ADRENERGIC CONTROL OF PROTEOLYSIS
IN
BROWN ADIPOCYTES

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ABSTRACT

Brown adipose tissue (BAT) is a sympathetically innervated tissue involved in control of thermoregulation and energy balance. BAT growth and/or atrophy occur in response to the need for energy dissipation. Fasting, deacclimation, and lactation result in tissue atrophy by loss of cells, mitochondrial proteins, and uncoupling protein 1 (UCP1), the molecular basis for thermogenesis. The overall objective is to gain a better understanding of the control and the mechanisms underlying BAT atrophy. Specific objectives are: (1) whether in vivo patterns of BAT atrophy can be reproduced in culture. (2) whether the loss of mitochondrial proteins is due to lysosomal proteolysis or proteolysis within mitochondria, under adrenergic influence. Mouse pre-adipocytes in culture differentiated to brown adipocytes. Increased expression of UCP1 was induced by norepinephrine (NE). NE was then removed and the pattern of change in thermogenic capacity evaluated. The number of cells and their protein content did not change, whereas the cell UCP1 content was decreased. Expression of cathepsin D (a lysosomal protease) was differentiation-dependent, but was not affected by NE. Direct estimates of turnover rates of UCP1 as well as mitochondrial translation products indicated that NE reduces the degradation of mitochondrial proteins as a whole. Addition of autophagic blockers reduced the loss of UCP1 upon NE removal. Therefore, in vitro cultures of brown adipocytes mimic some aspects of brown fat atrophy seen in vivo. Brown pre-adipocytes gain a large capacity for lysosomal protein degradation during differentiation. The activity of lysosomal proteases seems under the inhibitory control of NE, as its removal causes net loss of UCP1 preventable by inhibitors of autophagy.
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LIST OF ABBREVIATIONS:

aa, amino acid;
AA, ascorbic acid;
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;
CHX, cycloheximide;
COX, cytochrome oxidase;
cpm, count per minute;
CRE, cAMP response element;
CREB, cAMP response element binding protein;
DIT, diet-induced thermogenesis;
DMEM, Dulbecco's modified eagle medium;
DNA, deoxyribonucleic acid;
ECL, enhanced chemiluminescence luminol;
EDTA, ethylenediamineacetic acid;
FA, fatty acid;
FA's, fatty acids;
FADH, reduced flavin adenine dinucleotide;
GDP, guanosine 5'-diphosphate;
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;
3-MA, 3-methyladenine;
mRNA, messenger ribonucleic acid;
NADH, reduced nicotinamide adenine dinucleotide;
NE, norepinephrine;
NST, non-shivering thermogenesis;
PBS, phosphate-buffered saline;
PCA, perchloric acid;
PIK, phosphatidylinositol kinase;
Pi, inorganic phosphate;
PKA, protein kinase A;
PPAR, peroxisome proliferator-activated receptor;
SDS, sodium dodecyl sulfate;
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
SNS, sympathetic nervous system;
T3, 3,5,3'-triiodothyronine;
TCA, trichloroacetic acid;
TTBS, Tween 20 tris-buffered saline; ADP, adenosine 5’-diphosphate;

UCP, uncoupling protein;

W, wortmannin;
1.0 INTRODUCTION

1.1 BAT LOCATION, STRUCTURE AND INNERVATION

Brown adipose tissue (BAT) is mainly present in newborn and small mammals (Benito 1985; Vila et al 2000). In larger mammals, including humans, dogs, cows and primates, however, it apparently diminishes with age. BAT is located in many areas such as in the cervical, axillary, perirenal, perigonadal and interscapular regions (Vila et al 2000). Histological studies have demonstrated that BAT consists of 40% mature brown adipocytes, 50% endothelial cells forming the numerous capillaries surrounding brown fat cells and the remaining 10% being distributed between interstitial cells, mast cells and pre-adipocytes (D’ Allaire et al 1995; Atgie et al 1997). Mature brown adipocytes are characterized by the presence of a large regular/spherical nucleus and numerous lipid droplets surrounded by many mitochondria with parallel and densely packed cristae (Sbarbati et al 1987). Their mitochondria include low content of ATP synthase and high content of oxidative enzymes (enzymes involved in the respiratory chain within the mitochondria) paralleled by a high content of a tissue-specific protein called uncoupling protein 1 (UCP1) (Houstek et al 1990, 1995; Daikoku et al 2000; Denjean et al 1999). Pre-adipocytes are smaller than mature brown adipocytes. They are characterized by a large irregular nucleus, only a few small lipid droplets and a low content of mitochondria with a few cristae. Their mitochondria lack UCP1. The pre-adipocytes can replicate and differentiate into mature brown adipocytes under certain conditions in vitro and in vivo (Cannon and Nedergaard, 1996).
BAT is densely innervated by the sympathetic nervous system (SNS) that runs not only along the capillaries but also between the individual adipocytes (Atgie et al 1997). The two symmetrical pads of interscapular BAT are each supplied by 4 intercostal nerves as well as 1 nerve that enters along the thoracodorsal artery (Park and Himms-Hagen, 1989; Foster et al 1982). Pre- and mature brown adipocytes can respond to the sympathetic stimulation through the sympathetic receptors on their plasma membranes. Mature brown adipocytes contain several kinds of adrenergic receptors including $\alpha_1$ (1 and 2) and $\beta_1$ (1, 2 and 3) with the $\beta_3$-adrenergic receptor being the predominant one. In contrast, pre-adipocytes do not contain $\beta_3$-adrenergic receptor and $\beta_1$-adrenergic receptor is the predominant one (Atgie et al 1997; D’Allaire et al 1995; Cannon and Nedergaard, 1996).

1.2 BAT MITOCHONDRIA AND HEAT PRODUCTION

Mitochondria of all tissues other than BAT are the main site of ATP synthesis. The energy from the oxidation of substrates such as glucose and fatty acids (FA’s) is used by the respiratory chain to create a proton electrochemical potential difference across the inner mitochondrial membrane. Subsequently, as the cytosolic 5’-adenosine diphosphate (ADP) to 5’-adenosine triphosphate (ATP) ratio increases, proton re-entry is used to drive an inner membrane-bound ATP synthase that phosphorylates ADP to ATP (Nicholls and Locke, 1984; Branco et al 1999). Therefore, these mitochondria are coupled since ATP synthesis is dependent upon coupling between oxidation reactions and ADP phosphorylation (Figure 1.1 A) (Branco et al 1999).
Figure 1.1 Mitochondrial function in (A) all body tissues compared to (B) BAT. Whereas mitochondria of all tissues are well developed for ATP production, BAT mitochondria with high content of UCP1 are specialized for heat production.
In contrast, BAT function is nonshivering thermogenesis (NST, a type of heat production which does not involve muscular contraction) (Yahata et al 1999). The numerous mitochondria in mature brown adipocytes are the heat producing organelles. The molecular mechanism for heat generation is UCP1 as UCP1-ablated mice do not survive in cold temperatures (Enerback et al 1997; Jezek et al 1998; reviewed by Nedergaard et al 2001). UCP1 is a 32 kDa protein, a dimer of 307 amino acid (aa) residues, embedded in inner mitochondrial membrane (Rial and González-Barroso, 2001; Fernandez et al 1987; Klingenberg, 1990; Echtay et al 1999). UCP1 functions as a proton short-circuit that dissipates the proton electrochemical potential across the inner mitochondrial membrane (Palou et al 1998; Masaki et al 2000). As a result, it uncouples substrate oxidation from ATP synthesis (uncoupled mitochondria), increases O$_2$ consumption, and liberates substantial heat (Figure 1.1 B) (Branco et al 1999; Ricquier and Bouillaud, 1997; Klingenberg and Huang, 1999). Since the high O$_2$ consumption in BAT mitochondria is accompanied by heat generation, O$_2$ consumption in brown adipocytes is taken as an index of BAT thermogenesis in many studies (Matthias et al 1999; Ricquier et al 2000).

The proton-translocating activity of UCP1 within BAT mitochondria is regulated. It is inhibited by purine nucleotides such as 5’-guanosine diphosphate (GDP) and activated by fatty acids (FA’s) (Winkler et al 1997). Accordingly, UCP1 has been observed to have one GDP binding site per subunit (Lin et al 1980; Klingenberg, 1990). Furthermore, Matthias et al (1999) pointed out that the GDP-binding capacity observed in the isolated brown fat mitochondria from wild-type mice (UCP1-containing mice) was practically eliminated from UCP1-ablated mice
and was reduced to the level observed in liver mitochondria. In addition, low proton permeability and high membrane potential in BAT mitochondria from UCP1-ablated mice were not affected by GDP. Similarly, FA binding sites were also shown to exist on UCP1 (Jezek et al 1995, 1996; Ruzicka et al 1996). In wild-type cells, FA’s induced a thermogenic response, but were practically without effect in UCP1-ablated cells (Matthias et al 2000; Klingenberg and Echtay, 2001).
1.3 CAPACITY OF BAT FOR THERMOGENESIS IS VARIABLE

The thermogenic capacity of BAT is not constant. It is drastically affected by different environmental situations and by the physiological status of the animals (Himms-Hagen, 1990). Conditions such as cold temperature and certain types of diet have been shown to not only acutely activate the thermogenic activity of the tissue, but also to induce the growth of the tissue, and thereby increase its capacity for thermogenesis even more. In contrast, other conditions such as fasting, deacclimation (transfer of cold-acclimated animals to a thermoneutral temperature in which the animal does not need to produce heat as a mean of thermoregulation), and lactation, during which energy should be conserved, are known to cause atrophy of the tissue, and thus, decrease its thermogenic capacity. In general, BAT atrophy is characterized by loss of tissue cellularity, total cellular protein content, mitochondrial protein content and specifically UCP1 (Himms-Hagen, 1990). These conditions and their sympathetic/hormonal control are explained in more detail in the following sections.

1.3.1 INCREASED BAT THERMOGENIC ACTIVITY/CAPACITY

1.3.1.1 IMPORTANCE OF INCREASED TISSUE THERMOGENIC ACTIVITY DURING COLD EXPOSURE AND CERTAIN DIETS

Increased BAT thermogenic activity and capacity is important for thermoregulation in mammals exposed to cold, and for energy balance during feeding certain types of diet, preventing obesity (Smith et al 1998; West et al 1992; Harrold et al 2000). When rats were offered a palatable diet rich in fat, not all rats gained weight equally. About 18% of rats did not show weight gain in excess of controls (Rothwell
et al 1982). It was suggested that an ability to increase fat oxidation in response to increased fat intake might help to explain why some animals gain weight on high fat diets while others do not. Accordingly, several studies have reported that BAT, whose main function is to burn fat to produce heat, is important in this respect (Rothwell and Stock, 1979, 1980, 1981), and that variations in the rate of thermogenesis in the tissue can have a substantial effect upon energy balance and thermoregulation (Trayhurn, 1986; Rothwell and Stock, 1986).

Recently, Lowell et al (1993) used the regulatory elements of the gene for UCP1 to drive the expression of diphtheria toxin-A-chain in BAT of mice. Two lines of transgenic mice with primary deficiency of BAT were created. As a result of decreased brown fat, both lines of mice developed obesity. In one line of mice, however, brown fat was subsequently regenerated over time and interestingly, obesity was resolved. In the other line, on the other hand, deficiency persisted and as a consequence, obesity with its morbid complications advanced. When these mice were exposed to environmental cold, their core temperatures also dropped, indicating the importance of UCP1 and thus BAT in the regulation of body temperature. The latter results were confirmed using homologous recombination to inactivate the UCP1 gene in BAT (Enerback et al 1997).

The importance of UCP1 in thermoregulation and energy balance was also reported in transgenic mice expressing the UCP1 gene from the aP2-gene promoter (aP2-UCP mice) in brown and white fat (Kopecky et al 1995, 1996, 1996; and Stefl et al 1998). It was shown that transgenic mice with ectopic synthesis of UCP1 in white fat were resistant to obesity induced by a high fat diet. These animals exhibited
atrophy of BAT, as indicated by a decrease in the amount of brown fat and reduction of its total UCP1 and deoxyribonucleic acid (DNA) contents. BAT atrophy in these mice was presumably the result of increased thermogenesis in the white fat, reducing the need for heat production in BAT. Surprisingly, the transgenic mice exhibited a reduction in their capacity to maintain body temperature when exposed to 4°C environment. These results indicate that the role of brown fat in cold-induced thermogenesis can not be substituted by increased energy expenditure in other tissues.

1.3.1.2 CHANGES IN BAT DURING COLD EXPOSURE OF ANIMALS AND THEIR SYMPATHETIC/HORMONAL CONTROL

Several studies have demonstrated that acute cold exposure of various kinds of mammals (specially small rodents such as rats and mice) elicits heat production in BAT measured by either changes in BAT temperature or O₂ consumption (Bukowiecki et al 1982; Rothwell and Stock, 1981; Rothwell and Stock, 1980; Hoppeler et al 1995 and reviewed by Nedergaard et al 2001). In addition, chronic exposure of mice and rats to cold was observed to lead also to an increased BAT thermogenic capacity via the process of tissue recruitment (Mory et al 1984). There is remarkable BAT growth mainly due to the proliferation of brown pre-adipocytes during tissue recruitment (Bukowiecki et al 1980). It has been reported by several researchers that cold exposure of rats for 2 days markedly enhanced the proportion of pre-adipocytes relative to other cell types in BAT. The proliferation of brown pre-adipocytes continued to further increase so that after 3 weeks of cold exposure an increase of 6-8 times in total DNA content of BAT was observed.
(Bukowiecki et al 1980, 1986; Rothwell and Stock, 1984; Geloen et al 1992; Cousin et al 1996 and Fluorez-Duquet, 1998). Subsequent to proliferation, brown pre-adipocytes differentiate into mature brown adipocytes via fat accumulation, increasing the number of mitochondria per cell as measured by the activity of cytochrome oxidase (COX, a mitochondrial inner membrane enzyme involved in the respiratory chain), total cellular protein content, mitochondrial protein content, and specifically UCP1 (Mory et al 1984). Moreover, during the process of tissue recruitment, mature brown adipocytes present in BAT also increase their content of total cellular proteins, mitochondrial proteins and UCP1, but they do not go through proliferation (Figure 1.2).

Several researchers quantitated the amount of UCP1 in the mitochondria isolated from rats exposed to cold for different durations. While there was no change in the UCP1 content in 1 hour cold-adapted rats, a significant increase in the amount of the protein was observed after 48 hours, and an even greater increase in rats exposed to cold for 3 weeks (Figure 1.3) (Nedergaard and Cannon, 1985; Desautels et al 1978; Rial and Nicholls, 1984; Trayhurn et al 1987). UCP1 messenger ribonucleic acid (mRNA) was also increased during cold exposure of the animals (Ricquier et al 1984; Cousin et al 1996). There is an increase in total protein biosynthetic capacity of BAT during cold exposure of the animals along with an increased yield of total mRNA per unit mass of BAT (Figure 1.3). There is also increased translational activity within mitochondria (Figure 1.3) (Denjean et al 1999; Jingga et al 1996).

Both increased BAT thermogenesis as well as growth upon cold exposure of mice and rats are suggested to be mainly mediated by the release of norepinephrine
Figure 1.2  Brown fat proliferation and differentiation in conditions with increased tissue sympathetic activity. Brown pre-adipocytes proliferate and differentiate into mature brown adipocytes via fat accumulation, UCP1 expression and increasing their content of mitochondrial proteins in general.
Figure 1.3  Increased BAT sympathetic activity leads to the increased tissue thermogenic capacity. Tissue thermogenic capacity is elevated as a result of increased tissue cellularity as well as protein content. Increased tissue protein content is the result of increased both gene expression and mRNA translation. The half-life of UCP1 expression and increasing their content of mitochondrial proteins in general.
(NE) from the sympathetic nerves in BAT (Mory et al 1984; Jingenspor et al 1996; Cui and Himms-Hagen, 1992). Ashwell (1987) pointed out that NE turnover in BAT of mice and rats is elevated during cold (Figure 1.3). In addition, there are several studies suggesting the requirement of an intact sympathetic stimulation for BAT thermogenic activity and growth: (1) Stimulation of the sympathetic nerves supplying BAT resulted in the activation of thermogenic activity and growth (Rothwell and Stock, 1984). This is in agreement to the increased DNA content of interscapular BAT upon stimulation of sympathetic activity observed by Cousin et al (1996). (2) Surgical, chemical or immunological sympathectomy was shown to inhibit not only BAT thermogenesis but also its growth during cold (Geloen et al 1992). It was clearly observed that in the denervated BAT pads, the effects of cold exposure on cell proliferation were markedly inhibited compared to intact interscapular BAT pads. However, NE infusion markedly reversed that (Geloen et al 1988; Cannon and Nedergaard, 1996). It was also demonstrated that surgical denervation of rat BAT significantly inhibits cold-induced increases in protein content, COX activity and UCP1 content (Dulloo and Miller, 1984; Himms-Hagen et al 1990; Park et al 1988; Denjean et al 1999).

Several studies also examined the effects of NE on isolated brown adipocytes in culture. When added to the cultures of brown pre-adipocytes, NE accelerated DNA synthesis (Bronnikov et al 1992). Moreover, significant increases in the O$_2$ uptake (heat production) (Bukowiecki et al 1981) as well as in the rates of total protein synthesis (2-3 fold) and mitochondrial protein synthesis (2.6 fold), were observed upon NE treatment of isolated rat brown adipocytes (Figure 1.3) (Waldbillig
and Desautels, 1992). Among the mitochondrial proteins, UCP1 had the greatest increase. The NE-increased UCP1 synthesis was shown to be both at the level of gene transcription and protein translation (Figure 1.3) (Arch et al 1984; Kopecky et al 1990; Rehnmark et al 1989, 1990; Klaus et al 1991; Hernandez and Obregon, 2000). Other than being essential for the increased synthesis of UCP1 and its mRNA, NE has been demonstrated to stabilize the UCP1 mRNA and maintain the elevated level of UCP1 protein. NE has been reported to increase the UCP1 mRNA half-life from about 3 hours to at least 20 hours (Figure 1.3) (Pico et al 1994; Palou et al 1998; Nedergaard et al 2001; Hernandez and Obregon, 2000).

Although the thermogenic activity and growth of BAT are primarily controlled by NE, other hormones such as insulin and 3,5,3'-triiodothyronine (T3, thyroid hormone) may also play important roles (Rothwell and Stock, 1988; Palou et al 1998). Indeed, studies on rats with experimental diabetes induced by streptozotocin suggest that insulin is essential for the activity of BAT. In streptozotocin-diabetic rats, cold tolerance and NE-stimulated thermogenesis are markedly reduced (Seydoux et al 1984). Geloen and Trayhurn (1990) studied the effects of diabetes and insulin replacement on the UCP1 content of BAT. The content of UCP1 in BAT of diabetic animals was substantially decreased. The decrease in UCP1 in diabetic animals was the result of a fall in cell mitochondrial protein content and a reduction in the specific mitochondrial concentration of UCP1. At low replacement doses, insulin increased the specific mitochondrial concentration of the UCP1. Insulin also led to an increase in total mitochondrial proteins in BAT, indicating that there is a general effect of the hormone on the accumulation of mitochondrial proteins in the tissue. The argument
that insulin regulates the thermogenic capacity of BAT is further supported by the observation that there is a positive correlation between the dose of insulin replacement and both the total and mitochondrial protein contents of the tissue, in addition to the amount of UCP1. A stimulatory effect on the activity of particular enzymes in BAT mitochondria, for example COX and succinate dehydrogenase (an inner mitochondrial enzyme) can also be observed (Seydoux *et al* 1984). Whether the action of insulin on the thermogenic activity and capacity of BAT is primarily direct, or indirect via a central activation of the SNS, remains an open question (Geloen and Trayhurn, 1990). Evidence for the importance of direct effects of insulin on BAT is given in a report in which the reduction of COX activity observed in diabetic rats was attenuated by insulin, despite surgical denervation of the tissue (Geloen and Trayhurn, 1990). However, several studies have suggested that insulin can increase the activity of the SNS (Rothwell and Stock, 1988). In rats, streptozotocin-induced diabetes leads to a reduction in NE turnover in BAT (Yoshida *et al* 1985). The fact that insulin does not stimulate respiration in isolated brown adipocytes is a strong argument that, by itself, the hormone is not a direct activator of thermogenesis (Geloen and Trayhurn, 1990).

T3 is also important in regulating BAT thermogenic activity and growth (Reitman *et al* 1999). Hypothyroid animals are intolerant to the cold and even die when exposed to the cold for several hours (Bianco and Silva, 1987; Hernandez and Obregon, 2000). This deficiency can be restored by adequate replacement therapy with T3 (Silva and Matthews, 1988; Hernandez and Obregon, 2000). This is in accordance to a low mitochondrial UCP1 content of hypothyroid rats which can be
restored by T3 administration (Obregon et al 1987, 1996). In addition, T3 seems to be necessary for the expression of UCP1 *in vivo* (Hernandez and Obregon, 2000). Several research groups also include T3 as a differentiation factor of cultured brown adipocytes, necessary for the stimulation of UCP1 expression (Alvarez et al 1995; Klaus et al 1994; Guerra et al 1996; Masaki et al 2000). T3 appears to increase UCP1 mRNA half-life (Branco et al 1999; Bianco et al 1992).

### 1.3.1.3 CHANGES IN BAT UPON FEEDING ANIMALS CERTAIN TYPES OF DIET AND THEIR SYMPATHETIC/HORMONAL CONTROL

Certain types of diet are known to not only acutely stimulate BAT thermogenesis [known as diet-induced thermogenesis (DIT)], but also to induce the tissue growth (Figure 1.3) (Ricquier et al 2000; Bradford and Bruce, 2000; Giraudo et al 1994; Surwit et al 1998). Rippe et al (2000) showed an increased UCP1 mRNA expression in animals kept in room temperature but fed high fat diets. Brown fat UCP1 protein level and also mitochondrial protein content of BAT were increased by about 5 and 3 fold respectively by this type of diet (Curcio et al 1999). However, Nedergaard et al (1983) also demonstrated that not only the relative content of fat in the diet, but also the composition of the fat plays a role in DIT. Diets with a normal content of total fat, but with a relatively high content of polyunsaturated FA’s induce a real increase in the amount of UCP1 with a concomitant increase in the thermogenesis (Sadurskis et al 1995).
All the above changes occurring in BAT upon feeding certain types of diet seem to be mainly mediated by the release of NE from sympathetic nerves (Figure 1.3). It has been demonstrated that animals, which had been fed a high polyunsaturated fat diet had an increased turnover of NE in the sympathetic nerves innervating BAT (Sadurskis et al 1995). Moreover, intravenous injection of carbohydrates, such as glucose, also increased sympathetic activity (Rowe et al 1981; Sakaguchi et al 1988; Matsuo et al 1995). Accordingly, sympathetic activity is reduced in obese animals where BAT thermogenesis is reduced (Marette et al 1990). In addition to NE, insulin may be another important factor, which affects BAT thermogenesis and growth directly or indirectly upon eating diets high in carbohydrates as its level increases markedly under these conditions (Sakaguchi et al 1988).

So, the conclusion from the above studies is that the increased content of NE in BAT induced by either cold exposure of the animals or feeding them high fat diets leads to the increased tissue thermogenic activity and capacity. Increased tissue thermogenic capacity generally results from the increased tissue cellularity, total protein and mitochondrial protein contents and increased UCP1 content of mitochondria (Figures 1.2 and 1.3).
1.3.1.4 NE SIGNAL TRANSDUCTION PATHWAY LEADING TO INCREASED TISSUE THERMOGENIC ACTIVITY/CAPACITY

Knowing that NE is the main neurotransmitter responsible for all the changes that occur in BAT thermogenic activity and growth during cold exposure and diet, the question became "What is the NE signaling pathway for the induction of thermogenesis and tissue growth?" It has been demonstrated that NE stimulates both heat production and differentiation in BAT mainly via $\beta_3$-adrenoceptors on brown fat cells (Yoshida et al 1996; Zhao et al 1998). This has been demonstrated by the fact that the absolute $\beta_3$-agonist, CGP-12177, stimulates heat production as well as differentiation in brown adipocytes almost to the same extent as NE. In addition, adrenergic antagonists, ICI-89406 ($\beta_1$-selective), ICI-118551 ($\beta_2$-selective) and propranolol ($\beta_1/\beta_2$-non-selective) do not interfere with NE thermogenic activity and differentiation in brown fat cells (Tanaka et al 1995). However, it is noteworthy to mention that NE released from sympathetic nerves also interacts with $\alpha_1$-adrenergic receptors. Interaction with $\alpha_1$-adrenoceptors potentiates the $\beta_3$-mediated thermogenic effect of NE via the increased release of calcium from intracellular stores (Figure 1.4) (Cannon et al 1996).

Several studies carried out on whole animals, brown fat in situ, and brown adipocytes in vitro revealed the following chain of events involved in the thermogenic response and differentiation of the tissue, for example, to cold or diet (Figure 1.4): NE, which is released from sympathetic nerves adjacent to brown fat cells, binds mostly to $\beta_3$-adrenergic receptors that are coupled via $G_s$-proteins to adenylyl cyclase (Nedergaard et al 1999). Activation of adenylyl cyclase results in the conversion of
Figure 1.4  NE-signaling pathway which leads to thermogenesis and differentiation in mature brown adipocytes. CREB=cAMP-response element binding protein, CRE=cAMP-response element, cAMP=cyclic adenosine monophosphate, PKA=protein kinase A, HSL=hormone-sensitive lipase, FA=fatty acid.
ATP to cyclic adenosine monophosphate (cAMP), and thus increases the intracellular concentration of cAMP (Nedergaard et al 1999). There is activation of cAMP-dependent protein kinase A (PKA), which phosphorylates a serine residue on the hormone-sensitive lipase (HSL) and promotes its activation and translocation towards the lipid vacuoles. The activation of HSL is important in preparing the fuel for BAT thermogenesis. HSL catalyzes a rate-limiting step in the degradation of the stored triglycerides to FA’s (Nedergaard et al 1995). Then, free FA’s are released within the cytosol and play two important roles in the induction of thermogenesis by entering into the mitochondria: inside the mitochondria, FA’s (a) are oxidized by β-oxidation within matrix and therefore provide the fuel for the mitochondrial respiratory chain and, (b) activate UCP1 on the outside of the inner membrane, as mentioned before (Yamazaki et al 1995; Matthias et al 1999; Skulachev, 1999). Finally, the activated UCP1 dissipates the proton gradient across the inner mitochondrial membrane leading to heat generation (Figures 1.4 and 1.1 B) (Palou et al 1998; Strosberg and Pietri-Rouxel, 1996).

Another important event, which occurs within brown adipocytes upon β3-adrenoceptor stimulation and a concomitant increase in the intracellular concentration of cAMP following activation of PKA, is the process of brown fat differentiation (Atgie et al 1997). The mechanism of brown fat differentiation is still not fully understood, but it has been suggested that activation of PKA leads to the phosphorylation of the cAMP-response-element-binding protein (CREB) that is sufficient to increase the expression of UCP1 by binding to cAMP-response element (CRE) in the promoter region of UCP1 gene (Figure 1.4) (Nedergaard et al 1995).
The involvement of many transcription factors such as CCAAT/enhancer-binding protein β (C/EBP/β) and peroxisome proliferator-activated receptor-α and -γ (PPARα, PPARγ) also has been suggested in this process (Tanaka et al 1997; Barbera et al 2001).

In contrast to the involvement of the β3-adrenoceptors in the two processes of heat production and differentiation of brown adipocytes, β1-adrenoceptors are suggested to be involved in the process of pre-adipocyte proliferation induced by NE (Atgie et al 1997). This is confirmed by the fact that β3-specific agonist CGP-12177 is unable to stimulate DNA synthesis in pre-adipocytes whereas β1-agonist ICI89406 does (Atgie et al 1997). It is suggested that NE released from the sympathetic nerves binds to β1-adrenoceptors on the plasma membrane of pre-adipocytes. This leads to an increase in the intracellular concentration of cAMP by the same mechanism as binding of NE to β3-adrenoceptors. Increased cAMP level, then, in an unknown way, leads to the increased mitosis of brown pre-adipocytes. For example, it is suggested that acceleration of cell division would probably require increased activation of enzymes directly involved in DNA synthesis, such as ribonucleotide reductase (Atgie et al 1997). Preliminary experiments do indicate positive effects of adrenergic stimulation on the activity of ribonucleotide reductase in pre-adipocytes.
1.3.2 DECREASED BAT THERMOGENIC ACTIVITY/CAPACITY

1.3.2.1 CHANGES IN BAT UPON FASTING AND THEIR SYMPATHETIC/HORMONAL CONTROL

It was reported that fasting rats for 24-48 hours reduces BAT thermogenesis to subsequent exposure to cold, feeding high fat diets, and NE injection, measured by O₂ consumption as well as changes in BAT temperature (Avakian and Horvath, 1981; Rothwell et al 1983; Hayashi and Nagasaka, 1983). A 35% reduction in NE-stimulated O₂ consumption was observed in mice previously acclimated to 21°C and kept at 21°C during fasting (Desautels, 1985). The observed decrease in thermogenesis is consistent with several data showing BAT atrophy during fasting. BAT atrophy in rats and mice following 48 hours of fasting or even restriction of food intake to 65% of controls for 2-3 weeks was characterized by a rapid loss of brown fat mass, significant decrease in the tissue content of proteins in synchrony with tissue mitochondrial proteins, including content of UCP1 within mitochondria, without any change in BAT DNA content (Figure 1.5) (Rothwell and Stock, 1982; Desautels, 1985; Desautels et al 1986; Desautels et al 1990; Desautels and Dulos, 1988; Trayhurn and Jennings, 1988). These fasting-induced changes in BAT, however, have been shown to recover after the initiation of re-feeding. A full restoration of thermogenic capacity was evident after 15 days of re-feeding (Trayhurn and Jennings, 1988).

However, there are several important factors, which affect the extent of the nutritional stress and thus the rate of brown fat atrophy imposed by fasting (Figure 1.5). These factors include: environmental temperature in which the animals are
Several factors can influence the extent of tissue atrophy. These include lactation, deacclimation, fasting, and a decrease in sympathetic activity in BAT. Factors such as the number of animals per cage, environmental temperature, and feeding conditions can also contribute to the decrease in sympathetic activity.

- Lactation
- Deacclimation
- Fasting

These factors result in a decrease in sympathetic activity in BAT, leading to:
- Decrease in total protein content
- Decrease in mitochondrial protein content
- Decrease in mitochondrial UCP1
- Decrease in DNA content (except during fasting and lactation where DNA content does not change)

These changes ultimately lead to tissue atrophy.

Figure 1.5  Tissue changes leading to BAT atrophy in conditions with decreased tissue sympathetic activity. Tissue atrophy is generally associated with decreased tissue protein content and specifically mitochondrial UCP1 with or without loss of tissue cellularity.
acclimated and fasted. It was demonstrated that there was no protein loss or loss of tissue weight during fasting from BAT of mice acclimated and fasted at 33°C, in contrast to those acclimated and fasted at 4°C and 21°C. Moreover, the absolute and relative protein loss from BAT of fasting mice previously acclimated to 4°C far exceeded that of mice acclimated at 21°C (Desautels et al 1990). The tissue atrophy during a fast is also more pronounced in mice housed singly than at a higher caging density (Desautels and Dulos, 1990). This is probably because of the higher thermogenic requirement of mice housed singly in the absence of huddling or other activity. A significant fall in mitochondrial UCP1 after a 48 hour fast was observed when mice were housed singly or in pairs, as opposed to groups of 6-10 animals per cage (Trayhurn and Jennings, 1988, 1986; Desautels, 1985).

After evaluation of the effects of fasting on thermogenic activity and capacity of BAT, the question became “what are the agents controlling these effects?” As was mentioned before, since NE has been suggested to be the most important agent increasing BAT thermogenic activity and capacity, it was suggested that a decrease in SNS activity might be responsible for the effects of fasting. Several studies have evaluated SNS activity in BAT during fasting. There was a reduction in the rate of NE turnover in BAT following fasting (Young and Landsberg, 1977; Young et al 1982; Yoshida et al 1983). Moreover, measurement of electrical activity of sympathetic nerves to BAT during fasting demonstrated a decrease in the firing rate of sympathetic nerves, most of which occurred during the first 24 hours of fasting (Sakaguchi et al 1988). Consistent with these data, unilateral surgical denervation or chemical sympathectomy using 6-hydroxydopamine (which depletes the
catecholamine of BAT effectively), as well as propranolol injections, did cause BAT atrophy (Desautels and Dulos, 1988). BAT atrophy was characterized by some reduction in tissue content of proteins and succinate dehydrogenase activity, but a marked loss of UCP1 (Figure 1.5). However, the loss of tissue proteins and succinate dehydrogenase activity but not UCP1, caused by fasting was much greater than what was observed by denervation. Therefore, these data demonstrated that suppression of neural input to BAT is an important factor but may be not the only one in causing loss of BAT thermogenic capacity during a fast.

In searching for other factors responsible for BAT atrophy, Fernandez et al (1987) and Parks and Himms-Hagen (1989) explained that BAT production of T3 is markedly reduced by food deprivation. It was shown that in thyroxine-treated rats, the suppression of thermogenic response to NE caused by food deprivation is largely prevented (Hayashi and Nagasaka, 1983). Such an effect may be explained in part by a reduction of the tissue atrophy normally observed in fasting rats (Rothwell et al 1984). In hyperthyroid mice, the loss of tissue mitochondrial proteins after food deprivation or denervation, but not that of UCP1 from the mitochondria, was markedly suppressed (Desautels and Dulos, 1990). Insulin, as mentioned before, is another important factor, which enhances thermogenic capacity of BAT. Therefore, its decreased level during fasting may be another factor contributing to BAT atrophy, as streptozotocin-induced diabetic rats with decreased insulin levels showed BAT atrophy (Jourden et al 1984).
1.3.2.2 CHANGES IN BAT DURING DEACCLIMATION AND THEIR SYMPATHETIC/HORMONAL CONTROL

Similar to fasting, deacclimation also showed a decrease in thermogenic activity and capacity of BAT. Mice acclimated either to 4°C or 21°C and returned to a thermoneutral environment (33°C) showed a decrease in BAT thermogenic activity concomitant with tissue atrophy (Desautels, 1985). In contrast to fasting, deacclimation-induced atrophy was characterized by loss of tissue cellularity (DNA content) with a very rapid and significant loss of total cellular proteins, within 24-48 hours of exposure to 33°C. In synchrony with the tissue protein loss, there was a marked loss of mitochondrial proteins (Figure 1.5). The total tissue UCP1 content also decreased largely in proportion to the loss of mitochondrial proteins within the first 48 hours of exposure to 33°C (Figure 1.5). On a slower time course, there was a significant decrease in the concentration of UCP1 within mitochondria 8-20 days after transfer of cold-acclimated rodents to the warm environment (Trayhurn et al 1982; Desautels, 1985). Puigserver et al (1992) pointed out that when cold-acclimated mice were returned to thermoneutral temperature, mitochondrial UCP1 content slowly decreased, but even after 4 days it was still much higher than in controls. Keeping with the decrease in the UCP1 protein level during deacclimation, there is a rapid and striking loss of UCP1 mRNA upon deacclimation (Patel et al 1987).

NE was again suggested to be the main effector of all the changes occurred in BAT during the deacclimation period. It was demonstrated that when cold-exposed animals (mice and rats) are returned to a thermoneutral environment,
sympathetic activity to BAT stops or is greatly reduced (Figure 1.5) (Reichling et al 1987; Trayhurn and Jennings, 1988).

1.3.2.3 CHANGES IN BAT DURING LACTATION AND THEIR SYMPATHETIC/HORMONAL CONTROL

Similar to fasting and deacclimation, reduction in BAT thermogenic activity and capacity has been observed during lactation (Agius and Williamson, 1980; Williamson, 1980). This is, of course, because of the fact that increased food intake does not compensate for increased energetic cost of lactation and therefore reductions in BAT thermogenic activity and capacity make sure that the substrates, which would otherwise be dissipated in BAT as heat, are available for the synthesis of milk (Thurlby and Trayhurn, 1980). In 1983, Trayhurn measured BAT O₂ consumption in response to NE in mice at early, mid and late-lactation. The measurements were also made post-weaning. The study showed that NE-stimulated O₂ consumption is significantly decreased during lactation in mice, and that this reduction is greatest at mid-lactation when milk production is close to maximum. Trayhurn’s results also showed that the reduction in thermogenesis during lactation is only temporary, since a full recovery was observed 1 week after weaning. These results were also in agreement to the results obtained by Isler et al (1984) in rats.

The decrease in whole animal NE-stimulated O₂ consumption is consistent with the results of biochemical studies showing BAT atrophy during lactation. Total protein content of interscapular BAT pads was significantly lower at mid and late lactation than in the virgin controls, but returned to normal on weaning. In addition, a
marked fall in the mitochondrial protein content was evident between early and mid-lactation, and it was maintained until weaning (Figure 1.5). BAT mitochondrial protein content, however, was normalized 1 week after weaning. The mitochondrial concentration of UCP1 was not significantly altered in early lactation, but a marked fall in the concentration of the protein occurred between early and mid-lactation (Figure 1.5). The lowest mitochondrial concentration of UCP1 was found in late lactation (16% of the virgin controls) (Trayhurn, 1989; Trayhurn and Jennings, 1987). There was a marked increase in mitochondrial UCP1 concentration after weaning, such that by 3 weeks post-weaning the tissue content of UCP1 had returned to normal.

BAT atrophy in response to lactation appears to be the result of reduced sympathetic activity in BAT. There is 3 fold reduction in NE turnover rate during lactation in both rats and mice (Figure 1.5) (Villarroya et al 1987). The reduction in sympathetic tone in BAT was evident from early lactation. However, a rapid normalization of NE turnover in BAT has been observed following weaning and this would clearly account for the restoration of the thermogenic capacity of BAT (Trayhurn, 1989). Finally, it is noteworthy to mention that other than NE, the levels of several other hormones such as prolactin, corticosterone, and insulin are also altered in lactating rodents (Romsos, 1985; York et al 1985; Trayhurn, 1989).
1.3.2.4 PROTEOLYTIC PATHWAYS LEADING TO DECREASED TISSUE THERMOGENIC CAPACITY AND THEIR SYMPATHETIC/HORMONAL CONTROL

As was explained above, BAT atrophy caused by means of fasting, deacclimation of cold-acclimated animals to thermoneutral temperature, or lactation, is characterized by total tissue and mitochondrial protein loss per cell as well as loss of UCP1 specifically from mitochondria (Figure 1.5). Surprisingly, in contrast to the extensive studies concerned with the mechanisms underlying BAT growth and increased tissue thermogenic capacity, there have been few studies concerned with defining the pathways involved in mediating BAT atrophy, and protein loss in particular.

Changes in a tissue protein mass always depend on the balance between rates of protein synthesis and degradation. Cellular degradation of proteins is a process that facilitates the adaptation of cellular functions to the changes in environmental and physiological conditions. Proteolysis of cellular proteins occurs through a number of distinct pathways that hydrolyze different classes of proteins. Their relative importance depends on both cell type and different physiological or pathological conditions (Beynon and Bond, 1986; DeDuve, 1983). A major mechanism for degradation of cellular proteins is the non-selective bulk process known as autophagy (Plomp et al 1989). The process includes several steps: in the first step, autophagic sequestration, a cytoplasmic membrane envelops a region of cytoplasm into a closed vacuole called an autophagosome. Sequestration is highly regulated and is under the control of GTPases (enzymes which hydrolyze guanosine
5'-triphosphate), phosphatidylinositol kinases (PI K), and various phosphatases (Klionsky and Emr, 2000). Phosphatidylinositol 3-kinases (PI 3-K) are separated into 3 classes (Petiot *et al* 2000). In hepatocytes as well as in human colon cancer cells the product of the class III PI 3-K, phosphatidylinositol 3-phosphate (PI 3-P), is required for the formation of autophagosomes and is expressed constitutively within the cell (Blommaart *et al* 1997; Petiot *et al* 2000). PI 3-K inhibitors such as wortmannin (W), LY294002 [LY, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], and 3-methyladenine (3-MA) are potent inhibitors of autophagic sequestration (Blommaart *et al* 1997). The molecular mechanism for autophagosome formation is very poorly understood (Klionsky and Emr, 2000). In contrast, during hormonal stimulation (e.g. insulin) the increases in class I PI 3-K products, PI 3,4-bisphosphate and PI 3,4,5-triphosphate, inhibit autophagy (Klionsky and Emr, 2000). The inhibitory effect of insulin is strongly correlated with the phosphorylation of 31 kD protein, which is identified as ribosomal protein S6. Inhibition of S6 phosphorylation by rapamycin (an inhibitor of p70S6 kinase) increases autophagic sequestration significantly (Blommaart *et al* 1997). These results demonstrate that the increase in S6 phosphorylation caused by insulin results from the activation of p70S6 kinase. The products of class I PI 3-K activate a serine/threonine kinase known as PKB/Akt which results in activation of p70S6 kinase (Blommaart *et al* 1997). The mechanism by which a ribosomal protein S6 controls autophagy is perhaps the observation that in hepatocytes the autophagosomal membrane is derived from ribosome free regions of the rough endoplasmic reticulum. A high degree of phosphorylation of S6 may
promote the binding of ribosomes to such regions so that less membrane becomes available for autophagosome formation (Blommaart et al 1997).

In the second step, fusion of primary lysosome with autophagosome, proteases within lysosomes are transferred to the materials sequestered in the autophagosomes where the final hydrolysis (proteolysis) step takes place. Lysosomes are cellular organelles containing many hydrolytic enzymes, including cathepsins B, H, L, and D, which participate in the breakdown of extracellular phagocytosed materials of membrane proteins and of intracellular proteins with long half-lives (Dunn, 1994).

In addition to the major autophagic-lysosomal pathway, proteases have been identified in the cytosol and in various organelles, including the endoplasmic reticulum and mitochondria (Goldberg and Rock, 1992; Gottesman and Maurizi, 1992; Desautels and Goldberg, 1985; Desautels, 1986; Goldberg, 1992). Protein degradation by non-lysosomal proteases often depends on hydrolysis of ATP (Goldberg and Rock, 1992). In recent years, considerable progress has been made in the characterization of proteolytic systems of mitochondria. Degradation of mitochondrial proteins can occur via the lysosomal pathway by autophagy. This process is predominant under starvation conditions and results in the non-selective removal of mitochondrial proteins (Takeshige et al 1992; Langer and Neupert, 1996). On the other hand, observed differences in the turnover rates of mitochondrial proteins located in different compartments and of individual proteins within the same compartment have suggested rather early on of the existence of proteases within mitochondria (Walker et al 1978; Russel et al 1980; Lipsky and Pedersen, 1981).
More recently, a series of proteases have been identified in the mitochondrial matrix space and in the inner membrane which mediate the selective degradation of mitochondrial proteins with abnormal structures as may result from mutation, biosynthetic errors, and post-synthetic damages as well as degradation of newly synthesized non-assembled mitochondrial translation products (Langer and Neupert, 1996). The roles of mitochondrial proteases have been extensively studied in yeast. In addition to their proteolytic activity, they fulfill crucial functions in mitochondrial biogenesis (Van et al 1993; Suzuki et al 1994), and in the maintenance of mitochondrial genome integrity (Teichmann et al 1996). They also have chaperone-like activity that is involved during the assembly of respiratory and ATP-synthase complexes (Arlt et al 1996, 1998; Suzuki et al 1997; Leonhard et al 1999). This latter function ensures proper folding of newly synthesized proteins and helps the translocation of polypeptides across the membranes (Savel’ev et al 1998).

BAT possesses two major proteolytic pathways, which are important during tissue atrophy. The tissue has a great capacity for lysosomal proteolytic pathway and the relative proportion of lysosomal enzyme activities in BAT differs somewhat from that of other tissues (Desautels et al 1990). When compared with heart or skeletal muscle, BAT has greater specific activities of acid phosphatase and cathepsins D, B and, H. When compared with liver, BAT has a higher specific activity of cathepsin B, comparable activity of cathepsin D, and less acid phosphatase and cathepsins H and L activities (Desautels et al 1990). The lysosomal pathway of protein degradation was demonstrated to have an important role during tissue atrophy in vivo (Desautels et al 1990). There were significant increases in the specific
activities of cathepsins D and L in BAT homogenates of cold-acclimated mice fasted at 4°C compared with fed mice. Furthermore, in mice kept at 21°C protein degradation in brown fat of fasted mice was markedly inhibited by inhibitors of lysosomal proteolytic enzymes. The importance of the lysosomal pathway of proteolysis was also demonstrated in brown adipocytes differentiated in culture, as inhibitors of the autophagic process inhibited proteolysis (Desautels and Heal, 1999).

However, there are also evidences for the involvement of mitochondrial proteases in the degradation of mitochondrial proteins during BAT atrophy. Brown fat mitochondria contain numerous cristae. Electron microscopy of mitochondria during tissue atrophy revealed an irregular structure of mitochondria with infrequent cristae. These changes in brown fat mitochondria were accompanied with the selective loss of UCP1 relative to the other mitochondrial proteins (Desautels and Dulos, 1990; Desautels et al 1986; Muralidhara and Desautels, 1994; Desautels et al 1996). There are mitochondrial proteases in BAT. Isolated brown fat mitochondria have an ATP-stimulated proteolytic activity at pH 8 (a pH at which lysosomal proteases are inactive), and digitonin-washed BAT mitochondria (digitonin removes lysosomes) had also protease activity associated with both the membrane and soluble fractions (Desautels, 1992). There was no change in mitochondrial proteolytic activity in BAT of 24 hours food-deprived mice (Desautels, 1992). However, it is likely that mitochondrial proteolytic activity is involved in some of the selective changes in organelle protein composition, such as loss of UCP1 from BAT mitochondria in fasting mice. Accordingly, Desautels and Dulos (1993) isolated membranes from brown fat mitochondria and evaluated whether UCP1 present in the mitochondrial
membranes could be subject to endogenous proteolysis. There is a serine protease capable of degrading UCP1 in mitochondrial membranes of BAT. However, it is noteworthy to mention that the relative importance of lysosomal proteolysis versus proteolysis within mitochondria in reducing the BAT cell thermogenic capacity is still uncertain.

Finally, knowing the induction of the proteolytic pathways in BAT during tissue atrophy, the next question was to investigate the factors responsible for controlling protein degradation. All forms of BAT atrophy (fasting, deacclimation, and lactation) are associated with the suppression of sympathetic activity to BAT (Figure 1.5) (Himms-Hagen, 1989; Mantzoros et al 1996). NE was shown to inhibit proteolysis in BAT in vivo (Desautels and Dulos, 1990). Desautels and Heal (1999) also evaluated whether NE directly influences proteolysis in brown fat cells differentiated in culture. Addition of NE reduced total protein degradation significantly. Interestingly, inhibition of total proteolysis by NE was mediated mainly by inhibition of the lysosomal pathway, as the effect of NE was mimicked by addition of inhibitors of autophagy. In addition, when inhibitors of autophagy were added to brown fat cells together with NE, there was no marked additive effect on total proteolysis. However, there is no information regarding the adrenergic control of proteolysis of mitochondrial proteins and proteolysis within the mitochondria. Insulin was also an effective inhibitor of proteolysis in brown adipocytes differentiated in culture (Desautels and Heal, 1999). However, insulin inhibitory effect was only about one-half that seen with NE.
Compared to hepatocytes, there is very little unpublished information from our laboratory about the mechanism of inhibitory action of insulin, as well as that of NE, on proteolysis in brown adipocytes. There was a significant stimulation of immunoprecipitable PI 3-K activity with insulin but NE had no effect on either basal or insulin-stimulated PI 3-K activity. Rapamycin also abolished the inhibitory effect of insulin but had no effect on NE inhibition of proteolysis. Therefore, the mechanism of action of insulin on proteolysis in brown adipocytes seems to be similar to that in hepatocytes. However, the inhibitory effect of NE on proteolysis seems to be distinct from that of insulin in mature brown adipocytes.
2. OBJECTIVES

The overall objective is to gain a better understanding of the control and the mechanisms underlying atrophy of the BAT. Specific objectives are: (1) whether in vivo patterns of atrophy can be reproduced in brown adipocytes differentiated in culture. (2) to evaluate if withdrawal of NE (to mimic conditions for which there is suppression of SNS activity to trigger BAT atrophy) is accompanied by loss of UCP1 and if so, which pathway may be involved (lysosomal pathway of proteolysis, or proteolysis within mitochondria) (Figure 2.1).
Figure 2.1 If loss of UCP1 in culture occurs upon removal of NE, which is the proteolytic pathway involved (lysosomal or proteolysis within mitochondria).
3. METHODS

3.1 ISOLATION OF BROWN PRE-ADIPOCYTES AND CELL CULTURES

BAT from 8-12 CD1 strain newborn mice (2-4 days old) was removed under sterile conditions. Mice were killed by decapitation. All animal handling protocols were reviewed and approved by the Animal Care Center (U of S). The tissue was placed in a small volume of cell culture medium, DMEM (Dulbecco's Modified Eagle Medium), without fetal calf serum, supplemented with 33 μM biotin, 17 μM pantothenic acid, 100 μM ascorbic acid (AA), 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% O₂/5% CO₂ atmosphere. The tissue was minced with a sterile scalpel blade on a plastic petri dish and transferred to a 50 ml sterile centrifuge tube containing 6 ml of DMEM + BSA + 1 mg/ml collagenase (Worthington; CLS type 1). The tissue was incubated for 30 minutes in a 37°C water bath under 95% O₂/5% CO₂ gas atmosphere and vortex-mixed 10-15 shakes, at setting = 4, every 2.5 minutes. The cell dispersion was then filtered through sterile Nitex mesh (Thompson, 243 μm) and 30-40 ml DMEM + BSA was added. The cell dispersion was incubated for 20 minutes at room temperature to allow mature brown fat cells to float. The pre-adipocyte fraction was spun down at 2500 rpm for 5 minutes.

The cell pellet was resuspended in 10 ml DMEM + BSA by repeated pipetting, filtered through Nitex mesh and its volume was made up to 45 ml with DMEM + BSA. Two ml cell dispersion was added to 35 mm Falcon cell culture dishes in a biological cabinet (4 ml cell dispersion/60 mm cell culture dishes) and placed for 1 hour in a CO₂ incubator (95% air: 5% CO₂) at 37°C and 100% relative
humidity. After 1 hour, the medium was aspirated off. Two to four ml DMEM supplemented with antibiotics, biotin, pantothenic acid, AA, and 10% (w/v) fetal calf serum (ICN, CELLECT-Silver) was then added and cell culture dishes were returned to the CO₂ incubator. The cell culture medium was replaced every second to third day. Once the cell cultures reached confluence (about day 5), T₃ (1 nM), and insulin (50 nM) were added to the culture medium to trigger cell differentiation. The culture medium (with T₃/insulin) was replaced every second day until most cells had acquired the brown fat cell phenotype (about 5 more days). Brown fat cell differentiation was followed by the appearance of fat vacuoles, determined by light microscopy, and of UCP1, determined by immunostaining.

3.2 STAINING PROCEDURES FOR VISUALISATION OF FAT VACUOLES AND IMMUNOSTAINING OF UCP1 AND CATHEPSIN D

Brown fat cells were grown on coverslips coated with filter-sterilized Poly-L-Lysine [0.001% (w/v) in distilled water]. After 3 washes with phosphate-buffered saline (PBS), cells were fixed for 30 minutes in the presence of 4% (w/v) paraformaldehyde in PBS at room temperature. The cells were again washed 3 times with PBS and permeabilized with 0.1% Triton X100 in PBS + 1% BSA for 30 minutes. Cells were then washed 3 times in PBS and immunostained for UCP1 and cathepsin D.

Immunofluorescence microscopy was done as described by Malide (2001). Brown fat cells were incubated overnight at 4°C with 1:320 dilution of rabbit polyclonal antibodies against UCP1 or goat polyclonal antibodies against cathepsin D.
(SantaCruz, sc # 6487) in PBAL [PBS + 1% (w/v) BSA + 0.1 M lysine]. Antiserum against UCP1 was prepared in our laboratory (Desautels, 1985; Desautels and Dulos, 1990). Antiserum against UCP1 was pre-incubated for 2 hours at room temperature with sonicated heart mitochondria to absorb contaminating antibodies to other mitochondrial proteins and increase the specificity of the antiserum. Heart mitochondria do not have UCP1 but contain all other proteins associated with mitochondria.

Some cell monolayers were also incubated with non-immune serum (serum collected from the same animal prior to immunization) as control for non-specific binding. After washing 5 times with PBAL, cell monolayers were incubated for 2 hours in the dark and room temperature with 1:320 dilution of Texas Red-conjugated goat anti-rabbit IgG for UCP1 (Sigma, 401355) or 1:320 dilution of FITC-conjugated swine anti-goat IgG for cathepsin D (Cedarlane). The cells were washed 5 times with PBAL in the dark. The coverslips were dropped onto 10 μl of AF1 mounting solution (Citifluor) supplemented with 1 mg/ml p-phenylenediamine, an anti-fading agent, and sealed onto microscope slides using nail polish. Microscopic examination was done with an Olympus Ix70 microscope at 40X and 400X magnifications.

For staining of fat vacuoles, paraformaldehyde-fixed brown adipocytes were incubated for 30 minutes at room temperature with Oil Red O dye (4% w/v). The cells were washed 5 times with PBS to remove the dye and mounted as above. Light microscopy was done at 200X magnification.
3.3 ESTIMATION OF DNA, PROTEIN AND ATP CONTENT OF CULTURE

3.3.1 DNA ASSAY

Cell monolayers were washed 3 times and collected in 1 ml PBS. Cells were disrupted by sonication and the DNA content was quantified by the method described by Rafael and Vsiansky (1981) with calf thymus DNA (1 mg/ml) as standard. Briefly, 0.3 ml ice-cold NaOH (0.5 N) was added to 0.3 ml cell suspension in an Eppendorf centrifuge tube and centrifuged for 3 minutes at 12000 xg. One ml perchloric acid [PCA, 20% (v/v)] was added to 0.35 ml of supernatant in a clean Eppendorf centrifuge tube and kept on ice for 30 minutes. The precipitate containing DNA and protein was collected by centrifugation, and the supernatant was discarded. Nucleic acids were extracted by resuspension of the pellet in 0.25 ml PCA [5% (v/v)] by sonic disruption and incubation in a 70°C water bath for 20 minutes. Acid-insoluble precipitates (proteins) were removed by centrifugation. Aliquots of supernatant (0.15 ml) were added to tubes containing PCA [50 μl, 70% (v/v)], diphenylamine [4% (w/v)] in glacial acetic acid, 200 μl) and acetaldehyde [5 μl, 1.6% (w/v)]. Tubes were covered and kept in the dark at room temperature until the next day. After 24 hours, 200 μl aliquots of samples were transferred to a multi-well plate, and optical density was read at a wavelength of 600 nm. A standard curve (0-30 μg DNA) was used to determine DNA content of the samples. DNA standard curve was linear with $R^2 = 0.97$. 
3.3.2 PROTEIN ASSAY

Cell monolayers were washed 3 times with PBS, collected in 1 ml NaOH (0.5 N) and disrupted by sonication. Protein content was determined by the method of Bradford (1976), using fatty acid-free BSA (0.1 mg/ml) as standard. The protein standard curve (0-10 μg protein) was linear with \( R^2 = 0.99 \).

3.3.3 ATP ASSAY

Culture ATP content was evaluated using Sigma’s bioluminescent somatic cell assay kit # FL-ASC. Cell monolayers were washed 3 times with PBS and collected in 1 ml 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, pH 7.8, 5 mM MgSO4, 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 luciferase and luciferine), 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. The ATP content of samples was calculated using a standard curve attained by plotting the logarithm of the relative light intensity generated by the luciferine-luciferase reaction against the logarithm of the concentration of the ATP in the standard conditions. The ATP standard curve (0-3920 fmol ATP) was linear with \( R^2 = 0.97 \).
3.4 WESTERN BLOTS FOR UCP1 AND CATHEPSIN D

Cell monolayers were washed 3 times with 1 ml PBS, collected in 1 ml lysis buffer containing detergent, phosphatase, and protease inhibitors [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% (w/v) Nonidet P40, 0.2 mM Phenylmethylsulfonyl Fluoride, and 5 μg/ml of each of Leupeptin, Pepstatin, and Aprotinin], and disrupted by sonication to prepare a better sunciation. Prior to electrophoresis, the samples were mixed with an equal volume of 2X electrophoresis 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] and incubated at 95°C for 5 minutes. An aliquot of cell suspension corresponding to 20 μg protein for UCP1 and 50 μg protein for cathepsin D was used for protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were separated according to their size with a 15% acrylamide: 0.4% bis-acrylamide gel and transferred electrophoretically to nitrocellulose membrane. Bio Rad pre-stained markers (5 μl/well) were used to follow the protein separation during electrophoresis and to provide protein size markers. After electrophoretic transfer, the membrane was immersed in a blocking solution [5% defatted milk in TTBS (Tween 20 tris-buffered saline)] at 4°C overnight, to reduce non-specific binding. The membrane was incubated in primary antibody against UCP1 or cathepsin D (rabbit polyclonal antibody against UCP1 or goat polyclonal antibody against cathepsin D) with dilution of 1:1000 in 5% defatted milk/TTBS for 1 hour at room temperature. After extensive washings with TTBS, the
membrane was again incubated at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (HRP-conjugated goat anti-rabbit IgG for UCP1 and HRP-conjugated swine anti-goat IgG for cathepsin D) with a dilution of 1:3000 in 5% defatted milk/TTBS for 1 hour. After extensive washings with TTBS, the membrane was immersed for 1 minute in Chemiluminescence Luminol Reagent for visualization with a commercial ECL kit (Amersham enhanced chemiluminescence luminol). Gel images were scanned, digitized, and band densities quantified as pixel density with UN-SCAN-IT™ software. When pixel density of UCP1 is plotted against μg cell proteins, the curve is linear with $R^2 = 0.97$ (Figure 3.1). A 20 μg protein load was chosen for all subsequent western blots, since it falls within a linear range for UCP1 determinations (Figure 3.1).
Figure 3.1  UCP1 pixel density as a function of cell protein. An analysis of western blot is shown in (A), with its quantitative analysis shown in (B).
3.5 PROTEIN TURNOVER STUDIES

3.5.1 PROTEIN LABELING

Total cellular proteins including UCP1 were labeled for 2 days, from day 10-12, in the presence of the culture medium containing 10% (v/v) fetal calf serum, 10% (v/v) DMEM, 80% (v/v) methionine-free DMEM, and 10 $\mu$Ci/ml $[^{35}\text{S}]$ methionine. Two-three days of labeling was enough to uniformly label all cellular proteins (Desautels and Heal, 1999). A culture medium with reduced methionine content did not appear to affect the differentiation of the cells as estimated by the appearance of the cells by light microscopy. Supplementations with vitamins and antibiotics were as before. Mitochondrial translation products (subunits of mitochondrial respiratory chain complex, which are expressed by mitochondrial genome and synthesized on ribosomes within mitochondria) were labeled on day 12 for 2 hours using methionine-free DMEM containing 10 $\mu$Ci/ml $[^{35}\text{S}]$ methionine in the presence of 20 $\mu$g/ml cycloheximide (CHX), added 20 minutes prior to the labeling period. CHX prevents the synthesis of cytoplasmic proteins, but not synthesis of proteins on ribosomes within mitochondria. Two hours was chosen for labeling of mitochondrial translation products to minimize the cell cytotoxicity effect of CHX (Puigserver et al 1992).

3.5.2 UCP1 TURNOVER RATE

After 2 days of labeling, cells were washed 3 times to remove the labeled methionine and were incubated for up to 72 hours in the presence of standard culture medium supplemented with 2 mM unlabelled methionine to prevent re-incorporation
of the labeled aa into cellular proteins. All other treatments (presence or absence of hormones and inhibitors) are described in the results section.

After 24, 48 and 72 hours, the cell cultures were washed 3 times with PBS and collected in 1 ml lysis buffer described in section 3.4. After disruption by sonication, UCP1 protein in an aliquot of cell suspension corresponding to 500 μg of total cellular proteins was separated from the other labeled cellular proteins via immunoprecipitation as follows:

To approximately 500 μg of total cellular proteins, 10 μl of primary antibody against UCP1 was added and incubated at 4°C overnight with continuous mixing on a rotating platform. To recover UCP1 bound to antibody, 50 μl of pansorbin cells (Calbiochem, 10% suspension, binding capacity of 2.1 mg IgG/100 mg cells) was added to the cell lysates and incubated at 4°C on a rotating platform for 2 hours. The pellet was collected by centrifugation at 5000 rpm for 3 minutes at 4°C. The supernatant was carefully aspirated and discarded. The pellet was washed 3 times with 1 ml lysis buffer, each time repeating the centrifugation step above. After the final wash, the pellet was resuspended in 50 μl of electrophoresis sample buffer, boiled for 5 minutes at 95°C and loaded on a 15% acrylamide gel. Prior to the loading, 1 μg purified UCP1 was added as carrier protein for a better visualization of the corresponding bands to UCP1 detected by alkaline-phosphatase conjugate substrate kit. The separated proteins were then transferred electrophoretically to nitrocellulose membrane. Immunoblotting was done exactly as described under section 3.4, except the secondary antibody used was alkaline phosphatase-conjugated. UCP1 was detected by an alkaline phosphatase-conjugated substrate kit containing
Nitroblue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphatase (Puigserver et al 1992). The bands corresponding to UCP1 (molecular weight of 32 kDa) were cut, placed in a scintillation vial with 4 ml ACS scintillator fluid (Amersham). The UCP1 turnover rate was then estimated from the changes in $[^{35}\text{S}]$ radioactivity incorporated into UCP1 [as counts per minute (cpm)] over 72 hours after measurement of the labeled precursors. Control experiments with pre-immune serum, with or without pansorbin cells, were used to establish the specificity of the immunoprecipitations. $[^{35}\text{S}]$ methionine incorporation within immunoprecipitable UCP1 using antibody against UCP1 and pansorbin ranged from 400 to 700 cpm, while the background ranged from 90 to 100 cpm with pre-immune serum or when pansorbin was replaced with sansorbin, which does not bind IgG.

3.5.3 TURNOVER RATE OF MITOCHONDRIAL TRANSLATION PRODUCTS

After 2 hours of labeling in the presence of CHX (section 3.5.1), cultures were washed 3 times with DMEM containing 2 mM unlabeled methionine and incubated overnight in DMEM culture medium containing 10% fetal calf serum and 2 mM unlabeled methionine. This allows the degradation of short-lived mitochondrial proteins, which were not yet incorporated into the mitochondrial membranes. Thereafter, cultures were washed again and incubated up to 72 hours in the same culture medium. All the hormonal and drug treatments are as described in the results section. After 24, 48 and 72 hours, the cells were washed 3 times and collected in 1 ml lysis buffer (section 3.4). The total $[^{35}\text{S}]$ radioactivity at time 0 hour incorporated
into mitochondrial translation products was estimated as described before. The changes in \([S^{35}]\) radioactivity present within mitochondrial translation products were estimated over 72 hours as a percentage of radioactivity initially present.

3.5.4 RATE OF PROTEIN DEGRADATION IN THE ABSENCE OF SERUM

After the period of 2 days labeling, the cells were washed 3 times and incubated for 4 hours in the presence of 2.5 ml DMEM with 2 mM unlabeled methionine without serum. All the hormonal and drug treatments were as described in the section of results. Protein degradation was measured as % amount of \([^{35}S]\) radioactivity released after 4 hours relative to the amount of radioactivity incorporated into proteins at time 0. Therefore, to determine the amount of \([^{35}S]\) radioactivity released (acid-soluble released) after 4 hours, 0.25 ml of the culture medium was collected, added to 0.035 ml trichloroacetic acid (TCA, 100%) and 0.015 ml BSA [5% (w/v)] and was kept at 4°C. After ≥ 1 hour at 4°C, the samples were centrifuged, 0.15 ml of acid-soluble supernatant was added to 4 ml of Amersham ACS scintillator fluid and its content of \([^{35}S]\) radioactivity was counted. Thereafter, for the measurement of the amount of \([^{35}S]\) radioactivity incorporated into the proteins (acid-precipitable radioactivity) at time 0 hour, 1 culture was washed 3 times with PBS and collected in 1 ml lysis buffer (section 3.4). The cells were disrupted by sonication. The total \([^{35}S]\) radioactivity [acid-precipitable radioactivity + acid-soluble intracellular radioactivity (amount of \([^{35}S]\) released into the cell as a result of protein degradation)] was measured by placing 50 µl of cell suspension into a scintillator vial with 4 ml ACS scintillation fluid (Amersham), and counting. To
measure acid-soluble intracellular $[^{35}S]$ radioactivity, 0.035 ml TCA (100%) as well as 0.015 ml BSA (5%) were added to 0.25 ml of cell suspension and kept at 4°C. After ≥ 1 hour at 4°C, the samples were centrifuged and 0.15 ml of the acid-soluble supernatant was added to 4 ml of scintillation fluid and counted. However, acid-precipitable $[^{35}S]$ radioactivity at time 0 hour was calculated by subtraction of acid-soluble intracellular radioactivity at time 0 hour from total radioactivity at time 0 hour.

3.5.5 CALCULATION OF PROTEIN HALF-LIFE

For the calculation of half-lives of UCP1 and mitochondrial translation products, it was expected that the degradative processes follow a first order process; each protein has an equal and independent probability to be degraded. The turnover rate measurement was based on the loss of $[^{35}S]$ labeled protein over time according to

$$f(t) = f(0) e^{-Kt}$$

when $f(t)$ is the amount of labeled protein remaining at time $t$, $f(0)$ the initial amount of labeled protein and $K$ a rate constant (Cameron, 1986; Zak et al. 1979). If the fraction of the initial amount of labeled protein at time $t$ is designated as $F$, then, the above exponential equation would be $F = e^{-Kt}$ (Cameron, 1986; Zak et al. 1979). When $F$ is exactly one-half, the equation may be reduced to

$$\ln F = -Kt$$

$\ln F = -Kt$  $t = \ln F/-K$  $t = \ln 0.5/-K$  $t = 0.693/K$ and the value of $t$ is called the half-life for the degradative process (Cameron, 1986; Zak et al 1979). Therefore, the half-lives of UCP1 and mitochondrial translation products were calculated using the formula $t_{1/2} = 0.693/K$. $K = m$, the slope of the line $y = mx + b$, where $x = time$
and \( y = \ln \) of changes in \([S^{35}]\) labeled protein (UCP1 or mitochondrial translation products) over 72 hours (Cameron, 1986; Zak et al 1979).

### 3.6 STATISTICAL ANALYSIS

Differences between groups were examined by one-way and two-way analysis of variance (ANOVA) and Duncan’s multiple range test for comparisons between means. NCSS statistical software was used for computation. The results were considered statistically significant when \( P < 0.05 \). Results are presented as means ± standard error (S.E).
4.0 RESULTS

4.1 RATIONALE AND EXPERIMENTAL DESIGN

It was observed in vivo that cold exposure or feeding animals certain types of diet causes BAT growth, during which SNS activity increases (Rothwell and Stock, 1984; Park and Himms-Hagen, 1989). BAT growth is characterized by increased tissue cellularity, total protein content, mitochondrial protein content and UCP1. In contrast, conditions such as deacclimation, fasting, and lactation, which are associated with decreased SNS activity, cause BAT atrophy (Trayhurn, 1989; Trayhurn and Jennings, 1986). The pattern of tissue atrophy differs in response to specific changes in environmental conditions. During deacclimation, for instance, the number of cells decreases, as well as the cell content of protein, mitochondrial protein and UCP1, whereas during fasting there is not loss of cells. So, one objective of this study was to see whether in vivo patterns of BAT atrophy could be reproduced in brown adipocytes differentiated in culture? To this aim, we attempted to increase the cell thermogenic capacity with chronic exposure to NE, followed by removal of the neurotransmitter to mimic the suppression of sympathetic activity like in most conditions of BAT atrophy in vivo. In addition, the direct effect of NE removal on the brown fat cells differentiated in culture was evaluated.

The basic experimental design for these objectives is as follows: after isolation from interscapular BAT of mouse, brown pre-adipocytes were cultured at 37°C and incubated to proliferate in the presence of fetal calf serum and DMEM for 5 days, as was described in the section of methods. Cultures of brown adipocytes were then separated in 3 groups. Group 1, ascorbic acid (AA), received AA 1% (w/v) for 9
days (days 5-14). NE in Group (2) is dissolved in AA (1%) and equivalent volume of
AA was added to control cultures (Group AA). Group 2, (NE), received NE [10 μM,
the optimum concentration for increasing thermogenic capacity (Nedergaard et al
1993)] for 9 days (days 5-14). Group 3, (–NE), received NE (10 μM) for 7 days (days
5-12) followed by the removal of NE for 2 days (days 12-14) (Figure 4.1). The time
of treatment of the cultures with NE for 7 days, following its removal for 2 days was
chosen as 15-16 days is the maximum time brown adipocytes can be kept in culture
and 48 hours is sufficient to see BAT atrophy in vivo. The culture medium was
changed daily because of the rapid disappearance of NE from the culture medium
(Waldbillig and Desautels, 1992). The cultures in each group were collected for days
12-14. The cells were examined for viability and the state of differentiation. In
addition, changes associated with NE treatment and its removal from the cultures
were evaluated by measuring the culture DNA, total protein, and UCP1 content to
determine if brown fat cell atrophy results from NE removal, mimicking in vivo
conditions.
Experimental design for evaluation of the effect of NE and its removal on brown fat cell thermogenic capacity. \(T_3=3, 5, 3'-\text{triiodothyronine}, AA=\text{ascorbic acid},\) \(\text{NE}=\text{norepineprine}.)
4.2 BROWN FAT CELL DIFFERENTIATION AND VIABILITY IN PRIMARY CULTURES FOLLOWING NE TREATMENT AND ITS REMOVAL

To evaluate whether the brown pre-adipocytes differentiated into mature brown adipocytes, the cells on day 14 (AA, NE, -NE) were stained, using Oil Red O and an antibody against UCP1, for 2 markers of brown fat differentiation: appearance of fat vacuoles and the presence of UCP1, respectively. The cells in Group AA showed complete differentiation since Oil Red O stained-cells under light microscopy revealed the appearance of multiple fat vacuoles (Figure 4.2 A). The cells in Groups NE and -NE were not apparently different from those in Group AA (Figure 4.2 B and C). In addition, fluorescence microscopy with antibody against UCP1 of cells in Group AA also revealed the presence of UCP1 (Figure 4.3 B). The cells in Groups NE and -NE were similar to those in Group AA (Figures not shown). A culture incubated with non-immune serum was used as a control for the specificity of the antiserum and showed no UCP1 staining (Figure 4.3 A).

Brown fat cell viability in culture was also examined for all 3 groups of cells (AA, NE, and -NE). Visualization of brown fat cells using light microscopy showed that the cells in Groups NE and -NE do not differ in morphology from the cells in Group AA. The cells did not show any sign of abnormality such as shrinking or detachment from the culture dishes. The cell plasma membranes were also intact, as there was no nuclear staining when cells were exposed to 10 μl of 5 μg/ml ethidium bromide in PBS. Ethidium bromide is a cell impermeable dye that stains nuclear DNA only if the cell plasma membranes are damaged.
Figure 4.2  Light microscopy of Oil Red O stained-fat vacuoles in brown adipocytes differentiated in culture. Cultures were treated (A) 9 days (days 5-14) with AA (ascorbic acid), (B) 9 days (days 5-14) with NE (norepinephrine), and (C) 7 days (days 5-12) with NE followed by 2 days (days 12-14) of NE removal. Magnification is 200X.
Figure 4.3 Immunostaining of UCP1 in brown adipocytes differentiated in culture. Cultures were treated with (B) AA (ascorbic acid) for 9 days (days 5-14). (A) represents a culture incubated with non-immune serum which lacks antibody against UCP1. Magnification is 40X.
In addition, cells maintained their ATP content, a good indicator of cell viability. Culture ATP content was measured during 48 hours in all Groups (AA, NE, –NE) and expressed as femtomoles ATP/µg protein (Figure 4.4). Similar results were observed when ATP analysis was expressed as ATP content/culture dish. Figure 4.4 shows that addition of NE (P = 0.2), or its removal from the cultures (P = 0.9) for 48 hours does not change the ATP content of the cultures significantly compared to Group AA or NE, respectively. Cell viability, therefore, is maintained during treatment of the cultures with NE and its subsequent removal for up to 48 hours.
Figure 4.4  Changes in culture ATP content over 48 hours (days 12-14) in brown fat cell cultures. Brown fat cultures were treated with AA (ascorbic acid) for 9 days (days 5-14), NE (norepinephrine) for 9 days (+NE, days 5-14), and NE for 7 days (days 5-12) followed by 2 days (days 12-14) of its removal (-NE). The results are means of 3 separate experiments ± S.E. The statistical analysis is given in the text.
4.3 REMOVAL OF NE FROM CULTURES PRE-EXPOSED TO NE CAUSES NET LOSS OF UCP1

Cultures in 3 Groups of AA, NE, and -NE, were treated for changes in DNA content as a measure of cellularity (Figure 4.5). DNA content of the cultures did not change during NE treatment for 9 days ($P = 0.9$) and its removal ($P = 0.9$) for 48 hours.

Protein content of the cultures in Group AA did not change significantly over 48 hours ($P = 0.7$) (Figure 4.6). Addition of NE to the cultures for 9 days did not increase the amount of the protein/culture dish ($P = 0.7$). There was no change in the protein contents over 48 hours after withdrawal of NE in cells previously exposed to NE for 7 days ($P = 0.9$).

UCP1 contents of aliquots of cell suspensions (corresponding to 20 µg protein) were determined for cultures in each Group (AA, NE, and -NE) at times 0, 24 and 48 hours by Western blotting. Visual analysis of Western blots demonstrated that the amount of UCP1 is very much higher in Group NE compared to AA and is gradually decreased upon NE removal (Figure 4.7 A). For quantitative analysis, the UCP1 content of cultures of Group NE was taken arbitrary as control (100%). The UCP1 contents of Groups AA and -NE at different times were expressed as a percentage of the UCP1 content found in Group NE (Figure 4.7 B). The results are mean values of 3 separate experiments ± S.E. UCP1 amount in Group AA did not change significantly over 48 hours ($P = 0.6$). Similarly, the presence of NE did not affect the culture UCP1 content over 48 hours ($P = 0.6$). The UCP1 content of cells
treated with AA was significantly lower than in cultures treated with NE for the same period (P<0.0005). Furthermore, the amount of UCP1 gradually decreased upon NE removal in Group –NE during the 48 hours period. Forty eight hours after removal of NE, the UCP1 content was close to that found within cells treated with AA.
Changes in culture DNA content over 48 hours (days 12-14) in brown fat cell cultures. Brown fat cultures were treated with AA (ascorbic acid) for 9 days (days 5-14), NE (norepinephrine) for 9 days (days 5-14, and NE for 7 days (days 5-12) followed by its removal for 2 days (days 12-14). The results are means of 5 separate experiments ± S.E. The statistical analysis is given in the text.
Figure 4.6 Changes in culture protein content over 48 hours (days 12-14) in brown fat cell cultures. Brown fat cultures were treated with AA (ascorbic acid) for 9 days (days 5-14), NE (norepinephrine) for 9 days (days 5-14), NE for 7 days (days 5-12 followed by its removal for 2 days (days 12-14). The results are means values of 5 separate experiments ± S.E. The statistical analysis is given in the text.
Figure 4.7 Changes in UCP1 content over 48 hours (days 12-14) in brown fat cell cultures. Brown fat cultures were treated with AA (ascorbic acid) for 9 days (days 5-14), NE (norepinephrine) for 9 days (days 5-14), and NE for 7 days (days 5-12) followed by its removal for 2 days (days 12-14). The results are mean values of 3 separate experiments ± S.E. An analysis of western blot is shown in (A) with its quantitative analysis shown in (B). The amount of UCP1 in cells treated with NE is arbitrary taken as 100% control. The statistical analysis is given in the text.
4.4 CATHEPSIN D CONTENT INCREASES DURING DIFFERENTIATION, BUT IS NOT AFFECTED BY NE EXPOSURE AND ITS SUBSEQUENT REMOVAL

Loss of UCP1 could occur via autophagy or from proteolysis within mitochondria. Thus, it is of interest to evaluate what is the capacity of brown fat cells differentiated in culture for lysosomal proteolysis, and whether it is affected by chronic exposure of the cells to NE and withdrawal of neurotransmitter for 48 hours. For these purposes, cathepsin D was chosen as a marker for the presence of lysosomes in brown fat cells. Cathepsin D is an aspartyl protease with pH optimum of 4.0 (Seglen and Bohley, 1992). Thus, its actions will be limited to acidic vacuolar compartments, and is unlikely to serve any function in the cytosol or extracellularly.

Immunostaining of mature brown adipocytes (day 10) using antibody against cathepsin D revealed the intense staining within the cells (Figure 4.8 A). A culture incubated with non-immune serum was used as a control for specificity of the antiserum and showed no cathepsin D staining (Figure 4.8 B). For the evaluation of the time course of changes in lysosomal capacity of brown adipocytes, cells were collected on days 5, 7, 10, 13 and examined for their cathepsin D content. The results are expressed as the percent of the maximum cathepsin D expression observed in each of 3 separate experiments (Figure 4.9). The expression of cathepsin D is differentiation-dependent. Western blots showed a very low level of the enzyme in pre-adipocytes (day 5) that increased nearly 20 fold during differentiation (P<0.00001) (Figure 4.9 A and B).
Figure 4.8  Immunostaining for cathepsin D in mature brown adipocytes (day 10) differentiated in culture. (B) represents a culture with non-immune serum which lacks antibody against cathepsin D. Magnification is 400X.
Figure 4.9  Time course of changes in cathepsin D expression during brown adipocyte differentiation in culture. The data are the result of 3 separate experiments ± S.E. A typical western blot is shown in (A), with its quantitative evaluation shown in (B). The statistical analysis is given in the text.
To evaluate if expression of cathepsin D was influenced by the presence of NE, cells were treated exactly as shown in Figure 4.1. Cathepsin D contents of 3 groups of cultures (AA, NE and -NE) were evaluated with Western blots upon chronic exposure of the cells to NE and its withdrawal for 48 hours (Figure 4.10 A). Cathepsin D content in cultures exposed chronically to NE (Group NE) was taken arbitrary as control (100%). The cathepsin D contents of Groups AA and -NE at different times were expressed as a percentage of the cathepsin D content found in Group NE (Figure 4.10 B). There was no significant difference (P = 0.7) in cathepsin D expression in cells chronically treated with NE (Group NE) and AA (Group AA). Likewise, withdrawal of NE for 48 hours after 7 days of exposure to NE (Group -NE), had no effect on cell cathepsin D content (P = 1.0). Thus, brown adipocytes acquire a large content of lysosomes during differentiation whose expression is not under adrenergic control.
Figure 4.10 Changes in cathepsin D content over 48 hours (days 12-14) in brown fat cell cultures. Brown fat cultures were treated with AA (ascorbic acid) for 9 days (days 5-14), NE (norepinephrine) for 9 days (days 5-14), and NE for 7 days (days 5-12) followed by its removal for 2 days (days 12-14). The results are mean values of 3 separate experiments ± S.E. An analysis of western blot is shown in (A), with its quantitative analysis shown in (B). The amount of UCP1 in cells treated with NE is arbitrary taken as 100% control. The statistical analysis is given in the text.
4.5 COMPARISON OF THE EFFECT OF NE AND INHIBITORS OF AUTOPHAGIC SEQUESTRATION ON PROTEOLYSIS IN BROWN ADIPOCYTES

The observed loss of UCP1 in culture upon NE removal could be a result of decreased protein synthesis as well as increased rate of protein degradation. NE is assumed to be an inhibitory factor for protein degradation, since all conditions associated with decreased SNS activity cause loss of cellular proteins, and of UCP1, and thus BAT atrophy. This inhibitory effect of NE on proteolysis has been confirmed directly in brown fat cells differentiated in culture (Desautels and Heal, 1999). However, there is no direct evaluation of the effect of NE on the turnover rate of UCP1. Given the large lysosomal content of brown adipocytes (Figure 4.8), we first evaluated whether NE effect on proteolysis was via inhibition of lysosomal autophagy. The effect of NE on proteolysis was compared to that of 3 inhibitors of phosphatidylinositol 3-kinase (PI 3-K), wortmannin (W), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-1 (LY294002), and 3-methyladenine (3-MA), which were shown previously to inhibit the autophagic sequestration (Blommaart et al 1997; Davies et al 2000). A 4-hour exposure of brown adipocytes differentiated in culture to NE inhibited the degradation of cellular proteins significantly (P<0.0001, Figure 4.11). In addition, the effect of NE was similar to that observed with 3 inhibitors of PI 3-K. All 3 inhibitors significantly reduced proteolysis in the absence of NE (Figure 4.11). Furthermore, addition of PI 3-K inhibitors to brown fat cells together with NE, had no significant additive effect (P = 0.1).
Figure 4.11  Effect of NE on the degradation of cell proteins after 4 hours incubation in the presence or absence of 3 PI 3-K inhibitors. PI 3-K inhibitors are used at the concentrations of 200 nM for wortmannin (W), 10 μM for LY294002 (LY), and 2.5 mM for 3-methyladenine (3-MA). Proteolysis is measured in the absence of serum and is expressed as the percentage of $[^{35}\text{S}]$ radioactivity released after 4 hours relative to $[^{35}\text{S}]$ radioactivity incorporated at time 0 hour. The results are mean values of 3 separate experiments ± S.E. The statistical analysis is given in the text.
4.6 NE DECREASES THE TURNOVER RATE OF UCP1

NE is important in the control of proteolysis in mature brown adipocytes (Figure 4.11). The next purpose was to evaluate the direct effect of NE on the turnover rate of UCP1. Brown adipocytes in culture were treated exactly the same as Group AA and Group NE in Figure 4.1, except that the cellular proteins including UCP1 were labeled from day 10 to day 12 with $[^{35}\text{S}]$ methionine. On day 12, the cells were washed extensively to remove the excess label and unlabeled methionine was added to prevent re-incorporation of the labeled precursors into the proteins. Thereafter, the disappearance of labeled immunoprecipitated UCP1 was measured after 0, 24, 48, and 72 hours (Figure 4.12). When compared to the cultures in Group AA, addition of 1 $\mu$M NE decreased the rate of loss of labeled UCP1 significantly ($P<0.001$). The calculated half-life of UCP1 was $3.7 \pm 0.4$ days in Group AA and it increased significantly ($P<0.05$) to $8.3 \pm 0.9$ days in Group NE. Therefore, NE appears to stabilize UCP1 and lower its rate of turnover.
Figure 4.12 Effect of NE on UCP1 turnover rate and half-life over 72 hours (days 12-15). The cells were pre-treated with either AA (ascorbic acid) or NE (norepinephrine) for 7 days (days 5-12), and labeled using (³⁵S) methionine from day 10-12. The measurement of UCP1 turnover rate was done from day 12-15. The results are mean values of 3 separate experiments ± S.E. The statistical analysis is given in the text.
4.7 PI 3-K INHIBITORS REDUCE LOSS OF UCP1 UPON NE REMOVAL

According to the present data (Figure 4.11), NE inhibits proteolysis mainly by reducing autophagy. UCP1 is an inner mitochondrial membrane protein. Similar to the proteolysis of cytoplasmic proteins, degradation of mitochondrial proteins can also occur by autophagy of the whole organelle (Langer and Neupert, 1996). Therefore, we evaluated the importance of the lysosomal pathway in mediating the loss of UCP1 observed upon removal of NE from cultures previously exposed to NE for 7 days.

Brown fat cells in culture were treated as shown in Figure 4.1. However, cultures in Group -NE were divided into 3 subgroups receiving 3 structurally different PI 3-K inhibitors for up to 48 hours (days 12-14). One received W at the concentration of 200 nM (Group -NEW), the second one received LY294002 at the concentration of 10 μM (Group -NELY), and the third one received 3-MA at the concentration of 2.5 mM (Group -NE 3-MA) (Figure 4.13). If NE inhibits loss of UCP1 by inhibiting the lysosomal process, then treating the cultures with PI 3-K inhibitors during NE removal should prevent loss of UCP1. Because of the possible effects of the PI 3-K inhibitors on the basal rate of protein loss, cultures in Groups AA and NE were also divided into 3 subgroups receiving the 3 different PI 3-K inhibitors during 48 hours (days 12-14); AAW, AALY, AA 3-MA, NEW, NELY, and NE 3-MA (Figure 4.13). After 48 hours, the cells were collected and UCP1 contents were determined by Western blotting using antibody against UCP1. The amount of UCP1 in Group NE was taken arbitrary as control (100%) and the amount of UCP1 in other Groups was calculated as a percentage of that (Figure 4.14 B).
Figure 4.13  Experimental design for the evaluation of the importance of autophagic sequestration upon removal of NE on cell content of mitochondrial proteins. $T_3=3, 5, 3'$-triiodothyronine, AA=ascorbic acid, NE=norepinephrine, W=wortmannin, LY294002, 3-MA=3-methyladenine.
Figure 4.14 Effect of PI 3-K inhibitors on loss of UCP1 after removal of NE for 48 hours (days 12-14). The cells were pre-treated with either AA (ascorbic acid) or NE (norepinephrine) for 7 days (days 5-12). The amount of UCP1 in culture was then evaluated after 48 hours (days 12-14) in the presence or absence of PI 3-K (phosphatidylinositol 3-kinase) inhibitors and incubation with AA, NE, and -NE (removal of NE). PI 3-K inhibitors were used at the concentrations of 200 nM for wortmannin (W), 10 μM for LY294002 (LY), and 2.5 mM for 3-methyladenine (3-MA). The results are mean values of 4 separate experiments ± S.E. The statistical analysis is given in the text.
As was observed previously (Figure 4.7), the UCP1 content of Group NE is significantly increased compared to Group AA (P<0.0001), and the removal of NE for 48 hours in Group –NE caused significant loss of UCP1 (P = 0.02). Addition of PI 3-K inhibitors to cells chronically exposed to AA or NE did not cause significant change in the amount of UCP1 over 48 hours (P = 0.5). However, the addition of PI 3-K inhibitors upon removal of NE for 48 hours in cells previously exposed to NE (Group –NE) reduced the loss of UCP1 (P>0.1). These results suggest an important role for lysosomes in the loss of UCP1 upon NE removal from the cultures previously exposed to NE.
4.8 NE DECREASES DEGRADATION OF MITOCHONDRIAL TRANSLATION PRODUCTS

To evaluate if the effect of NE on the turnover rate of UCP1 is specific to UCP1 or applied to all mitochondrial proteins, the effect of NE and its removal on the turnover rate of mitochondrial translation products was examined. Mitochondrial translation products are proteins encoded by the mitochondrial genome and synthesized within mitochondria. These proteins are mostly subunits of mitochondrial inner membrane protein complexes (Desautels and Dulos, 1993).

Brown fat cells were treated as described in Figure 4.1, except that on day 12, the cells in each group were labeled using $^{35}$S methionine in the presence of CHX for 2 hours. CHX is an inhibitor of the synthesis of cytoplasmic proteins. In its presence, only mitochondrial translation products will be labeled (Puigserver et al. 1992). CHX inhibited $^{35}$S label incorporation into proteins by 96 ± 0.7%. The addition of chloramphenicol, which inhibits the synthesis of proteins by mitochondrial ribosomes, together with CHX inhibited protein synthesis somewhat further (98 ± 0.5%). Thus, in the presence of CHX, cells are markedly enriched with label mitochondrial translation products. After a labeling period of 2 hours, the cells were then extensively washed and incubated in the presence or absence of NE for 72 hours to estimate the turnover rate of mitochondrial translation products. Figure 4.15 shows there is significant degradation of mitochondrial translation products over 72 hours as estimated by (A) loss of acid precipitable radioactivity from the cell (P=0.03) or (C) from release of acid soluble radioactivity in the culture medium (P = 0.001).
Figure 4.15  Effect of NE and its removal on loss of mitochondrial translation products over 72 hours (days 12-15). The cells were pre-treated with either AA (ascorbic acid) or NE (norepinephrine) for 7 days, from day 5-12. On day 12, mitochondrial translation products were labeled for 2 hours using $[^{35}S]$ methionine in the presence of cycloheximide (CHX). Loss of mitochondrial translation products was measured over 72 hours (days 12-15) in the presence of AA, NE or removal of NE (-NE). Acid precipitable count represents the amount of $[^{35}S]$ methionine incorporated into mitochondrial translation products (CHX-independent protein labeling). Acid soluble intracellular and released represent the amounts of $[^{35}S]$ methionine remained into the cells or released into the medium, as a result of degradation of mitochondrial translation products, respectively. The results are mean values of 3 separate experiments ± S.E. The statistical analysis is given in the text.
Addition of NE diminishes the degradation of mitochondrial translation products significantly (P = 0.05). Removal of NE from cells previously exposed to NE for 7 days, however, increases the degradation of mitochondrial translation products measured as loss of acid precipitable radioactivity or from the release of incorporated-radioactivity into culture medium (P = 0.04, Figure 4.15 A and C).

The estimated half-life of mitochondrial translation products was 3.6 ± 0.8 days in Group AA which increased to 6.4 ± 0.3 days in Group NE (P<0.05) (Figure 4.16). These estimates were not significantly different (P>0.2) from the calculated half-lives of UCP1 in Groups AA and NE (Figure 4.12). Therefore, NE appears to inhibit the degradation of all mitochondrial membrane proteins, rather than having a selective effect on the turnover rate of UCP1.
Figure 4.16  Effect of NE on turnover rate and half-life of mitochondrial translation products over 72 hours (days 12-15). Prior to measurements, the cells were pre-treated with either AA (ascorbic acid) or NE (norepinephrine) for 7 days (days 12-12), and labeled on day 12 using $^{35}$S methionine for 2 hours in the presence of cycloheximide (CHX). The results are the mean values of 3 separate experiments ± S.E. The statistical analysis is given in the text.
4.9 PI 3-K INHIBITORS REDUCE LOSS OF MITOCHONDRIAL TRANSLATION PRODUCTS UPON WITHDRAWAL OF NE

NE reduces the turnover rate of UCP1 and of mitochondrial translation products in brown adipocytes differentiated in culture similarly (Figures 4.12 and 4.16). These data suggest that NE inhibits the loss of UCP1 mainly via inhibiting autophagic proteolysis (Figure 4.14). Thus, results suggest that lysosomal proteolysis should play an important role in the turnover rate of mitochondrial proteins generally. We examined the effect of inhibitors of autophagic sequestration on the turnover rate of mitochondrial translation products.

Brown fat cells in culture were treated as shown in Figure 4.13, except that on day 12, mitochondrial translation products were labeled using $^{35}$S methionine in the presence of CHX. Considering the amount of labeled mitochondrial translation products as 100% (shown as a dotted-blue line, Figure 4.17), there was a basal loss of 45% in brown fat cells over 72 hours (AA-). Addition of NE to the cells decreased loss of labeled mitochondrial translation products significantly ($P = 0.05$). Only 22% of labeled mitochondrial translation products were lost over 72 hours (NE-). Removal of NE for 72 hours from cells previously exposed to NE restored the loss of labeled mitochondrial translation products seen in the control condition (AA-, $P = 0.05$).

Addition of inhibitors of autophagic sequestration to Group AA (AAW, AALY, and AA 3-MA) reduced the loss of labeled mitochondrial translation products relative to Group AA-, although their effect did not reach statistical significance.
Figure 4.17  Effect of PI 3-K inhibitors on loss of mitochondrial translation products after removal of NE for 72 hours (days 12-15). The cells were pre-treated with either AA (ascorbic acid) or NE (norepinephrine) for 7 days (days 5-12), and labeled on day 12 using $[^{35}\text{S}]$ methionine for 2 hours in the presence of cycloheximide (CHX). The loss of mitochondrial translation products was then evaluated after 72 hours (days 12-15) in the presence or absence of PI 3-K (phosphatidylinositol 3-kinase) inhibitors and incubation with AA, NE, and –NE (removal of NE). PI 3-K inhibitors were used at the concentrations of 200 nM for wortmannin (W), 10 mM for LY294002 (LY), 2.5 mM for 3-methyladenine (3-MA). Acid precipitable counts present the amount of $[^{35}\text{S}]$ methionine incorporated into the mitochondrial translation products. The results are mean values of 3 separate experiments ± S.E. The statistical analysis is given in the text.
(P = 0.8). However, the effect of inhibitors (AAW, AALY, and AA 3-MA) was similar to the effect of NE (NE-). Combination of inhibitors and NE (NEW, NELY, and NE 3-MA) had not additive effect relative to Group NE without inhibitor (NE-) (P = 0.1). Furthermore, the presence of PI 3-K inhibitors upon removal of NE (-NEW, -NELY, and -NE 3-MA) decreased the loss of labeled mitochondrial translation products which was similar to the effect of NE (P = 0.8).

Therefore, these results suggest that NE reduces the loss of labeled mitochondrial translation products and the effect is similar to that observed with inhibitors of autophagy. However, there is a portion of labeled mitochondrial translation products (approximately 18%, red dotted-line in the Figure 4.17) that is not affected by NE or inhibitors of autophagy. They are probably degraded by means other than autophagy, likely proteolysis within mitochondria.
4.10 SUMMARY OF THE RESULTS

The results demonstrate that chronic treatment of brown fat cells in culture with NE for 7 days (days 5-12), followed by its removal for 48 hours (days 12-14) does not affect the cell morphology and viability. Brown adipocytes have a large capacity for the lysosomal pathway of protein degradation shown by intense immunostaining of brown adipocytes with antibody against cathepsin D. The great capacity of brown adipocytes for the lysosomal pathway of protein degradation is differentiation-dependent, but not affected by NE. Chronic treatment of the cells with NE increases the half-life of mitochondrial proteins, including UCP1, in general. This leads to the increased cell content of UCP1, and thus increased cell thermogenic capacity. In contrast, removal of NE for 48 hours (days 12-14) increases degradation of mitochondrial proteins, including UCP1, via activation of autophagy. This leads to the decreased cell content of mitochondrial proteins, including UCP1, and thus decreased cell thermogenic capacity. These observations are all summarized in Figure 4.18.
Brown pre-adipocytes in culture (Day 0)

Proliferation to confluence (day 5)

NE (10 μM) daily

Differentiation (day 12)

↑ cathepsin D expression

↑ UCP1 half-life

↑ Cell content of UCP1

↑ thermogenic capacity

No change in cell number, morphology, viability, and protein contents

↑ half-life of mitochondrial translation products

↑ Cell content of UCP1

↑ thermogenic capacity

Removal of NE for 48 h (day 12-14)

Activation of autophagy

↑ degradation of UCP1 and mitochondrial translation products

↓ cell content of UCP1 and mitochondrial translation products

↓ thermogenic capacity

No change in cell number, morphology, viability

Figure 4.18 Summary of the effects of prolonged exposure of brown fat cells in culture to NE (norepinephrine) followed by its removal.
5.0 DISCUSSION

BAT thermogenic capacity is not constant but varies with the need for thermogenesis (Himms-Hagen, 1989, 1990). Conditions such as fasting, lactation, and deacclimation are associated with atrophy of the tissue and decrease in its thermogenic capacity (Rothwell et al 1984; Trayhurn and Jennings, 1986). In rats and mice, changes in the sympathetic activity correlate well with changes in thermogenic capacity, and there is good evidence that NE is a major agent responsible for the tissue-associated changes during atrophy (Himms-Hagen, 1990). This is confirmed by the finding that most obesity models in mice and rats are associated with BAT atrophy and also a reduction of SNS activity (Marette et al 1990). Obesity and the reduction in the sympathetic activity can be reversed by cold exposure of the animals associated with increased sympathetic activity (Marette et al 1990). However, despite the importance of NE in the condition of BAT atrophy in vivo, the importance of other factors such as thyroid hormones and insulin can not be excluded as their levels also change during this condition. Several lines of investigation suggest that insulin, as well as thyroid hormones, are important for BAT function (Marette and Bukowiecki, 1989; McCormack, 1982; Mitchell et al 1992; Bianco and Silva, 1987; Bianco et al 1988). It was shown by Geloen and Trayhurn (1990) and Shibata et al (1987) that in mice made diabetic using streptozotocin, BAT undergoes atrophy, which can be reversed by insulin administration. Diabetic animal models have reduced SNS activity, and streptozotocin injections caused profound hypothyroidism (Himms-Hagen, 1989). Thus, it is difficult to resolve the importance of reduced BAT sympathetic activity itself causing the tissue atrophy from other factors present in vivo
conditions. Therefore, we have conducted experiments in brown fat cells differentiated in tissue culture to examine the effects of withdrawal of NE on the cell thermogenic capacity from cells previously exposed to NE. This approach was used to mimic in vitro the suppression of sympathetic activity known to occur in most conditions of BAT atrophy in vivo.

The main advantage is that the effect of a single factor (NE) in the induction of cell atrophy can be investigated. This work can have clinical significance in the treatment of many pathological conditions such as obesity and diabetes. However, in cell cultures there is no possibility to easily get rid of the waste products and to supply the adipocytes with enough nutrients when thermogenesis is activated.

5.1 WITHDRAWAL OF NE FROM CELLS PREVIOUSLY EXPOSED TO NE PARTIALLY MIMICS TISSUE ATROPHY SEEN IN VIVO

Brown fat atrophy has been observed in many physiological and pathological conditions. BAT atrophies when cold-acclimated rodents are returned to a thermoneutral environment (Desautels and Himms-Hagen, 1980; Desautels et al 1986), during fasting (Desautels, 1985; Rothwell et al 1984; Trayhurn and Jennings, 1988), and in the physiological states associated with increased energy demand, such as lactation (Trayhurn et al 1982). The pattern of BAT atrophy, however, differs among the stated conditions. While BAT atrophy caused by fasting, deacclimation, and lactation is characterized by cellular loss of proteins, mitochondrial proteins, and loss of UCP1 from mitochondria (Desautels, 1985; Desautels et al 1986; Trayhurn
and Jennings, 1988), loss of cells is only observed upon return of cold-acclimated rodents to warm environments (Desautels et al 1986) but not upon fasting and lactation (Desautels and Dulos, 1990; Desautels et al 1990; Muralidhara and Desautels, 1994; Trayhurn and Jennings, 1988; Trayhurn, 1989).

These results demonstrate that prolonged exposure of brown fat cells in culture to NE and its removal for 48 hours do not affect culture cellularity (Figure 4.5). In vivo, cold exposure of rats which is associated with an increased tissue sympathetic activity, or NE infusion in rats kept at 25°C through miniosmotic pumps are known to stimulate the mitotic activity of brown pre-adipocytes (Mory et al 1980; Geloen et al 1992; Bukowiecki et al 1982). In this study, brown adipocytes were grown in the presence of serum for 5 days prior to the addition of NE. In the presence of serum, the cells proliferate readily with a doubling time of about 24 hours until they reach apparent confluence (Nechad et al 1983; Lorenzo et al 1993). Therefore, at the time of NE addition (day 5), the culture dishes were already covered with a confluent cell monolayer and there was no more space for further proliferative response. Furthermore, addition of NE to post-confluent brown adipocytes does not lead to stimulation of DNA synthesis (Bronnikov et al 1992). If, however, NE is added to the cell cultures before confluence, ongoing DNA synthesis is accelerated (Bronnikov et al 1992).

There was no apparent loss of brown adipocytes upon removal of NE for 48 hours (Figure 4.5). This is somewhat similar to the conditions observed during fasting and lactation, but in contrast to the net cell loss from the tissue during deacclimation. Removal of NE did not appear to affect cell viability as visualization
of the cultures by light microscopy did not show altered cell morphology, or penetration of cell impermeable dyes such as ethidium bromide (data not shown). Furthermore, as another measure of cell viability, the cellular ATP level was also maintained upon removal of NE from the cultures compared to the cultures treated chronically with NE (Figure 4.4). NE is known to activate UCP1, via release of fatty acids from triglyceride stores, and to lower the cell ATP level (Nedergaard et al 2001; Pettersson and Vallin, 1976; Klingenberg and Echtay, 2001). However, NE is known to disappear from the culture medium within 24 hours (Waldbillig and Desautels, 1992). Measurements of the ATP content of the cultures were done 24 hours after addition and/or removal of NE. Thus, our measurement of ATP in the tissue culture reflects dominantly the cell number and permeability other than acute effect of NE.

The maintenance of cell viability in the cultures upon removal of NE may be due to a culture medium rich in serum, amino acids, pyruvate, and glucose. However, we did not measure the presence of apoptotic cells in our cultures. It was demonstrated that the amount of fragmented DNA, a measure of apoptosis, is markedly decreased (50%) after 1 day of cold exposure in BAT of mice (Lindquist and Rehnmark, 1998). The decreased rate of DNA fragmentation in the tissue was sustained as long as mice were kept in the cold environment (Lindquist and Rehnmark, 1998). When cold-adapted mice were transferred back to a thermoneutral environment, DNA fragmentation in the tissue increased significantly. Thus, it was suggested that NE acts as a survival factor, by preventing the onset of apoptosis in BAT. However, the inhibitory effect of NE on the onset of apoptosis appeared limited to pre-confluent proliferative brown adipocytes (Lindquist and Rehnmark, 1998).
Once the cells started the process of differentiation (day 5-6), NE was without effect (Lindquist and Rehnmark, 1998). *In vivo*, increased DNA fragmentation upon deacclimation of mice was not an immediate response (within 10 days, Lindquist and Rehnmark, 1998) which can not explain the rapid cell loss of almost 50% during 2-3 days of deacclimation. Thus, the mechanism underlying loss of adipocytes in certain conditions (e.g. deacclimation) remains unexplained.

Prolonged exposure of our brown fat cells differentiated in culture to NE and its removal for 48 hours did not affect the culture protein content (Figure 4.6). However, it was shown that cold exposure of rats for 7 days (Rothwell and Stock, 1984; Park and Himms-Hagen, 1989) causes a marked increase in tissue protein content. This effect of cold exposure on tissue protein content was totally prevented by surgical denervation (Himms-Hagen, 1989; Rothwell and Stock, 1984). Similarly, daily injections of NE for 9 days had the same effect on tissue protein content as cold exposure for 9 days (Mory et al 1984). Thus, increase in tissue protein may result from hyperplasia as well as increased cell content of proteins (hypertrophy). In contrast, fasting (2 days), deacclimation (2 days), lactation or BAT denervation of cold-acclimated animals (2-3 days) all cause a marked decrease in tissue protein content (Park and Himms-Hagen, 1989; Trayhurn and Jennings, 1988; Desautels, 1985). The absence of significant changes in cell protein content upon prolonged NE treatment, or upon removal of NE for 48 hours, could result from the degree of sympathetic stimulation. The duration and amount of exposure to NE (7 days) *in vitro* may have not been sufficient, given the rapid and exponential decline in NE concentration in tissue culture (Waldbillig and Desautels, 1992). In addition, although
NE is the most important trophic factor, the effect of other hormones possibly missing from our culture medium can not be excluded.

Loss of tissue proteins caused by denervation is somewhat reduced by repeated thyroid hormone injections (Desautels and Dulos, 1990). Similarly, loss of brown fat proteins after a 48-hour fast is markedly depressed in hyperthyroid mice (Desautels and Dulos, 1990). Therefore, the effect of suppression in SNS activity, as during fasting, on tissue protein content may be accelerated by changes in the level of other hormones such as thyroid hormones, or insulin, occurring at the same time (Fernandez et al 1987). Our brown fat cells in culture were incubated with supra-physiological concentrations of thyroid hormones and insulin during the time of NE treatment and removal. It is possible that the presence of these hormones could influence the effect of NE on the cell protein content. Another possible explanation for the absence of changes in cell protein content during prolonged NE treatment and its removal for 48 hours could be the balance between rates of protein synthesis and degradation, which may be different in vivo and in vitro.

Concerning UCP1, our cultures expressed a low basal amount of UCP1, even in the absence of NE. However, addition of NE markedly increased the culture content of UCP1 (Figure 4.7). This is in agreement with many previous studies both in vitro and in vivo where exposure of brown fat cells in culture to NE, or infusion of NE in vivo evoked increased expression of the UCP1 gene, measured as either UCP1 mRNA (Rehnmark et al 1989,1990; Tsukahara et al 1998; Denjean et al 1999) or as the amount of UCP1 protein (Herron et al 1990; Kopecky et al 1990; Houstek et al 1990, 1990; Puigserver et al 1992). NE also has been shown to have a stabilizing
effect on UCP1 mRNA in brown adipocytes in culture, increasing the half-life from about 3 hours to at least 20 hours (Pico et al 1994; Nedergaard et al 2001). Furthermore, the total BAT UCP1 content was low in animals, which had not been exposed to the cold, but increased at least 12-fold during 5 days of cold exposure (Puigserver et al 1992). Removal of NE for 48 hours from cultures previously exposed to NE for 7 days markedly decreases the culture UCP1 content back to the control level (Figure 4.7). These results emphasize the importance of NE as one of the most important factors in controlling the amount of UCP1.

Therefore, it is likely that removal of NE for 48 hours from cells previously exposed to NE causes a net decrease in the cell capacity for thermogenesis (Figure 4.18). The pattern of the tissue atrophy observed in vitro upon removal of NE, only partially mimics the tissue atrophy seen in vivo. While in vivo tissue atrophy is sometimes associated with decreased tissue cellularity, and always with reduced cell content of proteins and of UCP1, only the amount of UCP1/cell was decreased upon withdrawal of NE in culture (Figure 4.18).

5.2 NE INHIBITS DEGRADATION OF MITOCHONDRIAL PROTEINS

The lysosome is a major organelle by which cellular proteins are broken down. This compartment contains a range of proteolytic enzymes that are able to degrade essentially the variety of proteins, which are continually transported to those organelles from other regions of the cell (Seglen and Bohley, 1992; Klionsky and Emr, 2000). In addition, regulated turnover of organelles is confined to the lysosomes. The bulk of the material is delivered by the process known as autophagy,
which involves multiple steps and several pre-lysosomal vacuoles (Gordon et al 1989; Seglen and Bohley, 1992). This important route of delivery of cell constituents to the lysosomes is regulated by nutrients and hormones, allowing cells to match their degradative status to the environmental conditions (Blommaart et al 1997; Dunn, 1994; Seglen and Bohley, 1992). Brown fat atrophy, which is associated with reduced BAT sympathetic activity, is characterized by marked loss of mitochondrial proteins including UCP1 with or without changes in tissue cellularity (Himms-Hagen, 1989). However, very little is known about the biochemical pathways that bring about such marked alterations in BAT mitochondrial protein content including loss of UCP1 and how the proteolytic activity is regulated.

The final amount of UCP1 in a brown adipocyte is the outcome of a series of controlled processes. It may be controlled at the level of gene expression and mRNA stability (Nedergaard et al 2001). There are also translational aspects, and UCP1 protein stability and turnover may be altered (Nedergaard et al 2001). About half of the BAT content of UCP1 is lost within 7 days when cold-acclimated animals are returned to thermoneutral environment. In contrast, half of the cell content of UCP1 is lost within 2-3 days upon cessation of NE stimulation in cells, which had been treated with NE for 5 days in primary cultures (Puigserver et al 1992). A more recent estimation by Nedergaard et al (2001) suggested a 5-day half-life for UCP1 based on the rate of loss of the protein and a good correlation between changes in UCP1 mRNA and UCP1 protein. The latest estimates assume there is no effect of NE on translation. However, NE promotes protein synthesis (Waldbillig and Desautels, 1992) and inhibits protein degradation (Desautels and Heal, 1999). NE significantly
increased the incorporation of $[^{35}\text{S}]$ methionine into mitochondrial proteins and UCP1 (Waldbillig and Desautels, 1992). We made a direct measurement of turnover rate of UCP1 in the presence or absence of NE in brown fat cells differentiated in culture (Figure 4.12). The measurement was carried out between day 12 and 15, since there was no net change in culture UCP1 content, a steady-state condition. The half-life of UCP1 was markedly different in the presence or absence of NE. It was estimated to be about 3.6 days in the absence of NE, and about 8 days when brown adipocytes were incubated with NE (Figure 4.12). Therefore, these results suggest that the presence of NE is essential not only to increase the level of UCP1, but also to maintain its elevated level (Figure 4.18). Two pools of UCP1 have been reported to exist in brown adipocytes: Newly synthesized UCP1 which is not yet incorporated into mitochondria and is more susceptible to degradation and UCP1 present within mitochondria and protected against degradation (Puigserver et al. 1992). Our measurement of UCP1 turnover rate mostly consists of the fully incorporated UCP1 within mitochondria, with a very minor fraction being newly synthesized UCP1, as the labeling period was prolonged (48 hours), and over time, a larger fraction of labeled UCP1 becomes incorporated within mitochondria. The apparent difference between the half-life of UCP1 estimated in vivo and in vitro may be due to a more catabolic state of cells in tissue cultures.

NE does increase the half-life of mitochondrial translation products (Figure 4.16). In addition, the estimated half-life of mitochondrial translation products in the presence or absence of NE was not significantly different from those of UCP1 measured in the presence or absence of NE. This suggests that all
mitochondrial proteins including UCP1 are degraded at similar rates and possibly by
the same mechanism. Thus, the inhibitory effect of NE on protein degradation may
not be specific to UCP1, but possibly includes the whole mitochondria.

5.3 INVOLVEMENT OF AUTOPHAGY IN DEGRADATION OF
MITOCHONDRIAL PROTEINS

UCP1 half-life was 3.6 and 8 days in the absence and presence of NE
(Figure 4.12). Net loss of UCP1 to basal level occurs within 48 hours upon removal
of NE in cells previously exposed to NE for 7 days (Figure 4.7). This rapid loss of
UCP1 can not be explained only by basal turnover rate of UCP1, but suggests the
presence of a proteolytic activity within brown fat cells which becomes activated
upon removal of NE from cultures. There may be several proteolytic pathways in
BAT involved in degradation of UCP1 and thus BAT atrophy. Lysosomal proteolytic
pathway was clearly responsible for the marked protein loss from BAT of fasting
mice, as inhibitors of lysosomal enzymes such as chloroquine and leupeptin inhibited
protein degradation in brown fat of fasting mice (Desautels et al 1990). Chloroquine
is an acidotropic agent that raises the pH of lysosomes and is also an inhibitor of
cathepsin B, and leupeptin is a protease inhibitor that inactivates cathepsins B, H, and
L present in lysosomes (Desautels et al 1990; Desautels and Heal, 1999). NE inhibits
degradation of cellular proteins in brown fat cells differentiated in cultures (Figure
4.11, and Desautels and Heal, 1999). This inhibitory effect of NE is mediated at least
in part by inhibition of autophagy, as the effect of NE was mimicked by the addition
of inhibitors of autophagic sequestration (Figure 4.11, and Desautels and Heal, 1999).
However, there is little information on the expression and importance of lysosomal enzymes in brown fat function. The alternative pathway that could be responsible for rapid loss of UCP1 upon NE withdrawal is the presence of proteases within the mitochondria (Desautels and Dulos, 1994; Desautels, 1992).

There was intense immunostaining of brown fat cells differentiated in culture (day 10) with antibodies against cathepsin D (Figure 4.8). This suggests the presence of a large lysosomal proteolytic capacity in brown adipocytes. Cathepsin D is a good marker for the presence of lysosomes. It has a pH optimum of 4.0 and its activity is confined to acidic compartments such as lysosomes (Seglen and Bohley, 1992). The expression of cathepsin D in brown adipocytes is differentiation-dependent, with low expression in pre-adipocytes and high levels in differentiated cells (Figure 4.9). These results suggest an important function for lysosomes during differentiation and also in brown adipocytes. Lysosomes may participate in the turnover of the organelles which are no longer needed in differentiating pre-adipocytes, as well as in the breakdown of extracellular phagocytosed materials of membrane proteins, and of intracellular proteins with long half-life in both pre-adipocytes and mature brown adipocytes (Dunn, 1994; Desautels et al 1990). Cathepsin D behaves as a housekeeping gene and as a hormone-regulated gene that can be controlled. In a human breast cancer cell line, estrogens stimulate transcription of the cathepsin D gene by means of estrogen-responsive sequences located in the proximal region of the promoter (Cavailles et al 1993). In brown adipocytes, prolonged incubation of the cells with NE and its removal for 48 hours does not affect the cell content of cathepsin D (Figure 4.10). Therefore, although NE has been
demonstrated to be involved in the expression of many genes during differentiation of brown adipocytes including UCP1 (Cannon et al. 1996), it is not involved in cathepsin D expression during differentiation. In agreement, the specific activities of lysosomal enzymes including cathepsin D were similar in BAT of mice acclimated at 4, 21, and 33°C (Desautels et al. 1990). Cold exposure of mice, which increases the sympathetic activity to BAT, was without effect on the specific activity of cathepsin D (Desautels et al. 1990).

Although cathepsin D expression markedly increased in brown adipocytes during differentiation, autophagic proteolysis is likely inactive and under inhibitory control of NE. Removal of NE from cultures likely triggers formation of autophagic vacuoles and thus increases the rate of loss of mitochondrial proteins including UCP1 (Figure 4.18). Our results indicate that removal of NE increases the loss of mitochondrial translation products and of UCP1. However, in the presence of W, LY, and 3-MA, the loss was reduced (Figures 4.14 and 4.17). W, LY, and 3-MA are potent inhibitors of PI 3-K and are known to inhibit the sequestration step in autophagy (Blommaart et al. 1997). W covalently binds to the enzyme, which is then irreversibly inhibited (Blommaart et al. 1997). By contrast, LY binds to the enzyme in a reversible manner, inhibition being competitive with ATP, presumably because LY and ATP have structural resemblance (Blommaart et al. 1997). Although W is a potent inhibitor of PI 3-K, this compound may not be entirely specific. W also inhibits PI 4-K (Blommaart et al. 1997). It is unlikely, however, that inhibition of PI 4-K is responsible for inhibition of autophagic sequestration by W because LY, which is very specific and does not affect PI 4-K, similarly inhibits autophagic sequestration.
However, the mechanism of action of 3-MA on PI 3-K is still unclear (Blommaart et al 1997). It is noteworthy to mention that the doses of W, LY, and 3-MA used in this study are the lowest doses causing maximal inhibition of proteolysis (according to the dose response curves, unpublished data from our laboratory), and are consistent with doses required to inhibit PI 3-K (Blommaart et al 1997; Desautels and Heal, 1999).

As it was mentioned before (introduction section), 2 enzymes of PI 3-K and p70S6 kinase are involved in the control of proteolysis in hepatocytes (Blommaart et al 1997; Klionsky and Emr, 2000). Both enzymes are part of the insulin signal transduction pathway in hepatocytes and as insulin is an effective inhibitor of proteolysis in mature brown adipocytes (Desautels and Heal, 1999), these enzymes may be involved in mediating its inhibitory effect in these cells. There was a significant stimulation of immunoprecipitable PI 3-K activity with insulin in mature brown adipocytes differentiated in culture. Rapamycin (an inhibitor of p70 S6 kinase) also abolished the inhibitory effect of insulin on proteolysis (unpublished data from our laboratory). However, despite of similar inhibitory effect of NE on proteolysis to the inhibitors of PI 3-K (Figures 4.11, 4.14, and 4.17), NE had no effect on either basal or insulin-stimulated PI 3-K activity in brown adipocytes differentiated in culture (unpublished data from our laboratory). Rapamycin had also no effect on NE inhibition of proteolysis in mature brown adipocytes (unpublished data from our laboratory).

Formation of autophagic vacuoles and the increased activities of lysosomal proteases upon cessation of NE stimulus could be beneficial to the cells and the tissue. Removal of NE from brown fat cells in culture causes loss of UCP1
and presumably a decrease in the cell thermogenic activity. Mitochondria are well known to produce reactive damaging $O_2$ species and the effect is dominant when $O_2$ is present and the organelle has a lower rate of $O_2$ consumption. Consequently, a decrease in brown fat cell thermogenic activity (from decreased SNS activity, or in culture from removal of NE) is likely accompanied by a decrease in the rate of $O_2$ consumption, simultaneously with an increase in the formation of reactive $O_2$ species (Skulachev, 1996). Inhibition of mitochondrial respiratory activity is known to result in the over-production of reactive $O_2$ species. These may damage the cells in many ways, but could alone act as activators of apoptosis (Mignotte and Vayssiere, 1998). The formation of autophagic vacuoles upon cessation of sympathetic activity could help to prevent or remove damaged mitochondria and thus prevent cell damages and loss by apoptosis.
6. CONCLUSIONS

Brown fat cells in culture acquire a large capacity for lysosomal proteolysis during differentiation, which is not affected by NE. Chronic exposure of the cells to NE increases the thermogenic capacity. This may be due in part to inhibition of proteolysis resulting in increased half-life of mitochondrial proteins, including UCP1. Activation of autophagy occurs in response to the withdrawal of NE from cells previously exposed to NE. Rapid loss of mitochondrial proteins and UCP1 may serve to have a protective role in BAT. This effect in BAT cell culture is similar to what is observed in many situations of BAT atrophy in vivo. However, our culture does not reproduce all aspects of BAT atrophy in vivo, such as loss of tissue cellularity.
7.0 REFERENCES


protein mRNA after high-carbohydrate or high-fat diets. Am. J. Physiol. 266, R1578-R1583.


