferation of brown pre-adipocytes via interaction with β_1-adrenoceptors (Geloen *et al* 1992; Bronnikov *et al* 1999). Furthermore, NE is an effective lipolytic agent in 3T3-L_1 adipocytes via interaction with β_3-adrenoceptors (Lafontan and Berlan, 1993; Chernick *et al* 1986).

The inhibition of proteolysis by NE in mature brown adipocytes is due to its interaction with β_3-adrenoceptors, and a subsequent increase in intracellular cAMP (Desautels and Heal, 1999). Addition of forskolin, which activates AC, inhibits protein degradation to the same extent as NE (Desautels and Heal, 1999). However, the mechanism by which NE inhibits proteolysis beyond cAMP generation is not understood. Cyclic AMP is an important signal transduction molecule that participates in the regulation of many intracellular processes. Elevation of intracellular cAMP level (via activation of AC, by inhibition of phosphodiesterase, or using a cAMP analog) was shown to inhibit hepatocytic protein degradation (Holen *et al* 1996). It was suggested that there might be a cAMP-sensitive control element at the sequestration step of the autophagic pathway, probably involving PKA as well as other protein kinases (Holen *et al* 1996). However, changes in intracellular concentration of cAMP did not appear to participate in protein turnover in white adipose tissue, skeletal or cardiac muscles (Sugden and Fuller, 1991; Tischler *et al* 1984). Our results in 3T3-L1 adipocytes confirm lack of adrenergic control of proteolysis in white adipocytes.

Therefore, the main objective of this study is to evaluate how the neurotransmitter NE can influence protein degradation in brown adipocytes differentiated in primary culture. The broader aspect of this study is to gain a better understanding of the signal transduction mechanisms controlling protein degradation in brown adipocytes, and to explain how loss of proteins and brown fat atrophy occurs in many pathological conditions such as diabetes and obesity.

Use of a primary cell culture to evaluate what controls protein degradation in brown adipocytes, has some advantages over *in vivo* studies: (a) measurement of rates of proteolysis are easy to handle; (b) only one cell type, brown adipocytes, is being studied, as opposed to many cell types present in intact tissue; and (c) the complex *in vivo* changes in the levels of many hormones that occur under physiological conditions known to trigger BAT atrophy can be eliminated. There are many factors other than NE, such as insulin and thyroid hormones whose levels change during *in vivo* conditions of brown fat atrophy (Himms-Hagen, 1990; Geloen and Trayhurn, 1990). Furthermore, Insulin is an important growth factor for BAT, as suggested by the tissue atrophy observed in rats and mice made diabetic with streptozotocin, that can be reversed with insulin administration (Geloen and Trayhurn, 1990). Insulin was shown to increase rate of protein synthesis (Desautels *et al* 1996) and to inhibit protein degradation (Desautels and Heal, 1999). Thyroid hormones have permissive or direct actions on the expression of many genes important for thermogenesis in BAT (Bianco and Silva, 1987; Bianco *et al* 1988). On the negative side, use of tissue culture may not adequately mimic *in vivo* conditions, where there is rapid delivery of O_2 and substrates for thermogenesis and removal of

waste products. The use of fetal calf serum may not be the same as the hormonal balance present *in vivo*.

4.2 NE DECREASES ATP CONTENT IN BROWN ADIPOCYTES

Both extra- and intra-lysosomal pathways are responsible for the breakdown of cellular proteins. Extra-lysosomal proteolytic pathways include all the soluble proteases present in the cytosol, nucleus and organelles such as mitochondria and endoplasmic reticulum (Jennissen, 1995; Blommaart *et al* 1997). Lysosomes, organelles containing 15-20 proteolytic enzymes, are well equipped to degrade the variety of proteins and other macromolecules which are continually transported to these organelles from other regions of the cell (Seglen and Bohley, 1992). An important feature of protein degradation in animal cells (extra- and intra-lysosomal) is a requirement for ATP (Hershko and Ciechanover, 1982; Hershko, 1988). In animal cells, ATP is required to tag cytosolic proteins for degradation by ubiquitination (Hershko, 1988). ATP is also required for the formation of the active protease complex that degrades ubiquitinated proteins (Ganoth *et al* 1988) and for the activity of a large multifunctional protease complex, the "proteasome" (Driscoll and Goldberg, 1989). Mitochondria also possess an ATP-dependent proteolytic pathway that can degrade proteins to their amino acid constituents (Desautels and Goldberg, 1985; Desautels, 1986). The energy requirement for intramitochondrial proteolysis is presumably due to ATP-dependent proteases reported first in liver mitochondria (Desautels and Goldberg, 1982) and purified from adrenal cortex mitochondria (Watabe and Kimura, 1985).

Furthermore, changes in the intracellular concentration of ATP could be an important factor in the control of protein degradation (Plomp *et al* 1987). In hepatocytes, autophagy is a main pathway of protein degradation, and it has been reported that the reduction of intracellular ATP concentration in isolated rat hepatocytes led to a significant decrease of proteolytic flux. A variety of chemical blockers of ATP synthesis or transport from mitochondria were used to cause progressive decrease in hepatocyte ATP, and it remains uncertain whether such changes are under physiological conditions. Nontheless, proteolytic flux in rat hepatocytes showed a linear relationship with change in intracellular ATP (Schellens and Meijer, 1991). Although the various steps of the autophagic process (sequestration, fusion, and digestion) may differ in their response to the energy status of the cell, the evidence indicates that all three steps in the pathway are sensitive to relatively small changes of intracellular ATP (Schellens and Meijer, 1991; Schellens *et al* 1988). In contrast, the ATP requirement of ATP-dependent proteases for mitochondrial proteolytic pathway is in the low micromolar range and may be less susceptible to changes in cell ATP content.

There is no study relating to the possible relationship between changes in ATP level and rate of protein degradation in brown adipocytes. The primary function of BAT is heat production stimulated by SNS (Himms-Hagen, 1990). There are some findings, which suggest that NE activation of UCP1, and thermogenesis leads to a decrease in cell ATP. Williamson (1970), and Pettersson and Vallin (1976) showed that ATP level decreased after NE addition to a suspension of isolated hamster brown adipocytes. There was a concurrent increase in adenosine 5'-monophosphate (AMP)

level. There is very limited data, but NE-induced decrease in BAT ATP may also occur *in vivo*. Ma and Foster (1984) infused NE in rats and as the dose of NE increased, BAT content of ATP declined while ADP and AMP increased.

The present results showed a decrease of 40-50% in the cell ATP level upon NE addition to brown adipocytes (Figures 3.6 and 3.7). Decrease in cell ATP level by NE was specific to differentiated brown adipocytes, since NE was not able to decrease cell ATP level in brown pre-adipocytes and 3T3-L_1 adipocytes (Figure 3.8). Decrease in cell ATP in cells isolated by collagenase digestion often occurs as an artifact of the isolation process. Some nucleotides may be lost due to the action of nucleotidases during the isolation process. Also, the greater the length and severity of anaerobiosis during isolation, the more severe the loss of cellular nucleotides. Cell breakage after the isolation is complete would also lower the level of nucleotides (LaNoue *et al* 1982). The decrease in cell ATP level shown in the brown adipocytes differentiated in culture upon NE addition in this study, is unlikely an artifact of the cell culture, or cytotoxic effect of the drug. It is well known that the primary function of BAT is the production of heat. The thermogenic function of BAT results from the expression in the mitochondria of UCP1 (Klaus *et al* 1991; Klingenberg 1993; Jezek *et al* 1998). When BAT is stimulated by NE, UCP1 is activated and acts as a proton translocator (Klingenberg, 1990), dissipating the electrochemical gradient across the inner mitochondrial membrane, producing heat. UCP1 acts as a by-pass to the proton-translocating ATP synthetase, causing reduction in ATP production (Himms-Hagen, 1990). Therefore, the decrease in the cell ATP level observed upon NE addition to brown adipocytes differentiated in culture is likely due to the activation of

UCP1 and represents a physiological event, not an artifact of the cell culture. Moreover, chronic stimulation of brown adipocytes in culture by NE increases the cell thermogenic capacity demonstrated by increase in the amount of mitochondrial proteins and of UCP1 (Figure 3.15) (Lowell and Spiegelman, 2000; Bouillaud *et al* 1984; Herron *et al* 1990; Kopecky *et al* 1990). In addition, there is evidence that NE also acts to prevent apoptosis in BAT (Briscini *et al* 1998; Lindquist and Rehnmark, 1998). No signs of cytotoxicity upon addition of NE to brown adipocytes in culture for 4 hours or 7 days were observed (Figure 3.16). Examination of the culture by light microscopy did not reveal loss of cells or changes in the appearance of the cells. Furthermore, there was no difference in the cellular ATP level after chronic NE treatment (7 days) compared to non-treated cells (Figure 3.17 B). Cell ATP content in our experiments was measured 24 hours after the last addition of NE. This suggests that the decrease in cell ATP showed after 4 hours of addition of NE is transient and an acute effect of NE, in keeping with the rapid disappearance of NE from cell culture medium (Waldbillig and Desautels, 1992).

Is the 40-50% decrease in the cell ATP content after addition of NE observed *in vitro* seen *in vivo*? There is only one report on this issue. Ma and Foster (1984) showed significant decreases in BAT ATP following NE infusion in rats. NE at the highest concentration used, lowered BAT ATP level by 50%. Brown adipocytes represent about 40% of the total cell population of the intact tissue (D'Allaire *et al* 1995). Assuming the decrease in ATP level occurs mainly in brown adipocytes, the decrease in ATP level in brown adipocytes *in vivo* could be as high as 75%. Thus, the decrease in cell ATP level observed *in vitro* may also occur *in vivo*, but this requires

further investigation under more physiological conditions (e.g. cold exposure slightly after birth, etc.).

Present results showed that there is a correlation between NE-dependent decrease in cell ATP and NE-dependent inhibition of proteolysis in brown adipocytes (Figure 3.6). However, the inhibition of proteolysis by NE may be an indirect effect of the decrease in cell ATP level. As mentioned before, AMP is elevated under conditions of energy depletion by NE addition to isolated brown adipocytes (Williamson, 1970; Pettersson and Vallin, 1976). Increases in intracellular AMP in hepatocytes have been shown to be a potent, novel physiological inhibitor of autophagic pathway of protein degradation (Kovacs *et al* 1998). Furthermore, a link between NE-dependent activation of thermogenesis, decrease in cell ATP, and inhibition of proteolysis has yet to be established.

4.3 FATTY ACIDS ARE THE LIKELY MEDIATORS OF NE-DEPENDENT DECREASES IN CELL ATP LEVEL AND INHIBITION OF PROTEOLYSIS

NE binding to β_3-adrenergic receptor on the brown fat cell membrane activates AC resulting in increased production of cAMP (Pettersson and Vallin, 1976). Cyclic AMP switches on the PKA cascade resulting in the phosphorylation and activation of HSL, and thus liberation of FAs from triglyceride stores (Pettersson and Vallin, 1976). FAs provide the substrates for electron transport chain and act to activate UCP1 (Locke and Nicholls, 1981; Locke *et al* 1982; Bukowiecki *et al* 1981). Addition of FFAs increases respiration of isolated brown adipocytes and decreases membrane potential of BAT mitochondria (Bukowiecki *et al* 1981). Addition of FAs

to cells or mitochondria from UCP1-ablated mice fails to stimulate respiration and to decrease mitochondrial membrane potential (Hofmann *et al* 2001; Nedergaard *et al* 2001). In addition, BAT mitochondria are more prone to the uncoupling effect of FFAs compared to liver mitochondria that lack UCP1 (Wojtezak and Schonfeld, 1993). Finally, experiments with reconstituted UCP1 in proteoliposomes (Winkler and Klingenberg, 1994) and with ectopic expression of UCP1 in yeast (Gonzalez-Barroso *et al* 1996) have shown that FFAs are necessary for UCP1 to act as a proton carrier.

Therefore, if NE's effects on cell ATP level and protein degradation are mediated via release of FAs from triglyceride stores within the cells and subsequent activation of UCP1, one can expect to mimic the effects of NE on ATP level and proteolysis by addition of exogenous FAs to brown adipocytes. Moreover, the addition of FAs to cells lacking UCP1 should not result in a decrease in cell ATP and inhibition of proteolysis. If addition of FAs can mimic the inhibitory effects of NE on proteolysis, it may rule out the involvement of cAMP-dependent activation of PKA. The concentrations of exogenously added FAs used in this study were similar to those used in previous work that were effective in affecting BAT cell respiration (Bukowiecki *et al* 1981; Matthias *et al* 2000).

Increasing concentrations of palmitic acid decreased both protein degradation and cell ATP level in differentiated brown adipocytes (Figure 3.9). As palmitic acid had no effect on protein degradation and ATP content in brown pre-adipocytes (Figure 3.10) and 3T3-L_1 adipocytes (Figure 3.11), its action is suggested to be UCP1-dependent. The effects of palmitic acid on protein degradation

and ATP level in brown adipocytes, were much smaller than those of NE (Figure 3.9). Likewise, Cunningham *et al* (1986) suggested that NE caused a greater stimulation of respiration than exogenously added palmitic acid.

The present data also showed that oleic acid and linoleic acid were as potent as palmitic acid in decreasing cell ATP level, and thus protein degradation (Figure 3.12). These results are in agreement with those of Bukowieki *et al* (1981) that palmitic acid, oleic acid, and linoleic acids are equivalent in stimulating brown adipocyte respiration. Arachidonic acid, however, proved to be more effective than the other three FAs (Figure 3.12). Arachidonic acid almost mimicked the inhibitory effect of NE on cell ATP level and protein degradation. Arachidonic acid is a precursor for prostaglandines and many other biologically active lipid metabolites. It is probable that metabolites of arachidonic acid may play a role in control of proteolysis.

The smaller inhibitory effect of palmitic acid on proteolysis and ATP level compared to that of NE, as well as the differences between the effects of different FAs on protein degradation and ATP level, compared to each other could have several explanations. NE causes the release of large amounts of FAs from triglyceride stores into the cytosol. The ability of exogenously added FAs to uncouple mitochondria, and thus to reduce ATP level and protein degradation may depend on mainly a critical intracellular concentration. In turn, this would depend upon the FA extracellular concentrations and the relative solubility of the FA in aqueous media and lipid phase of the membrane.

FAs were added complexed with albumin at equivalent molar ratios. Affinity of albumin is not the same for all FAs. Thus, the calculated extracellular FFA concentrations corresponding to the molar ratios of FA:albumin markedly differ for different FAs, which likely influence the rate of permeation and intracellular concentration. However, at the same molar ratio of FA:albumin of 6, for instance, while the extracellular FFA concentration of linoleic acid was higher than oleic and arachidonic acids, the inhibitory effect of linoleic acid on proteolysis and ATP level was the same as oleic acid and smaller than arachidonic acid (Figure 3.12). Therefore, it is likely that different FAs differ in the interaction with UCP1. It was previously reported that long chain FAs are not equivalent in terms of their abilities to interact with UCP1 (Klingenberg and Huang, 1999).

Furthermore, the intracellular concentration of exogenously added FAs would be dependent upon the balance between entry into the cell and their utilization as fuel for β-oxidation and respiration in the mitochondria. Exogenously added FAs may also be utilized for lipogenesis and stored in the triglyceride stores within cytosol. Bromopalmitic acid, a non-hydrolysable FA which can not be used as substrate for thermogenesis (Fong *et al* 1997; Declercq *et al* 1987), was shown to be the most effective FA in reducing both intracellular ATP level and protein degradation (Figure 3.13). While palmitic acid:albumin molar ratio of 6 decreased protein degradation to 86±7%, bromopalmitic acid in the same molar ratio decreased protein degradation to 61±3% of the control. Regarding the ATP level, the same kind of result was obtained. While palmitic acid/albumin molar ratio of 6 decreased the cell ATP level to 89±5% of the control, bromopalmitic acid at the same molar ratio

reduced the cell ATP level to 43±9%. However, bromopalmitic acid at high concentrations seems to be toxic to the cells, as it reduced cell ATP level almost to zero.

4.4 ARE EFFECTS OF NE ON CELL ATP AND PROTEOLYSIS AFFECTED BY CHANGES IN THE CELL CONTENT OF UCP1?

That the effects of NE and FAs on reduction of cell ATP and proteolysis are only observed in mature brown adipocytes containing UCP1, but not in brown pre-adipocytes and 3T3-L_1 adipocytes lacking UCP1, suggests that NE acts at least in part via activation of UCP1. There are marked alterations in brown fat cell content of UCP1 *in vivo*. Thus, it is of interest to evaluate if the effect of NE on proteolysis is dependent upon the concentration of UCP1 in the cells. Chronic exposure of the cells to NE for 7 days significantly increased the expression of UCP1 (Figure 3.15). However, there was no further effect of NE on proteolysis and cell ATP content compared to brown adipocytes with basal expression of UCP1 (Figure 3.17 A and B). The results could be explained by some desensitization or down-regulation of β_3-adrenergic receptors following chronic exposure of brown adipocytes to NE (Vicario *et al* 1998). Alternatively, ATP can be produced in brown fat cells by substrate-level phosphorylation from glycolysis in cytosol. BAT has a large glycolytic capacity and chronic NE treatment of brown adipocytes may stimulate glucose uptake via an increase in the intrinsic transport activity of glucose transporters through cAMP (Marette and Bukowieki, 1989; Shimizu *et al* 1996, 1998). Furthermore, chronic NE treatment of brown adipocytes stimulates the

expression of glycolytic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (Barroso *et al* 1999). Thus, despite of increase in UCP1 content, ATP from the glycolysis could be used as an energy source for the degradation of cellular proteins in cells chronically exposed to NE. It is therefore likely that cellular changes associated with chronic NE treatment of brown adipocytes prevent the further decrease in cell ATP level, and thus protein degradation upon acute exposure of brown adipocytes to NE.

Finally, these results demonstrate a linear relationship between changes in intracellular ATP level and extent of inhibition of proteolysis (Figure 3.14). Therefore, it seems that changes in intracellular ATP level induced by NE *in vitro* may play an important role in the regulation of protein degradation in mature brown adipocytes. Thus, NE-dependent increase in protein synthesis with concurrent NE-dependent inhibition of proteolysis would markedly increase the rate of protein accumulation in brown adipocytes in conditions such as cold exposure.

However, suppression of SNS activity as occurs in many *in vivo* conditions of BAT atrophy is unknown if it affects ATP level, yet there is net protein loss from activation of autophagic process.

5.0 CONCLUSION

Inhibition of proteolysis by NE is specific to differentiated brown fat cells. The neurotransmitter exerts its inhibitory action on proteolysis at least in part from a reduction in the cell ATP. The reduction in cell ATP is likely the result of FA-induced uncoupled metabolism from activation of UCP1. NE-dependent decrease in cell ATP and subsequent inhibition of proteolysis may play a significant role in affecting the cell content of protein under certain physiological and pathological conditions, known to activate thermogenesis and stimulate BAT growth.

6.0 REFERENCES

Agius, L. and Williamson, D. H. (1980) Lipogenesis in interscapular brown adipose tissue of virgin, pregnant, and lactating rats. The effects of intragastric feeding. Biochem. J. 190, 477-480.

Arechaga, I., Raimbault, S., Prieto, S., Levi-Meyrueis, C., Zaragoza, P., Miroux, B., Ricquier, D., Bouillaud, F., Rial, E. (1993) Cysteine residues are not essential for uncoupling protein function. Biochem. J. 296, 693-700.

Atgie, C., D'Allaire, F., and Bukowiecki, L. J. (1997) Role of β_1- and β_3- adrenoceptors in the regulation of lipolysis and thermogenesis in rat brown adipocytes. Am. J. Physiol. 273, C1136-C1142.

Bamshad, M., Song, C. K. and Bartness, T. J. (1999) CNS origins of the sympathetic nervous system outflow to brown adipose tissue. Am. J. Physiol. 276, R1569-R1578.

Barbera, M. J., Schluter, A., Pedraza, N., Iglesias, R., Villarroya, F. and Giralt, M. (2001) Peroxisome proliferator-activated receptor α activates transcription of the brown fat uncoupling protein-1 gene. J. Biol. Chem. 276 (2), 1486-1493.

Barrett, A. J. and Kirschke, H. (1981) Cathepsin H, and cathepsin L. Methods Enzymol. 80, 535-561.

Barroso, I., Benito, B., Garcia-Jimenez, C., Hernandez, A., Obregon, M. J. and Santisteban, P. (1999) Norepinephrine, tri-iodothyronine and insulin upregulate glyceraldehyde-3-phosphate dehydrogenase mRNA during brown adipocyte differentiation. Eur. J. Endocrinol. 141, 169-179.

Beynon, R. J. and Bond, J. S. (1986) Catabolism of intracellular proteins: molecular aspects. Am. J. Physiol. 251, C141-C152.

Bianco, A. C. and Silva, J. E. (1987) Intracellular conversion of thyroxine to triiodothyronine is required for the optimal thermogenic function of brown adipose tissue. J. Clin. Invest. 79, 295-300.

Bianco, A. C., Sheng, X. and Silva, J. E. (1988) Triiodothyronine amplifies norepinephrine stimulation of uncoupling protein gene transcription by a mechanism not requiring protein synthesis. J. Biol. Chem. 263, 18168-18175.

Blommaart, E. F. C., Luiken, J. J. F. P. and Meijer, A. J. (1997) Autophagic proteolysis: control and specificity. Histochem. J. 29, 365-385.

Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Saydoux, J., Muzzin, P. and Giacobino, J-P.(1997) Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. FEBS Lett 408, 39-42.

Bouillaud, F., Ricquier, D., Mory, G., Thibault, J. (1984) Increased level of mRNA for the uncoupling protein in brown adipose tissue of rats during thermogenesis induced by cold exposure or noradrenaline infusion. J. Biol. Chem. 259, 11583-11586.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of micrograms of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-257.

Brand, M. D., Brindle, K. M., Buckingham, J. A., Harper, J. A., Rolfe, D. F. S., Stuart, J. A. (1999) The significance and mechanism of mitochondrial proton conductance. Int. J. Obes. Relat. Metab. Disord. 23 (Suppl 6), S4-S11.

Briscini, L., Tonello, C., Dioni, L., Carruba, M. O., Nisoli, E. (1998) Bcl-2 and Bax are involved in the sympathetic protection of brown adipocytes from obesity-linked apoptosis. FEBS Lett. 431, 80-84.

Bronnikov, G., Bengtsson, T., Kramarova, L., Golozoubova, V., Cannon, B. and Nedergaard, J. (1999) β_1 to β_3 switch in control of cyclic adenosine monophosphate during brown adipocyte development explains distinct β-adrenoceptor subtype mediation of proliferation and differentiation. Endocrinology 140, 4185-4197.

Bronnikov, G., Houstek, J., Nedergaard, J. (1992) β-adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. Mediation via β_1 but not via β_3 receptors. J. Biol. Chem. 267, 2006-2013.

Bukowiecki, L., Collet, A. J., Follea, N., Guay, G. and Jahjah, L. (1982) Brown adipose tissue hyperplasia: a fundamental mechanism of adaptation to cold and hyperphagia. Am. J. Physiol. 242, E353-E359.

Bukowiecki, L., Follea, N., Lupien, J. and Paradis, A. (1981) Metabolic relationships between lipolysis and respiration in rat brown adipocytes. J. Biol. Chem. 256, 12840-12848.

Cannon, B. and Nedergaard, J. (1996) Adrenergic regulation of brown adipocyte differentiation. Biochem. Soc. Trans. 24, 407-412.

Cannon, B., Jacobsson, A., Rehnmark, S. and Nedergaard, J. (1996) Signal transduction in brown adipose tissue recruitment: noradrenaline and beyond. Int. J. Obes. 20 (Suppl. 3), S36-S42.

Cao, Z., Umek, R. M., McKnight, S. L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L_1cells. Genes Dev. 5, 1538-1552.

Champigny, O. and Ricquier, D. (1990) Effects of fasting and refeeding on the level of uncoupling protein mRNA in rat brown adipose tissue: evidence for diet-induced and cold-induced responses. J. Nutr. 120, 1730-1736.

Champigny, O., Holloway, B. R. and Ricquier, D. (1992) Regulation of UCP gene expression in brown adipocytes differentiated in primary culture. Effects of a new β-adrenoceptor agonist. Mol. Cell. Endocrinol. 86, 73-82.

Chernick, S. S., Spooner, P. M., Garrison, M.M. and Scow, R. O. (1986) Effect of epinephrine and other lipolytic agents on intracellular lipolysis and lipoprotein lipase activity in 3T3-L_1 adipocytes. J. Lipid Res. 27, 286-294.

Cousin, B., Bascands-Viguerie, N., Kassis, N., Nibbelink, M., Ambid, L., Casteilla, L. and Penicaud, L. (1996) Cellular changes during cold acclimation in adipose tissues. J. Cell Physiol. 167, 285-289.

Cunningham, S. A., Wiesinger, H. and Nicholls, D. G. (1986) Quantification of fatty acid activation of the uncoupling protein in brown adipocytes and mitochondria from the guinea-pig. Eur. J. Biochem. 157, 415-420.

D'Allaire, F., Atgie, C., Mauriege, P., Simard, P. M. and Bukowiecki, L. J. (1995) Characterization of β_1- and β_3-adrenoceptors in intact brown adipocytes of the rat. Br. J. Pharmacol. 114, 275-282.

Daikoku, T., Shinohara, Y., Shima, A., Yamazaki, N., Terada, H. (2000) Specific elevation of transcript levels of particular protein subtypes induced in brown adipose tissue by cold exposure. Biochim. Biophys. Acta 1457, 263-272.

Danforth, E. and Himms-Hagen, J. (1997) Obesity and diabetes and the beta-3 adrenergic receptor. Eur. J. Endocrinol. 136, 362-365.

Declercq, P. E., Falck, J. R., Kuwajima, M., Tyminski, H., Foster, D. W. and McGarry, J. D. (1987) Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. J. Biol. Chem. 262 (20), 9812-9821.

DeDuve, C. (1983) Lysosomes revisited. Eur. J. Biochem. 137, 391-397.

DeMartinis, F. D., Ashkin, K. T. and Lampe, K. T. (1987) Fluorescence detection of mitochondrial clusters in mammalian white fat cells *in vivo*. Am. J. Physiol. 253, C783-C791.

Denjean, F., Lachuer, J., Geloen, A., Cohen-Adad, F., Moulin, C., Barre, H., Duchamp, C. (1999) Differential regulation of uncoupling protein-1, -2, and –3 gene expression by sympathetic innervation in brown adipose tissue of thermoneutral or cold-exposed rats. FEBS Lett. 444, 181-185.

Desautels, M. (1985) Mitochondrial thermogenin content is unchanged during atrophy of BAT of fasting mice. Am. J. Physiol. 249, E99-E106.

Desautels, M. (1986) Mitochondrial proteolysis. In: Mitochondrial physiology and pathology. P 40-65. Van Nostrand Reinhold Company Inc.

Desautels, M. (1992) ATP-stimulated protease activity in brown fat mitochondria: response to a 24-h fast in mice. Biochem. Cell Biol. 70, 765-769.

Desautels, M. and Dulos, R. A. (1988) Effects of repeated cycles of fasting-refeeding on brown adipose tissue composition in mice. Am. J. Physiol. 255, E120-E128.

Desautels, M. and Dulos, R. A. (1990) Importance of neural input and thyroid hormones in the control of brown fat atrophy in mice. Can. J. Physiol. Pharmacol. 68, 1100-1107.

Desautels, M. and Dulos, R. A. (1993) Nucleotide requirement and effects of fatty acids on protein synthesis and degradation in brown adipose tissue mitochodria. Can. J. Physiol. Pharmacol. 71, 17-25.

Desautels, M. and Dulos, R. A. (1994) Temperature and phenylmethylsulfonyl fluoride sensitive loss of uncoupling protein in isolated brown adipose tissue mitochondrial membranes. Biochem. Cell Biol. 72, 1-7.

Desautels, M. and Goldberg, A. L. (1982) Demonstration of an ATP-dependent, vanadate-sensitive endoprotease in the matrix of rat liver mitochondria. J. Biol. Chem. 257, 11673-11679.

Desautels, M. and Goldberg, A. L. (1985) The ATP-dependent breakdown of proteins in a mammalian mitochondria. Biochem. Soc. Trans. 13, 290-293.

Desautels, M. and Heal, S. (1999) Differentiation-dependent inhibition of proteolysis by norepinephrine in brown adipocytes. Am. J. Physiol. 277, E215-E222.

Desautels, M., Bhaumick, B. and Ram, J. I. (1996) Comparison of trophic effects of norepinephrine, insulin and IGF-1 in mouse brown adipocytes. Am. J. Physiol. 270, R1287-R1295.

Desautels, M., Dulos, R. A. and Mozaffari, B. (1986) Selective loss of uncoupling protein from mitochondria of surgically denervated brown adipose tissue of cold-acclimated mice. Biochem. Cell Biol. 65, 1125-1134.

Desautels, M., Michalska, E. and Mozaffari, B. (1990) Role of acid proteases in brown adipose tissue atrophy caused by fasting in mice. Bichem. Cell Biol. 68, 441-447.

Driscoll, J. and Goldberg, A. L. (1989) Skeletal muscle proteasome can degrade proteins in an ATP-dependent process that does not require ubiquitin. Proc. Natl. Acad. Sci. USA 86, 787-791.

Dunn, W. A., Jr. (1994) Autophagy and related mechanisms of lysosome-mediated protein degradation. Trends Cell Biol. 4, 139-144.

Echtay, K. S., Bienengraeber, M., Winkler, E., Klingenberg, M. (1998) In the uncoupling protein (UCP-1) His-214 is involved in the regulation of purine nucleoside triphosphate but not diphosphate binding. J. Biol. Chem. 273, 24368-24374.

Emilsson, V., Summers, R. J., Hamilton, S., Liu, Y. L. and Cawthorne, M. A. (1998) The effects of the β_3-adrenoceptor agonist BRL 35135 on UCP isoform mRNA expression. Biochem. Biophys. Res. Commun. 252, 450-454.

Enerback, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Harper, M.-E. and Kozak, L. P. (1997) Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature 387, 90-94.

Fleury, C., Neverova, M., Collins, S., Surwit, R. S., Ricquier, D., Warden, C. H., Raimbault, S., Champigny, O., Levimeyrueis, C., Bouillaud, F., Seldin, M. F. (1997) Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat. Genet. 15, 269-272.

Foellmi-Adams, L. A., Wyse, B. M., Herron, D., Nedergaard, J. and Kletzien, R. F. (1996) Induction of uncoupling protein in brown adipose tissue. Synergy between norepinephrine and pioglitazone, an insulin-sensitizing agent. Biochem. Pharmacol. 52, 693-701.

Fong, C. J., Leu, S-J. and Chai, S-P. (1997) Differential inhibition of lipolysis by 2-bromopalmitic acid and 4-bromocrotonic acid in 3T3-L_1 adipocytes. Biochim. Biophys. Acta 1344, 65-73.

Ganoth, D., Leshinsky, E., Eytan, E. and Hershko, A. (1988) A multicomponent system that degrades proteins conjugated to ubiquitin. J. Biol. Chem. 263, 12412-12419.

Geloen, A. and Trayhurn, P. (1990) Regulation of the level of uncoupling protein in brown adipose tissue by insulin. Am. J. Physiol. 258, R418-R424.

Geloen, A., Collet, A. J., Bukowiecki, L. J. (1992) Role of sympathetic innervation in brown adipocyte proliferation. Am. J. Physiol. 263, R1176-R1181.

Geloen, A., Collet, A. J., Guay, G. and Bukowiecki, L. J. (1988) β-adrenergic stimulation of brown adipocyte proliferation. Am. J. Physiol. 254, C175-C182.

Gonzalez-Barroso, M. M., Fleury, C., Arechaga, I., Zaragoza, P., Levi-Meyrueis, C., Raimbault, S., Ricquier, D., Boillaud, F., Rial, E. (1996) Activation of the uncoupling protein by fatty acids is modulated by mutations in the C-terminal region of the protein. Eur. J. Biochem. 239, 445-450.

Granneman, J. G. (1995) Expression of adenylyl cyclase subtypes in brown adipose tissue: neural regulation of type III. Endocrinology 136, 2007-2012.

Green, H. and Meuth, M. (1974) An established pre-adipose cell line and its differentiation in culture. Cell 3, 127-133.

Guardiola-Diaz, H. M., Rehnmark, S., Usuda, N., Albrektsen, T., Feltkamp, D., Gustafsson, J-A. and Alexson, S. E. H. (1999) Rat peroxisome proliferator-activated receptors and brown adipose tissue function during cold acclimatization. J. Biol. Chem. 274 (33), 23368-23377.

Guerra, C., Kozak, R. A., Walsh, K., Kurtz, D. M., Wood, P. A. and Kozak, L. P. (1998) Abnormal nonshivering thermogenesis in mice with inherited defects of fatty acid oxidation. J. Clin. Invest. 102, 1724-1731.

Hamann, A., Felier, J. S. and Lowell, B. B. (1996) Decreased brown fat markedly enhances susceptibility to diet-induced obesity, diabetes, and hyperlipidemia. Endocrinology 137, 21-29.

Harper, M-E. and Himms-Hagen, J. (2001) Mitochondrial efficiency: lessons learned from transgenic mice. Biochimica. Biophysica. Acta 1504, 159-172.

Herron, D., Rehnmark, S., Néchad, M., Loncar, D., Cannon, B. and Nedergaard, J. (1990) Norepinephrine-induced synthesis of the uncoupling protein thermogenin (UCP1) and its mitochondrial targeting in brown adipocytes differentiated in culture. FEBS Lett. 268, 296-300.

Hershko, A. (1988) Ubiquitin-mediated protein degradation. J. Biol. Chem. 263, 15237-15240.

Hershko, A. and Ciechanover, A. (1982) Mechanisms of intracellular protein breakdown. Annu. Rev. Biochem. 51, 335-364.

Himms-Hagen, J. (1984) Nonshivering thermogenesis. Brain Res. Bull. 12, 151-160.

Himms-Hagen, J. (1989) Brown adipose tissue thermogenesis and obesity. Prog. Lipid Res. 28, 67-115.

Himms-Hagen, J. (1990) Brown adipose tissue thermogenesis: interdisiplinary studies. FASEB J. 4, 2890-2898.

Hofmann, W. E., liu, X., Bearden, C. M. Harper, M-E. and Kozak, L. P. (2001) Effects of genetic background on thermoregulation and fatty acid-induced uncoupling of mitochondria in UCP1-deficient mice. J. Biol. Chem. 276, 12460-12465.

Holen, I., Gordon, P. B., Stromhaug, O. E. and Seglen, P. O. (1996) Role of cAMP in the regulation of hepatocytic autophagy. Eur. J. Biochem. 236, 163-170.

Houstek, J., Kopecky, J., Baudysova, M., Janikova, D., Pavelka, S. and Klement, P. (1990) Differentiation of brown adipose tissue and biogenesis of

thermogenic mitochondria *in situ* and in cell culture. Biochim. Biophys. Acta 1018, 243-247.

Jacobsson, A., Nedergaard, J. and Cnnon, B. (1986) α- and β-adrenergic control of thermogenin mRNA expression in brown adipose tissue. BioSci. Rep. 6, 621-631.

Jagus, R. (1987) Characterization of *in vitro* translation products. Methods Enzymol. 152, 296-304.

Jennissen, H. P. (1995) Ubiquitin and the enigma of intracellular protein degradation. Eur. J. Biochem. 231, 1-30.

Jezek, P., Bauer, M., Trommer, W. E. (1995) EPR spectroscopy of 5-DOXYL-stearic acid bound to the mitochondrial uncoupling protein reveals its competitive displacement by alkylsulfonates in the channel and allosteric displacement by ATP. FEBS Lett. 361, 303-307.

Jezek, P., Engstova, H., Zacova, M., Vercesi, A. E., Costa, A. D. T., Arruda, P., Garlid, K. D. (1998) Fatty acid cycling mechanism and mitochondrial uncoupling proteins. Biochim. Biophys. Acta 1365, 319-327.

Jezek, P. and Freisleben, H-J. (1994) Fatty acid binding site of the mitochondrial uncoupling protein: demonstration of its existence by EPR spectroscopy of 5-DOXYL-stearic acid. FEBS Lett. 343, 22-26.

Jezek, P., Hanus, J., Semrad, C., Garlid, K. D. (1996) Photoactivated azido fatty acid irreversibly inhibits anion and proton transport through the mitochondrial uncoupling protein. J. Biol. Chem. 271 (11), 6199-6205.

Klaus, S., Casteilla, L., Bouillaud, F., Ricquier, D. (1991) The uncoupling protein UCP: a membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue. Int. J. Biochem. 23, 791-801.

Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K. and Evans, R. M. (1994) Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc. Natl. Acad. Sci. USA. 91, 7355-7359.

Klingenberg, M. (1990) Mechanism and evolution of the uncoupling protein of brown adipose tissue. Trends Biochem. Sci. 15, 108-112.

Klingenberg, M. (1993) Dialectics in carrier research: the ADP/ATP carrier and the uncoupling protein. J. Bioenerg. Biomembr. 25, 447-457.

Klingenberg, M. (1999) Uncoupling protein- a useful energy dissipator. J. Bioenerg. Biomembr. 31, 419-430.

Klingenberg, M. and Huang, S-G. (1999) Structure and function of the uncoupling protein from brown adipose tissue. Biochim. Biophys. Acta 1415, 271-296.

Klingenberg, M. and Winkler, E. (1985) The reconstituted isolated uncoupling protein is a membrane potential driven H^+ translocator. EMBO. J. 4, 3087-3092.

Kopecky, J., Baudysova, M., Zanotti, F., Janikova, D., Pavelka, S., Houstek, J. (1990) Synthesis of mitochondrial uncoupling protein in brown adipocytes differentiated in cell culture. J. Biol. Chem. 265, 22204-22209.

Kopecky, J., Clarke, G., Enerback, S., Spiegelman, B. and Kozak, L. P. (1995) Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. J. Clin. Invest. 96, 2914-2923.

Kopecky, J., Hodny, Z., Rossmeisl, M., Syrovy, I. and Kozak, L. P. (1996) Reduction of dietary obesity in aP2-Ucp transgenic mice: physiology and adipose tissue distribution. Am. J. Physiol. 270, E768-E775.

Kopecky, J., Rossmeisl, M., Hodny, Z., Syrovy, I., Horakova, M. and Kolarova, P. (1996) Reduction in dietary obesity in aP2-Ucp transgenic mice: mechanism and adipose tissue morphology. Am. J. Physiol. 270, E776-E786.

Kovacs, A. L., Gordon, P. B., Grotterød, E. M. and Seglen, P. O. (1998) Inhibition of hepatocytic autophagy by adenosine, adenosine analogs and AMP. Biol. Chem. 379, 1341-1347.

Kozak, U. C., Kopecky, J., Teisinger, J., Enerback, S., Boyer, B. and Kozak, L. P. (1994) An upstream enhancer regulating brown-fat-specific expression of the mitochondrial uncoupling protein gene. Mol. Cell. Biol. 14, 59-67.

Lafontan, M. and Berlan, M. (1993) Fat cell adrenergic receptors and the control of white and brown fat cell function. J. Lipid Res. 34, 1057-1091.

Langer, T. and Neupert, W. (1996) Regulated protein degradation in mitochondria. Experientia 52, 1069-1076.

LaNoue, K. F., Koch, C. D. and Meditz, R. B. (1982) Mechanism of action of norepinephrine in hamster brown adipocytes. J. Biol. Chem. 257, 13740-13748.

LeBlanc, J. and Labrie, A. (1997) A possible role for palatability of the food in diet-induced thermogenesis. Int. J. Obes. 21, 1100-1103.

Lindquist, J. M. and Rehnmark, S. (1998) Ambient temperature regulation of apoptosis in brown adipose tissue. J. Biol. Chem. 273 (46), 30147-30156.

Locke, R. M. and Nicholls, D. G. (1981) A re-evaluation of the role of fatty acids in the physiological regulation of the proton conductance of brown adipose tissue mitochondria. FEBS Lett. 135, 249-252.

Locke, R. M., Rial, E., Scott, I. D. and Nicholls, D. G. (1982) Fatty acids as acute regulators of the proton conductance of hamster brown fat mitochondria. Eur. J. Biochem. 129, 373-380.

Lowell, B. B. and Spiegelman, B. M. (2000) Towards a molecular understanding of adaptive thermogenesis. Nature 404, 652-660.

Lowell, B. B., S-Susulic, V., Hamann, A., Lawitts, J. A., Himms-Hagen, J., Boyer, B. B., Kozak, L. P. and Flier, J. S. (1993) Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. Nature, 366, 740-742.

Ma, S. W. and Foster, D. O. (1984) Redox state of brown adipose tissue as a possible determinant of its blood flow. Can. J. Physiol. Pharmacol. 62, 949-956.

Manchado, C., Yubero, P., Vinas, O., Iglesias, R., Villaroya, F., Mampel, T. and Giralt, M. (1994) CCAT/enhancer-binding proteins α and β in brown adipose tissue: evidence for a tissue-specific pattern of expression during development. Biochem. J. 302, 695-700.

Marette, A. and Bukowiecki, L. J. (1989) Stimulation of the glucose transport by insulin and norepinephrine in isolated rat brown adipocytes. Am. J. Physiol. 257, C714-C721.

Matsuo, T., Shimomura, Y., Saitoh, I., Tokuyama, K., Takeuhci, H., Suzuki, M. (1995) Sympathetic activity is lower in rats fed a beef tallow diet than in rats fed a safflower oil diet. Metabolism 44, 934-939.

Matthias, A., Jacobsson, A., Cannon, B. and Nedergaard, J. (1999) The bioenergetics of brown fat mitochondria from UCP1-ablated mice. J. Biol. Chem. 274, 28150-28160.

Matthias, A., Ohlson, K. B. E., Fredriksson, M., Jacobsson, A., Nedergaard, J. and Cannon, B. (2000) Thermogenic responses in brown fat cells are fully UCP1-dependent. J. Biol. Chem. 275 (33), 25073-25081.

Melnyk, A., Harper, M-E. and Himms-Hagen, J. (1997) Raising at thermoneutrality prevents obesity and hyperphagia in brown adipose tissue-ablated transgenic mice. Am. J. Physiol. 272, R1088-R1092.

Monemdjou, S., Kozak, L. P. and Harper, M. E. (1999) Mitochondrial proton leak in brown adipose tissue mitochondria of UCP1-deficient mice is GDP insensitive. Am. J. Physiol. 276, E1073-E1082.

Morrison, S. F., Sved, A. F. and Passerin, A. M. (1999) GABA-mediated inhibition of raphe pallidus neurons regulates sympathetic outflow to brown adipose tissue. Am. J. Physiol. 276, R290-R297.

Morroni, M., Barbatelli, G., Zingaretti, M. C. and Cinti, S. (1995) Immunohistochemical, ultrastructural and morphometric evidence for brown adipose tissue recruitment due to cold acclimation in old rats. Int. J. Obes. 19, 126-131.

Mory, G., Bouillaud, F., Combes-George, M. and Ricquier, D. (1984) Noradrenaline controls the concentration of the uncoupling protein in brown adipose tissue. FEBS Lett. 166, 393-396.

Mory, G., Ricquier, D. and Hemon, P. (1980) Effects of chronic treatments upon the brown adipose tissue of rats. II. Comparison between the effects of catecholamine injections and cold adaptation. J. Physiol. 76, 859-864.

Mory, G., Ricquier, D., Nechad, M. and Hémon, P. (1982) Impairment of trophic response of brown fat to cold in guanethidine-treated rats. Am. J. Physiol. 242, C159-C165.

Muralidhara, D. V. and Desautels, M. (1994) Changes in brown adipose tissue composition during fasting and refeeding of diet-induced obese mice. Am. J. Physiol. 266, R1907-R1915.

Murdza-Inglis, D. L., Patel, H. V., Freeman, K. B., Jezek, P., Orosz, D. E. and Garlid, K. D. (1991) Functional reconstitution of rat uncoupling protein following its high level expression in yeast. J. Biol. Chem. 260 (18), 11871-11875.

Nedergaard, J., Becker, W. and Cannon, B. (1983) Increase in thermogenin content of rat brown adipose tissue due to essential fatty acid surplus. J. Nutr. 113, 1717-1724.

Nedergaard, J., Golozoubova, V., Matthias, A., Asadi, A., Jacobsson, A., Cannon, B. (2001) UCP1: the only protein able to mediate adaptive non-

shivering thermogenesis and metabolic inefficiency. Biochim. Biophys. Acta 1504, 82-106.

Nedergaard, J., Bronnikov, G., Golozouboba, V., Rehnmark, S., Bengtsson, T., Thonberg, H., Jacobsson, A. and Cannon, B. (1993) Brown adipocyte differentiation: an innate switch in adrenergic receptor endowment and in adrenergic response. In: "Obesity in Europe". P 73-80. John Libbey and Company Ltd.

Nedergaard, J., Herron, D., Jacobsson, A., Rehnmark, S., Cannon, B. (1995) Norepinephrine as a morphogen? –its unique interaction with brown adipose tissue. Int. J. Dev. Biol. 39, 827-837.

Nedergaard, J., Matthias, A., Golozoubova, V., Jacobsson, A. and Cannon, B. (1999) UCP1: the original uncoupling protein- and perhaps the only one. J. Bioenerg.Biomembr. 31 (5), 475-491.

Oudart, H., Groscolas, R., Calgari, C., Nibbelink, M., Leray, C., Le Maho, Y. and Malan, A. (1997) Brown fat thermogenesis in rats fed high-fat diets enriched with n-3 polyunsaturated fatty acids. Int. J. Obes. 21, 955-962.

Oya, A., Asakura, H., Koshino, T. and Araki, T. (1997) Thermographic demonstration of nonshivering thermogenesis in human newborns after birth: its relation to umbilical gases. J. Perinat. Med. 25, 447-454.

Palou, A., Pico, C., Bonet, M. L., Oliver, P. (1998) The uncoupling protein, thermogenin. Int. J. Bichem. Cell Biol. 30, 7-11.

Park, I. R. A. and Himms-Hagen, J. (1988) Neural influences on trophic changes in brown adipose tissue during cold acclimation. Am. J. Physiol. 255, R874-R881.

Patel, H. V., Freeman, K. B. and Desautels, M. (1987) Selective loss of uncoupling protein mRNA in brown adipose tissue on deacclimation of cold-acclimated mice. Biochem. Cell Biol. 65, 955-959.

Pettersson, B. and Vallin, I. (1976) Noradrenaline-induced shift in levels of adenosine 3':5'-monophosphate and ATP parallel to increased respiratory rate and lipolysis in isolated hamster brown fat cells. Eur. J. Biochem. 62, 383-390.

Plomp, P. J. A. M., Gordon, P. B., Meijer, A. J., Hoyvik, H. and Seglen, P. O. (1989) Energy dependence of different steps in the autophagic-lysosomal pathway. J. Biol. Chem. 264, 6699-6704.

Plomp, P. J. A. M., Wolvetang, E. J., Groen, A. K., Meiger, A. J., Gordon, P. B. and Seglen, P. O. (1987) Energy dependence of autophagic protein degradation in isolated rat hepatocytes. Eur. J. Biochem. 164, 197-203.

Puigserver, P., Herron, D., Gianotti, M., Palou, A., Cannon, B. (1992) Induction and degradation of the uncoupling protein thermogenin in brown adipocytes *in vitro* and *in vivo*. Biochem. J. 284, 393-398.

Rehnmark, S., Antonson, P., Xanthopoulos, K. G. and Jacobsson, A. (1993) Differential adrenergic regulation of C/EBPα and C/EBPβ in brown adipose tissue. FEBS. Lett. 318, 235-241.

Rehnmark, S., Kopecky, J., Jacobsson, A., Nechad, M., Herron, D., Nelson, B. D., Obregon, M-J., Nedergaard, J. and Cannon, B. (1989) Brown

adipocytes differentiated in culture *in vitro* can express the gene for the uncoupling protein thermogenin: effects of hypothyroidism and norepinephrine. Expt. Cell Res. 182, 75-83.

Rehnmark, S., Nechad, M., Herron, D., Cannon, B. and Nedergaard, J. (1990) α- and β-adrenergic induction of the expression of the uncoupling protein thermogenin in brown adipocytes differentiated in culture. J. Biol. Chem. 265, 16464-16471.

Reichling, S., Ridley, R. G., Patel, H. V., Harley, C. B. and Freeman, K. B. (1987) Loss of brown adipose tissue uncoupling protein mRNA on deacclimation of cold-exposed rats. Biochem. Biophys. Res. Commun. 142, 696-701.

Rial, E. and Gonzalez-Barroso, M. M. (2001) Physiological regulation of the transport activity in the uncoupling proteins UCP1 and UCP2. Biochim. Biophys. Acta 1504, 70-81.

Rial, E., Poustie, A. and Nicholls, D. G. (1983) Brown-adipose-tissue mitochondria: the regulation of the 32000-M_r uncoupling protein by fatty acids and purine nucleotides. Eur. J. Biochem. 137, 197-203.

Ribeiro, M. O., Lebrun, F. L. A. S., Christoffolete, M. A., Branco, M., Crescenzi, A., Carvalho, S. D., Negrao, N. and Bianco, A. C. (2000) Evidence of UCP1-independent regulation of norepinephrine-induced thermogenesis in brown fat. Am. J. Physiol. 279, E314-E322.

Richieri, G. V., Anel, A. and Kleinfeld, A. M. (1993) Interactions of long-chain fatty acids and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB. Biochemistry 32, 7574-7580.

Ricquier, D., Bouillaud, F., Toumelin, P., Mory, G., Bazin, R., Arch, J., Penicaud, L. (1986) Expression of uncoupling protein mRNA in thermogenic or weakly thermogenic brown adipose tissue. Evidence for a rapid β-adrenoreceptor-mediated and transcriptionally regulated step during activation of thermogenesis. J. Biol. Chem. 261, 13905-13910.

Ricquier, D., Barlet, J-P., Garel, J-M., Combes-Georges, M. and Dubois, M. P. (1983) An immunological study of the uncoupling protein of brown adipose tissue mitochondria. Biochem. J. 210, 859-866.

Ricquier, D., Casteilla, L., Bouillaud, F. (1991) Molecular studies of the uncoupling protein. FASEB J. 5, 2237-2242.

Ricquier, D., Miroux, B., Larose, M., Cassard-Doulcier, A-M. and Bouillaud, F. (2000) Endocrine regulation of uncoupling proteins and energy expenditure. Int. J. Obes. 24 (Suppl 2), S86-S88.

Rippe, C., Berger, K., Boiers, C., Ricquier, D. and Erlanson-Albertsson, C. (2000) Effect of high fat diet, surrounding temperature, and enterostatin on uncoupling protein gene expression. Am. J. Physiol. 279, E293-E300.

Rohlfs, E. M., Daniel, K. W., Premont, R. T., Kozak, L. P. and Collins, S. (1995) Regulation of uncoupling protein gene (UCP) by β_1-, β_2-, and β_3-adrenergic receptor subtypes in immortalized brown adipose cell lines. J. Biol. Chem. 270, 10723-10732.

Rothwell, N. J. and Stock, M. J. (1981) Influence of noradrenaline on blood flow to brown adipose tissue in rats exhibiting diet-induced thermogenesis. Pfluegers Arch. 389, 237-242.

Rothwell, N. J. and Stock, M. J. (1984) Effects of denervating brown adipose tissue on the responses to cold, hyperphagia and noradrenaline treatment in the rat. J. Physiol. 355, 457-463.

Rothwell, N. J., Saville, M. E. and Stock, M. J. (1984) Brown fat activity in fasted and refed rats. Biosci. Rep. 4, 351-357.

Ruzicka, M., Borecky, J., Hanus, J., Jezek, P. (1996) Photoaffinity labeling of the mitochondrial uncoupling protein by [^{3}H] azido fatty acid affects the anion channel. FEBS Lett. 382, 239-243.

Sadurskis, A., Dicker, A., Cannon, B. and Nedergaard, J. (1995) Polyunsaturated fatty acids recruit brown adipose tissue: increased UCP content and NST capacity. Am. J. Physiol. 269, E351-E360.

Sakaguchi, T., Arase, K., Fisler, J. S. and Bray, G. A. (1988) Effects of starvation and food intake on sympathetic activity. Am. J. Physiol. 255, R284-R288.

Schellens, J. P. M. and Meijer, A. J. (1991) Energy depletion and autophagy. Cytochemical and biochemical studies in isolated rat hepatocytes. Histochem. J. 23, 460-466.

Schellens, J. P. M., Vreeling-Sindelarova, H., Plomp, P. J. A. M. and Meijer, A. J. (1988) Hepatic autophagy and intracellular ATP. A morphometric study. Exp. Cell Res. 177, 103-108.

Schwartz, J. H., Young, J. B. and Landsberg, L. (1983) Effect of dietary fat on sympathetic nervous system activity in the rat. J. Clin. Invest. 72, 361-370.

Seglen, P. O. and Bohley, P. (1992) Autophagy and other vacuolar protein degradation mechanisms. Experientia 48, 158-171.

Shima, A., Shinohara, Y., Doi, K., Terada, H. (1994) Normal differentiation of rat brown adipocytes in primary culture judged by their expression of uncoupling protein and the physiological isoform of glucose transporter. Biochim. Biophys. Acta 1223, 1-8.

Shimizu, H., Tsuchiya, T., Sato, N., Shimomura, Y., Kobayashi, I., Mori, M. (1998) Troglitazone reduces plasma leptin concentration but increases hunger in NIDDM patients. Diabetes Care 21, 1470-1474.

Shimizu, Y., Kielar, D., Minokoshi, Y. and Shimizu, T. (1996) Noradrenaline increases glucose transport into brown adipocytes in culture by a mechanism different from that of insulin. Biochem. J. 314, 485-490.

Sirover, M. A. (1996) Emerging new functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. Life Science 58, 2271-2277.

Skulachev, V. P. (1999) Anion carriers in fatty acid-mediated physiological uncoupling. J. Bioenerg. Biomembr. 31 (5), 431-445.

Stefl, B., Janovska, A., Hodny, Z., Rossmeisl, M., Horakova, M., Syrovy, I., Bemova, J., Bendlova, B. and Kopecky, J. (1998) Brown fat is essential for cold-induced thermogenesis but not for obesity resistance in aP2-Ucp mice. Am. J. Physiol. 274, E527-E533.

Strieleman, P. J., Schalinske, K. L. and Shargo, E. (1985) Fatty acid activation of the reconstituted brown adipose tissue mitochondria uncoupling protein. J. Biol. Chem. 260 (25), 13402-13405.

Sugden, P, H. and Fuller, S. J. (1991) Regulation of protein turnover in skeletal and cardiac muscle. Biochem. J. 273, 21-37.

Surwit, R. S., Wang, S., Petro, A. E., Sanchis, D., Raimbault, S., Ricquier, D., Collins, S. (1998) Diet-induced changes in uncoupling proteins in obesity-prone and obesity-resistant strains of mice. Proc. Natl. Acad. Sci. USA 95, 4061-4065.

Tanaka, T., Yoshida, N., Kishimoto, T. and Akira, S. (1997) Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. EMBO J. 16 (24), 7432-7443.

Thomas, S. A. and Palmiter, R. D. (1997) Thermoregulatory and metabolic phenotypes of mice lacking noradrenaline and adrenaline. Nature 387, 94-97.

Thonberg, H., Zhang, S-J., Tvrdik, P., Jacobsson, A., Nedergaard, J. (1994) Noradrenaline utilizes α_1- and β-adrenoreceptors synergistically to maximally induce *c-fos* expression in brown adipocytes. J. Biol. Chem. 269, 33179-33186.

Tischler, M. E., Ost, A. H., Spina, B., Cook, P. H. and Coffman, J. (1984) Regulation of protein turnover by glucose, insulin and amino acids in adipose tissue. Am. J. Physiol. 247, C228-C233.

Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I. and Spiegelman, B. M. (1994) PPAR2γ:Tissue specific regulator of an adipocyte enhancer. Genes Dev. 8, 1224-1234.

Trayhurn, P. (1989) Thermogenesis and energetics of pregnancy and lactation. Can. J. Physiol. Pharmacol. 67, 370-375.

Trayhurn, P. and Jennings, G. (1986) Evidence that fasting can induce a selective loss of uncoupling protein from brown adipose tissue mitochondria in mice. Biosci. Rep. 6, 805-810.

Trayhurn, P. and Jennings, G. (1988) Nonshivering thermogenesis and the thermogenic capacity of brown fat in fasted and/or refed mice. Am. J. Physiol. 254, R11-R16.

Trayhurn, P., Ashwell, M., Jennings, G. (1987) Effect of warm or cold exposure on GDP binding and uncoupling protein in rat brown fat. Am. J. Physiol. 252, E237-E243.

Trayhurn, P., Douglas, J. B. and McGuckin, M. M. (1982) Brown adipose tissue thermogenesis is suppressed during lactation in mice. Nature 298, 59-60.

Vicario, P. P., Candelore, M. R., Schaeffer, M-T., Kelly, L., Thompson, G. M., Brady, E. J., Saperstein, R., MacIntyre, D. E., Tota, L. M. and Cascieri, M. A. (1998) Desensitization of β_3-adrenergic receptor-stimulated adenylyl cyclase activity and lipolysis in rats. Life Science 62 (7), 627-638.

Villa, M., Gamez, J., Solano, A., Playan, A., Schwartz, S., Santorelli, F. M., Cervera, C., Casali, C., Montoya, J. and Villarroya, F. (2000) Uncoupling

protein-1 mRNA expression in lipomas from patients bearing pathogenic mitochondrial DNA mutations. Biochem. Biophys. Res. Commun. 278, 800-802.

Villarroya, F., Felipe, A. and Mampel, T. (1987) Reduced noradrenaline turnover in brown adipose tissue of lactating rats. Comp. Biochem. Physiol. A. 86, 481-483.

Waldbillig, D. and Desautels, M. (1992) Characterization of norepinephrine stimulated protein synthesis in rat brown adipocytes. Am. J. Physiol. 263, R1003-R1012.

Wang, N-D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R. and Darlington, G. J. (1995) Impaired energy homeostasis in C/EBPα knockout mice. Science 269, 1108-1112.

Watabe, S. and Kimura, T. (1985) Adrenal cortex mitochondrial enzyme with ATP-dependent protease and protein-dependent ATPase activities. J. Biol. Chem. 260, 14498-14504.

Williamson, J. R. (1970) Control of energy metabolism in hamster brown adipose tissue. J. Biol. Chem. 245, 2043-2050.

Williamson, D. H. (1980) Integration of metabolism in tissues of the lactating rat. FEBS Lett. (Suppl.) 117, K93-K105.

Winkler, E. and Klingenberg, M. (1992) Photoaffinity labeling of the nucleotide-binding site of the uncoupling protein from hamster brown adipose tissue. Eur. J. Biochem. 203, 295-304.

Winkler, E. and Klingenberg, M. (1994) Effect of fatty acids on H^+ transport activity of the reconstituted uncoupling protein. J. Biol. Chem. 269, 2508-2515.

Wojtczak, L. and Schonfeld, P. (1993) Effect of fatty acids on energy coupling processes in mitochondria. Biochim. Biophys. Acta 1183, 41-57.

Yamashita, H., Oh-ishi, S., Kizaki, T., Nagasawa, J., Saitoh, D., Ohira, Y. and Ohno, H. (1995) Insulin stimulates the expression of basic fibroblast growth factor in rat brown adipocyte primary culture. Eur. J. Cell. Biol. 68, 8-13.

Yeh, W-C., Cao, Z., Classon, M. and McKnight, S. L. (1995) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper protein. Genes Dev. 9, 168-181.

Yoshida, T., Kemnitz, J. W. and Bray, G. A. (1983) Lateral hypothalamic lesions and norepinephrine turnover in rats. J. Clin. Invest. 72, 919-927.

Yoshitomi, H., Yamazaki, K., Tanaka, I. (1998) Cloning of mouse uncoupling protein 3 cDNA and 5'-flanking region, and its genetic map. Gene 215, 77-84.

Young, J. B. and Landsberg, L. (1977) Suppression of sympathetic nervous system during fasting. Science 196, 1473-1475.

Young, J. B., Saville, E., Rothwell, N. J., Stock, M. J. and Landsberg, L. L. (1982) Effect of diet and cold exposure on norepinephrine turnover in brown adipose tissue of the rat. J. Clin. Invest. 69, 1061-1071.

Zhao, J., Cannon, B. and Nedergaard, J. (1997) α_1-Adrenergic stimulation potentiates the thermogenic action of β_3-adrenoreceptor-generated cAMP in brown fat cells. J. Biol. Chem. 272 (52), 32847-32856.

Zhao, J., Cannon, B., Nedergaard, J. (1998) Thermogenesis is β_3- but not β_1-adrenergically mediated in rat brown fat cells, even after cold-acclimation. Am. J. Physiol. 275, R2002-R2011.

Zhao, J., Unelius, L., Bengtsson, T., Cannon, B., Nedergaard, J. (1994) Coexisting β-adrenoceptor subtypes: significance for thermogenic process in brown fat cells. Am. J. Physiol. 267, C969-C979.