

Silver Medal Lecture

High-fat diet, muscular lipotoxicity and insulin resistance

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A high dietary fat intake and low physical activity characterize the current Western lifestyle. Dietary fatty acids do not stimulate their own oxidation and a surplus of fat is stored in white adipose tissue, liver, heart and muscle. In these organs intracellular lipids serve as a rapidly-available energy source during, for example, physical activity. However, under conditions of elevated plasma fatty acid levels and high dietary fat intake, conditions implicated in the development of modern diseases such as obesity and type 2 diabetes mellitus, fat accumulation in liver and muscle (intramyocellular lipids; IMCL) is associated with the development of insulin resistance. Recent data suggest that IMCL are specifically harmful when combined with reduced mitochondrial function, both conditions that characterize type 2 diabetes. In the (pre)diabetic state reduced expression of the transcription factor PPAR γ co-activator-1 α (PGC-1 α), which is involved in mitochondrial biogenesis, has been suggested to underlie the reduced mitochondrial function. Importantly, the reduction in PGC-1 α may be a result of low physical activity, consumption of high-fat diets and high plasma fatty acid levels. Mitochondrial function can also be impaired as a result of enhanced mitochondrial damage by reactive oxygen species. Fatty acids in the vicinity of mitochondria are particularly prone to lipid peroxidation. In turn, lipid peroxides can induce oxidative damage to mitochondrial RNA, DNA and proteins. The mitochondrial protein uncoupling protein 3, which is induced under high-fat conditions, may serve to protect mitochondria against lipid-induced oxidative damage, but is reduced in the prediabetic state. Thus, muscular lipotoxicity may impair mitochondrial function and may be central to insulin resistance and type 2 diabetes mellitus.

Intramyocellular lipids: Mitochondrial function: Oxidative damage: Type 2 diabetes: Insulin resistance

The prevalence of obesity continues to increase in affluent societies. For example, in the USA the percentage of individuals >20 years of age with obesity (defined as a BMI of >30 kg/m²) increased from 13% in 1960 to 30% in 2000, according to the latest National Health and Nutrition Examination Survey (Flegal *et al.* 2002). Moreover, according to this survey the overall prevalence in individuals >20 years with a BMI of >25 kg/m², which is considered to represent overweight, was 64%. This increase in the prevalence of obesity in adults has not only been seen in affluent societies, but has also been observed recently in developing countries. Even more dramatically,

the prevalence of obesity in US children between 6 and 19 years of age increased from 4% in the 1960s to 15% in 2000 (Ogden *et al.* 2002). Obesity increases the risk for a number of health-threatening diseases and disorders, such as type 2 diabetes mellitus, high blood pressure, high cholesterol, asthma, arthritis and cardiovascular complications. In line with the increasing prevalence of obesity, the incidence of type 2 diabetes mellitus has increased dramatically. In 2003 about 194 million individuals, 5.1% of the global population in the age-group 20–79 years, were estimated to have diabetes. It is estimated that the number of individuals suffering from type 2 diabetes

worldwide will be approximately 330 million in 2025, with approximately 10% (472 million) of the global population suffering from impaired glucose tolerance. The continuous increase in the prevalence of type 2 diabetes contributes considerably to illness and mortality rates.

Obesity, energy balance and fat balance

By definition, the development of obesity and overweight is characterized by a positive energy balance. Numerous investigations (Schutz *et al.* 1989; Bennett *et al.* 1992) have shown that in the long term an imbalance between energy intake and energy expenditure is reflected in a positive fat balance. In the last 20–30 years many food products have become available that are cheap, palatable and high in fat content. Since dietary fat is the most energy-dense macronutrient, about 38 kJ/g (in comparison, carbohydrate and protein only provide about 17 kJ/g), an increase in dietary fat intake profoundly increases dietary energy intake and thus results in a positive energy balance. In addition, in human subjects there is evidence for a clear substrate hierarchy for the utilization of macronutrients, in which fat balance is least regulated. For example, the human body responds only very slowly by increasing fat oxidation when fat intake is increased (Thomas *et al.* 1992; Schrauwen *et al.* 1997a), leading to a deposition of dietary fat in the fat stores. On the other hand, the storage capacity for carbohydrate and protein in the human body is limited and therefore carbohydrate and protein oxidation are very well and rapidly adjusted to their respective intake (Abbott *et al.* 1988). As a consequence, a positive energy balance will be reflected in a positive fat balance.

Fat oxidation on a high-fat diet

Although there is ample evidence that the adaptation of fat oxidation to increased fat intake is slow in man, the reason for this slow adaptation is relatively unknown. According to the two-compartment model of Flatt (1987), whole-body fat oxidation can be increased via an expansion of fat mass, leading to increased plasma NEFA levels available for oxidation. In this model the body is divided into two compartments, fat mass and glycogen stores, and the oxidation mixture of fatty acids and glucose depends on the size of these two compartments. As the glycogen stores are very limited in size, small changes in the size of the glycogen stores will affect glucose oxidation. In contrast, as the fat mass can be relatively unlimited in size, a large expansion of fat mass is needed before changes in fat oxidation will occur. This model can explain why the addition of a surplus of fat to a single meal, which will not result in a change in fat mass, does not affect fatty acid oxidation rates, and why obese subjects have relatively high fat oxidation. In fact, expansion of fat mass (obesity) could be considered as an adaptation of the body to increase fat oxidation to a level that matches a high dietary fat intake. However, it has been shown (Schrauwen *et al.* 1997a) that healthy human volunteers who consume a high-fat diet for 7 d, while being in energy balance, are able to slowly increase their fat oxidation to a level that

equals the high-fat intake. As no substantial expansion of fat mass can be expected after 7 d of a high-fat diet, these results seem to contradict Flatt's (1987) model. These findings have, however, been explained in terms of the changes in glycogen stores that may have occurred (Schrauwen *et al.* 1997b, 1998). During the first days on a high-fat diet, when fat oxidation does not equal fat intake, subjects are in negative carbohydrate balance (carbohydrate oxidation > carbohydrate intake), leading to a decrease in the body's glycogen stores. According to Flatt's (1987) two-compartment model, a decrease in glycogen stores would result in a decrease in glucose oxidation and would therefore be another way to increase fat oxidation. Indeed, it has been shown (Schrauwen *et al.* 1997b, 1998) that lowering glycogen stores by exhaustive exercise markedly improves the rate at which subjects are able to adapt their fat oxidation to an increased fat intake.

In order to further investigate the mechanisms by which fat oxidation increases on a high-fat diet, a more detailed determination of fatty acid oxidation has been conducted in subjects consuming high-fat diets (Schrauwen *et al.* 2000). Interestingly, it was observed that the increase in fat oxidation after 7 d of a high-fat diet is completely accounted for by an increase in TAG-derived fatty acid oxidation. Thus, using labelled fatty acids plasma-derived fatty acid oxidation can be distinguished from the oxidation of TAG-derived fatty acids (mainly intramyocellular lipids (IMCL)). IMCL are small lipid droplets that are located in the sarcoplasm and predominantly found in the vicinity of mitochondria, suggesting that they may serve as a rapidly-available energy source for the muscle. Using proton magnetic resonance spectroscopy to quantify these IMCL non-invasively, it has recently been shown (Schrauwen-Hinderling *et al.* 2005) that the amount of IMCL is already markedly increased after 7 d of a high-fat diet in healthy lean subjects. In analogy with the model of Flatt (1987), which predicts that an increase in body fat mass is needed to increase whole-body fat oxidation, these combined observations of an increased IMCL mass and increased IMCL oxidation indicate that increases in IMCL content are also needed to drive increased IMCL oxidation. Thus, the slow rate at which fat oxidation adapts to increased fat intake when a high-fat diet is consumed may also be attributed to the time needed to increase IMCL content. Interestingly, it has been found (Schrauwen *et al.* 2002c) that when sedentary middle-aged subjects follow an endurance training programme for 3 months whole-body fat oxidation increases, and again this increase is completely accounted for by an increase in TAG-derived fatty acid oxidation. In accordance with the earlier mentioned hypothesis, endurance training is also known to increase IMCL content (Goodpaster *et al.* 2001; Schrauwen-Hinderling *et al.* 2006a), suggesting that similar mechanisms may be involved in the training- and diet-induced increase in fat oxidation.

Intramyocellular lipid and insulin sensitivity

As mentioned earlier, the steadily-increasing prevalence of obesity is accompanied by a steady increase in the

prevalence of type 2 diabetes mellitus. Central to the aetiology of type 2 diabetes is insulin resistance, an early detectable characteristic preceding the development of full-blown and clinically-overt type 2 diabetes. Insulin resistance indicates a state of reduced responsiveness of the insulin-sensitive tissues to circulatory levels of insulin, i.e. a reduced glucose disposal and impaired inhibition of hepatic glucose output at a given concentration of insulin (McGarry, 2002). A major contribution to whole-body insulin resistance comes from the skeletal muscle, as revealed by classical work by DeFronzo *et al.* (1981), who have shown a 45% reduction in leg glucose uptake following a hyperinsulinaemic euglycaemic clamp in patients with type 2 diabetes and a strong positive correlation ($r = 0.70$, $P < 0.001$) between leg and total body glucose uptake.

Evidence gathered in recent decades has pointed towards an important causal role of disturbed fatty acid metabolism in the development of type 2 diabetes mellitus. Not only are plasma glucose levels increased in uncontrolled type 2 diabetes, but also plasma NEFA, and the storage of fatty acids in non-adipose tissues such as pancreas, liver and muscle is elevated in patients with type 2 diabetes (Schalch & Kipnis, 1965). Moreover, a strong negative correlation has been found between the level of IMCL and insulin sensitivity in non-trained subjects (Perseghin *et al.* 1999), and levels of IMCL are increased in first-degree relatives of patients with type 2 diabetes who are insulin resistant, but not diabetic (Jacob *et al.* 1999). These data suggest that IMCL accumulation may be a primary factor in the development of type 2 diabetes. Remarkably, this suggested role differs from the putative physiological function of IMCL as an important energy source that drives muscular fatty acid oxidation. On the other hand, the high levels of IMCL in the endurance-trained state contradict the suggestion that IMCL may have detrimental effects on insulin sensitivity, as endurance training is well known to improve whole-body and muscular insulin sensitivity. To unravel this apparent paradox the molecular adaptations that occur in skeletal muscle have been examined in subjects who were either trained for 2 weeks or consumed a high-fat diet for 7 d. As expected, both interventions increased IMCL levels to a similar extent (Schrauwen-Hinderling *et al.* 2006a,b). Furthermore, consistent with the hypothesis that elevated IMCL levels may serve as an important energy source, it has been found (Schrauwen-Hinderling *et al.* 2006a) that after 2 weeks of endurance training in previously untrained subjects there are already molecular adaptations in skeletal muscle that point towards an improved fat oxidative capacity. In contrast, however, the consumption of a high-fat diet for 1 week is accompanied by molecular changes that would favour fat storage in muscle rather than oxidation (Schrauwen-Hinderling *et al.* 2005). These findings suggest that IMCL may be increased for two different reasons: (1) a functional increase, whereby IMCL serves as a rapidly-available energy source; in these conditions (e.g. as with endurance training) fat oxidative capacity improves, followed by the storage of more muscular lipids that can serve as rapidly-available energy source; (2) a pathophysiological increase, whereby the increase in IMCL is merely the result of a continuous

over-supply of fat to the muscle and is not accompanied by an increased fat oxidative capacity; in this condition muscular fat accumulation results from passive uptake of excessive fatty acids, and the increased IMCL content may ultimately drive IMCL oxidation. Thus, in the latter condition the increase in IMCL can be seen as an 'adaptation' to allow increased muscular fat oxidation when fat intake is high, similar to the concept of obesity being an adaptation to increase whole-body fat oxidation. However, as with obesity, the 'adaptive' increase in IMCL in the latter condition is accompanied by negative side effects, which is indicated by the relationship between IMCL and insulin resistance.

Thus, it can be deduced that high IMCL content is only harmful in the absence of adaptations in oxidative capacity, such as with high-fat diet consumption and/or type 2 diabetes mellitus. To examine this hypothesis a comparison has been made of the IMCL content and mitochondrial oxidative capacity in patients with type 2 diabetes and in normoglycaemic controls matched for BMI (Schrauwen-Hinderling *et al.* 2007). Thus, a ^{31}P magnetic resonance spectroscopy method was used to investigate *in vivo* mitochondrial function by measuring the phosphocreatine kinetics during recovery from exercise (Meyer, 1988; Kemp & Radda, 1994). During exercise phosphocreatine content decreases transiently but recovers rapidly after exercise. In the post-exercise state phosphocreatine resynthesis is driven almost purely oxidatively (Sahlin *et al.* 1979) and the resynthesis rate reflects *in vivo* mitochondrial function (Kemp & Radda, 1994). It was found that despite similar IMCL levels, phosphocreatine resynthesis rate is reduced by approximately 50% in patients with type 2 diabetes, indicating mitochondrial dysfunction. Thus, the combination of high IMCL and low oxidative capacity is important in the aetiology of type 2 diabetes mellitus. Along the same line of reasoning Goodpaster *et al.* (2001) have suggested that fat oxidative capacity might be more important than IMCL content in determining insulin sensitivity, whereas Petersen *et al.* (2004) have reported decreased mitochondrial function (oxidative capacity) in the insulin-resistant offspring of patients with type 2 diabetes when compared with insulin-sensitive controls. Together, these findings indeed point towards the importance of mitochondrial oxidative capacity in the aetiology of type 2 diabetes. Although beyond the scope of the present review, a functional explanation is provided by the observation that lipid metabolites such as fatty acyl-CoA or diacylglycerol are more likely to intervene with insulin signalling than IMCL *per se*. Diacylglycerol is the intermediate between fatty acyl-CoA, which can enter the mitochondria for oxidation, and TAG, the form in which lipids are stored in muscle. If an increase in IMCL is not accompanied by a sufficiently increased fat oxidative capacity (i.e. a low mitochondrial oxidative capacity), levels of lipid metabolites will rise and interfere with insulin signalling (for review, see Shulman, 2000).

What causes mitochondrial dysfunction?

Although the finding of a reduced mitochondrial oxidative capacity in both first-degree relatives and patients with

type 2 diabetes is intriguing, the mechanism underlying this reduction remains to be determined. One likely explanation for the mitochondrial dysfunction observed in the (pre)diabetic state is a reduced expression of genes involved in mitochondrial oxidative metabolism, as has been observed in type 2 diabetes (Mootha *et al.* 2003; Patti *et al.* 2003). Many of the oxidative genes are under transcriptional control of PPAR γ coactivator-1 α (PGC-1 α), which stimulates oxidative phosphorylation, mitochondrial biogenesis and the generation of oxidative type 1 muscle fibres (Lin *et al.* 2002). In addition to the down-regulation of the so-called OXPHOS gene set, it has been shown that the expression of PGC-1 α itself is also reduced in patients with type 2 diabetes (Mootha *et al.* 2003; Patti *et al.* 2003). Strikingly, the same observations have been reported in subjects who do not have type 2 diabetes, but have a family history of type 2 diabetes (Patti *et al.* 2003). Recently, these findings have been confirmed (M Mensink, MKC Hesselink, AP Russell, G Schaart, J-P Sels and P Schrauwen, unpublished results) in patients with type 2 diabetes, showing an approximately 60% reduction in PGC-1 α gene expression compared with BMI and age-matched controls. Interestingly, it has been found (M Mensink, MKC Hesselink, AP Russell, G Schaart, J-P Sels and P Schrauwen, unpublished results) that treating these patients with the anti-diabetic agent Rosiglitazone restores muscular PGC-1 α levels, along with an improvement in muscular insulin sensitivity. In addition, it has been shown (Russell *et al.* 2005) that acute exercise, which is well known to improve muscular oxidative capacity and insulin sensitivity, up regulates the expression of PGC-1 α in healthy subjects. Also, prolonged endurance training increases PGC-1 α protein content (Russell *et al.* 2003a). These data indicate that PGC-1 α may indeed play a crucial role in the development of mitochondrial dysfunction, insulin resistance and type 2 diabetes mellitus.

If PGC-1 α is indeed a crucial factor in relation to mitochondrial function, the question arises of why PGC-1 α is reduced in the prediabetic state?

Although it is tempting to speculate that genetic factors underlie the reduced expression of PGC-1 α in the prediabetic state, the impact of environmental factors should not be ruled out. As mentioned earlier, acute exercise (Russell *et al.* 2005), as well as endurance training (Russell *et al.* 2003a), can increase the expression and protein content of PGC-1 α . Whether the opposite effect, a reduction in PGC-1 α as a result of physical inactivity, also occurs is presently unknown, but it is tempting to suggest that the low level of physical activity that characterizes Westernized society is partly responsible for the reduction in PGC-1 α levels. In that context, the study of Mootha *et al.* (2003) has observed that the reduction in OXPHOS genes is strongly correlated with whole-body aerobic capacity. Although this result can be interpreted as PGC-1 α being a determinant of oxidative capacity, it is also known that whole-body aerobic capacity is mainly determined by an individual's physical activity history (Katzel *et al.* 2001; Nagaya *et al.* 2001). Thus, the strong correlation between OXPHOS genes and aerobic capacity may also support the suggestion that physical (in)activity underlies the reduction in PGC-1 α and oxidative gene expression.

Very interestingly, consumption of a high-fat diet for 3 d reduces PGC-1 α protein levels by approximately 20% in human volunteers, whereas there is a 40% reduction in c57Bl/6J mice after 3 weeks on a high-fat diet (Sparks *et al.* 2005). Again, this finding suggests that the reduction of PGC-1 α in the prediabetic state may very well be associated with Westernized lifestyle factors. To investigate whether a high fatty acid supply is indeed able to down regulate PGC-1 α a study has been conducted to establish whether the acute elevation of plasma NEFA during a hyperinsulinaemic euglycaemic clamp is associated with a down-regulation in the expression of skeletal muscle genes involved in oxidative metabolism (Hoeks *et al.* 2006a). Thus, when healthy volunteers receive an infusion of Intralipid (a fat emulsion; Fresenius Kabi, Bad Homburg, Germany) over a period of 6 h there is an approximately 2.7-fold increase in plasma NEFA levels, a 31% decrease in insulin sensitivity and an approximately 1.6-fold increase in muscular fat content. Under these conditions of high circulating and muscular fat content, PGC-1 α is acutely down regulated. Again, this finding suggests that the decrease in PGC-1 α expression observed in the (pre)diabetic state may be the result of diabetogenic environmental factors, and PGC-1 α may therefore provide a link between an unhealthy lifestyle and the development of mitochondrial dysfunction and ultimately type 2 diabetes mellitus.

A role for oxidative stress in the development of insulin resistance

Since the finding that mitochondrial function may be impaired in the (pre)diabetic state, most studies have focused on PGC-1 α and mitochondrial biogenesis. However, mitochondrial function is not only determined by mitochondrial biogenesis, but also by mitochondrial quality. In the latter context, it has been shown (Kelley *et al.* 2002) that mitochondria from patients with type 2 diabetes are smaller and show morphological aberrations when compared with controls, and mitochondrial area correlates positively with insulin sensitivity. The smaller and damaged mitochondria in skeletal muscle of patients with diabetes also result in an impaired functional capacity (Kelley *et al.* 2002). Thus, mitochondria of patients with type 2 diabetes have a reduced electron transport chain capacity, as measured by NADH:O₂ oxidoreductase activity, as well as a reduced citrate synthase activity (Kelley *et al.* 2002).

To explain the observed mitochondrial damage, elevated production of reactive oxygen species (ROS) and its by-products (e.g. lipid peroxides) has been suggested. In addition to the production of ATP, mitochondria are also the major contributor to the production of ROS. Mitochondrial ROS can react rapidly with DNA, protein and lipids, thereby leading to so-called oxidative damage. Recent evidence points towards a causal role for ROS in the development of insulin resistance. Thus, Houstis *et al.* (2006) have found that cellular ROS levels are elevated in two cell models of insulin resistance in which insulin resistance is either induced by treatment with the cytokine

TNF α or by the glucocorticoid dexamethasone. To provide evidence for a causal relationship between the elevated ROS levels and insulin resistance, the authors over-expressed a variety of antioxidant genes in their model, and it was found that the over-expression of each of these genes could reverse the insulin resistance induced by both TNF α and dexamethasone (Houstis *et al.* 2006).

Fatty acids are especially very prone to ROS-induced oxidative damage, resulting in the formation of lipid peroxides, which in turn can induce damage to proteins and DNA. Thus, accumulation of fatty acids in the vicinity of the mitochondrial matrix, where ROS are formed, increases the likelihood of lipid peroxidation. As discussed earlier, patients with type 2 diabetes are characterized by the accumulation of IMCL and these lipid droplets are located close to the mitochondria. To prevent simple diffusion of fatty acids into the mitochondria their entry is regulated by the enzyme carnitine palmitoyltransferase 1, which serves as an inward transporter of oxidizable fatty acids. However, this system cannot completely prevent the diffusion of fatty acid into the mitochondria. Mitochondrial membranes consist of lipid bilayers and therefore fatty acids can still enter the mitochondria matrix via a so-called 'flip-flop' mechanism over the membrane (Ho *et al.* 2002). It can easily be imagined that this 'passive diffusion' is more likely to occur under conditions of a high IMCL concentration, as is the case with insulin resistance and type 2 diabetes mellitus. Consistent with this notion, skeletal muscle of subjects who are obese and insulin resistant not only contains a higher amount of IMCL, but these lipids also show a higher extent of lipid peroxidation (Russell *et al.* 2003b). Potentially, these lipid peroxides could lead to oxidative damage to mitochondrial structures and explain the increased mitochondrial damage observed in patients with type 2 diabetes (Kelley *et al.* 2002).

Mitochondrial uncoupling protein-3 to prevent lipid-induced oxidative damage

If mitochondria are vulnerable to their own ROS and the resulting lipid peroxides, these organelles should be equipped with a protective mechanism. Recently, it has been proposed (Schrauwen *et al.* 2006) that the mitochondrial uncoupling protein (UCP) 3 fulfils such a protective role against lipid-induced oxidative damage. UCP3 was discovered in 1997 as a human homologue of the brown adipose tissue-specific UCP1. However, after several years of research it became evident that in contrast to UCP1, UCP3 has no major role in the regulation of energy metabolism, but is rather involved in fatty acid metabolism. In several physiological experiments it has been observed that UCP3 is up regulated under conditions in which the supply of fatty acids to the muscle exceeds the fat oxidative capacity, such as fasting, high-fat feeding (Hesseling *et al.* 2003) and acute exercise (Schrauwen *et al.* 2002a), and in type 2b muscle fibres (Hesseling *et al.* 2001). On the other hand, UCP3 is down regulated when the fat oxidative capacity is high, as in type 1 muscle fibres (Hesseling *et al.* 2001) and with endurance training (Schrauwen *et al.* 2005). When fatty acid supply does not match fatty acid

oxidation, fatty acids will accumulate in the cytosol and the possibility of fatty acids entering the mitochondrial matrix via 'flip-flop' will increase. Based on these findings, it has been postulated (Schrauwen *et al.* 2001) that UCP3 could function as a fatty acid anion exporter. Fatty acids that 'flip-flop' over the inner mitochondrial membrane will become deprotonated as a result of a proton gradient over the inner mitochondrial membrane, resulting in fatty acid anions. Importantly, fatty acid anions are not able to 'flip-flop' back across the membrane (Ho *et al.* 2002), nor can they undergo β -oxidation because the enzyme that converts long-chain fatty acids to fatty acyl-CoA is absent in the mitochondrial matrix (Tol, 1975). Thus, the inner mitochondrial membrane would accumulate fatty acid anions on the side of the mitochondrial matrix. Moreover, these fatty acid anions could potentially leave the inner mitochondrial membrane and accumulate inside the mitochondrial matrix; these anions could accumulate either dissolved in the matrix at very low concentrations or bound to matrix-associated fatty acid-binding proteins (Unterberg *et al.* 1990). In the matrix (or at the inner side of the inner mitochondrial membrane), these fatty acid anions would be prone to peroxidation by ROS. As peroxidized fatty acids are highly reactive (Yagi, 1987), they could damage DNA, RNA and proteins of the mitochondrial machinery, which are mainly present in the mitochondrial matrix. It has been postulated (Schrauwen *et al.* 2001) that UCP3 may serve as an exporter of non-metabolizable fatty acid anions, protecting mitochondria from oxidative damage. This hypothesis is consistent with those postulated by other researchers, which also predict that UCP3 is a fatty acid anion exporter (Skulachev, 1991; Garlid *et al.* 1998; Jezek *et al.* 1998).

Subsequent to postulating this hypothesis several experiments were carried out to test its validity. First, use was made of the potential of Etomoxir to inhibit carnitine palmitoyltransferase 1, the rate-limiting step in the mitochondrial uptake of fatty acyl-CoA. Healthy human subjects were given Etomoxir for 36 h, while consuming a high-fat diet to increase fatty acid supply (Schrauwen *et al.* 2002b). According to the hypothesis, this condition would be one of the most formidable in which UCP3 is needed, as both the oxidative capacity is low and the supply of fat is high. As anticipated, there is a 67% induction of UCP3 at the protein level after only 36 h of Etomoxir treatment. These findings have been confirmed in rats (Schrauwen *et al.* 2003), where it was found that Etomoxir treatment for 8 d results in an approximately 2-fold decrease in carnitine palmitoyltransferase 1 activity and an approximately 2-fold increase in UCP3 protein levels.

A second study (Hoeks *et al.* 2003) has utilized the unique ability of medium-chain fatty acids to pass the mitochondrial bilayer independently of carnitine palmitoyltransferase 1. An important aspect of the hypothesis is that neutral fatty acids that enter the mitochondrial matrix cannot be oxidized because they cannot be converted to fatty acyl-CoA inside the matrix (Kerner & Hoppel, 2000). However, this premise is not true for medium-chain fatty acids; these fatty acids can be activated inside the mitochondrial matrix and do not rely on carnitine palmitoyltransferase 1 for mitochondrial uptake (Tol, 1975;

Rasmussen *et al.* 2002). Thus, even when neutral medium-chain fatty acids reach the mitochondrial matrix they can be activated to fatty acyl-CoA and directed to β -oxidation (Fujino *et al.* 2001). Obviously, these fatty acids do not need to (and should not) be exported from the matrix, eliminating a role for UCP3 in the handling of these specific medium-chain fatty acids. Thus, there is no need for UCP3 to control increased levels of medium-chain fatty acids inside the matrix, in contrast to the anticipated increase in UCP3 with a high-fat diet containing predominantly long-chain fatty acids. To test this notion, rats were fed high-fat diets containing either long-chain fatty acids or medium-chain fatty acids for 14 d (Hoeks *et al.* 2003). UCP3 protein content was found to increase 2-fold on feeding the long-chain fatty acid high-fat diet, but to be unaffected by feeding the medium-chain fatty acid high-fat diet. Furthermore, this differential UCP3 response is accompanied by a similar rise in plasma NEFA levels and also cannot be explained by a PPAR effect, as other PPAR-responsive genes are similarly affected by the diets (Hoeks *et al.* 2003). Again, these data support the hypothesis that UCP3 is involved in the export from the mitochondrial matrix of fatty acids that cannot be metabolized.

According to the hypothesis UCP3 prevents the accumulation of fatty acid anions inside the mitochondrial matrix and membranes, which otherwise would be vulnerable to ROS-induced oxidative damage, resulting in lipid peroxides. It can therefore be predicted that lack of UCP3 would lead to increased lipid peroxide levels and/or oxidative damage. Indeed, mice lacking UCP3 are characterized by increased levels of lipid peroxides (Hoeks *et al.* 2006b) and damage to mitochondrial proteins (Brand *et al.* 2002). Very interestingly, it has recently been shown (Echtay *et al.* 2003) that UCP3 can be activated by 4-hydroxy-2-nonenal, an important byproduct of lipid peroxidation, suggesting that a negative feedback loop is involved in the regulation of mitochondrial lipid peroxide production, whereby elevated lipid peroxide levels activate UCP3, thereby limiting further accumulation of these toxic lipid products.

Uncoupling protein 3 and type 2 diabetes mellitus

The increased content of muscular fat, the higher extent of lipid peroxidation of these fats and the observed mitochondrial damage in patients who are insulin resistant and/or patients with type 2 diabetes could be linked if the previously mentioned negative feedback loop is disturbed. Although the possibility of a link clearly needs further investigation, it is intriguing to note that patients with type 2 diabetes, as well as subjects with impaired glucose tolerance who are often considered as being 'prediabetic', have a 50% reduction in UCP3 protein levels (Schrauwen *et al.* 2006). It is tempting to speculate that in patients with type 2 diabetes the reduction in UCP3 is a pathological condition in which low levels of UCP3 fail to sufficiently protect mitochondria against lipid-induced mitochondrial damage, indicating a defective feedback mechanism between lipid peroxides and mitochondrial uncoupling. This notion is further supported by the finding (Mensink

et al. 2006) that in subjects who are prediabetic (i.e. impaired glucose tolerant) a lifestyle intervention programme results in a 2-fold increase in UCP3 protein after 1 year, thereby restoring UCP3 to normal physiological levels. In addition, UCP3 protein content is also restored in patients with type 2 diabetes after 1 year of an endurance training programme, in parallel with improvement in insulin sensitivity (Mensink *et al.* 2006). Finally, it has recently been shown (Schrauwen *et al.* 2006) that the insulin-sensitizing agent Rosiglitazone up regulates the protein content of UCP3 in skeletal muscle of patients with type 2 diabetes, in parallel with an improved oxidative capacity, mitochondrial succinate dehydrogenase activity and muscular insulin sensitivity. In addition, evidence for an important role for UCP3 in the aetiology of type 2 diabetes also comes from the recent finding that genetic variation in the *UCP3* gene is associated with the 10- and 15-year risk for developing type 2 diabetes mellitus (Gable *et al.* 2006). Obviously, these findings in human subjects do not yet prove that reduced levels of UCP3 are causally related to the development of lipid-induced oxidative damage and insulin resistance, and further studies are needed to prove a functional role for UCP3 in type 2 diabetes.

Conclusion

The consumption of high-fat diets is associated with an excessive storage of fatty acids in white adipose tissue, but also in non-adipose tissues such as liver, heart and skeletal muscle. Muscular fat accumulation is strongly associated with insulin resistance, especially when a high muscular fat content is accompanied by a low mitochondrial oxidative capacity, as in patients with type 2 diabetes. The transcription factor PGC-1 α , which is involved in mitochondrial biogenesis, has been shown to be reduced in patients with type 2 diabetes. Recent data indicate a role for physical inactivity and high dietary fat intake in the reduction of PGC-1 α . In addition to a putative reduction in mitochondrial biogenesis, patients with diabetes display increased mitochondrial damage. Such damage could be a result of the detrimental effects of ROS produced in the mitochondria. Fat accumulation inside muscle cells may lead to the entry of fatty acids into mitochondria where they are prone to ROS-induced peroxidation. In turn, these lipid peroxides may damage mitochondria and cause a further deterioration in oxidative capacity. UCP3 may play an important role in the protection of mitochondria against these lipid peroxides, by exporting fatty acid anions and/or peroxides from the mitochondrial matrix. UCP3 is activated by lipid peroxides, which suggests a negative feedback loop that limits lipid-induced oxidative damage. Of note, UCP3 protein levels are decreased by 50% in patients with type 2 diabetes mellitus as well as in subjects who are 'prediabetic' compared with age- and BMI-matched controls. This finding may suggest that the negative feedback loop is indeed disturbed in pre-diabetes, and ultimately could lead to lipid-induced mitochondrial damage and insulin resistance. Interestingly, insulin-sensitizing interventions such as treatment with Rosiglitazone or a lifestyle intervention restore UCP3 levels to control values.

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