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UNCOUPLING PROTEINS mRNA LEVELS IN MICE LACKING ACYLATION-STIMULATING PROTEIN

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Abstract

The etiology of obesity involves imbalanced energy intake and utilization. ASP is an adipose tissue hormone that facilitates adipocyte uptake of serum fatty acids and their storage. Mice lacking ASP have less adipose tissue mass, despite increased food intake, than wild-type littermates. We hypothesize that the unstored fuels are oxidized through UCP (thermogenic mitochondrial carriers).

In male ASP-deficient mice mRNA levels were measured by semi-quantitative RT-PCR and the following changes were observed: UCP-1 decreased in all tested tissues, UCP-2 increased by 15% and 6 fold in muscle and white adipose tissue and UCP-3 increased 2.5 and 10 fold in muscle and epididymal adipose tissue, respectively. In female ASP-deficient mice UCP-1 decreased in all tissues, UCP-2 increased by 10% and 40% in inguinal and brown adipose tissue, respectively, and UCP-3 remained stable in all tissues. High fat diet nullified these differences, and decreased all wild-type UCP levels.

We propose that UCP-2 and 3 assume the role of UCP-1 in fuel utilization, thus helping mice face an increased energy load in the absence of ASP.

Abrégé

L'obésité résulte du déséquilibre entre l'apport et la dépense d'énergie. L'ASP est une hormone qui stimule l'incorporation des lipides dans la cellule. Les souris déficientes en ASP ont moins de tissu adipeux, en dépit d'une consommation alimentaire élevée. Nous proposons que l'énergie diététique non incorporée serait dissipée par les UCP (protéines mitochondriales thermogéniques).

Chez les souris mâles déficientes en ASP, la mesure des niveaux d'ARNm par la méthode de RT-PCR semi-quantitative démontre que: UCP-1 apparaît réduite dans tous les tissus testés, UCP-2 est élevée de 15% et 6 fois la normale dans les tissus musculaire et adipeux, et la UCP-3 est élevée 2.5 et 10 fois la normale dans les tissus musculaire et adipeux épididymal, respectivement. Chez les souris femelles déficientes en ASP, UCP-1 est réduite dans tous les tissus, UCP-2 augmente de 10% et de 40% la normale dans les tissus adipeux inguinal et brun, tandis que UCP-3 reste stable dans tous les tissus. Globalement, une diète riche en lipides atténue ces différences et décroît les protéines UCP comparativement à une diète normale.

Donc, UCP-2 et 3 se substituent la fonction de la protéine UCP-1, facilitant ainsi l'utilisation des calories non incorporées par les souris manquantes d'ASP.

Background.

This study has stemmed from the observation that transgenic mice that lack acylation-stimulating protein (ASP) are hyperphagic, yet lean, with reduced adiposity and increased insulin sensitivity. There is no evidence of malabsorption or dylipedimia, and metabolic rate is increased.

Since Uncoupling Proteins (UCP) are postulated players in metabolism and thermogenesis, we investigated their expression in ASP-deficient mice, hypothesizing that their upregulation may explain the increased metabolism of those mice.

Review of the literature

I] Acylation-Stimulating Protein

1.1. Identity of Acylation-stimulating protein (ASP)

ASP was originally isolated from human plasma based on its cellular functional activity. It was found to stimulate triglyceride synthesis (TGS) in human skin fibroblasts and adipocytes (15). The same effect was reproduced in murine fibroblasts and adipocytes. Analysis of ASP showed it to be a small basic protein composed of 76 amino acids, resulting in a molecular weight of 8932 Daltons. ASP is identical in sequence to des-arginated C3a, and this identity was established by amino terminal sequence analysis, ion spray ionization mass spectroscopy and amino acid analysis (44). Experimental confirmation came through the addition of the factors required for the production of complement 3a in the medium of cultured adipocytes, which resulted in increased TG synthesis, as would be expected in the presence of ASP (44). Lastly, recombinant ASP was reported to have the same effect on TGS as plasma ASP, and this was blocked by immunoprecipitation (44). ASP is distinct from the anaphylatoxin C3a, as recombinant ASP activity is sensitive to solubilization conditions, in contrast to C3a activity (44).

1.2. ASP secretion

Adipose tissue possesses endocrine functions, secreting hormones such as leptin and ASP. Both human and murine adipocytes synthesize and secrete complement 3 (a member of the alternate complement pathway of the immune system), factor B (a necessary cofactor that binds C3) and adipsin (factor D, a serine protease that acts as a catalyst to the above-mentioned reaction) (15, 44). The result of the interplay between these factors and the C3 substrate at the surface of the adipocyte is the 77-amino acids C3a, which, if released systemically, acts as a chemotactic agent and member of the complement cascade. The N-terminal arginine of C3a is rapidly cleaved by a carboxypeptidase, resulting in C3adesArg, or ASP (15, 44).

C3 is the only member of the classical complement system that is secreted by adipocytes, as neither C2, nor C5, other important immune players, are produced in adipose tissue.

1.3. ASP function

ASP has been shown to increase TGS in cultured murine adipocytes by two mechanisms. The first is induction at a cellular level of diacylglycerol-acyl transferase (DGAT), which is the final enzyme in the incorporation of free fatty acids (FFA) into a triglyceride backbone. Kinetic analysis indicates that ASP alters DGAT activity, not substrate binding, as it increases *Vmax*, not *Km* (4). The second mechanism of ASP effect is the increase specific membrane glucose transport in the adipocyte, where it can be used to synthesize the glycerol backbone of triglycerides. This is achieved through translocation of GLUT1 and GLUT4 glucose carriers from intracellular vesicles to the plasma membrane (4, 15). Thus, ASP accelerates the storage of FFA into triglycerides, by facilitating substrate entry into the cell and utilization by the enzymes. This results in efficient trapping of dietary FFA by the adipocytes (64).

The effects of ASP on TGS were demonstrated to be independent of, and additive with, the action of insulin on TG metabolism (15). They are presumably

mediated by a surface receptor-signal transduction cascade that involves protein kinase C (PKC). Indeed, the effect of ASP on TGS was replicated by known activators of PKC, inhibited by inhibitors of PKC and stimulation by ASP was associated with an increase in PKC activity (4).

The effects of ASP are observed mainly in adipocytes. Despite its high homology to the immune-acting C3a, ASP has no activity on macrophages and is inert in the complement pathway.

1.1.3. ASP regulation

The production of ASP by adipose tissue is markedly accelerated in the second half (3-6 hrs) of the postprandial period. This peak in ASP secretion coincides with maximal TG clearance and FFA uptake by the adipocytes.

Of all components that are increased in the bloodstream after a meal, chylomicrons, which are large lipoprotein complexes carrying FFA from the intestine to the peripheral tissues, seem to be responsible for induction of ASP secretion. This phenomenon was reproduced in culture: when chylomicron preparations alone were added to cultured human and adipocytes (59), ASP production was shown to increase. It was subsequently shown that the main factor responsible for this effect was not an apoprotein, rather a loosely associated protein identified as transthyretin (TTR) (59).

TTR associates with retinal binding protein to transport retinol and thyroxine in the circulation. Molecular studies confirmed this experimental relationship, by demonstrating a functional retinoid receptor-inducible element in the promoter sequence of the C3 gene.

II] ASP-deficient mice

2.1. ASP-deficient mice

Given the importance of ASP in facilitating FFA trapping by the adipocyte in the post-prandial period, and in order to better dissect out the dependence of lipid metabolism on ASP, a strain of mice was genetically engineered to lack ASP. Since there did not exist an adipsin knockout animal model (that would

nullify the enzyme needed for conversion of C3 to ASP), ASP-deficiency was achieved by silencing the C3 gene (45, 46, 47).

It was expected that, in the absence of ASP, the adipose tissue of knockout animals would be less efficient at trapping FFA, hence causing delayed postprandial triglyceride clearance, and reduced adipose tissue mass. The following phenotypes were observed.

2.1.1. Male ASP deficient mice (C3KO)

The male C3 KO have a 26 % decrease in adipose tissue weight as compared to their wild-type littermates (45, 46). This occurred on both a low fat (LF) and high fat (HF) diet, despite increased energy intake (16%) and with no evidence of malabsorption as tested by fecal fat analysis (Figure 1). On the LF diet, the mice had a markedly decreased feed efficiency, needing 33% more calories per g weight than the wild-type counterparts. As expected from the decreased adipose tissue mass, there were also decreased leptin levels, but more

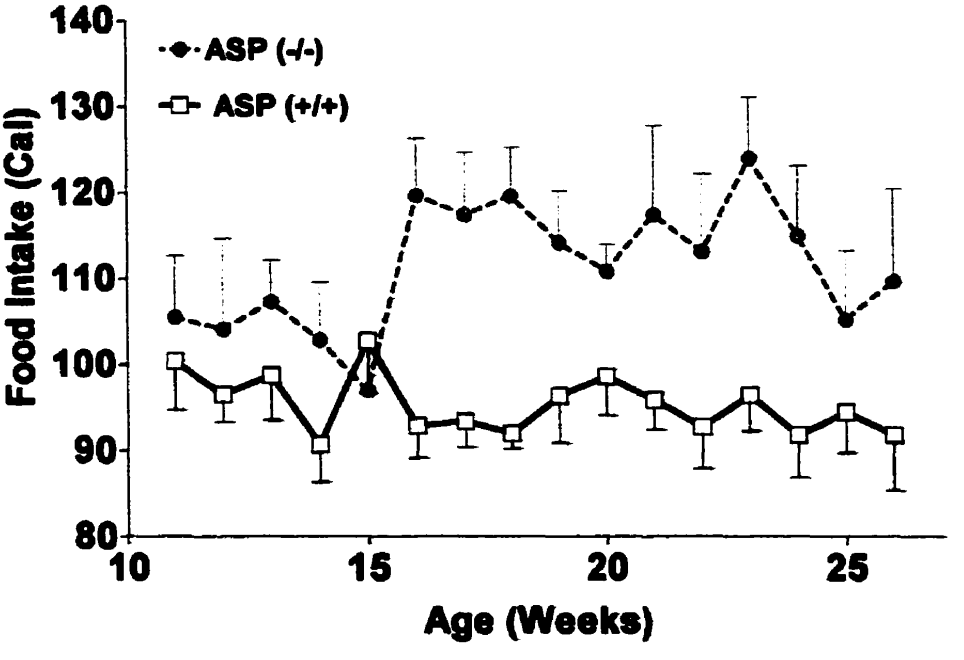

Fig.1. Food intake of ASP-deficient mice (ASP -/-) compared to wild-type mice (+/+) on a low-fat diet (from Murray I, 1999, *J. Biol. Chem.* 274 (45)).

so that could be explained by the loss of adiposity (a 29 % downward shift in leptin/body weight ratio as compared to control).

The mice did exhibit significant delays in post-prandial TG clearance (80-120% increase in clearance time compared to control) as well as increased post-prandial non-esterified fatty acids (from 37% to 73% increase on the LF and HF diet, respectively, compared to the diet-matched wild-type mice). These increases could not be explained by higher fasting lipid levels, as there was no difference in this regard between ASP KO mice and wild-type mice, on either LF or HF diet (45, 46). Finally, despite delayed clearance of serum lipids, the KO animals had a 30-40% lower insulin-glucose product, denoting increased insulin sensitivity (45).

2.1.2. Female ASP-deficient mice (C3 KO)

The female C3 KO mice have a 10% decrease in body weight, both on LF and HF diet, compared to controls (Figure 2), which is mostly accounted for by decreased adipose tissue mass (58% reduction on the LF diet) (47). Leptin levels are also reduced, even on the HF diet, with an 18% drop in plasma concentration after adjusting for the reduced body weight and adipose tissue mass (47).

Post-prandial TG clearance is delayed at 3 and 4 hrs after a meal, but the differences are not significant and not as marked as in the males. Again, fasting TG levels were identical to those of wild-type littermates (47). Female mice also exhibit lower basal glucose and insulin levels (12.8% and 41% lower, respectively, than controls, on HF diet).

2.2. Calorimetric Studies of ASP-deficient mice

Calorimetric studies of ASP deficient mice confirmed 10-15% lower body weight compared to wild-types, increased food intake, and a consequently decreased feed efficiency. There was no difference in amount and timing of peak physical activity between C3 KO and WT. However, oxygen consumption, as well as respiratory quotient (RQ) were increased by approximately 10%, suggesting increased metabolic rate (unpublished data, Dr. P. Havel). Elevated

RQ parallels the relative proportion of carbohydrate oxidation to carbon dioxide and it suggests preferential use carbohydrates as body fuel.

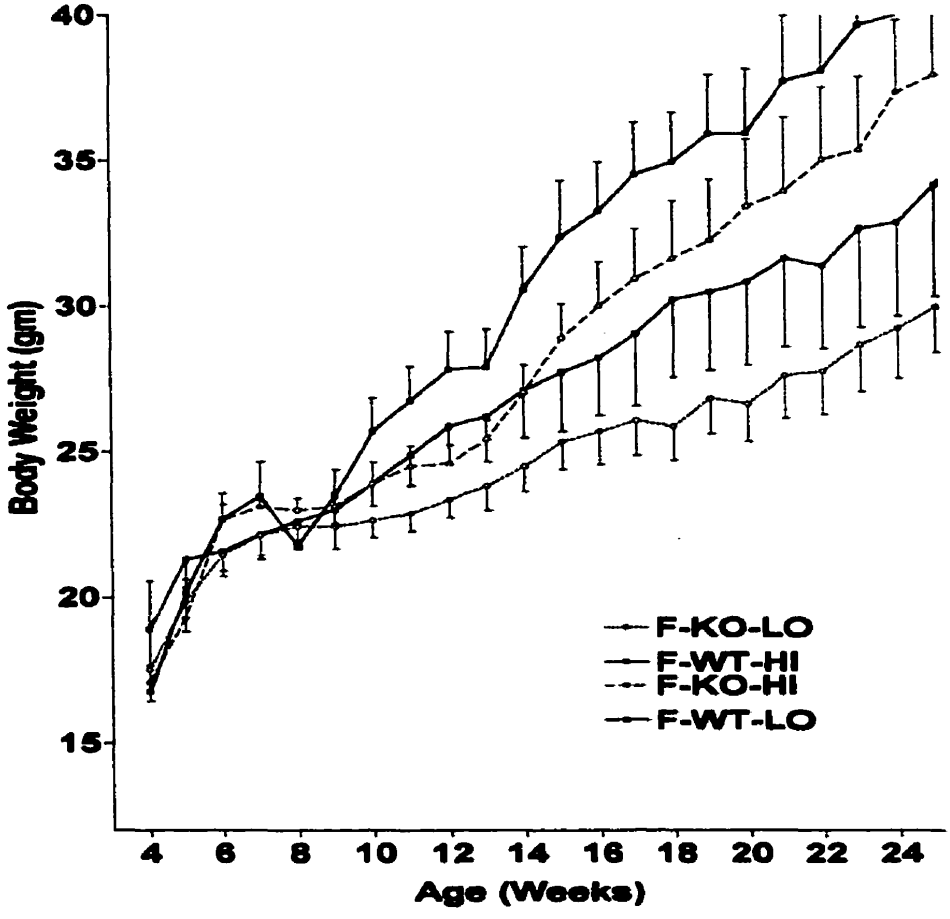

Fig. 2. Body weight in ASP-deficient (KO) and wild-type (WT) female mice placed on a high-fat (HI) or low-fat (LO) diet (from Murray I, 2000, Endocrinol. ((47))

III] Experimental rationale:

ASP-deficient mice were found to have an increased metabolism, leading to decreased feed efficiency despite lack of malabsorption or increased energy expenditure. Therefore, metabolism must have increased at the cellular and mitochondrial level, in order to dispose of the excess calories taken in. Uncoupling proteins are candidates for this process, and this project examined their mRNA expression.

IV] Uncoupling Proteins

4.1. Uncoupling proteins (UCP) and the mitochondria

Uncoupling proteins were discovered following studies on the metabolic function of mitochondria. Mitochondria have been known to be the site of ATP synthesis since the 1940s (9, 35). It was then widely believed that there was perfect coupling between oxygen consumption (via electron flow from the reduced NADH) and the pumping of protons from the mitochondrial matrix, which generates the electrochemical gradient necessary to ATP synthesis from ADP (9). However, despite careful isolation techniques, a basal proton leak across the mitochondrial membrane continued to be observed, along with the observation that oxygen consumption persists even with inhibition of ADP phosphorylation (9). This meant that, in order to conserve the same rate of ATP production, oxygen consumption had to increase to a higher level than anticipated. Estimates place the magnitude of oxygen consumption dissipated by the hydrogen ions leak to 20-30% of the overall metabolic rate, making it a major player in energy homeostasis (7, 9, 72).

The significance of this flux of hydrogen ions has been widely debated, with explanations ranging from control of NAD+/NADH ratio, to protection from oxygen radicals, to dissipation of the energy stored in the proton gradient to generate heat (thermogenesis). This latter hypothesis was based on the observation that this non-ohmic proton leak had an inducible component, catalyzed by a specific inner mitochondrial protein acting as a hydrogen ion carrier (7, 9, 53). This carrier was induced in cold temperatures and uncoupled the oxidative production of a proton gradient from the synthesis of ATP, dissipating that energy as heat (7).

This "Uncoupling Protein-1" was found primarily in brown adipose tissue, which was known to be the site of thermogenesis in rodents (7). Thermogenesis, however, occurs in all mammals, even in those, like humans, that lack distinct depots of brown adipose tissue. The search for related uncoupling proteins (UCP) in other tissues culminated with the discovery in 1997 of UCP-2, another

mitochondrial carrier with 59% homology to UCP-1 in humans, that seemed to be ubiquitously expressed (28, 40). Moreover, ectopic expression of UCP-2 in transformed yeast cells or liposomes increases proton flux and heat production, as would be expected if it had an uncoupling function similar to that of UCP-1. A third member of the family, UCP-3, is expressed mainly in skeletal muscle and has 57% and 73% identity to UCP-1 and 2, respectively, in humans (7). Despite the similarities in both structure and effect on proton leak that exist among these three proteins, UCP-2 and 3 are not induced by cold temperatures and seem to be regulated differently from UCP-1 (7, 17). This raises a question regarding their role in thermogenesis and suggests that they may modulate some of the other physiologic functions of the mitochondrial hydrogen ions flux, such as fatty acid cycling and maintenance of a favourable ATP/ADP balance.

4.1.1. Mechanism of action of UCP-1

The uncoupling activity of UCP-1 is modulated by several agents, including free fatty acids and nucleotides, as described below. These modulators allow UCP-1 to work at times at very high transport rates, almost like a channel, and at others, at lower rates, acting more like a carrier. The explanation may be that the inner mitochondrial membrane UCP contain two distinct domains: a channel-like structure and a gating domain (53). Based on these characteristics, there are four postulated models of the mechanism of action of UCP-1 (Figure3).

The first model proposes that UCP-1 is an anion carrier. This is based on observations that UCP-1 is able to transport anions such as chloride (Cl-), bromide (Br-) and nitrate (NO3-). The protons in this case would be part of an incidental co-transport that would ensure electroneutrality, and UCP-1 would operate as a proton channel in the absence of fatty acids (Figure 3.a).

A second model takes into account the observed stimulatory action of non-esterified fatty acids (NEFA) on UCP-1 uncoupling action. It proposes that the channel pore mechanism is altered by the presence of NEFA, which provide an essential free carboxyl group (COO-) that would somehow facilitate transport of protons into the mitochondrial matrix (Figure3.b). The requirements for

successful activation by NEFA seem to be a chain length between 10 and 14 carbon atoms, with variable degree of saturation and hydrophilicity (35).

Yet a third model views UCP-1 essentially as a fatty-acid cycler, capable of flipping a neutral FFA from the inner mitochondrial membrane to the matrix, and returning a deprotonated FFA after it has released its hydrogen ion into the matrix (Figure 3.c).

The fourth model is an additional modification on the previous three, in that free (not Mg2+-bound) nucleotides (GDP more so than GTP, ADP or ATP) induce a conformational change in the gating mechanism of UCP-1 that blocks all transport modes, and thus inhibits UCP-1 specific uncoupling of the mitochondria (Figure3.d). The affinity of UCP-1 for nucleotides is highly sensitive to pH, varying inversely with pH (therefore an acidic milieu provides the highest degree of inhibition). Interestingly, a low ATP/ADP ratio or the addition of cAMP increase UCP-1 activity, suggesting that intracellular phosphorylation potential acts as a monitor of environmentally imposed thermogenesis.

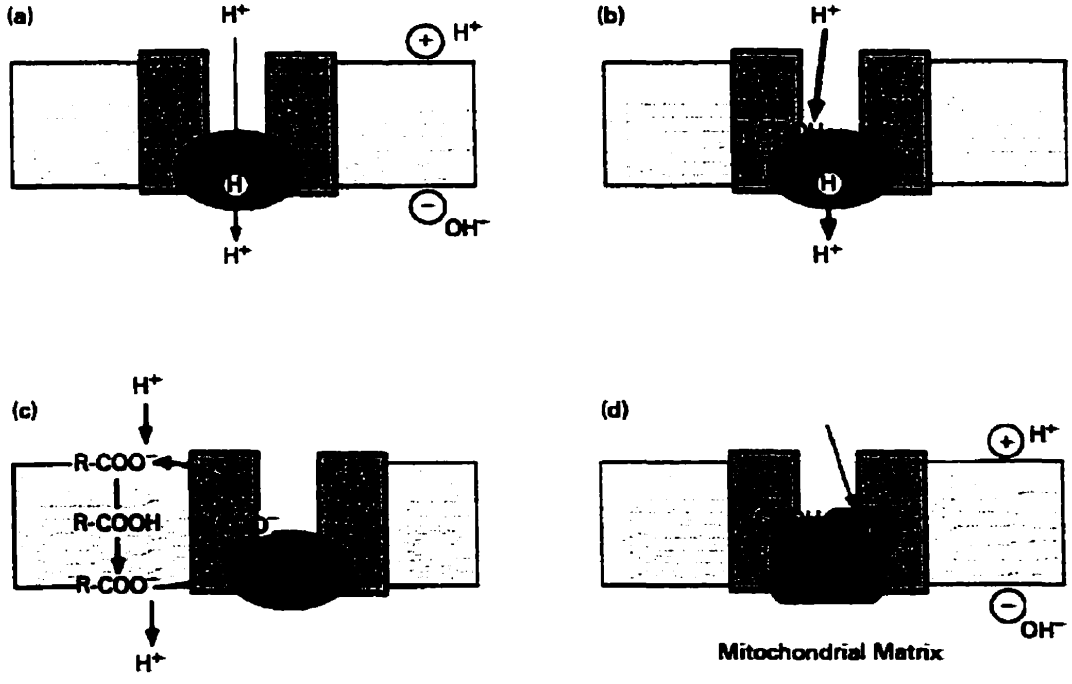

Fig.3. Mechanism of function of UCP-1 (from Ricquier D, 2000, *Biochem. J.* 345 (53))

The observation that UCP-1 mediated Cl- transport is subject to nucleotide regulation suggests that a combination of models 1 and 4 describes the UCP-1 carrier appropriately. It is also known the UCP-1 proton, but not halide, transport is activated by fatty acids, supporting model 2. The fatty acid cycling hypothesis, however, does not seem to apply to UCP-1, because studies in which fatty acids were trapped by albumin showed that UCP-1 continued to uncouple; conversely, addition of fatty acids to mitochondria lacking UCP-1 induced uncoupling that could not be blocked by nucleotides (53). These findings suggest that UCP-1 possesses a separate pathway that is not dependant on FFA cycling. Moreover, substitution of FFA with glucose at the ω-position, which anchored that end of the lipid to the surface of the membrane, still activated hydrogen ion transport, arguing against a flip-flop mechanism (53).

Therefore, we can conclude that UCP-1 is a mixed proton and halide mitochondrial carrier, inhibited by nucleotides and activated by fatty acids.

4.1.2. Mechanism of action of UCP-2 and 3

Several studies support the view that UCP-2 and 3 have the ability to uncouple oxidative respiration from ATP synthesis in the same way that UCP-1 does. First, liposomes containing human UCP-2 and 3 exhibit a proton flux (53). Second, recombinant yeast expressing UCP-2 and 3 have a decreased membrane potential, as is observed in mitochondria uncoupled by UCP-1 (53). Vesicles reconstituted with UCP-3 had measurable Cl- flux, suggesting that UCP-3, like UCP-1 has a dual proton-halide transport capability (53).

Regulation of this activity by ligands is different, however: fatty acids do not seem to be co-factors or activators of UCP-2 and 3 and nucleotides fail to inhibit UCP-2 uncoupling unless they attain very high levels (53). UCP-3, in contrast to UCP-1, is activated by high ATP/ADP ratios (22, 29). Given the preponderant expression of UCP-3 in muscle tissue, this regulation underscores the secondary importance of energy dissipation compared to muscle contraction in that tissue. Indeed, rising ADP levels should inhibit UCP-3 in order to conserve ATP for the contractile system. Overall, as will be discussed later, the patterns of

expression and regulation of UCP-2 and 3 support fatty acid cycling as the preferred mechanism for these two carriers (31).

4.2. UCP structure

UCP belong to the family of mitochondrial anion carrier proteins (MACP) that includes the ADP/ATP carrier, the phosphate carrier and the oxoglutarate/malate carrier (53). Structurally, these 300-amino acids long proteins share a triplicated structure with three internal signature hydrophobic motifs (31, 36, 52). The predicted secondary structure includes six membrane-spanning α-helices linked by polar loops, arranged such that the C- and N-terminals face the cytosolic side, along with four other loops, while the other three loops display characteristic motifs on the matrix side (31). UCP-1's regulatory nucleotide binding site is postulated to lie between amino acids 255 and 273, a region located on the matrix side (52, 53). The residues responsible for the activator effect of fatty acids remain unknown. Sequencing of UCP genes reveals that the proteins do not posses an N-terminal signal peptide, which is generally needed for post-translational targeting of UCP to the mitochondrial membrane. While targeting remains poorly understood, regulation of UCP expression is reflected in the regulatory elements present in the genes sequences (62). In fact, identification of cAMP response element (CRE), thyroid hormone response element (TRE) and retinoic acid response element (RARE) in the rat UCP gene (62) confirms experimental findings of such hormonal regulation, as will be discussed later.

4.3. UCP expression

UCP-1 was originally described in brown adipose tissue (BAT) in rodents, and has traditionally believed to be expressed solely in that tissue (30, 36). Recent studies, using more sensitive techniques, have shown that UCP-1 is also expressed in rodent white adipose tissue (WAT), and can be induced in rodent skeletal muscle after treatment with β3-adrenergic agonists (19, 52, 74). Relative expression, however, is maximal in BAT. UCP-2 is expressed in most tissues, across species, but its levels are higher in WAT. UCP-2 is surprisingly absent in

hepatocytes, whose mitochondria exhibit a high degree of uncoupling. Rather, most accounts of UCP-2 expression in the liver are within liver parenchymal cells (68). UCP-3 was originally found in skeletal muscle, but it is also expressed at lower levels in rodent BAT and WAT.

4.4. UCP regulation

Regulation of UCP gene expression is differential for each UCP subtype and involves multiple agents. While cold has traditionally been the main signal for thermogenesis in brown adipose tissue and for induction of UCP-1, UCP-1 also responds to thyroid hormone, circulating leptin levels and β3-adrenergic stimulation (7, 17, 53). These are all metabolic signals, linking energy expenditure and energy intake. UCP-2 and-3, however, are minimally influenced by ambient temperature and their expression is more sensitive to circulating free fatty acids levels, prolonged high-fat diet, PPAR-γ agonists and other signals of fuel homeostasis (6, 17, 27, 32).

4.4.1. Temperature

As the body's principal thermogenin, UCP-1 expression expectedly increases upon exposure to cold temperatures. Lean mice acclimated at 19°C for 10 days show an increased in UCP-1 mRNA in BAT compared to littermates exposed to a temperature of 28°C for the same duration (23, 42). Conversely, lack of UCP-1, as in UCP-1 knockout mice, results in an inability of the animals to maintain body temperature in a cold environment.

An up-regulation of UCP-2 was observed in BAT UCP-1 knockout mice, but his did not correct the animals excessive sensitivity to cold (23) and it is unclear if this occurred as a compensatory mechanism for absence of UCP-1 or as a direct response to the temperature challenge.

UCP-3 mRNA expression in skeletal muscle or in BAT is not influenced by cold exposure.

4.4.2. Leptin

Leptin is an adipose tissue hormone, the product of the *ob* gene, secreted by adipocytes in direct proportion to their tissue mass. This 16-kDa protein acts at the level of hypothalamic nerve centres to mediate neuroendocrine responses to energy supply or deprivation, presumably by regulating appetite (25). Leptin knockout mice (*ob/ob*) are hyperphagic, obese, diabetic, have hypertrophic WAT and their thermogenic response is decreased, resulting in a lower core temperature. These observations point to a link between leptin control of energy intake and thermogenesis, perhaps at the level of UCP (12, 21).

Three days administration of leptin to both lean and *ob/ob* mice results in decreased body weight, restoration of UCP-1 mRNA and protein deficits in BAT and retroperitoneal WAT, with little change in UCP-2 expression (19). In an attempt to differentiate between direct effects of leptin on UCP expression as opposed to secondary effects mediated by decreased food intake, rats were fasted for 2 days while exogenous leptin was administered in pulses every 8 hours. Fasting resulted in a decrease of BAT UCP-1 and 3 mRNA levels, no change in UCP-2 expression and an increase in UCP-3 expression in skeletal muscle. Leptin administration restored BAT UCP-1 and 3 levels to those seen in fed controls, while having no effect on UCP-2 and muscle UCP-3 (60).

In order to clarify whether leptin modulation of UCP occurs centrally or peripherally, lean rats received 4 days continuous intracerebroventricular leptin infusion, with no peripherally measurable serum leptin levels (21). Leptin treatment resulted in decreased food intake, decreased weight gain, insulinemia and increased muscle and BAT glucose utilization index. At a cellular level, the decrease in BAT UCP-1, 2 and 3 mRNA levels observed in pair-fed controls was prevented by leptin, while both treatment groups showed decreased liver and WAT UCP-2 and decreased muscle UCP-3 mRNA (21).

In a similar study, denervation of BAT and skeletal muscle yielded a loss of responsiveness to leptin. This is in contradiction to other experiments (60) that show a 30% increase in rat BAT UCP-2 and UCP-3 mRNA levels, even after

denervation of the tissue. This may be due to incomplete denervation (unilateral in the latter study) or differences in leptin administration (peripheral vs central).

Overall, these studies suggest that leptin acts centrally to effect a neurally mediated increase in BAT UCP-1 and 3 levels, with little or no change in UCP-2 and 3 expression in WAT and muscle.

4.4.3. Fat diet

Considering the thermogenic role of UCP-1 and its strong modulation by leptin, the observation that UCP-2 and 3 map to trait loci associated with obesity (resting metabolic rate, body fat) in human chromosome 11q13, as well as the putative fatty acid cycling mechanism of UCP-2 and 3, this raises the question of fatty acid intake modulation of UCP (58).

In fact, 4 weeks feeding of rats with a diet containing 60% fat resulted in 1.6-fold increase in UCP-2 in epididymal WAT and 2-fold increase in UCP-3 mRNA in skeletal muscle compared to low fat (12% fat) fed controls (33). Rats fed a low fat (96%) diet for two weeks had decreased skeletal muscle UCP-2 and 3 expression and reduced energy expenditure that were abolished by refeeding with high-fat (63%) diet, and similar reports come from studying C57Bl/6J mice (28). Given that high fat diet induces hyperinsulinemia and hyperglycemia, the observed elevated expression of UCP-2 and 3 after dietary fat ingestion may point towards a role in insulin resistance (56).

In an attempt to differentiate between hormonal effects of chronic high fat diet versus direct effects of free fatty acids (FFA) on UCP expression, rats were fasted for 12-72 hours in order to obtain lipolysis and elevation of serum FFA. In the quadriceps muscle, UCP-3 mRNA levels increased 10-fold and UCP-2 levels 2-fold (71). Administration of leptin did not eliminate the effect, nor did administration of cortisone to fed animals (in order to mimic starvation) reproduce the observation. However, administration of Intralipid and heparin to fed rats did cause and increase in UCP-3/actin mRNA ratio compared to saline-infused fed controls (71). These results suggest that FFA regulate UCP-3 levels; perhaps the consequent elevation in uncoupling facilitates oxidation of excess plasma FFA. A

confirmatory study performed in humans showed a direct correlation between UCP-3 mRNA expression and non-esterified FFA serum levels and lipid oxidation rate (33). The effect of triglycerides infusion on UCP-3 and their correlation were nullified by administration of a hyperinsulinemic clamp that prevented an increased in serum FFA levels and in FFA oxidation rates (33).

Interestingly, not all muscle fibre groups respond to FFA as an interorgans signalling mechanism. Fasted rats that exhibited increased UCP-2 and 3 mRNA levels in fast and slow-twitch fibres muscle groups lost this response after administration of nicotinic acid (inhibitor of lipolysis) in slow-twitch muscle fibres only (55). Yet, glycolytic/fast-twitch fibres make up the great majority of muscle bulk in the body and have the highest susceptibility towards developing insulin resistance. In conclusion, signals other than FFA serum levels may contribute to enhance UCP-2 and 3 expression in skeletal muscle.

Finally, UCP-1 seems to respond little to dietary manipulations, despite BAT stimulation by food intake (as evidenced by increased blood flow, GDP binding and water content post-prandially) and UCP-1 dependence on intracellular high FFA concentrations (72). It is possible that the experimentally obtained, stable mRNA levels fail to reflect increased UCP-1 translation and uncoupling activity independent of the degree of gene expression (62).

4.4.4. Sympathetic Nerve Supply and Insulin

Adaptive thermogenesis in rodents is primarily mediated by sympathetically innervated β3-adrenergic receptors. These receptors are expressed most abundantly in BAT and skeletal muscle. It has been previously shown that specific β3-adrenergic agonists promote energy expenditure, decrease weight gain and improve insulin sensitivity in obese and diabetic animals (27, 34, 48). Their effect may be mediated by upregulation of UCP.

UCP-1 expression is increased 4.5-fold after β3-adrenergic agonist (βAA) treatment of cultured BAT adipocytes. This adrenergic action is mediated through intracellular activation of cAMP, which then binds to a putative CRE element to activate UCP-1 gene transcription (62). Interestingly, this effect is diminished in

the presence of insulin. This may be due to the opposing actions of βAA and insulin on intracellular transducers such as Mitogen-Activated Protein kinase (MAPK), which has a downregulatory effect on UCP-1 and is inhibited by βAA but activated by insulin (34). Ectopic expression of UCP-1 is also induced (or enhanced) by chronic treatment with βAA, both UCP-1 at the mRNA and protein levels in WAT and skeletal muscle of mice with genetically or environmentally acquired obesity (74).

All UCP are upregulated by βAA in BAT: UCP-1, 2 and 3 rise 14, 6 and 16-fold, respectively; in WAT UCP-1 and 3 mRNA levels increase 12 and 9-fold, but there is no effect on UCP-2 expression (75). However, UCP-2 and 3 mRNA levels fall in skeletal muscle and heart in mice treated with a 21 days course of βAA, concomitantly with a decrease in insulin and FFA levels. This may suggest a more complex regulation of UCP-2 and 3, with tissue-differentiated action of sympathetic innervation, insulin and circulating FFA as metabolic regulators (75).

To the support of this hypothesis comes a latter study, in which obese Zucker rats treated with βAA exhibit increase BAT UCP-1 and 3 mRNA levels, but no change in UCP expression in WAT and skeletal muscle. When compared to a control group treated with Metformin to achieve parallel control of weight and insulin levels, WAT UCP-2 and 3 expression is positively correlated to insulin levels (58).

In conclusion, sympathetic stimulation plays a large role in BAT UCP induction, may induce or enhance UCP-1 expression in ectopic tissues and has little effect on WAT and muscle UCP 2 and 3 expression. The latter seems to correlate with serum insulin levels, while UCP-1 is downregulated by insulin.

4.4.5. Peroxisome Proliferator Activated Receptor (PPAR) agonists

PPAR-γ, a member of the family of orphan receptors, has been implicated in the expression of fat-specific genes and adipocytes differentiation, as well as linked to increased insulin sensitivity (32, 70). PPAR-γ is most abundantly expressed in WAT, with minimal expression in skeletal muscle. It is decreased by

fasting, obesity and insulin-dependent diabetes mellitus (this being reversed upon treatment with insulin). Its expression is enhanced on a high-fat diet in rodents.

Thiazolidinediones (TZD) are agents used in non insulin-dependent diabetes mellitus to sensitize the tissues to insulin and increase energy expenditure (70). They are presumed to act by activating PPAR-γ, which may then activate other energy-related genes, such as UCP. In fact, Wistar fatty rats treated with TZD for two weeks have an increased UCP-3 expression in WAT and BAT. In a similar study, histological inspection of db/db mice BAT tissue showed an increase in its size and increase in lipid vacuoles per adipocyte. This was associated with elevated UCP-1, 2 and 3 mRNA levels, while UCP-2 and 3 remained unaffected in WAT and muscle (32). Interestingly, acute exposure in culture of white and brown adipocytes and skeletal myocytes to TZD resulted in several fold increase in UCP-2 mRNA, suggesting differential effect with respect to duration of treatment (11).

However, it is to PPAR-α agonists, which are known to increase fatty acids oxidation in tissues, that UCP-2 and 3 are more responsive to. Mice fed a diet rich in N-3 fatty acids (as are found in fish oil and fibrate) had increased expression of UCP-2 in BAT and liver (with a decrease in WAT) and increased UCP-3 mRNA levels in skeletal muscle (with a decrease in BAT). While FFA intake was shown to be necessary for induction of UCP-3 expression in skeletal muscle of neonatal mice, this dependency was abolished by administration of PPAR-α agonists (10). It is unclear, however, if PPAR-α is necessary for initial UCP-3 induction in skeletal muscle at birth, and how this ties to the newborn's requirements for thermogenesis. The results of these studies point to differential and tissue-dependent regulation of the UCP by PPAR-α and γ and to a potential role for UCP in insulin-resistance and sensitization.

4.4.6. Retinoic Acid (RA)

Retinoic acid (RA) is a derivative of vitamin A that plays a crucial role in the development and differentiation of mammalian cells. RA exerts its functions by diffusing through the cell membrane lipid bilayer and binding to cytosolic

receptors that then dimerize and translocate to the nucleus to activate receptor element sequences of specific genes (51, 61). Given the fat-soluble characteristics of vitamin A, RA is largely stored in adipose tissue, both WAT and BAT. Yet it is only in the past few years that a direct action of RA on UCP gene transcription was postulated, and demonstrated (2).

In fact, exogenous administration of RA causes a 7-fold increase in UCP mRNA levels in cultured BAT adipocytes. Administration of compounds that block protein synthesis did not abolish RA-induced expr4esssion of UCP-1, suggesting a direct regulation of the gene (62). Moreover, specific RA receptors response elements (RARE) were found to be present in the UCP-1 sequence, upstream of the transcription site, suggesting RA activation of UCP-1 expression *in vivo*. (3, 14).

Expression of UCP-1 gene, like expression of leptin, may thus be a marker for the degree of adipocyte differentiation. As such, it would explain the observed action of RA, a known morphogen, on UCP-1 transcription. Interestingly, however, it has been shown that acquisition of the WAT phenotype is blocked when preadipocytes are exposed to RA. In this context, RA may specifically act in BAT act to preserve the thermogenic phenotype to the detriment of lipid-storing phenotype, by upregulating genes such as UCP-1 and perhaps down-regulating other, more WAT-specific genes (2).

RA is also postulated to act on UCP activity. Recent studies have demonstrated increased proton transport by UCP-1 and 2 in BAT mitochondria upon addition of RA, to a greater extent than the uncoupling obtained with fatty acids stimulation alone (51). Regulation of BAT UCP-2 and 3 expression, however, is negatively regulated by RA (61). If UCP-2 and 3 lack a definite uncoupling/thermogenic role in BAT, such inhibition by RA would support the hypothesis of RA-facilitated thermogenesis in BAT.

4.4.7. Other regulators

Thyroid hormone, corticosteroids and glucose levels have all been reported to play a role in UCP regulation, although the extent to which this is true

in vivo, under normal conditions, is debatable (17, 62).

Thyroid hormone (TH) is a natural candidate for the role of UCP regulator, by virtue of being the principal metabolism-controlling hormone. Thyroid hormone is released by the pituitary gland in response to neuroendocrine stimuli and to levels of TH in the serum. There are two forms of TH: triiodothyronine (T3) and thyroxine (T4), the former being the biologically active form at the level of the tissue. Cellular uptake of T4 is followed by transformation to T3 in the cytosol by the enzyme type II thryoxine 5'-deiodinase (5'D-II). T3 then binds to T3 receptors and the complex translocated to the nucleus to activate the thyroid receptor response element (TRE) of specific genes, in analogous fashion to RA. Lack of TH, or hypothyroidism, is associated with decreased energy expenditure, weight gain, decreased sympathetic stimulation and reduced thermogenesis. Such clinical observations would support a positive effect of T3 on UCP expression. Studies performed in rodents have found an abundance of T3 receptors in BAT, as well as the presence of the enzyme 5'D-II (8), and the TRE was identified in the UCP gene (14, 62).

Other regulators include: corticosteroids, that have been shown to downregulate UCP-1, 2 and 3 expression in BAT (62); glucose, where an overexpression of its transporter channel, GLUT-4, in skeletal muscle has been associated with upregulated UCP-3 (69); Growth Hormone (GH), where 4 months long treatment of GH deficient adults with GH has yielded a 3-fold increase in muscle UCP-3 mRNA (17). Their mode of action has not been well described and the extent of their direct or indirect effect on UCP has been controversial.

4.5. Function of UCP

One of the ways to disentangle the function of each UCP from the myriad of metabolic variables that change with different UCP levels is to disrupt that gene in a transgenic animal model and to observe the resulting phenotype.

The role of UCP-1 was examined in two ways: one, by ablating brown adipose tissue, which is the major site of UCP-1 expression, and two, by inactivating the UCP-1 gene itself. In the first model, the diphteria toxin A chain

(DTA) was placed under control of regulatory elements of UCP-1 gene. Given the high level of UCP-1 expression in BAT, the DTA was produced in amounts sufficient to destroy the BAT of those transgenic mice (42). The resulting phenotype was one of obesity, hyperphagia and insulin resistance with the consequent hyperglycemia, hyperinsulinemia and dyslipedemia. Interestingly, in these thermogenically-challenged (BAT-lacking) animals, this phenotype was expressed solely upon exposure to environmental temperatures below the mouse thermoneutrality range (33-35°C) (42). These results suggested that BAT is a major site of fuel utilization for the purposes of core temperature maintenance, in the absence of BAT energy intake increases, with concomitant increased storage in white adipose tissue, while energy dissipation remains relatively inefficient. This supports the postulated uncoupling and thermogenic activity of UCP-1 *in vivo*, which had been previously demonstrated at a cellular level in mitochondrial preparations (9). Drawbacks of this study include incomplete BAT destruction (as it depends on the levels of UCP-1 transcriptional activity), as well as the confounding systemic effects of BAT ablation, including possible upregulation of UCP-1, and perhaps UCP-2 and 3, in other tissues that would assume more thermogenic characteristics. Indeed, DTA mice are not completely cold intolerant: rather, they can survive for at least 2 days at 4°C, suggesting a back-up thermogenic mechanism (42).

The second model is a true UCP-1 knockout, in which the UCP-1 gene was disrupted through insertion of the neomycin gene, leading to undetectable UCP-1 protein levels, but not affecting BAT development. The phenotype of these mice is one of severe cold-sensitivity, where time necessary for the core body temperature to drop by 10°C varied from 1.5 to 9.5 hours (23). Despite normal resting oxygen consumption levels, the UCP-1 knockouts had a blunted response to a known upregulator of oxygen metabolism, namely β3-adrenergic stimulation (23). Hence, UCP-1 plays a role in thermogenesis, maintenance of core temperature in different environments and upregulation of basal metabolic rate (reflected by oxygen consumption) under sympathetic stimulation.

Interestingly, however, the UCP-1 knockout mice were neither hyperphagic, nor obese, but they had increased BAT mass, with histological changes suggestive of lipid deposition. Additionally, BAT levels of UCP-2 were 5 fold higher in knockout animals compared to wild-type ones, approaching levels of expression in white adipose tissue (23). The interpretation of these findings may be that of a possible role of UCP-2 in maintaining energy balance and protecting against obesity. That ablation of BAT nullifies this protection may point to important UCP-2 action in BAT.

Epidemiological and genetic analysis in humans showed a strong linkage between UCP-2 loci and resting metabolic rate and UCP-2 is negatively correlated with body fat. Despite the upregulation of UCP-2 observed in UCP-1 knockout mice, thermogenesis was deficient. Thus, uncoupling mediated by UCP-2 may not contribute as much to heat production, but rather may help maintain energy balance, since the animals do not become obese.

The function of UCP-2 in this case, might be to regulate cellular energetics: by cycling mitochondrial fatty acids from the mitochondria into the cytosol for storage, UCP-2 would be establishing an equilibrium between lipolysis (with secondary NADH generation) and lipogenesis (causing NADH oxidation). In fact, regeneration of coenzymes such as NADH through the concomitant proton leakage that occurs with fatty acid cycling, would maintain a stable cellular ATP/ADP ratio (53) that would not risk inhibiting the respiratory chain and that would be matched to the body's energy state (Figure 4).

A few studies propose a diametrically opposite action of UCP-2, as a facilitator of the obese phenotype. Overexpression of UCP-2 in the rat insulin-secreting pancreatic cells abolishes insulin secretion in response to a high glucose stimulus, mimicking, in effect, physiological resistance to hyperglycemia, eventually leading to hyperinsulinemia and peripheral insulin resistance (14). Obese *ob/ob* or *db/db* mice have increased UCP-2 transcripts in WAT or liver, yet this does not reverse their phenotype (1), in fact it may even be contributory, by facilitating lipogenesis and energy storage. Nonetheless, overexpression models are far from reflecting the normal steady state, *in vivo* reality, where UCP-2

expression in pancreatic islets may be ectopic, rather than physiological, thus questioning the proposed link between UCP-2 and insulin resistance.

The function of UCP-3 is still poorly understood. Originally, the observations that UCP-3 expression is sensitive to sympathetic stimulation, thyroid hormone levels (27), and other signals of thermogenesis, as well as the high levels of expression in BAT and skeletal muscle, seemed to suggest a thermogenic role for UCP-3. However, mutations in human UCP-3 gene had either no metabolic effect, or reduced fat oxidation (1).

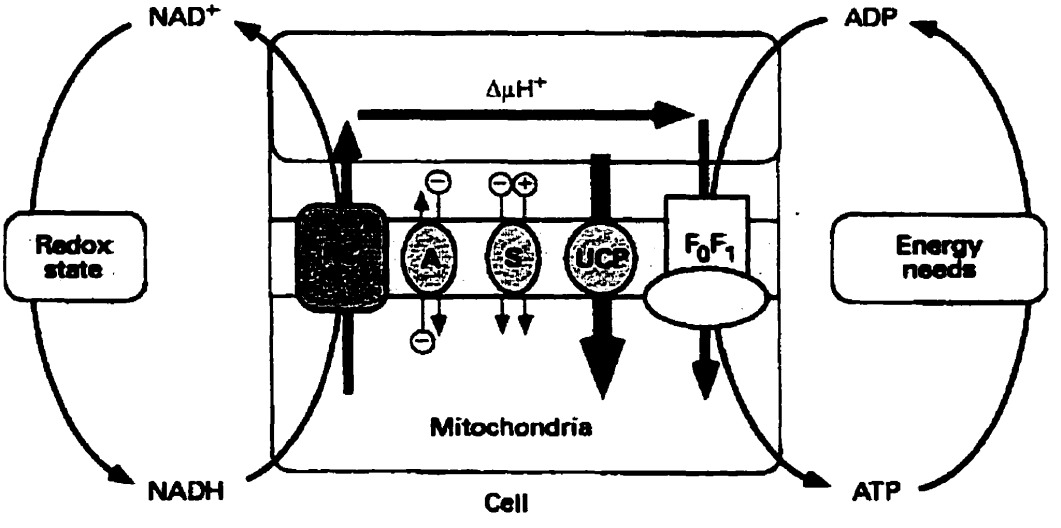

Fig.4. Function of UCP-2 as the link between the redox state of coenzymes and ATP production by mitochondria (Ricquier D et al, 2000, *Biochem. J.* 345 (53))

Moreover, the location of the UCP-3 locus in a region linked to metabolic rate and hyperinsulinemia (1), as well as its upregulation in response to dietary fat augmentation (6, 17, 28, 55, 56), argues towards its function in controlling lipid metabolism.

Only recently were the effects of UCP-3 overexpression documented. Placement of the human UCP-3 gene under the skeletal α-actin promoter, which is known to become activated post-partum in skeletal muscle, led to

overexpression of the human UCP-3 gene in mice, to a total of 66-fold increase in mRNA levels in that tissue (16). The transgenic phenotype was one of hyperphagia, with a 50% increase in food consumption, of decreased adipose tissue mass, and of markedly elevated oxygen consumption (with a 77 to 91% increase). Interestingly, UCP-3 overexpression also affected markers of metabolism, causing decreased insulin levels and increased glucose clearance, suggestive of increased insulin sensitivity (16).

In keeping with the observations that GLUT4 overexpression in skeletal muscle causes upregulation of UCP-3 mRNA levels (69) and that UCP-3 upregulation in response to circulating fatty acid levels occurs in slow-twitch more than in fast-twitch muscle fibbers (55, 56), these results suggest a role for UCP-3 as a switch in the utilization of different fuels (glucose vs fat) for purposes of energy production. Possibly, UCP-3 responds to high lipid levels by switching on fatty acid cycling and promoting mitochondrial oxidation of the excess fatty acids, as well as cellular uptake of those lipids from the circulation by virtue of a favourable gradient. At the same time, it would facilitate increased glucose uptake for temporary triglyceride formation in the cytosol, thus lowering serum glucose levels and decreasing insulin secretion. That the same upregulation occurs when glucose transport is artificially increased by overexpressing GLUT4 transporter, may be explained by altered cellular energetics, as the excess glucose is used in the Krebs's cycle to produce ATP, and NAD+ accumulation may drive the need for proton leak and regeneration of NADH, in a fashion analogous to that described for UCP-2 (53). Other proposed functions for UCP-3 exist, including that of a regulator and protector against oxidative damage by oxygen radicals generated by excessive mitochondrial reactions (53). We can conclude that the overall data support a role for UCP-3 in maintaining energy balance, rather than core body temperature, in humans and rodents.

Based on this review, it becomes apparent that UCPs play an extensive role in metabolism, thermogenesis and nutritional homeostasis, being regulated by a vast array of hormones and exogenous regulators. It is thus plausible that up-regulation of UCP activity may explain the increased metabolism of ASP-deficient mice.

Hypothesis

We hypothesize that:

1) in mice lacking ASP, FFA have decreased conversion to triglycerides because of the absence of ASP;
2) this accumulation is dealt with by the adipocytes and other tissues by effecting an **increase in UCP-2 and 3 transcription/activity in order to efficiently cycle the extraneous fuel, which raises the metabolism of the knockout animals;**
3) **UCP-1 mRNA levels will be decreased** in those mice compared to the wild-type animals because of the lower leptin levels (major upregulator of UCP-1) that may be secondary to the decreased adiposity of ASP-lacking mice.

Methods

I] Primer design:

Murine UCP-1, 2 and 3 mRNA sequences were searched for on the UniGene website (http://www.ncbi.nlm. nih.gov/UniGene/index.html) and were used to generate multiple sense-antisense primer pairs by web primer design programs (Oligos-U-like: http://www.path.cam.ac.uk). Each primer pair generated was tested for homology with each other UCP gene by comparing the alignment of desired sequences (http://www2.igh.cnrs.fr/bin/align-guess.cgi).

Care was taken to avoid G and C rich regions of homology at the 3' end, as the bond would have permitted transcription despite less than ideal matching of the remainder of the primer. Specifications included: length between 18 and 24 base pairs, a GC content of approximately 50% and ideal melting temperature between 60 and 70°C. The following murine primer pairs were chosen based on their high specificity to each murine uncoupling protein gene and satisfaction of the above-mentioned specifications:

UCP-1: sense 5'-AGCAA GAGGAAGGGACGCTC-3' (38-57 bp), antisense 5'-TTCGGAAGTTGTCGGGTT TC-3' (255-237 bp).

UCP-2: sense 5'-GTTTCGTCTCCCAGCCATTTT-3' (61-80 bp), antisense 5'-TGATTTCCTGCTACCTCCCA-3' (290-271 bp).

UCP-3: sense 5'-ACTGTATGCTGAAGA TGGTGGCT-3' (925-946 bp), antisense 5'-AATGTTAGGCATCCAACCG-3' (1315-1287 bp).

Each primer pair was tested against all three, purified, UCP-specific plasmids obtained from Dr. Helen Harper (University of Ottawa) and obtained no cross-reactivity indicated high primer specificity and reliability.

For each primer pair, a standard curve with different cycle numbers (between 25 and 35) was constructed (Fig. 5), and MgCl2 concentrations (between 1.5 and 2.5 mM, the optimal concentrations for *Taq* polymerase activity) were varied (Fig. 6). The conditions chosen were those that allowed linearity of cDNA production with maximal amount of the product without causing signal saturation (Figures 5, 6). For example, in Figure 5, the peak observed at 30 cycles was associated with loss of linearity of quantified PCR product and signal saturation compared to the standard curve. These variables were 25, 30 and 35 PCR cycles and MgCl2 concentrations were 2.0, 1.5 and 2.0 mM for UCP-1, 2 and 3, respectively.

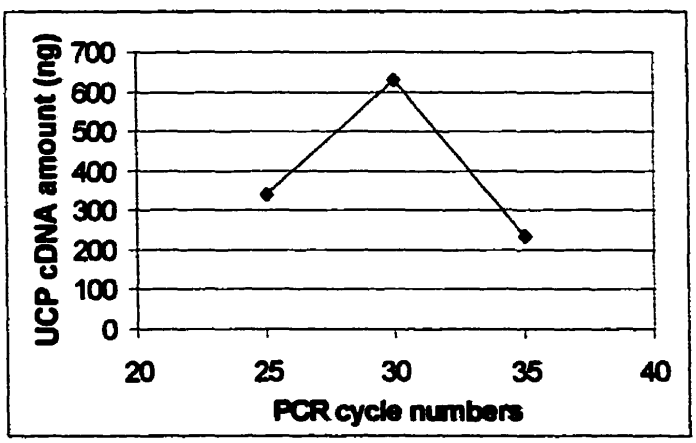

Fig.5. PCR cycles standard curve (UCP-1 PCR product after 25, 30 and 35 PCR cycles, with 2 mM MgCl2)

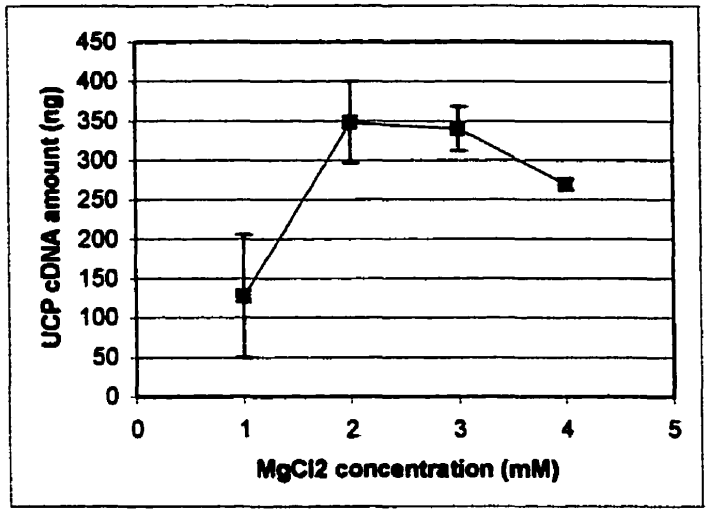

Fig.6. Magnesium chloride standard curve (UCP-1 PCR product after 25 PCR cycles, with 1, 1.5, 2 and 2.5 mM MgCl2)

18-S was chosen as a cellular control because of its stability of expression across tissues. 18-S primers were commercially obtained from Ambion (QuantumRNA 18S internal standards), and their ideal PCR conditions were demonstrated to be 25 cycles and 2 mM MgCl2.

II] Experiments:

a) Ethics:

All experimental protocols were approved by the Royal Victoria Hospital Ethics Committee and were in concordance with the guidelines set out by the Canadian Committee on Animal Care.

b) Preparation of the animals:

Original C3 knockout mice (strain 129Sv x C57Bl/6) were kindly provided by Drs Colten and Wetsel. Heterozygous mice were mated to produce the knockout and wild-type littermates used for the present experiments. The mice were weighed once weekly from weaning to 4 weeks of age and at 8 weeks the mice were housed individually for a 2-weeks acclimatization. Finally, 10 weeks

old C57BL/6 female and male mice were gender-specifically, randomly divided into two groups (6 animals each) and assigned to one of two diets (45, 46, 47).

The high-fat diet (with fat accounting for 45% of daily calories intake) consisted of 22.9% protein, 45.8% carbohydrate and 20.3% fat w/w and was obtained as diet D12478 from Research Diets Inc (New Brunswick). The low-fat diet (D12477, with 10% of daily calories intake being provided by fat), which represents a normal mouse chow diet, contained 19.3% protein, 67.3% carbohydrate and 4.3% fat w/w. The food was weighed three times weekly over 16 weeks and food intake determined over the period of 10 to 26 weeks of age. The fat content of feces was determined by extraction, after a 24 hours collection from mice on normal chow or mice having received a fat load (45, 47). Mice were housed in sterile barrier facilities with equal day/night periods.

c) Tissue preparation:

At the age of 32 or 48 weeks, the mice were anaesthetized with a mixture of 5 ml Ketamine (100 mg/ml), 2.5 ml xylazine (20 mg/ml), 1 ml aceprozamine (10 mg/ml) and 1.5 ml sterile saline amounting to 0.01 ml/10 g body weight. They were then sacrificed by cervical dislocation. The following tissues were excised: white adipose tissue (from inguinal and gonadal sites), brown adipose tissue (intrascapular site) and skeletal muscle (quadriceps). The samples were weighed and frozen in liquid nitrogen at –80°C for later analysis.

d) RNA extraction and Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR):

Total RNA was isolated from the four tissue samples for each animal by an experienced graduate fellow in Dr. Cianflone's lab, using the TRIzol method (Gibco-BRL). Reverse-transcription was performed using Moloney murine leukemia virus reverse transcriptase in buffer containing dNTPs, RNAse inhibitor and oligo$(dT)_{15}$ primer (Gibco BRL). The resulting cDNA (RT mixture) was subjected to PCR. Briefly, 4 µl or 2 µl of cDNA and 2 µl of UCP-1, 2, or 3 primers (1 µM final concentration) were mixed with 0.5 units *Taq* DNA

polymerase/ reaction tube in standard buffer (0.2 mM dNTPs, 100 μM TMAC and MgCl2 diluted to either 1.5, 2 or 2.5 mM). The cDNA was amplified using 25, 30 or 35 cycles of: 1 min denaturation at 95°C, 1 min hybridization at 60°C and 1 min extension at 72°C, preceded by 5 min of denaturation at 95°C, and followed by 7 min final extension at 72°C. The PCR products (Taq polymerase, MgCl2, PCR buffer, primers, TMAC 10-4M) were all obtained from Gibco BRL.

e) Polyacrylamide gel electrophoresis and Silver Staining:

The amplified cDNA sample was loaded in gel wells (usually 5 μl of 24: 20 μl PCR product mixed with 4 μl loading buffer) and separated by electrophoresis on 7.5 % polyacrylamide gel in 1.5 mM Tris-HCl, pH 8.8 buffer at 200 V. The gels were then stained using successive washes of 40% methanol/ 10% acetic acid (10 min), 10% ethanol (2x5 min), oxidizer solution (5 min), silver reagent solution (20 min), and developer (5 min), all obtained from Biorad. The developing reaction was stopped by washing with 5% acetic acid for 5 min and the gels were then washed with sterile water, placed between cellophane layers and dried for 2 hours at 80°C under vacuum (Figure 7).

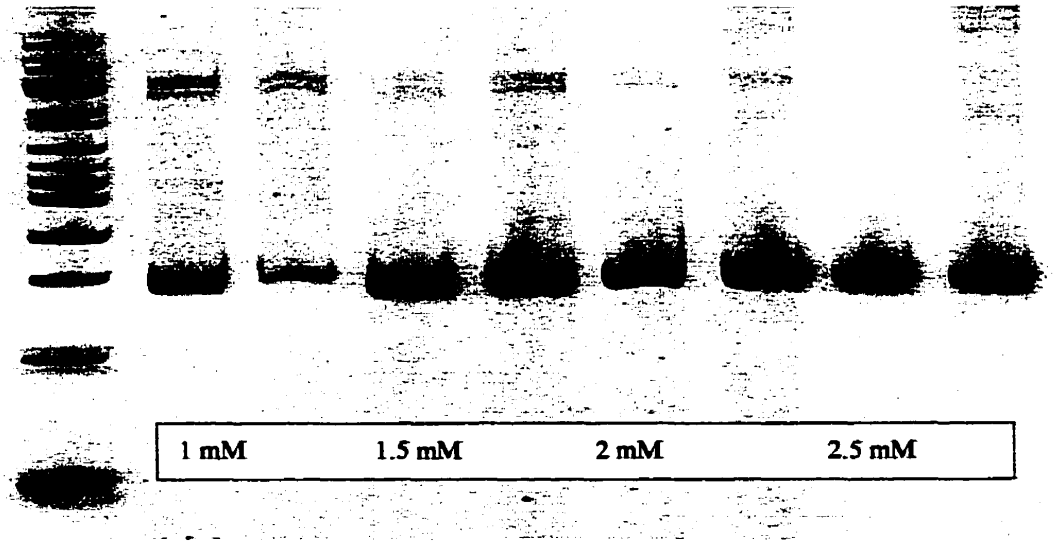

Fig.7. Polyacrylamide gel showing the standard curve on the left, and duplicate UCP-1 cDNA samples amplified at 25 PCR cycles

f) Results processing:

Quantification was done by gel scanning, using the Molecular Analyst kit (Biorad), referenced against a DNA standard that was electrophoresed with every gel. Results were expressed as UCP cDNA amount over 18-S cDNA amount. The quantified data was graphed using Microsoft Excel and statistical analysis was performed using Sigma Stat's One-Way ANOVA test. Where values did not obey a normal distribution, a ranked One-Way ANOVA test was performed.

Results

I] Male mice

Brown Adipose tissue (Fig.8 a). On a normal, low-fat (LF) diet, knockout (KO) mice show a 5-fold drop ($p<0.05$) in UCP-1 mRNA levels compared to controls (WT), with no change in UCP-2 or 3 levels. On the high-fat (HF) diet, KO UCP-1 expression remains unchanged, UCP-2 mRNA levels drop 5-fold ($p<0.05$) and UCP-3 expression is 2.5-fold lower than in the pair-diet controls (HF WT). High fat diet causes a 2-fold drop in WT UCP-1 expression compared to LF WT, and a 2.5-fold drop in UCP-2 and 3 expression in KO, compared to LF KO.

Muscle tissue (Fig.8.b). On LF diet, KO UCP-1 expression drops 3-fold, UCP-2 remains unchanged and UCP-3 rises 2.5-fold ($p<0.05$). On HF diet, KO UCP-1, 2 and 3 mRNA levels are unchanged compared to HF WT. UCP-1 levels are 8 times lower ($p<0.05$) in HF WT compared to LF WT. Expression of UCP-1, 2 and 3, respectively, is 2-fold, 1.5-fold and 2-fold ($p<0.05$) lower, compared to LF KO.

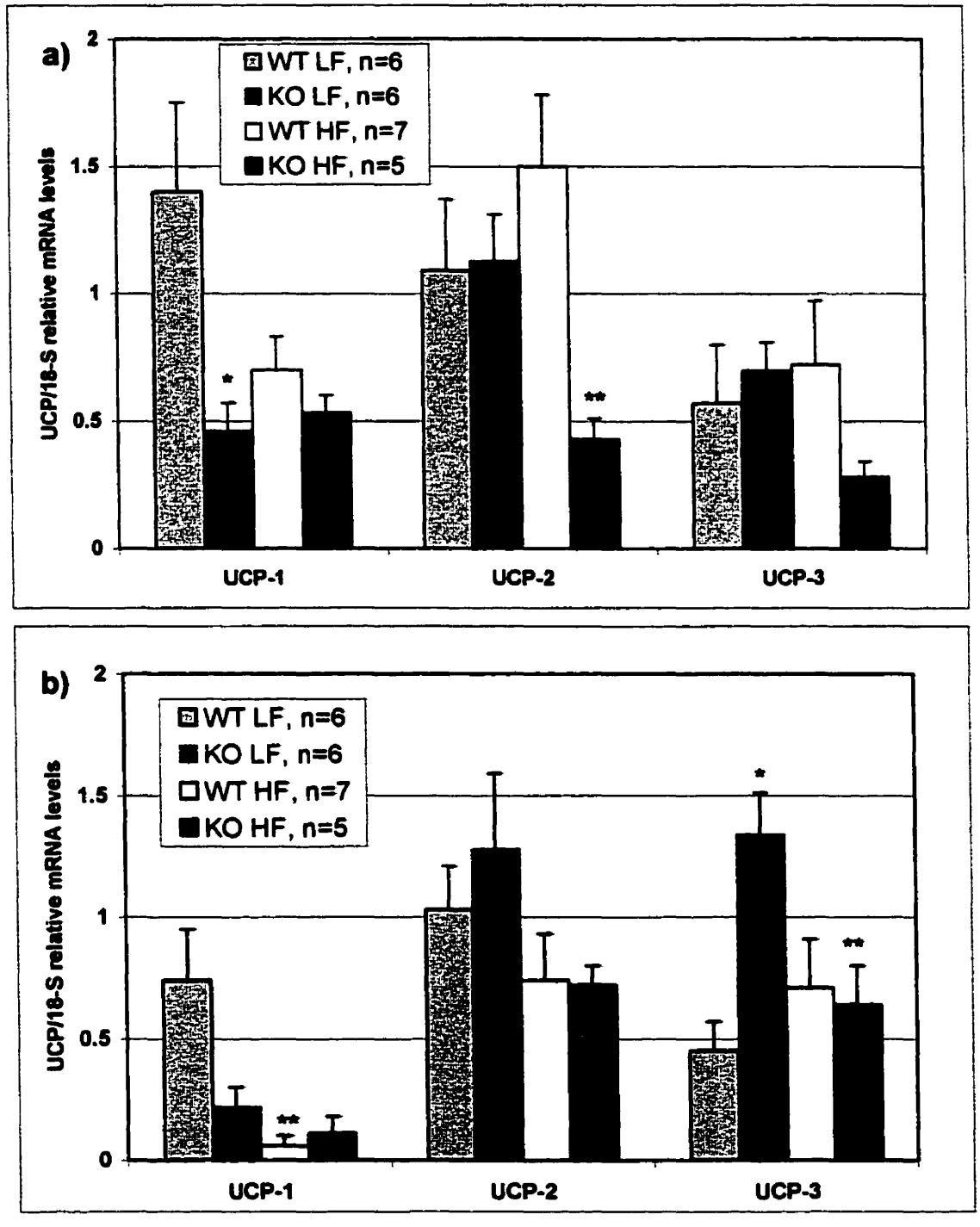

Fig.8. a) BAT (brown adipose tissue) UCP/18-S mRNA ratio in WT (wild-type) and KO (knock-out) mice placed on LF (low-fat) and HF (high-fat) diet; **b)** Muscle UCP/18-S mRNA ratio in WT and KO mice placed on LF and HF diet.

*: $p<0.05$ KO vs WT; **: $p<0.05$ HF vs LF (Dunn's and Tukey's tests)

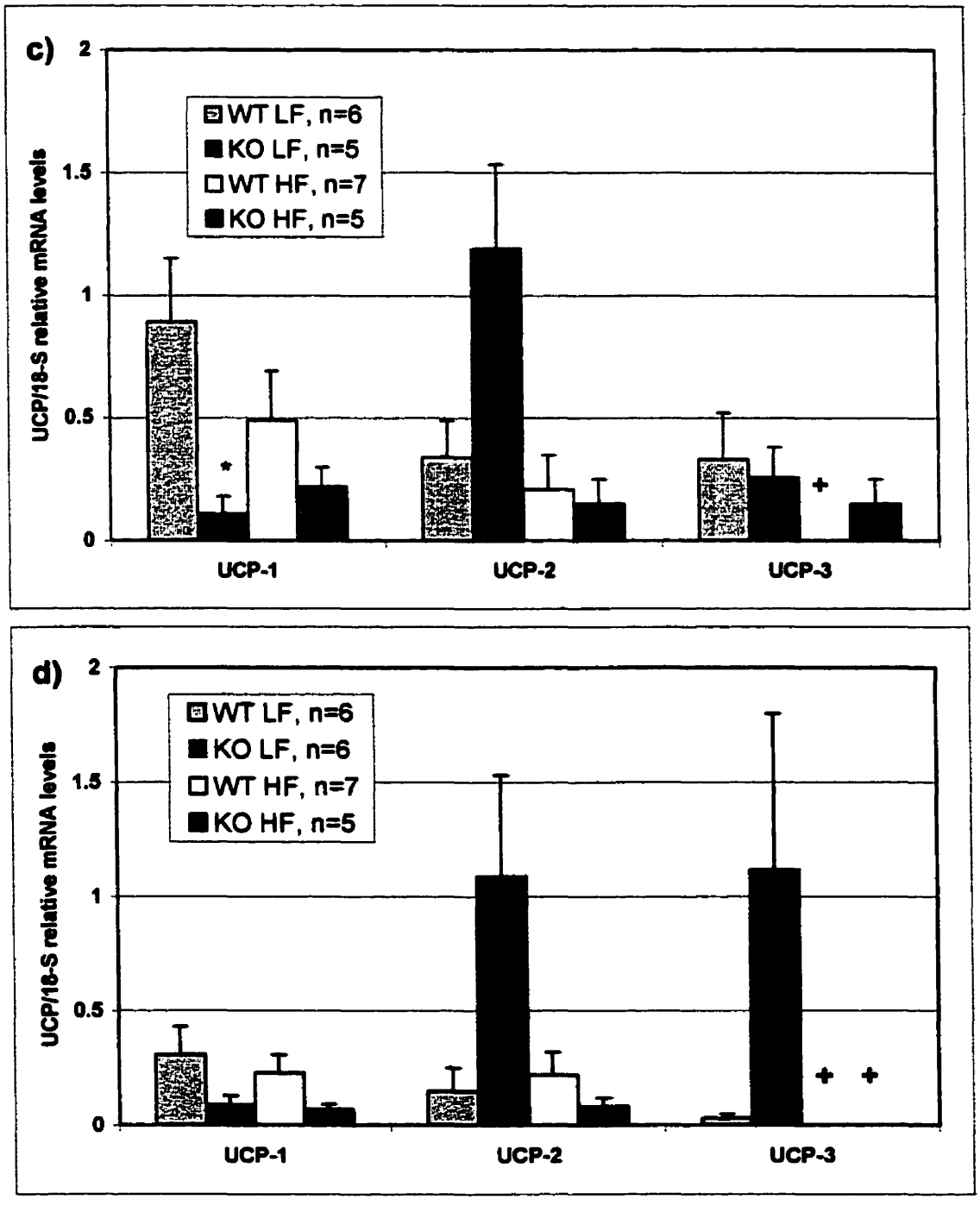

Fig.8. c) Inguinal UCP/18-S mRNA ratio in WT (wild-type) and KO (knock-out) mice placed on LF (low-fat) and HF (high-fat) diet; **d)** Epididymal UCP/18-S mRNA ratio in WT and KO mice placed on LF and HF diet.

*: $p<0.05$ KO vs WT (Dunn's test), +: undetectable levels

Inguinal white adipose tissue (Fig.8.c). On LF diet, KO UCP-1 expression drops 6.5-fold ($p<0.05$), UCP-2 rises 3-fold and UCP-3 remains unchanged. On HF diet, UCP-1 levels also drop 2-fold, UCP-2 remains unchanged and UCP-3 levels rise compared to HF WT. The HF diet causes a 1.5-fold drop in WT UCP-1 expression, no change in uCP-2 and a significant drop in UCP-3. In HF KO, UCP-2 expression falls 6-fold compared to LF KO.

Epididymal white adipose tissue (Fig.8.d). On LF diet, KO UCP-1 expression drops 3-fold, UCP-2 rises 6-fold and UCP-3 rises 30-fold. On HF diet, UCP-1 levels drop2.5-fold, UCP-2 fall 2.5 fold and UCP-3 levels are undetectable. There is no difference in UCP-1, 2,3 expression in HF WT mice compared to LF WT ones. HF diet in KO mice causes a 12-fold and immeasurable drop in UCP-3 expression compared to LF KO counterparts.

II] Female mice

Brown Adipose tissue (Fig.9.a). On a normal, low-fat (LF) diet, knockout (KO) mice show a 2-fold drop in UCP-1 mRNA levels compared to controls (WT), with a 1.5-fold rise in UCP-2 and no change in UCP-3 levels. On the high-fat (HF) diet, KO UCP-1 expression is 2-fold higher, UCP-2 is unchanged and UCP-3 expression is 1.3-fold higher than in the pair-diet controls (HF WT). High fat diet causes a 2-fold drop in WT UCP-1 expression compared to LF WT, and a 1.3-fold increase in KO UCP-1, a 1.4-fold drop in UCP-2 and no chance in UCP-3 expression, compared to LF KO.

Muscle tissue (Fig.9.b). On LF diet, KO UCP-1 and 2 expression remains unchanged and UCP-3 levels drop slightly. On HF diet, KO UCP-1 and 3 levels remain stable, while UCP-2 mRNA levels drop 1.4-fold compared to HF WT. UCP-1 levels are 3 times lower in HF WT compared to LF WT, UCP-2 levels are 1.3- fold higher and UCP-3 are unchanged. Expression of UCP-1 is 4-fold lower in KO HF compared to LF KO, while UCP-2 and 3 are unchanged.

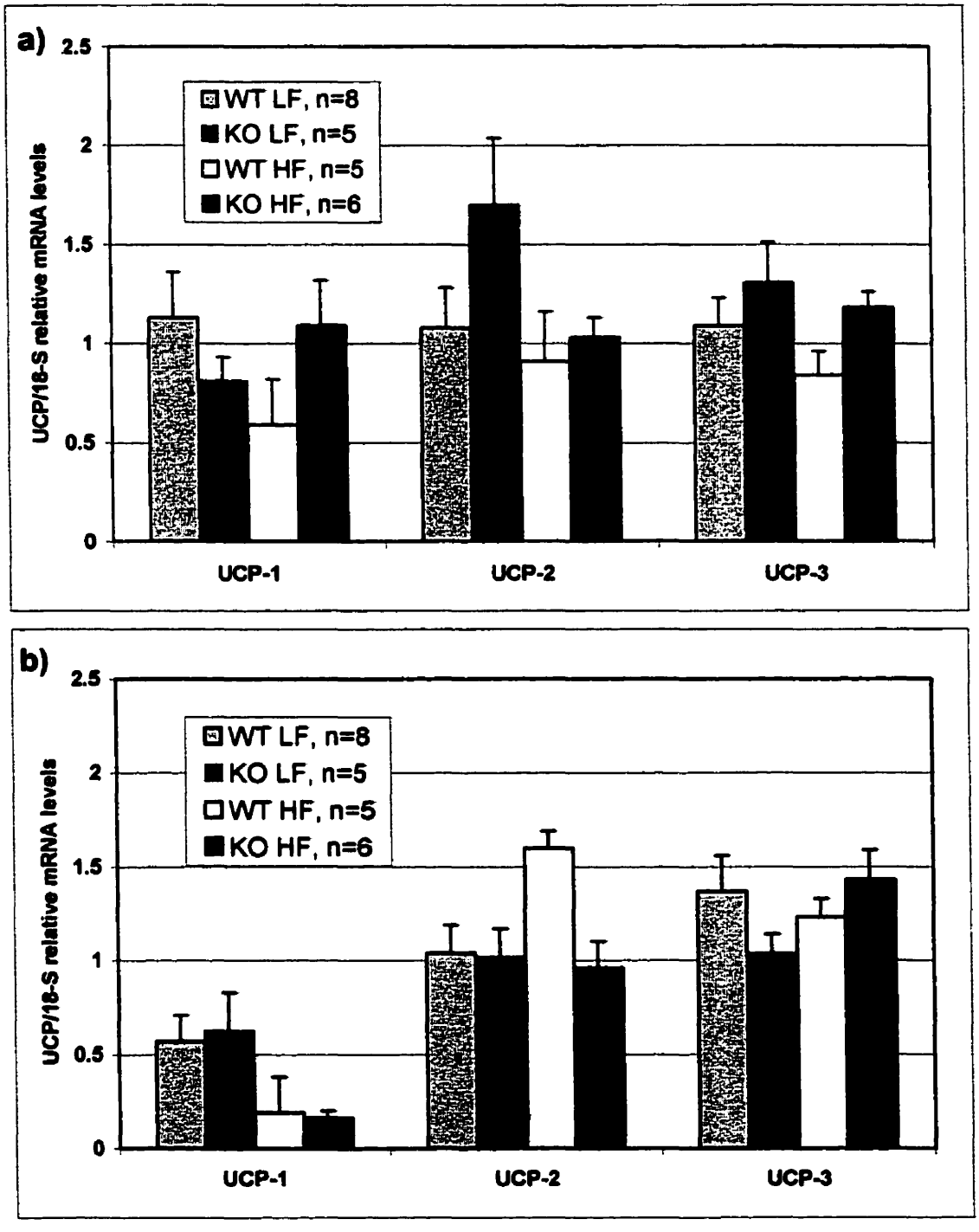

Fig.9. a) BAT UCP/18-S mRNA ratio in WT (wild-type) and KO (knock-out) female mice placed on LF (low-fat) and HF (high-fat) diet; **b)** Muscle UCP/18-S mRNA ratio in WT and KO female mice placed on LF and HF diet.

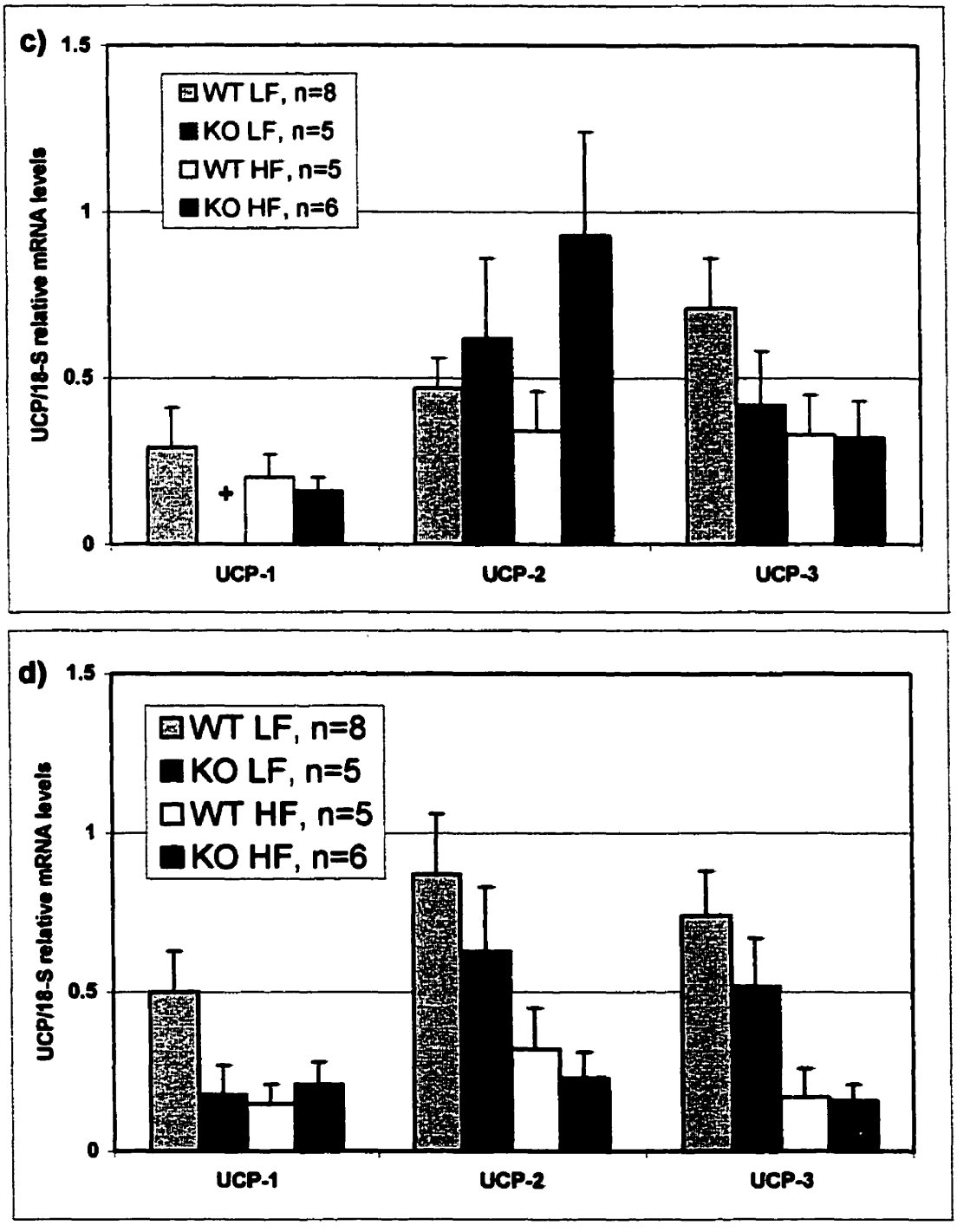

Fig.9. c) Inguinal UCP/18-S mRNA ratio in WT (wild-type) and KO (knock-out) female mice placed on LF (low-fat) and HF (high-fat) diet; **d)** Ovarian UCP/18-S mRNA ratio in WT and KO female mice placed on LF and HF diet.

+: undetectable levels

Inguinal white adipose tissue (Fig.9.c). On LF diet, KO UCP-1 expression drops to undetectable levels, UCP-2 and 3 remain unchanged. On HF diet, UCP-1 and 3 levels are stable and UCP-2 rises2.5-fold compared to HF WT. The HF diet causes a 2-fold drop in WT UCP-3 expression, no change in UCP-1 and 3. In HF KO, UCP-1 expression rises, while UCP-2 and 3 remain stable compared to LF KO.

Ovarian white adipose tissue (Fig.9.d). On LF diet, KO UCP-1 expression drops 3-fold, while UCP-2 and3 remain unchanged. On HF diet, there is no change in UCP-1, 2 or 3 levels as compared to HF WT. HF diet causes a 3, 2.5 and 4-fold drop in UCP-1, 2 and 3 levels, respectively, in WT compared to LF WT. HF diet in KO mice elicits no change in UCPs expression compared to LF KO counterparts.

Summary

In male ASP-deficient mice on a regular, low-fat, mouse diet UCP-1 mRNA levels decrease in all tissues, UCP-2 levels increase in muscle and white adipose tissue, while UCP-3 levels increase in muscle and epididymal adipose tissue. In female ASP-deficient mice, UCP-1 expression drops or remains unchanged in all tissues, UCP-2 expression increases slightly in inguinal and brown adipose tissues, while UCP-3 expression remains unchanged across tissues. The differences between knockout and wild-type animals are largely negated by placement on a high-fat diet, which also causes a decrease in wild-type UCP expression compared to low-fat diet.

Discussion

Reliability and accuracy of the RT-PCR method of UCP mRNA levels quantification. Uncoupling proteins, while theoretically responsible for up to 30% of the resting metabolic rate in many mammals, are probably not expressed in large amounts. Given the lack of reliable antibodies for UCP protein detection at the time this study commenced, we have, like the majority of researchers before us, decided to follow UCP mRNA levels and to correlate any changes with the observed animals'phenotype.

The preferred method of UCP mRNA detection has, for a number of years, been Northern blotting of RNA samples. The probes varied from study to study and among classes of rodents (as rat UCP sequence is slightly different from mouse sequence). A downside of this method is the requirement of relatively large amounts of RNA, leaving to us little opportunity to test that same tissue for multiple gene expression. Moreover, given the introduction in the system of a genetic alteration, with unknown effects on gene levels, we needed a very sensitive technique. For these reasons we decided to employ semi-quantitative RT-PCR, which requires relatively small amounts of tissue and RNA, with amplification to a detectable level of gene product and is quite free of errors.

The elevated (60-80%) level of homology among the three UCP genes investigating posed a significant challenge. There is no mention in studies that have used murine primers (PCR) or Northern probes (Northern) to quantify UCP expression of the degree of cross-reactivity between any single UCP primer and the two other UCP gene sequences. We have designed our primers, as mentioned in the Methods section, to satisfy very stringent requirements and to avoid areas of high homology with other genes.

As apparent in our results, we have detected, albeit at a low level, detectable UCP-1 mRNA in tissues not previously thought to express this gene. Although a few recent studies now confirm this finding, it is unclear why UCP-1 Northern probes and PCR primers used in other studies that had cross-reactivity to UCP-2 and 3, failed to detect those UCP in the place of UCP-1, increasing the

likelihood of UCP-1 expression in ectopic tissues. A possible explanation may be the low sensitivity of the Northern blot technique for genes expressed in small amounts in tissues other than BAT, which is known to possess elevated levels of UCP-1. We are confident that the UCP-1 detected in skeletal muscle and WAT represents true, indigenous UCP-1, especially given that its pattern of expression differ from those of UCP-2 and 3 in the same tissue.

The silver staining technique is sensitive and accurate and quantification of the UCP gel bands was performed by analysis against a known standard curve, obtaining usual regression coefficient ranging between 0.96 and 0.99, with a linear range between 10 and 150 ng of DNA.

The failure of observed differences to reach statistical significance is likely a reflection of the small number of animals, rather than an effect of technique dispersal and poor precision.

Finally, we questioned the relevance of observed changes in mRNA levels as compared to UCP protein expression and protein activity. UCP-3 mRNA expression and immunohistochemically detected UCP-3 protein levels have been reported to correlate, at least when both are upregulated by leptin treatment (63). In the same study, UCP-1 transcriptional and translational levels were discordant, suggesting UCP-1-specific post-translational control. Unfortunately, there is no similar data available on UCP-2, and we must rely on studies that have shown parallel measurements of UCP-2 mRNA levels and UCP-2 uncoupling action, and extrapolate our mRNA measurements, and the conclusions based on them, to protein level and activity.

UCP expression in male mice and ASP-deficient phenotype. ASP-lacking male mice are hyperphagic yet fail to gain as much weight as their wild-type littermates, exhibit decreased adipose tissue mass, delayed post-prandial triglyceride clearance and elevated insulin sensitivity, with low basal insulin and glucose serum levels.

UCP-1. Despite the absolute drop in adipose tissue mass, BAT is visually larger and yellower looking than the controls. At a cellular level, the decrease in

BAT UCP-1 levels suggests that UCP-1-mediated thermogenesis does not account for the oxidation of the vanishing (not stored, nor excreted) caloric intake. This decrease is in keeping, however, with the observation that leptin levels, in ASP knockouts, are lower than expected from the difference in adipose tissue mass. Since leptin is a potent activator (either directly or through increased sympathetic discharge) of UCP-1, lower leptin levels in thermoneutral ambient conditions should result in lower UCP-1 expression. Moreover, circulating FFA were never shown to cause an increase in BAT UCP-1 mRNA levels. Hence, in the absence of proper storage signals (no ASP, therefore, decreased GLUT-mediated glucose transport across the membrane and decreased intracellular DGAT activity mean less TAG production and storage in the adipocyte), the NEFA that do enter the BAT adipocyte are not oxidized to produce heat since UCP-1 levels are low. Rather they eventually accumulate as lipid droplets, increasing BAT mass and altering its histological appearance to resemble WAT.

Interestingly, presumably thanks to the same decreased leptin signal, UCP-1 expression falls in other knockout mice tissues, such as skeletal muscle and WAT. We observed that UCP-1 levels fall even in wild-type mice placed on a high-fat diet. This may be explained by the relative hyperinsulinemia associated with that dietary manipulation, as it has been shown that insulin activates MAPK, a downregulator of UCP-1.

UCP-2. A true oxidative/thermogenic role has not yet been ascribed to UCP-2 and 3, which are currently seen as intracellular energy and fuel regulators rather than systemic metabolic proteins. However, in the face of the decreased UCP-1 expression, yet successful disposition of excess FFA that we observe in ASP-deficient mice, it is tempting to propose that UCP-2 and 3 have the ability to compensate and assume an oxidative role in the right metabolic conditions.

In fact, in knockout mice placed on a regular mouse chow, low-fat diet, UCP-2 expression increases in inguinal and epididymal WAT. This occurs in a fashion similar to the rise observed in UCP-1-deficient transgenic mice, suggesting perhaps a compensatory mechanism needed for maintenance of energy balance.

The observed UCP-2 rise is negated, at a transcriptional level, as a result of chronic exposure high-fat dietary intake. So if excess, unstored, cytosolic FFA are indeed the signal for UCP-2 upregulation, why doesn't that increased UCP-2 expression occur in the much needful setting of high fat diet challenge? Unexpectedly, UCP-2 decreases in BAT, muscle and epididymal WAT of knockout mice fed a HF diet. These results contradict findings of other studies, where FFA or dietary fat had little augmentatory or no effect on UCP-2 mRNA levels. Looking at hormonal consequences of ASP suppression, decreased leptin and insulin levels are the only salient and measured players that could explain UCP-2 behavior. However, we know from other studies that leptin administration has no effect of UCP-2 levels. We ignore whether endogenously lower leptin levels (due, as in this case, to decreased adipose tissue mass and perhaps lack of ASP) have a permissive effect on UCP-2 expression, explaining the results observed on LF diet. Is this permissive role removed once leptin levels rise with rising adipose tissue mass in HF diet-fed mice? Or does the relative hyperinsulinemia that occurs with chronic HF diet have a down-regulatory effect, similar to the one it exercises on UCP-1? Conversely, if the direct, positive correlation observed between UCP-2 levels and insulin sensitivity is correct, and if UCP-2 expression is insulin-dependent, could the decreased insulin sensitivity observed with increased dietary fat intake explain that decreased expression?

Irrespective of the actual mechanism involved in these changes, the differences in UCP-2 expression are not statistically significant, and may be physiologically too slight to explain the apparently elevated metabolism in the ASP-deficient mice.

UCP-3. The last UCP investigated, UCP-3, is preferentially expressed in skeletal muscle. Induced at birth by the first meal and FFA intake -an effect reproducible by PPAR-α agonists- (10), UCP-3 has been seen as an important local regulator of intracellular fuel balance and response to various metabolic signals. Because muscle is the major suspect implicated in peripheral insulin-resistance, it is tempting to correlate UCP-3 expression and the insulinemic state of the body. Moreover, FFA have been shown to directly upregulate UCP-3

mRNA levels, and serum NEFA elevation is the major phenotypic side-effect of ASP deficiency. However, in ASP knockout mice on LF diet, that obtain most of their calories from carbohydrates, UCP-3 expression rises in skeletal muscle, and to a lower extent, in epididymal WAT. With the concomitant rise in UCP-3, the phenotype of the ASP-deficient mice approximates that of UCP-3-overexpressing mice: lean, hyperphagic and insulin-sensitive (16).

To understand these observations, we must consider the consequences of using primarily glucose as a fuel. Its oxidation through the Krebs' cycle produces massive amounts of protonated co-enzymes, such as NADH2 and FADH that are then used in the respiratory chain to drive ATP production. Eventually, the ATP/ADP ratio would rise to the extent of depleting ADP and NAD stores, leading to arrest of mitochondrial oxidation and perturbance of many cellular reactions. However, as was discussed earlier, UCP-3 is activated by high ATP/ADP ratios, and its uncoupling activity provides the means for dissipating the proton gradient, regenerating co-enzymes and ADP stores and maintaining cellular energetic stability. Evidence that glucose overload of the cell may cause UCP-3 upregulation comes from research on overexpression of GLUT-4 in skeletal muscle that resulted in increased glucose flux and elevated UCP-3 mRNA levels.

Finally, as with UCP-2, addition of high-fat diet disturbs the knockout compensation for lack of ASP and UCP-3 levels fall in the major tissues, muscle and BAT, to rise only slightly in inguinal WAT.

In the light of these observations, we must question whether chronic elevation of circulating FFA does indeed signal UCP-3 upregulation, since we did not observe this in HF KO. For, the same elevation should have occurred in HF KO, as evidenced by the delayed post-prandial clearance and by the high serum NEFA that are accentuated on the high-fat diet (45, 46). Leptin has been shown not to affect UCP-3, and we will hence not consider it a player in this signaling game. Another explanation, as in the case of UCP-2, remains in the shape of the hyperinsulinemia caused by the dietary alteration. Yet previous studies have linked UCP-3 and insulin as positively correlated markers, which would

contradict our findings, unless that hyperinsulinemia was insufficient to overcome insulin-resistance at the tissue level. Before turning to altered insulin sensitivity as the reason for the observed changes in UCP-3 behavior, we should examine other factors that may have been modified by the high-fat diet. Indeed, a low carbohydrate intake (as seen in high-fat diet) may have led to down-regulation of GLUT-4 receptors and decreased use of carbohydrate as intracellular fuel. UCP-3 would then act not as a permissive agent for FFA oxidation, but rather as a link between mitochondrial ATP/ADP and co-enzymes levels and fuel balance. In mice fed a high-fat diet, as the cell would become saturated with storable FFA in the HF-fed mouse, and lipogenesis-derived NAD would ensure stable energetics, the role of UCP-3 as guardian of storage and arbiter of fuel utilization would decrease, irrespective of the presence or absence of ASP. While such explanation supports a fatty-acid cycling/energetics maintainer function of UCP-3, the lean, hypermetabolic phenotype of ASP–deficient mice favors a thermogenic/oxidizing activity for UCP-3, perhaps even as a by-product of the proposed primary function.

UCP expression in female mice and ASP knockout phenotype. Female rodents, similarly to female humans, are characterized by relatively larger adiposity, yet a paradoxical protection from insulin resistance, presumably explained by estrogenic hormonal effects that are lacking in males. Even when placed on a high-fat diet, female mice do not become insulin resistant as male mice do. While ASP-deficient female exhibit greater reduction in adipose tissue compared to their wild-type littermates, the changes in UCPs expression brought on by ASP-deficiency are not as great.

UCP-1. While ectopic expression of UCP-1 in skeletal muscle and white adipose tissue is still observed, the relative drop in mRNA levels in ASP-deficient mice is not statistically significant. Given the activating effect of leptin on UCP-1, and the better preserved amounts of adipose tissue in females, it is possible that the higher levels of leptin in female mice succeed in maintaining higher UCP-1 levels despite the effect of ASP deficiency. Even in the absence of detectable

differences in cross-sectional leptin concentrations between males and females, it has been shown in humans that the amplitude of human leptin pulses is greater in females, perhaps resulting in a stronger effect at the level of the tissues, without causing increased serum leptin (38). As the animals are placed on a high fat diet, however, the leptin-potentiating effect does not seem to be maintained and UCP-1 expression drops to the same extent in knockout and wild-type female mice. Again, one possible explanation may be the hyperinsulinemia observed with chronic dietary fat intake, downregulating UCP-1 through the effect of insulin on MAPK.

UCP-2. Interestingly, UCP-2 expression remains remarkably unperturbed by the genetic or dietary alterations imposed on female mice, except in ovarian adipose tissue, where mRNA levels drop in animals placed on a high-fat diet. If we examine the physiological difference between inguinal and ovarian (or even epididymal, its male counterpart) adipose tissues, we may suggest that the former is an example of subcutaneous fat depots, whereas the latter may behave more like visceral fat depots. The importance of visceral adiposity in insulin resistance and dyslipidemias has clinically been demonstrated in humans, with a higher risk for cardiovascular disease and diabetes being associated with a preponderance of visceral adiposity (as measured by waist to hip ratio). More interestingly, at the cellular level, visceral adipocytes have been found to express more insulin receptors, yet less GLUT4, PPAR-γ and leptin than the subcutaneous adipocytes, suggesting a relative resistance to insulin (37). Hence, if ovarian adipose tissue is insulin-resistant, and if that resistance is exaggerated on a high-fat diet (Boden, Mason) then it would explain why the presumably insulin-dependent UCP-2 expression drops. Nonetheless, lower UCP-2 mRNA levels in ASP-deficient knockout mice do not exclude UCP-2 from being a player in increased metabolic rate that could explain the observed phenotype. Indeed, retinoic acid, whose levels could be higher due to larger fat depots in females, can increase UCP-2 activity independently of transcriptional events (51).

UCP-3. The female body habitus, molded by the ratio of estrogens to androgens, has poorer muscle development compared to that seen in males. While

metabolically, less skeletal muscle mass may translate into less peripheral insulin resistance, it also means less energy utilization, be that fatty fuel or glucose. Interestingly, this clinical hypothesis seems to be confirmed at the cellular level by stable UCP-3 expression in female mice, unaffected by either the absence of ASP or high-fat diet. Lack of response to the increased insulin sensitivity seen in ASP-deficient mice may be due to concomitantly stable serum glucose levels, or to the lack of post-prandial delay in triglycerides clearance seen in knockout male mice (45, 46, 47). If there is no perceived fuel abundance, either as carbohydrate or lipid, UCP-3 may not become upregulated. Given the previously described concordance between UCP-3 mRNA and protein levels (63), we propose that UCP-3 activity is not altered by either ASP-deficiency or high-fat diet in female mice, and that it probably does not contribute to increased metabolism in female Asp-deficient mice.

ASP and insulin: their action at a cellular level. One of the most interesting phenotypic characteristics of ASP-deficiency is the increased insulin sensitivity (expressed as the product of insulin and glucose concentrations) compared to wild-types, in both male and female mice. Considering the previously described action of Thiazolidinediones (TZD) on UCPs, and the proposed link with TZD as insulin-sensitizing agents, we can postulate that either directly, or through PPAR-γ, insulin has a potentiating effect on UCP-2 and 3 expression. Indeed, it is reasonable to suspect that a hormone involved in energy storage postprandially should somehow communicate with agents that maintain cellular energy balance. While it is seemingly paradoxical that a signal favoring glucose storage and inhibiting lipolysis should facilitate energy expenditure through futile cycling of much-needed fuels, from the cellular energetic balance it makes perfect sense. As described in the discussion of the function of UCP-2, lipogenesis consumes NADH2, which, if not opposed, would lead to depletion of that co-enzyme stores and arrest of the respiratory oxidative cycle (53). The futile proton leak mediated by uncoupling proteins, with or without actual fatty acid flipping across the mitochondrial membrane, could play an important role in

replenishing NADH2 stores and maintaining ATP/ADP stability. The source of protons, in this case, would be the donating fatty acids, before or during their oxidation inside the mitochondrion (16).

The initial question, why are ASP-deficient mice more insulin-sensitive, remains. Since both ASP and insulin are storage hormones, acting one exclusively, the other principally, at the level of the adipocyte, why should the absence of one potentiate the other?

Simplistically, any disruption of adipose tissue may translate to changes in insulin sensitivity. Targeted disruption of the RIIβ subunit of protein kinase A, which is a holoenzyme that mediates cAMP cellular effects, results in lean mice that have reduced white adipose tissue and that are protected against diet-induced obesity (20). This presumably occurs because of the switch to the RI isoform and thus a better mediation of PKA activity, which itself promotes UCP mRNA expression, explaining the observed increased metabolism. Similarly, overexpression of GLUT-4, the glucose transporter, in adipose tissue leads to increased insulin-sensitivity in those mice (26).

At the other end of the spectrum, complete ablation of either brown adipose tissue (discussed previously) or white adipose tissue (43) will precipitate an insulin-resistant or frankly diabetic phenotype, with or without accompanying obesity. Interestingly, the transgenic mice that lack adipose tissue are heavier than their normal littermates, with fatty livers and large internal organs, reduced leptin levels and elevated glucose, insulin and triglycerides levels.

Hence, either excessive (as in leptin knockout mice) or absent adipose tissues will cause insulin-resistance or diabetes. The range of adipose tissue disturbances between these extremes corresponds to a range of alterations in insulin-sensitivity. Thus, disruption of adipose tissue storage secondary to ASP absence may cause insulin-sensitivity simply by reduction of body lipid depots.

Alternatively, if insulin is viewed more as a glucose hormone, trying to promote cellular storage of glucose, while ASP is more of a triglyceride hormone, favoring NEFA uptake from chylomicrons and their cytosolic storage as triglycerides, then there may be some competition for transporters and cellular

signaling agents. More importantly, if ASP action occurs first, then the fuel-replete adipocyte may exhibit some resistance to the delayed action of insulin. In humans, FFA administration was shown to inhibit whole-body insulin-stimulated glucose uptake and intracellular glucose utilization (5). Such resistance will necessarily cause increased insulin levels to overcome the partial tissue unresponsiveness. Studies performed in skeletal muscle demonstrated that the FFA effect in that tissue occurs in at least two ways: by diminishing GLUT4 translocation to the membrane and by inhibiting elements of the insulin signal transduction cascade (39).

Hence, in the absence of ASP, either because of delayed NEFA uptake and inadequate storage capacity, or because of accelerated NEFA utilization by upregulated UCP, there is low adipose tissue mass. This translates to a reduced cellular lipid signal that would normally oppose insulin-mediated glucose uptake and utilization. This decreased resistance would thus lead to reduced insulin levels and enhanced insulin-sensitivity.

Conclusion

In conclusion, male ASP-deficient mice have elevated UCP-2 and UCP-3 expression, while ASP-deficient female mice express slightly elevated UCP-2, which correlate with the elevated metabolism of knockout animals. The downregulation of UCP-1 expression may be secondary to lowered leptin levels and to hyperinsulinemia on the high-fat diet.

The upregulation of UCP-2 and 3 may be secondary to insulin activity, to intracellular fuel abundance, or to other unidentified factors. This upregulation, if translated in increased uncoupling activity, would explain the improved tissue insulin sensitivity in ASP-deficient mice, through reduced adipose tissue mass.

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REFERENCES

1. Adams SH et al. 2000. UCP homologs: emerging views of physiological function. *J. Nutr.* 130: 711-4
2. Alvarez R et al. 1995. A novel regulatory pathway for brown fat thermogenesis : retinoic acid is a transcriptional activator of the mitochondrial UCP gene. *J. Biol. Chem.* 270 (10): 5666-73
3. Alvarez R et al. 2000. Both RAR and RXR-dependent signaling pathways mediate the induction of the brown adipose tissue UCP-1 gene by retinoids. *Biochem. J.* 345: 91-7
4. Baldo A et al. 1995. Signal transduction pathway of Acylation-Stimulating Protein: involvement of protein kinase C. *J. Lipid Res.* 36: 1415-26
5. Boden G & Chen X. 1995. Effects of fat on glucose uptake and utilization in patients with non-insulin-dependent diabetes. *J. Clin. Invest.* 96 (3): 1261-8
6. Boss O et al. 1998. UCP-3 expression in skeletal muscle and free fatty acids in obesity. *The Lancet* 351: 1933
7. Boss O. 1998. The uncoupling proteins: a review. *Eur. J. of Endocrinol.* 139: 1-9
8. Branco M et al. 1999. 3,5,3'-triiodothyronine actively stimulates UCP in brown fat under minimal sympathetic activity. *Am. J. Physiol.* 276: E 179-87
9. Brand MD et al. 1999. The significance and the mechanism of the mitochondrial proton conductance. *Int. J. Obesity* 23 (suppl. 6): S4-11
10. Brun S et al. 1999. Activators of PPAR-γ induce the expression of the UCP-3 gene in skeletal muscle: a potential mechanism for the lipid intake-dependent activation of UCP-3 gene expression at birth. *Diabetes* 48: 1217-22
11. Camirand A et al. 1998. Thiazolidinediones stimulate UCP-2 expression in cell lines representing white and brown adipose tissue and skeletal muscle. *Endocrinol.* 139 (1): 428-31
12. Cancello R et al. 1998. Leptin and UCP-1 genes are reciprocally regulated in brown adipose tissue. *Endocrinol.* 139 (11): 4747-50
13. Cassard-Doulcier A et al. 1992. In vitro interactions between nuclear proteins and UCP gene promoter reveal several putative transactivating factors including EtS1, Retinoid X Receptor, thyroid hormone receptor, and a CACCC box-binding protein. *J. Biol. Chem.* 260 (39): 24335-42

14. Chan CB et al. 1999. Overexpression of UCP-2 inhibits glucose-stimulated insulin secretion from rat islets. *Diabetes* 48: 1482-6

15. Cianflone K. 1997. Acylation stimulating protein and the adipocyte. *J. Endocrinol.* 155: 203-6

16. Clapham JC et al. 2000. Mice overexpressing human UCP-3 in skeletal muscle are hyperphagic and lean. *Nature* 406: 415-8

17. Collins S et al. 1999. Regulation of the uncoupling proteins genes (UCP-1, 2, 3) by nutrients and hormones. Progress in Obesity Research, Ed. 8, Editors Guy-Grand B & Ailhaud G, John Libby & Company Ltd. 373-9

18. Combatsiaris TP et al. 1999. Downregulation of UCP-2 mRNA in white adipose tissue and UCP-3 mRNA in skeletal muscle in the early stages of leptin treatment. *Diabetes* 48: 128-30

19. Commins SP et al. 1999. Induction of UCP expression in brown and white adipose tissue by leptin. *Endocrinol.* 140: 292-300

20. Cummings DE et al. 1996. Genetically lean mice result from targeted disruption of the RIIβ subunit of protein kinase A. *Nature* 382: 622-6

21. Cusin I et al. 1998. Chronic central leptin infusion enhances insulin-stimulated glucose metabolism and favors the expression of uncoupling proteins. *Diabetes* 47: 1014-9

22. Echtay KS et al. 1999. Regulation of UCP-3 by nucleotides is different from regulation of UCP-1. *FEBS letters* 450:8-12

23. Enerbäck et al. 1997. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* 387: 90-3

24. Esterbauer H et al. 1998. UCP-1 mRNA expression in obese human subjects: the role of sequence variations at the UCP-1 gene locus. *J. Lipid. Res.* 39: 834-44

25. Friedman JM. 1998. Leptin and the regulation of body weight in mammals. *Nature* 395: 763-70

26. Gnudi L et al. 1996. Overexpression of GLUT4 selectively in adipose tissue in transgenic mice: implications for nutrients partitioning. *Proc. Nutr. Soc.* 55: 191-9

27. Gong D et al. 1997. UCP-3 is a mediator of thermogenesis regulated by thyroid hormone, β3-adrenergic agonists and leptin. *J. Biol. Chem.* 272 (39): 24129-32

28. Gong D et al. 1999. Genomic organization and regulation by dietary fat of the UP-3 and UCP-2 genes. *BBRC* 256: 27-32

29. Hagen T et al. 2000. UCP-1 and 3 are regulated differently. *Biochem.*39: 5845-51
30. Jacobsson A et al. 1985. Mitochondrial UCP from mouse brown fat : molecular cloning, genetic mapping and mRNA expression. *J. Biol. Chem.* 260 (30): 16250-4
31. Jezek P et al. 1998. Fatty acid cycling mechanism and mitochondrial uncoupling proteins. *Bioch. Et Biophys. Acta.* 1365: 319-27
32. Kelly LJ et al. 1998. PPAR-γ and α mediate in vivo regulation of uncoupling proteins (UCP-1, UCP-1, UCP-3) gene expression. *Endocrinol.* 139: 4920-7
33. Khalfallah Y et al. 2000. Regulation of UCP-2 and UCP-3 mRNA expression during lipid infusion in human skeletal muscle and subcutaneous adipose tissue. *Diabetes* 49: 25-31
34. Klein J et al. 2000. Insulin and the β3-adrenoreceptor differentially regulate UCP-1 expression. *Mol. Endocrinol.* 14: 764-73
35. Klingenberg M et al. 1999. Structure-Function relationship in UCP-1. *Int. J. Obseity.* 23 (suppl. 6): S 24-9
36. Kozak LP et al. 1988. The mitochondrial UCP gene: correlation of exon structure to transmemebrane domains. *J. Biol. Chem.* 25 (5): 12274-7
37. Lefebvre AM et al. 1998. Depot-specific differences in adipose tissue gene expression in lean and obese subjects. *Diabetes* 47: 98-103
38. Licinio J et al. 1998. Sex differences in circulating human leptin pulse amplitude : clinical implications. *J. Clin. Endocrinol. Metab.* 83: 4140-7
39. Mason TM et al. 1999. Prolonged elevation of plasma free fatty acids desensitizes the insulin secretory response to glucose in vivo in rats. *Diabetes* 48: 524-30
40. Matsudo J et al. 1997. Cloning of rat UCP-3 and UCP-2 cDNAs: their gene expression in arts fed a high-fat diet. *FEBS letters* 418: 200-4
41. Matsudo J et al. 1998. Increased adipose expression of the uCP-3 gene by thiazolidinediones in Wistar fatty rats and in cultured adipocytes. *Diabetes* 47: 1800-14
42. Melnyk A &Himms-Hagen J. 1998. Temperature-dependent feeding: lack of role for leptin and defect in brown adipose tissue-ablated obese mice. *Am. J. Physiol.* 274: R 1131-5
43. Moitra J et al. 1998. Life without white fat: a transgenic mouse. *Gen. & Develop.* 12 3168-81

44. Murray I et al. 1997. Functional bioactive recombinant acylation stimulating protein is distinct from C3a anaphylatoxin. *J. Lipid Res.* 38: 2492-501

45. Murray I et al. 1999. Acylation-Stimulating Protein (ASP) deficiency alters post-prandial and adipose tissue metabolism in male mice. *J. Biol. Chem.* 274 (51): 36219-25

46. Murray I, Sniderman AD & Cianflone K. 1999. Mice lacking ASP have delayed postprandial triglyceride clearance. *J. Lipid. Res.* 40: 1671-6

47. Murray I et al. 2000. Reduced body weight, adipose tissue, and leptin levels despite increased energy intake in female mice lacking ASP. *Endocrinol.* 141 (8): 1041-9

48. Nagase I et al. 1996. Expression of uncoupling protein in skeletal muscle and white fat of obese mice treated with thermogenic β3-adrenergic agonist. *J. Clin. Invest.* 97: 2898-904

49. Nordfors L et al. 1998. Reduced gene expression of UCP-2 but not UCP-3 in skeletal muscle of human obese subjects. *Diabetologia* 41: 935-9

50. Quevedo S et al. 1995. Quantitative measurement of rat UCP-mRNA by competitive polymerase chain reaction. *Analyt. Biochem.* 226: 379-82

51. Rial E et al. 1999. Retinoids activate proton transport by the uncoupling proteins UCP-1 and UCP-2. *EMBO J.* 18 921): 5827-32

52. Ricquier D & Cassard-Doulcier A. 1993. The biochemistry of white and brown adipocytes analyzed from a selection of proteins. *Eur. J. Biochem.* 218: 785-96

53. Ricquier D & Bouillaud F. 2000. The uncoupling protein homologues: UCP-1, UCP-1, UCP-3, StUCP and AtUCP. *Biochem J.* 345: 161-79

54. Rouru J et al. 1999. Effect of intravenously infused leptin on insulin sensitivity and on expression of UCPs in brown adipose tissue. *Endocrinol.* 140: 3688-92

55. Samec S et al. 1998. Interorgan signaling between adipose tissue metabolism and skeletal muscle UCP homologs: a role for circulating free fatty acids? *Diabetes* 47: 1693-8

56. Samec S et al. 1999. Post-starvation gene expression of skeletal muscle UCP-2 and UCP-3 in response to dietary fat levels and fatty acid composition: a link with insulin resistance. *Diabetes* 48: 436-41

57. Sarmiento U et al. 1997. Morphology and molecular changes induced by recombinant human leptin in the white and brown adipose tissues of C57Bl/6 mice. *Lab. Invest.* 77: 243-56

58. Savantaus E et al. 1998. Differential regulation of uncoupling proteins by chronic treatment with β3-adrenergic agonist BRL 35135 and Metformin in obese fa/fa Zucker rats. *BBRC* 246: 899-904

59. Scantlebury T, Maslowska M & Cianflone K. 1998. Chylomicron-specific enhancement of Acylation Stimulating Protein and precursor protein C3 production in differentiated human adipocytes. *J. Biol. Chem.* 273 (33): 20903-9

60. Scarpace PJ et al. 1998. UCP-2, UCP-3 and leptin gene expression modulation by food restriction and leptin. *J. Endocrinol.* 159: 349-57

61. Scarpace PJ et al. 2000. Modulation of UCP-2 and UCP-3: regulation by denervation, leptin and retinoic acid treatment. *J. Endocrinol.* 164: 331-7

62. Silva JE. 1997. Regulation of the UCP gene expression. *Eur. J. Endocr.* 136 : 251-64

63. Sivitz WI et al. 1999. Fasting and leptin modulate adipose and muscle uncoupling proteins : divergent effects between mRNA and protein expression. *Endocrinol.* 140: 1511-9

64. Sniderman AD et al. 1998. The adipocyte, fatty acid trapping, and atherogenesis. *Arterioscler. Throm. Vasc. Biol.* 18: 147-51

65. Surwit RS et al. 1997. Low plasma leptin in response to dietary fat in diabetes- and obesity-prone mice. *Diabetes* 46: 1516-20

66. Tonello C et al. 1998. SR 59230A blocks β3-adrenoreceptor-linked modulation of UCP-1 and leptin in rat brown adipose tissue. *Eur. J. Pharmac.* 352 : 125-9

67. Tsuboyama- Kasaoka N et al. 1998. Upregulation of UCP-3 mRNA by exercise training and downregulation of UCP-3 by denervation in skeletal muscle. *BBRC* 247: 498-503

68. Tsuboyama- Kasaoka N et al. 1999. Upregulation of liver UCP-2 mRNA by either fish oil feeding or fibrate administration in mice. *BBRC* 257: 879-85

69. Tsuboyama- Kasaoka N et al. 1999. Overexpression of GLUT4 in mice causes upregulation of UCP-3 mRNA in skeletal muscle. *BBRC* 258: 187-93

70. Vidal-Puig A et al. 1996. Regulation of PPAR-γ gene expression by nutrition and obesity in rodents. *J. Clin. Invest.* 97: 2553-61

71. Weigle DS et al. 1998. Elevated free fatty acids induce UCP-3 expression in muscle. A potential explanation for the effect of fasting. *Diabetes* 47: 298-302

72. Winkler E & Klingenberg M. 1994. Effect of fatty acids on H+ transport activity of the reconstituted UCP. *J. Biol. Chem.* 269 (4): 2508-15

73. Yamada M et al. 1998. Genomic organization and promoter function of the mouse UCP-2 gene. *FEBS letters* 432: 65-9

74. Yoshida T et al. 1998. β3-adrenergic agonist induced a functionally active uncoupling protein in fat and slow-twitch muscle fibres. *Am. J. Physiol.* 274: E469-75

75. Yoshitomi H et al. 1998. Differential regulation of mouse uncoupling proteins among brown adipose tissue, white adipose tissue and skeletal muscle in chronic β3-adrenergic receptor agonist treatment. *BBRC* 253: 85-91