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**"Estudio funcional de la proteína desacopladora
mitocondrial UCP3 en relación con la apoptosis y
las especies reactivas de oxígeno"**

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Gene expression of leptin and uncoupling proteins: molecular end-points of fetal development

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Abstract

Uncoupling proteins (UCPs) are considered to be major determinants of energy expenditure in mammals. During development in rodents, the expression of the UCP genes occurs sequentially. UCP2 mRNA is expressed long before birth. UCP1 mRNA expression in brown adipose tissue (BAT) starts in the late fetal period, and the expression of UCP3 mRNA begins immediately after birth in BAT and skeletal muscle. The postnatal induction of UCP1 gene expression is due mainly to cold stimuli, whereas the switch-on of UCP3 mRNA expression after birth requires the stimulus of food intake, specifically of lipids in the mother's milk. However, UCP3 mRNA expression after birth is also highly sensitive to leptin, and administration of a single injection of leptin to neonatal mice that were not allowed to suckle partly mimicked the natural induction of UCP3 gene expression in BAT and skeletal muscle. The speed of the effects of leptin on UCP3 mRNA expression suggests a direct action on skeletal muscle and BAT. The injection of leptin produced minor effects on UCP1 mRNA expression, and no effects were observed on UCP2 mRNA. In summary, leptin appears to contribute to the regulation of UCP3 gene expression in the perinatal period. Whatever the mechanism of action of leptin in BAT and skeletal muscle, it is already functional at birth.

Introduction

The uncoupling proteins (UCPs) confer proton permeability on the mitochondrial inner membrane and promote uncoupling of the respiratory chain. They have been considered to have a role in the regulation of thermogenesis and, in general, in the energy expenditure of mammals. UCP1, which is expressed only in brown adipose tissue

(BAT), is a key component of the thermogenic machinery of mammals in response to cold, and it may also be involved in diet-induced thermogenesis. The more recently discovered UCP2, which is expressed almost ubiquitously, and UCP3, which is predominant in skeletal muscle and BAT, have a less well defined function (for a review, see [1]). The fact that the expression of the UCP3 gene is mainly regulated by non-esterified fatty acids has implicated it in fatty acid oxidation [2,3] and, although mice with targeted disruption of the UCP3 gene do not show major metabolic abnormalities [4,5], transgenic mice that over-express UCP3 in muscle show reduced fat accumulation, despite increased food intake [6]. Hormonal factors such as adrenergic stimuli, retinoids or fatty acid-derived molecules influence the expression of UCP genes [3,7,8]. Several reports have shown that leptin activates the expression of the UCP genes by direct or indirect mechanisms [7,9,10], although other authors have described a down-regulation of the UCP2 and UCP3 genes by short-term treatment with leptin [11].

Leptin, the product of the *ob* gene, has a major function in controlling food intake behaviour, acting as a satiety factor. Moreover, it promotes energy expenditure in peripheral tissues. This effect may be mediated by activation of the sympathetic nervous system due to the central effects of leptin, but it has also been reported that a direct action of leptin on peripheral tissues, such as BAT and muscle, promotes metabolic fuel usage and energy expenditure [12,13]. Leptin is present in the blood of human and rodent fetuses, especially just before birth [14,15]. In humans, the development of white adipose tissue in the late fetal period may account for this. In rodents, white adipose tissue develops only after birth, and the placenta may be a major source of leptin in the late fetal period [16]. After birth there is a decline in circulating leptin levels, although in rodents a close time-course analysis has shown a transient peak in leptin levels in the blood on the first day after birth [14,17]. Leptin is unlikely to be involved in the control of energy balance during the

Key words: birth, development, UCPs.

Abbreviations used: BAT, brown adipose tissue; UCP, uncoupling protein.

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fetal period, but it may be involved in developmental regulation [12]. However, just after birth, the mechanisms for the regulation of energy intake and expenditure must be at work. The initiation of suckling, which constitutes a novel means of nutrient intake after delivery, and the thermogenic requirements at birth are major metabolic challenges to the neonate. Accordingly, expression of the UCP genes, especially those encoding UCP1 and UCP3, is activated just after birth [3,18,19]. The role of leptin in the control of gene expression in relation to energy metabolism at birth is not known, and nor has it been established whether the mechanisms of response to leptin are developed at birth. We have addressed this issue by examining the effects of leptin on the expression of the UCP genes in two major tissues involved in energy expenditure, i.e. BAT and skeletal muscle, just after birth.

Materials and methods

Newborn Swiss mice were studied at 0 h (considered to be time at which all the pups had been born, but none had started suckling) and 16 h after birth. For studies on postnatal starvation, pups were separated from dams at 0 h and placed in a humidified thermostatted chamber (30 °C). When indicated, starved pups were given 100 µl of a triacylglycerol emulsion (Intralipid; Pharmacia, Sant Cugat, Spain) or 100 µl of 0.1 g/ml glucose solution by intragastric gavage. Other starved pups were injected intraperitoneally with murine recombinant leptin (2 µg/g body wt; Preprotech, London, U. K.) dissolved in PBS; controls were injected with equal amounts of vehicle. Treatments were initiated 2 h after birth, mice were killed by decapitation 14 h later, and interscapular BAT and skeletal muscle from the two hind legs were dissected out and frozen in liquid nitrogen. Blood was collected and serum prepared. Controls and treated animals were taken from the same litter in each experiment, and samples from two or three pups were pooled for each experimental situation.

Total RNA from tissues was extracted using the guanidine isothiocyanate method. For Northern blot analysis, 20 µg of total RNA was denatured, electrophoresed on 1.5 % formaldehyde/agarose gels, and transferred to positively charged nylon membranes (N⁺; Boehringer Mannheim). A 0.2 µg portion of ethidium bromide was added to the RNA samples in order to check for equal loading of the gels and transfer efficiency. Pre-hybridization and hybridization were carried out

at 55 °C using a solution containing 0.25 M Na₂HPO₄ (pH 7.2), 1 mM EDTA, 20 % SDS and 0.5 % blocking reagent (Boehringer, Mannheim, Germany). Blots were hybridized using the following DNA probes: rat cDNA for UCP1 [20], human cDNA for UCP3 [21] and a mouse UCP2 probe obtained after coupled reverse transcription and PCR amplification [19]. The DNA probes were labelled with [α -³²P]dCTP using the random oligonucleotide primer method. Hybridization signals were quantified using a GS-525 Molecular Image System (Bio-Rad). Serum non-esterified fatty acids were quantified using a colorimetric method (Wako Chemicals, Neuss, Germany). Statistical analyses were performed using Student's *t* test.

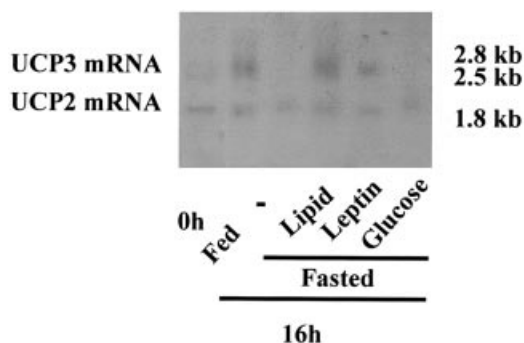
Results

UCP2 mRNA was expressed in the skeletal muscle of mice at birth, whereas the expression of UCP3 mRNA was hardly detectable by Northern blot at this stage of development (Figure 1). However, by 16 h after birth, UCP3 mRNA was highly expressed. Whether or not suckling was initiated did not have a major influence on UCP2 mRNA levels in the first 16 h of life. However, suckling was an absolute requirement for the postnatal induction of UCP3 mRNA; intake of lipid, but not of glucose, had the same effect on UCP3 mRNA expression as did milk intake. These findings were in agreement with previous reports [3]. When starved mice were treated with leptin, this caused

Figure 1

Effects of leptin on UCP2 and UCP3 mRNA expression in skeletal muscle after birth

Shown is a representative Northern blot analysis of the relative abundance of UCP2 and UCP3 mRNAs in the skeletal muscle of mice at birth (0 h) and in neonates 16 h after birth that were allowed (Fed) or not (Fasted) to suckle. Starved pups were treated with a lipid emulsion, glucose or a single injection of leptin, or acted as controls (-). The sizes of the corresponding transcripts are shown on the right.



activation of UCP3 mRNA expression to a lesser extent than that elicited by suckling or lipid intake ($62 \pm 14\%$ compared with lipid-treated pups). However, leptin had no effect on UCP2 mRNA levels. The changes in UCP3 mRNA expression in skeletal muscle were associated with serum levels of non-esterified fatty acids, which were low in pups at birth ($245 \pm 37 \mu\text{mol/l}$) and in starved or glucose-treated pups at 16 h (219 ± 28 and $257 \pm 38 \mu\text{mol/l}$ respectively), but were high in fed and lipid-treated pups at 16 h (1276 ± 124 and $1178 \pm 314 \mu\text{mol/l}$ respectively). The only exception was the leptin-treated group, in which UCP3 mRNA levels were high but serum levels of non-esterified fatty acids remained low ($228 \pm 19 \mu\text{mol/l}$).

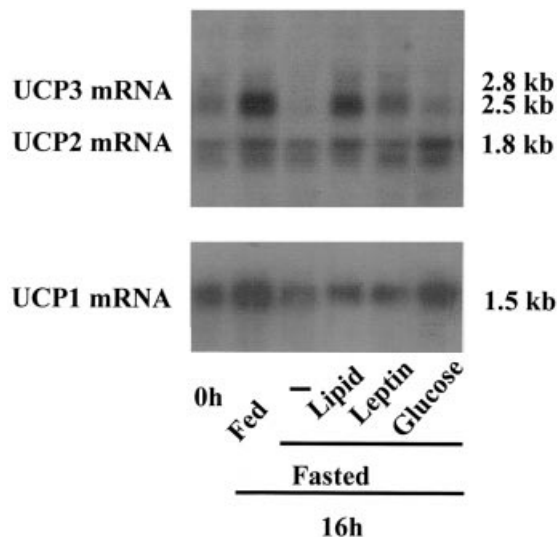
A parallel study was performed using interscapular BAT (Figure 2). UCP2 and UCP1 mRNAs were substantially expressed at birth, whereas UCP3 mRNA levels were very low. This is in agreement with our previous study on UCP gene expression in BAT during mouse development [19]. Pups that had been suckled for

16 h showed a burst of UCP3 mRNA expression (11.0 ± 2.2 -fold induction; $P \leq 0.05$) and more modest rises in the levels of UCP1 mRNA (3.1 ± 0.5 -fold induction; $P \leq 0.05$) and UCP2 mRNA (2.7 ± 0.3 -fold induction; $P \leq 0.05$) when compared with 0 h neonates. When pups did not suckle, effects on UCP3 mRNA similar to those reported for skeletal muscle were observed: complete impairment of UCP3 mRNA expression, which became almost undetectable. The lack of suckling also blunted the small increases in UCP2 and UCP1 mRNA levels, which were not significantly different from those in 0 h pups. Treatment with lipids, but not with glucose, induced UCP3 mRNA expression to levels similar to those in naturally suckled pups, similar to what was observed in skeletal muscle. A slight rise was observed in UCP2 mRNA levels following lipid intake (2.1 ± 0.3 -fold induction with respect to starved pups; $P \leq 0.05$), but no significant effects of glucose treatment were observed. In contrast, UCP1 mRNA levels were unaffected by lipid intake, but a significant rise (3.7 ± 0.4 -fold, $P \leq 0.05$), similar to that observed following suckling, was observed as a consequence of glucose treatment. When starved pups were treated with leptin, a dramatic rise was observed in UCP3 mRNA levels when compared with starved controls, although levels did not reach those in suckling or lipid-treated pups (67% of those in lipid-treated pups). Neither UCP1 mRNA nor UCP2 mRNA levels were modified significantly by leptin treatment.

Figure 2

Effects of leptin on UCP1, UCP2 and UCP3 mRNA expression in BAT after birth

Shown is a representative Northern blot analysis of the relative abundance of UCP1, UCP2 and UCP3 mRNAs in interscapular BAT of mice at birth (0 h) and in neonates 16 h after birth that were allowed (Fed) or not (Fasted) to suckle. Starved pups were treated with a lipid emulsion, glucose or a single injection of leptin, or acted as controls (-). The sizes of the corresponding transcripts are shown on the right. The band just below the 1.8 kb UCP2 mRNA is due to cross-reactivity of the UCP2 and UCP3 cDNA probes with UCP1 mRNA. The specific UCP1 mRNA signal, as assessed by the use of the UCP1 cDNA probe, is shown in the lower panel.



Discussion

The present findings show that UCP3 mRNA is induced after birth in skeletal muscle, and that initiation of food intake (particularly lipids) is required for this induction, in agreement with previous findings [3]. The regulation of UCP3 mRNA in the BAT at birth is essentially similar: there is a rapid postnatal burst that does not occur if there is no initiation of suckling, and this can also be elicited by lipid intake, but not by glucose intake. Thus both in skeletal muscle and in BAT the induction of UCP3 mRNA was associated positively with changes in the circulating levels of non-esterified fatty acids. As there is practically no white adipose tissue in the newborn mouse, non-esterified fatty acid levels must change in parallel with lipid intake; there is no rise in non-esterified fatty acids due to lipolytic activity [22]. UCP2 mRNA is induced after birth to a moderate extent, and is slightly sensitive to food intake.

UCP1 mRNA is induced after birth and, in conditions of low cold stress (30 °C), food intake is also required for this induction. However, for UCP1 mRNA the postnatal induction is mimicked more effectively by intake of glucose rather than lipid. This is in agreement with previous reports indicating that UCP1 gene expression is sensitive to glucose [23].

UCP3 mRNA expression in skeletal muscle and BAT from neonates was highly sensitive to leptin. However, no major effects of leptin were observed on UCP2 or UCP1 mRNA expression in either of the tissues analysed. The up-regulation of UCP3 mRNA by leptin was not associated with any change in non-esterified fatty acid levels, and therefore must occur via a distinct mechanism. In contrast with other experimental systems of leptin administration, the effects of leptin in the present experiment could not be mediated by modifications to food intake. Leptin may act through activation of the sympathetic nervous system [24] (which is active at this period of development [18]), especially in mediating UCP3 mRNA induction in BAT. However, the design of the experiments, in which mice were at a high environmental temperature close to thermoneutrality, and the lack of a significant effect of leptin on the expression of the UCP1 gene, which is extremely sensitive to sympathetic stimulation after birth [18], makes this possibility unlikely. The capacity of leptin to affect skeletal muscle or adipose cells directly is controversial, and different reports have provided positive [25,26] and negative [27,28] evidence. A direct action of leptin on BAT and skeletal muscle remains the most plausible explanation for the effects of leptin on UCP3 mRNA expression in the neonate. Further research will be needed in order to establish why high levels of leptin in the late fetal period do not lead to activation of UCP3 gene expression. Leptin receptors have been detected in several tissues of the mouse fetus [29], but specific assessment of the ontogeny of leptin receptor expression in muscle and BAT during the perinatal period will require further studies. Moreover, the role of myogenic or adipogenic transcription factors in determining the 'switching on' of UCP3 gene transcription [8] needs to be established *in vivo*.

The role of leptin in the induction of UCP3 mRNA at birth may have a relationship with food intake. After birth, there is a transient peak in circulating leptin levels that has been attributed to BAT [15,17]. However, high levels of leptin after birth require food intake, and circulating leptin is

undetectable if neonates are not allowed to suckle [15]. Thus leptin may contribute to the suckling-dependent induction of UCP3 gene expression after birth. Moreover, the two mechanisms of induction of UCP3 gene expression may be interrelated since, under the present conditions of treatment, leptin up-regulates the expression of peroxisome proliferator-activated receptor α , the main mediator of the effects of fatty acids on UCP3 gene expression [3] in BAT (M. Giralt, R. Iglesias and F. Villarroya, unpublished work). Previous findings have shown that young mice were sensitive to chronic leptin treatment, especially the effects of leptin in promoting energy expenditure [30].

Our present findings indicate that the UCP3 gene is the most sensitive of the UCPs to the effects of leptin in the neonatal period. If this protein is involved in energy expenditure, it may be a major target of leptin immediately after birth. Minor effects of leptin on UCP1 and UCP2 gene expression in BAT may also contribute. Moreover, our results indicate that, whatever the mechanism of action of leptin in BAT and skeletal muscle, it is already functional at birth.

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Fetal and neonatal adipose maturation: a primary site of cytokine and cytokine-receptor action

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Abstract

During late gestation, the maturation of fetal adipose tissue is geared towards the synthesis of high levels of uncoupling protein 1 (UCP1), which is unique to brown adipose tissue. At birth, rapid activation of UCP1 ensures a large increase in heat production. These adaptations are nutritionally sensitive, and may be mediated in part by rapid changes in prolactin and leptin secretion after birth. Restriction of maternal nutrition reduces adipose tissue deposition, with no effect on UCP1. Increased maternal food intake results in increases in levels of UCP1 and the short form of the prolactin receptor, but in a decrease in adipose tissue content per kg of fetus. The ontogeny of the

long and short forms of the prolactin receptor follows that of UCP1, to peak at birth. Then, during postnatal life, UCP1 disappears in parallel with the loss of prolactin receptors. Treatment of neonatal lambs with prolactin increases body temperature and the thermogenic potential of brown adipose tissue. In contrast, acute leptin treatment results in maintenance of colonic temperature, but chronic leptin treatment accelerates UCP1 loss. Increasing our understanding of the interaction between prolactin and leptin during perinatal development may enable the establishment of strategies aimed at maximizing adipose tissue development in order to promote metabolic adaptation to the extra-uterine environment.

Introduction

Adipose tissue development commences *in utero*, with the adipocyte lineage being derived from stem cell precursors which have the potential to become brown adipose tissue (BAT) or white

Key words: adipose tissue, fetus, leptin, prolactin receptor, uncoupling protein.

Abbreviations used: BAT, brown adipose tissue; UCP, uncoupling protein.

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The cross-talk among mitochondrial activity, reactive oxygen species production and apoptosis in skeletal muscle. A role for uncoupling protein-3?

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Abstract

UCP3 is a mitochondrial protein preferentially expressed in skeletal muscle mitochondria. Like other uncoupling proteins, it lowers mitochondrial membrane potential and reactive oxygen species (ROS) production as well as it favours fatty acid oxidation. Both its amount and activity may control responsiveness of skeletal muscle cells to challenges involving

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mitochondrial activity. ROS are major by-products of mitochondrial respiratory activity. Excessive ROS production results in oxidative stress, which usually damages cell function and may elicit apoptosis. However, ROS can also act as a physiological signal in relation to multiple cell functions, including promotion of cell survival. Skeletal muscle is a highly specialized tissue in which mitochondrial ROS production is relevant to physiological (contraction, exercise) or pathological situations. Differentiated muscle cells are particularly resistant to apoptosis. However, oxidative stress appears to be associated with apoptosis activation in skeletal muscle in such conditions as ageing, muscle dystrophy, ischemia/reperfusion, and others. UCP3 alters the sensitivity of muscle cells to apoptosis activation, although the relationship of this effect with the regulation of ROS production is unclear. UCP3 might serve as a potential target for pharmacological or nutritional therapies attempting to influence skeletal muscle via modulation of mitochondrial respiration, ROS production and apoptotic cell death.

Abbreviations

ROS, reactive oxygen species, SOD, superoxide dismutase, TNF α , tumor necrosis factor alpha, UCP, uncoupling protein, NF κ B, nuclear factor kappa B

1. Introduction

Physiological uncoupling of the mitochondrial respiratory chain with respect to oxidative phosphorylation has been studied extensively during the last decades within the wider context of thermogenesis. The mitochondrial uncoupling protein-1 (UCP1) was the first member of the uncoupling protein family to be identified. Its ability to permeabilize the mitochondrial inner membrane to protons leads to the thermogenic capacity of mitochondria in brown adipose tissue, the only tissue in which UCP1 is present. Since the 1997 discovery of other mitochondrial uncoupling proteins, particularly UCP2 and UCP3, which are expressed in a variety of human tissues, a complete re-evaluation of the functional role for this family of proteins has been undertaken. Claims have been made linking such mitochondrial UCPs as UCP1 to thermogenesis, but the evidence accumulating so far indicates these proteins are involved in controlling the production of reactive oxygen species (ROS) in mitochondria.

UCP3 is a mitochondrial uncoupling protein preferentially expressed in the skeletal muscle of humans and rodents. Skeletal muscle is a highly specialized tissue, and only a comprehensive understanding of the roles of mitochondrial metabolic activity and ROS production, as well as their relationships to oxidative stress and cell viability in this tissue, can provide the appropriate context to consider the biological relevance of UCP3 activity. This

review describes present knowledge of ROS biology in skeletal muscle, the role of mitochondrial activity in ROS production, how this may influence cell death in muscle, and, finally, the effects of mitochondrial uncoupling in these processes. Advances in this field are of the outmost relevance for biomedical research in identifying targets to influence skeletal muscle mitochondrial activity and establishing the potential role of UCP3 in therapeutic approaches to muscle diseases.

2. Reactive oxygen species production (ROS) by mitochondria. What happens in muscle?

2.1. The chemistry of ROS

The reactive oxygen species (ROS) includes all molecular oxygen-derived molecules. Although ROS have often been categorized as free radicals, this is not strictly true. A free radical is defined as an atomic or molecule species capable of independent existence and containing one or more unpaired electrons in one of its molecular orbitals [1]. As reviewed in a previous chapter, the first ROS generated in biological systems is the superoxide anion $O_2^{\cdot-}$ a free radical generated by direct one-electron reduction of molecular oxygen. $O_2^{\cdot-}$ is short-lived and poorly reactive, its “instability” the result of rapid dismutation of $O_2^{\cdot-}$ in H_2O_2 , hydrogen peroxide [2]. This reaction can be spontaneous or enzymatic, involving the superoxide dismutase, which is present in cells (in mitochondrial matrix, inter-membrane space and cytosol) and in extracellular compartments [3]. Although dismutation of $O_2^{\cdot-}$ probably accounts for much of the H_2O_2 produced by eukaryotic cells, H_2O_2 can also be formed by a direct two-electron reduction of O_2 , a reaction mechanism shared by many flavoprotein oxidases [4]. Unlike $O_2^{\cdot-}$, H_2O_2 is not a free radical and is a much more stable molecule. H_2O_2 can diffuse across biological membranes, whereas $O_2^{\cdot-}$ cannot. Although H_2O_2 is a weaker oxidizing agent than $O_2^{\cdot-}$, in the presence of transition metals it can give rise to the indiscriminately reactive and toxic hydroxyl radical (OH^{\cdot}), through the Fenton chemical reaction. OH^{\cdot} can react with polyunsaturated fatty acids lending the radicals alkoxy (RO^{\cdot}) and peroxy (ROO^{\cdot}). Still another family of reactive molecules exists in biological systems: the reactive nitrogen species (RNS), which also includes some free radicals. Nitric oxide (NO^{\cdot}), the nitrogen dioxide (NO_2^{\cdot}), and the highly reactive peroxynitrite ($ONOO^{\cdot}$), which result from the reaction between NO^{\cdot} and $O_2^{\cdot-}$, are examples of RNS.

The high reactivity and relative instability of ROS and RNS make them extremely difficult to detect and measure in biological systems. Thus, assessments of ROS and free radical generation are usually achieved by indirect measurement of various end products resulting from the interaction of ROS with such cellular components as lipids, DNA and proteins [5,6]. Most

methods for the identification of specific ROS are based on reactions with various “detector” molecules that are oxidatively modified to elicit luminescent or fluorescent signals [2].

2.2. Mitochondria: The main source of ROS in nonphagocytic cells

Cellular production of ROS stems from both enzymatic and non-enzymatic processes. Any electron-transferring protein or enzymatic system can generate ROS as “by-products” of the electron transfer reaction. This is the case for cytochrome P450, found in the endoplasmic reticulum, peroxisomes, xanthine oxidase or NADPH oxidase. However, the principal source of ROS in non-phagocytic cells is mitochondria. Quantitative data, obtained on isolated mitochondria, suggest that about 2-6% of oxygen consumption is due to superoxide anion generation, with about 80 % of cellular O_2^- generated by mitochondria.

Molecular oxygen (dioxygen: O_2) is essential for the survival of aerobic organisms. Aerobic energy metabolism is dependent on oxidative phosphorylation, a process by which the oxido-reduction energy of mitochondrial electron transport (via a multi-component NADH dehydrogenase enzymatic complex) is converted to a high-energy phosphate bond of ATP. This process occurs in the mitochondrial respiratory chain. O_2 acts as the final electron acceptor for cytochrome c-oxidase, the last component in this mitochondrial enzymatic complex, which catalyzes the reduction of O_2 to H_2O . However, under physiological conditions, incomplete reduction of molecular oxygen may occur. Indeed, superoxide anion is generated as a by-product of electron transfer through the respiratory chain complexes, which are organized in the order of their redox potential. Some participate in one-electron transfers, whereas others can react in one- or two-electron transfers, resulting in the formation of free radicals. Among these, complexes I and III are the major sites of ROS production [7,8].

There is growing evidence that complex I produces most of the O_2^- generated by intact mammalian mitochondria *in vitro*, although the generation mechanism is less clear than in complex III. Results obtained on isolated mitochondria have shown that in state 4 (without ADP), and in the absence of electron transport chain inhibitors, the rate of O_2^- production by the respiratory chain is high when succinate is used as substrate. However, this elevated rate of O_2^- production is blocked by the inhibitor of complex I rotenone, suggesting that in these conditions most O_2^- production occurs at complex I and not at complex III [9,10]. Therefore, high O_2^- generation from complex I occurs during reverse electron transport, from succinate to NAD^+ . This electron transport is driven by the high proton motive force generated from proton pumping by complexes III and IV. This ROS production is inhibited by the addition of uncoupling molecules or ADP, suggesting that reverse electron

transport occurs only when membrane mitochondrial potential is high [10]. When the respiratory chain is working normally, and membrane mitochondrial potential is weak, electrons are transferred from complex II to complex III (see below). The site of O_2^- production at complex I remains the subject of debate: it could result from an Fe-S centre or from an FMN group [11,10]. In any case, it seems likely that this O_2^- production occurs primarily in the matrix side of the inner membrane [12,13].

O_2^- generation by complex III is due to ubisemiquinone, a free radical intermediate (QH^\cdot), formed during the Q cycle at the Q_o site of complex III [12,14,15]. The generation of ROS is accelerated by complex III inhibitors distal from this point, such as antimycin A. Most O_2^- from Q_o is produced facing the intermembrane space [12] suggesting that O_2^- may be present only in the mitochondrial matrix. Alternatively, O_2^- might be released in the intermembrane space via mitochondrial membrane pores since it is not capable of crossing biological membranes. The local availability of Cu-Zn superoxide dismutase (SOD) in the mitochondrial matrix, as well as in the intermembrane space, results in the dismutation of O_2^- into H_2O_2 . Mitochondrial H_2O_2 can cross biological membranes, and therefore it can circulate far from its site of production.

An important regulator of ROS generation by mitochondria is the mitochondrial membrane potential. The generation of ROS is exponentially dependent of this potential. Thus, both chemical uncouplers [16] and UCPs appear to decrease mitochondrial ROS [17-19] (see below).

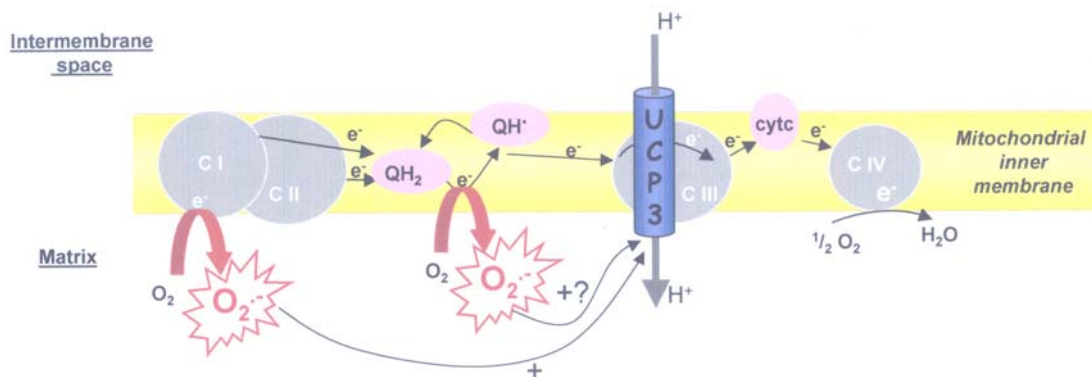


Figure 1. Schematic representation of the main sites of ROS production in respiratory chain and localization of UCP3. Complex I produce most of the O_2^- generated by intact mammalian cells, although the mechanism of generation is less clear than for complex III. The O_2^- generation by complex III is due to the ubisemiquinone radical intermediate, formed during the ubiquinone cycle. The O_2^- is produced in the mitochondrial matrix where it will be dismutated in H_2O_2 . O_2^- could also be able to activate UCP activity, particularly, O_2^- generated by complex I could activate UCP3. This scheme also illustrates recent reports of ubiquinone of an obligatory partner of UCPs and the proposed placement of UCP3 (see last part of the chapter for details)

2.3. The cellular effects of ROS

In mitochondria, O_2^- content must be efficiently controlled in order to protect itself and the cell from oxidative damage. A cascade of enzymatic systems (SOD, catalase, glutathione peroxidase) can scavenge O_2^- and its metabolites as soon as they are produced. Moreover, there are antioxidant molecules able to directly scavenge ROS, as reviewed in a preceding chapter.

ROS have been historically regarded as toxic by-products of metabolism, implicated in widespread damage to lipids, proteins (see previous chapter) and numerous pathologies. However, the accumulated evidence suggests that ROS, regardless of origin, may serve as subcellular messengers in gene regulatory and signal transduction pathways. [20,21] In fact, cellular ROS depends on the balance between generated ROS and the antioxidant capacity of the cell. While, under physiological conditions, the damaging effects of ROS are counteracted by antioxidants, changes in cellular ROS can elicit modifications in both gene expression and regulatory signalling pathways. This usually involves alterations in the activity of the MAP kinase and $TNF\alpha$ signalling pathways, as well as in $NF\kappa B$ -dependent transcriptional regulation (for review see [20]). Indeed, $TNF\alpha$ acts on many cell types via multiple secondary messengers, in particular ceramide [22] which induces mitochondrial ROS formation by inhibition of complex III [23-26].

A new relationship between energy metabolism and mitochondrial ROS has emerged in recent studies [27,28] showing that high levels of metabolic substrates (fatty acids, glucose) may induce increases in metabolite flow to the mitochondria and subsequent release of ROS to the cytosol. This would involve detection by as yet unidentified cytosolic regulators and metabolic flow would be redirected towards storage pathways, thus decreasing the metabolic pressure towards mitochondria and consequently preventing ROS release. Therefore, ROS production could be one of the signals controlling the balance between storage and oxidation in the maintenance of cellular energetic homeostasis. In this proposed mechanism of metabolic sensing, mitochondrial ROS might function as signalling molecules facilitating communication between the mitochondria and the cytosol.

On the other hand, an imbalance (excessive generation of oxidants or decrease in antioxidant capacity) in which the effects of ROS are more potent than the compensatory capacity of antioxidants results in what is called "oxidative stress". In this scenario, oxidant effects of ROS induce irreversible modifications of biological molecules resulting in tissue damage. For instance, ROS can alter protein structure and function by modifying critical amino acids residues [29,30], causing lipid peroxidation and subsequent production of toxic by-products such as pentane, malondialdehydes, lipid hydroperoxides, isoprostanes, and conjugated dienes [31]. ROS can even damage DNA,

particularly mitochondrial DNA, due to its close proximity to the respiratory chain and lack of protection by histones.

Finally, a critical process involving ROS in the cell, either at non-toxic levels or in relation to oxidative stress, is the control of cell death mechanisms, principally apoptosis, as discussed below.

2.4. ROS and disease

Oxidative stress is associated with several pathologies. A multitude of theories exists attempting to explain the relationship between ROS-mediated damage and the initiation and progression of disease. In many diseases, primary mitochondrial involvement is profound and plainly evident, whereas in many others settings, mitochondrial participation is only suspected or suggested. Similarly, etiopathology in numerous disease settings is hampered by a limited understanding of the relevance of mitochondrial-dependent activation of apoptotic or necrotic pathways due to oxidative stress.

In the case of mitochondrial diseases, in which alterations in mitochondrial DNA are relevant to the origins of the pathology, increases in ROS production and decreases in ATP production can cause oxidative phosphorylation deficiency, resulting in mitochondrial disturbances [32]. In addition, a primary defect in oxidative phosphorylation may increase O_2^- production, thereby increasing oxidative stress and consequent damage to mitochondrial DNA. This, in turn, results in more deficient oxidative phosphorylation and ROS production, thereby initiating a cascade of amplifying disturbances that lead ultimately to oxidative stress. In the last 15 years, several clinical syndromes have been associated with mitochondrial DNA mutations, although the extent of ROS involvement in the pathogenicity of each diseases has not been clearly established (see preceding chapter).

Oxidative damage is also a common occurrence in neurodegenerative diseases. Evidence of oxidative damage in Parkinson's disease [33,34], Alzheimer's disease [35,36], amyotrophic lateral sclerosis [37,38], Huntington's disease [39], and Friedrich's ataxia [40,41], has been extensively reported. However, the involvement of mitochondrial dysfunction and ROS production as primary agents in the etiopathogenesis of these diseases remains unclear. Enzymatic deficiencies in the electron transport chain were identified in Alzheimer's disease, amyotrophic lateral sclerosis (complex IV), Huntington's disease, and Friedrich's ataxia (complex II and III), but any observed respiratory deficiencies are most likely secondary to the pathogenic initiating factors at work [42].

Oxidative processes have also been implicated in atherosclerosis and the resulting cardiovascular alterations. There is a consensus of opinion that atherosclerosis represents a state of heightened oxidative stress characterized by lipid and protein oxidation: i.e. low-density lipoprotein oxidation is an early

event in atherosclerosis and oxidized LDL contributes to atherogenesis [43]. The exact source of ROS implicated in LDL oxidation remains the subject of debate [44-47]. Nonetheless, mitochondria could play an indirect role in LDL oxidation as shown in a recent study [48].

The study of oxidative DNA damage is another clinically important parameter [1], particularly because it links ROS production to the pathogenesis of cancer. Chemical carcinogenesis consists of a multi-step process involving both mutation and increased cell proliferation. Prolonged production of cellular ROS is not only related to DNA mutation, but also to modifications of gene expression as mediated by such transcription factors as AP-1 and NF κ B, known to be activated by ROS [49].

2.5. ROS in skeletal muscle physiology. Contraction and exercise

Over the past decade, ROS have been established as physiological modulators of skeletal muscle function. Existing data indicate that ROS are permanently generated in the muscles of healthy individuals, modulating processes ranging from development to metabolism and from blood flow to contractile function. It is known that resting skeletal muscle produces $O_2^{\cdot-}$ at a low rate, but that this dramatically accelerates during contractile activity. Thus, as an important site of ROS production, skeletal muscle must be protected from ROS damage and this is accomplished by the presence of antioxidant enzymes (SOD, GPx, catalase,...). Among non-enzymatic antioxidants, reduced glutathione appears as among the more important due to its high concentration (0.5 to 3 mM) in skeletal muscle.

ROS have biphasic effects on the contractile function of unfatigued skeletal muscle. The low ROS levels present under basal conditions are essential for normal force production. Selective depletion of ROS from unfatigued muscle by way of SOD or catalase causes a fall in force. Conversely, modest ROS supplementation causes force to increase. This positive effect is reversed at higher ROS concentrations, when force production falls in a time- and dose-dependent manner. Negative effects can be inhibited by pre-treating muscles with antioxidants or even be reversed by post-hoc administration of reducing agents [50]. The rise in ROS production that occurs during strenuous exercise contributes to the development of acute muscle fatigue. Muscle-derived ROS are generated faster than they can be buffered by endogenous antioxidants, and, as ROS accumulate in the working muscle, they inhibit force production. This is comparable to the drop in force that occurs when unfatigued muscle is exposed to high levels of exogenous ROS.

A single source of ROS cannot be responsible for all the ROS generated by exercising muscle [51]. The rise in intracellular ROS during repetitive

muscle contraction appears to be mediated by the 14 kDa isoform of phospholipase A₂, PLA₂ [52] and is dependent on the influx of intracellular calcium [53]. To a large extent, physical exercise increases energy demand and, to support it, oxygen flux through active muscle may increase approximately 100-fold above resting values. It has therefore been argued that substantial increases in mitochondrial O₂⁻ production cannot be avoided since O₂⁻ generation in mitochondria is linearly dependent on oxygen tension. Moreover it has been observed that mitochondria isolated from muscles of normal animals release H₂O₂. This signal is stimulated by calcium, diminished by PLA₂ inhibitors, and exaggerated in mitochondria from septic animals [54]. These observations implicate mitochondria as the major source of ROS in skeletal muscle and identify PLA₂ as a regulator of ROS production. This model is consistent with previous reports indicating that mitochondria are the source of ROS in TNF α -stimulated muscle [55].

An alternative mechanism by which exercise may promote ROS production involves ischemia-reperfusion. Intense exercise is associated with transient tissue hypoxia in several organs as blood is shunted away to cover the increased blood supply in active muscle and skin. In addition, muscles fibers may undergo relative hypoxia, as oxygen supply is unable to match energy demand [56]. Re-oxygenation of these tissues occurs after cessation of exercise, and is tied to production of ROS [57,56]. One way in which reperfusion could lead to increased ROS production is through the conversion of xanthine dehydrogenase to xanthine oxidase. Both enzymes catalyze the degradation of hypoxanthine into xanthine, and subsequently into urate. However, only xanthine oxidase produces O₂⁻ during the final step of this reaction. Production of ROS via this mechanism leads to oxidative stress several hours after exercise, and is not restricted to skeletal muscle [56]. The involvement of ROS in physiological adaptations to exercise also implicates gene expression [58]. More than twenty years ago, it has already been hypothesized that ROS produced in exercising muscle might stimulate mitochondrial biogenesis [59]. Growing evidence supports the theory that gene regulation in muscle might be redox sensitive. Muscles adapt to exercise by up-regulating gene expression for such antioxidant enzymes as glutathione peroxidase, SOD and catalase [60]. Oxidative signalling may mediate this response since it is blocked by antioxidant supplementation [61,62]. It is therefore to be expected that any modification in the mechanisms of ROS production or scavenge will impact overall gene expression in skeletal muscle.

2.6 ROS in skeletal muscle pathologies

Multiple factors other than contraction and exercise may alter ROS production in skeletal muscle and, under the oxidative stress settings, elicit pathological conditions. Aging, as reviewed in a previous chapter, appears to

increase the oxidant load to which muscles are exposed [63]. Muscle injury, due to reperfusion or straining, also results in oxidative stress and can be linked to loss of function. Indeed, following mechanical loading or injury, skeletal muscle cells become an important source of soluble factors influencing neutrophil chemotaxis and neutrophil-derived ROS production in situ [64]. Finally, muscles may experience oxidative stress in such inflammatory disease processes, as hyperthyroid myopathy, sepsis, heart failure and chronic obstructive pulmonary disease [50]. ROS formation is markedly enhanced during diaphragm contraction in endotoxin-treated septic animals [65]. Indeed, it has been suggested that ROS generated in this situation plays a central role in sepsis-related skeletal muscle dysfunction [65]. It has been shown that limb skeletal muscles from mice suffering heart failure following myocardial infarction exhibit increased ROS generation and preserved antioxidant enzyme activity. This may well explain the metabolic abnormalities and exercise intolerance commonly observed in patients with heart failure [66]. Patients with dystrophinopathy [67] or chronic alcoholic myopathy [68] have also reportedly show oxidative stress in skeletal muscle, and, in the latter condition, antioxidants were effective in preventing some muscle disturbances [69].

3. Mitochondria, ROS and apoptosis

3.1. The role of mitochondria in apoptosis

Cell death is a common process within a living tissue and may occur either by necrosis or apoptosis. Although both processes may co-exist within the same tissue, apoptosis is a genetically programmed process, differing in morphology, regulation and even in biological significance from necrosis. While necrosis and apoptosis account for numerous destructive effects associated with pathological conditions, apoptosis is also a necessary process ensuring normal development and tissue homeostasis. In fact, a misregulation of apoptosis can lead to severe defects due either to excess or a lack of cell death. Stereotypical morphological features of the apoptotic process include cell shrinkage, membrane blebbing, cytoplasm condensation, nuclear condensation, and DNA fragmentation. Membrane budding produces membrane-enclosed apoptotic bodies containing intact organelles. This allows their processing by phagocytes without the occurrence of inflammation [70].

Apoptosis can be triggered both by external or internal stimuli. The external pathway starts via ligand-mediated activation of specific plasma membrane death receptors, while the internal pathway originates within mitochondria.

Apoptosis can be induced by the binding of ligands to one or more of the extracellular receptors of the tumour necrosis factor receptor superfamily. The most important ligands are the cytokine TNF α and Fas-ligand which are

present in immune cells. The binding of ligands to their receptor leads to activation of caspases. Caspases are a set of proteases that are activated by proteolytic cleavage and can activate other caspases in a 'cascade-like' way consequently amplifying cell death signal. Eventually, caspases are the effectors responsible for the biochemical breakdown of cytosolic and nuclear targets leading to the morphological features of apoptosis [71].

The mitochondrial pathway for induction of apoptosis is defined by a crucial event termed mitochondrial outer membrane permeabilization (MOMP). MOMP occurs suddenly in apoptosis, leading to the release of proteins normally located in the mitochondrial intermembrane space. MOMP is often accompanied by a dissipation of inner mitochondrial membrane potential that takes place before, during, or as a consequence of permeabilization. MOMP causes cell death by three general mechanisms: a release of proteins activating caspases responsible for downstream events often associated with apoptosis, the release of molecules involved in caspase-independent cell death, and the loss of mitochondrial functions essential for cell survival [72]. Which primary signals lead to MOMP remains controversial but two general mechanisms have been described, one involving the inner mitochondrial membrane and the other involving only the outer mitochondrial membrane. The former involves the formation of a pore in the mitochondrial membrane, known as the permeability transition pore (PT), which allows passage of water and solutes up to 1.5 kDa. The classical hypothetical model for the formation of this pore suggested primary roles for both the adenine nucleotide translocator (ANT), at the inner mitochondrial membrane, and the voltage-dependent anion channel (VDAC), at the outer mitochondrial membrane. However, the actual constituents of the PT pore remain a matter of debate due to recent evidence on pore formation in the absence of ANT [73]. The PT pore opening is favoured by Ca^{2+} and open-closed transitions are modulated by such factors as trans-membrane electrical potential, matrix pH, redox potential, adenine nucleotides, and divalent metal anions. Finally, the PT pore can be induced or inhibited by a variety of drugs [74]. Opening of the PT pore causes dissipation of membrane potential perhaps sufficient to produce swelling of the matrix and, ultimately, outer membrane breakdown. However, loss in membrane potential is not enough to guarantee PT pore involvement since there exist other triggering events. Furthermore, the PT opening can be transient [75], and is far from being an irreversible process.

The second mechanism for mitochondrial-dependent apoptosis is mediated by members of the Bcl-2 family of apoptosis-regulating proteins, which act directly on the outer mitochondrial membrane. This family includes both pro-apoptotic (i.e. Bax, Bak) and anti-apoptotic (i.e. Bcl-2, Bcl-X_L) members that promote or block MOMP, respectively [76, 77]. These proteins may affect the function of one another by forming heterodimers. The relative proportion of

pro- and anti-apoptotic proteins appears to be important in determining cellular fate when challenged with pro-apoptotic stimuli. Activated pro-apoptotic proteins such as Bax or Bak can generate large lipidic pores by interaction with lipids prone to negative curvature in membranes, thereby inducing MOMP [78]. However, PT-independent MOMP can be followed by secondary PT [79]. Bcl-2 members not only interact functionally and/or physically with proteins believed to comprise the PT pore, but also known participate in intermediary metabolism (i.e. VDAC, hexokinase) [80]. These interactions most likely establish a “cross-talk” between metabolism and apoptosis, tying control of the cell death process to specific metabolic demands.

The release of proteins from the intermembrane space, as well as loss of mitochondrial functions due to MOMP, promotes downstream events that ultimately lead to cell death. Whether or not all of the proteins are released simultaneously is not clear [81]. Cytochrome c induces caspase-dependent cell death whereas other released proteins act as caspase-independent death effectors. Cytochrome c participates in electron transference through the respiratory chain, but, when released into the cytoplasm, interacts with both Apaf-1 protein and activated caspase-9 generating an apoptosome, the catalytic complex eliciting caspase-cascade activation. Effectors of caspase-independent cell death are mitochondrial proteins that can translocate to the nucleus and promote DNA breakdown (i.e. apoptosis inducing factor AIF and Endo G) [82].

Programmed cell death is a complex process highly regulated not only by Bcl-2 family members but also by different apoptosis repressors. These repressors can be regarded as ‘death checkpoints’ for the cell. There are inhibitors primarily responsible for blocking receptor-mediated apoptosis via interaction with initiator caspases (i.e. ARC and FLIP). This is a group of proteins termed inhibitors of apoptosis proteins (IAPs) which have both upstream (caspase-9) and downstream (caspase-3) inhibitory targets to prevent apoptosis [83]. Some of the pro-apoptotic factors released by mitochondria during MOMP act through inhibition of several IAPs (i.e. Smac/Diablo, Htra2) [82].

3.2. ROS and the mitochondrial control of apoptosis

Apoptosis is an active cellular self-destruction process that depends on ATP availability. In fact, when ATP levels are insufficient, a cell that was initially dying by apoptosis will follow the necrotic pathway. The susceptibility of cells to either apoptosis or necrosis is highly influenced by levels of oxidative stress, which, as mentioned above, results from imbalances between ROS production and antioxidant capacity. As previously shown, the mitochondrial respiratory chain is a primary site of ROS production within the cell. Other sources of ROS include radiation, cytotoxic chemicals and drugs.

While cells contain different anti-oxidant mechanisms to counteract excessive ROS production, when this production overwhelms anti-oxidants defense, oxidative stress occurs.

Increases in ROS are associated with both the early and late stages of apoptosis regulation, often as side effects of other changes. Major increases in ROS can be detected during the early stages of apoptosis when caused by p53 activation, ceramide release and under the action of staurosporine or TNF α . Moreover, free radicals such as hydrogen peroxide or superoxide, as well as free radical amplifiers such as paraquat, can act as instigators of apoptosis [84, 85]. Thus have antioxidants been proven to impair programmed cell death in different cell systems [86, 87, 88] and defective antioxidant mechanisms been shown to trigger or sensitize cells to apoptosis [89, 90].

Oxidative stress provokes a global shut down of mitochondrial function, thereby contributing to cell death through decreases in cellular energy supply. Transient and limited ATP deprivation correlates to apoptosis while dramatic ATP depletion is more related to necrosis. However, while inhibitors of respiration, ATP synthase and chemical uncouplers do not necessarily affect cell viability, apoptosis has been induced in HeLa cells by a combined action of respiratory inhibitors and uncouplers, a setting whereby large amounts of ROS are formed, indicating possible pro-apoptotic action of ROS as generated by mitochondria [91].

Maximal ROS production is associated with high mitochondrial membrane potential and low electronic flux. Rotenone, an inhibitor of Complex I in the respiratory chain, has been shown to independently induce apoptosis by enhancing the amount of mitochondrial ROS [92]. Furthermore, inhibition of Complex II in the respiratory chain has recently been proven to induce Ca²⁺ release from mitochondria through the PT pore opening, probably due to increases in ROS production, and thus leading to motor alterations observed in several neurodegenerative disorders [93].

In addition to the bioenergetic activity of mitochondria, Ca²⁺ homeostasis plays a pivotal role in the cross-talk of ROS with mitochondrial-dependent signaling of apoptosis. Intra-mitochondrial Ca²⁺ is known to promote ROS production [94], which is thought to play a role in Ca²⁺-induced PT pore opening [95]. One mechanism proposed for Ca²⁺-induced apoptosis is related to cardiolipin oxidation. Cardiolipin is a phospholipid located in the inner mitochondrial membrane binding cytochrome c to the outer surface of this membrane during respiration. It has been reported that Ca²⁺-induced ROS production may involve cardiolipin oxidative damage with consequent alterations in membrane solubility and cytochrome c release following MOMP [96].

Moreover, it should be recalled that maintenance of mitochondrial membrane integrity is a dynamic process and, under severe oxidative stress, PT pore opening occurs. MOMP can be induced by pro-oxidant agents acting

directly on PT pore constituents, on members of the Bcl-2-family, or as a consequence of lipid and protein peroxidation which modify membrane constitution. This leads to mitochondrial swelling and release of pro-apoptotic factors [97]. Moreover, ROS derived from mitochondrial permeability transition may influence apoptotic events downstream from MOMP, e.g., apoptosome formation during Fas-mediated apoptosis [98].

ROS are involved in the modulation of apoptosis as exerted by members of the Bcl-2 family. In fact, ROS regulate the phosphorylation and ubiquitination of Bcl-2 family proteins resulting in increased levels of proapoptotic proteins [99]. Furthermore, Bcl-2 (or Bcl-x_L) might act as an antioxidant agent to block a putative ROS-mediated step in the cascade of events required for apoptosis either affecting ROS production [100,101] or preventing oxidative damage to cellular constituents [102] and thereby inducing endogenous cellular antioxidants [103].

Finally, ROS can independently promote apoptosis via their interaction with biological macromolecules (i.e. lipids, proteins, nucleic acids and carbohydrates), as exemplified above by cardiolipin. ROS interactions with these molecules generate new secondary radicals, initiating a continuum of chain reactions that can lead to lipid peroxidation, DNA damage, growth inhibition, or cell death, as already mentioned [104]. DNA damage may be particularly relevant to mitochondrial DNA. Compared with nuclear DNA, the mitochondrial genome appears to be more sensitive to oxidative damage due to a lack of DNA-protecting proteins, a low efficiency reparation mechanism, and the proximity of the respiratory chain [105]. Indeed, alterations of mitochondrial DNA can progressively diminish mitochondrial function (see below).

It is important to note that ROS should not solely be identified as promoters of cell death. In fact, ROS have been shown to possess beneficial effects on cell survival when not exceeding their detoxification capacity. These beneficial properties of ROS on cell survival are essentially related to their capacity to act as signalling messengers inside the cell [106, 107]. Important evidence suggesting a role for ROS as secondary messengers is their ability to activate NFκB. This transcription factor regulates the inducible expression of several genes involved in cell survival and execution. NFκB is known to control both anti-apoptotic genes such as Bcl-2, and pro-apoptotic factors such as Bax and p53 [108]. Recently, ROS removal has been shown to promote cell death under different situations, suggesting that oxidative stress may promote signalling mechanisms in cell survival [109-111]. In fact, exposing the heart to ROS by intermittent ischemia, a process commonly known as preconditioning, initiates different signalling pathways (i.e. NFκB, MAP kinase cascades...) which may convert an initial cell death signal into a survival signal, thereby diminishing subsequent ischemia/reperfusion injury [112]. Moreover, ROS

induce the expression of antioxidant defenses such as thioredoxin [113], which has been shown to protect myocardial cells against hypoxia-reoxygenation injury [114].

3.3. Lipoapoptosis: ROS involvement in fatty acid effects on apoptosis

Long-chain fatty acids, particularly palmitate, behave as major activators of apoptosis in several cell types, from pancreatic beta-cells to cardiomyocytes. The term “lipoapoptosis” has been coined to describe the apoptotic process elicited by fatty acids [115, 116]. It is believed that, when fatty acid influx into a cell exceeds its oxidation capacity, fatty acids enter other metabolic pathways, principally esterification to triglycerides. Triglycerides in low amounts are not particularly harmful to most cell types, but they are a source of fatty acids through lipolysis, and intracellular free fatty acids promote deleterious pathways, including apoptosis. A major intracellular mediator of fatty acid action on apoptosis is ceramide, which similarly mediates apoptotic responsiveness to TNF α or staurosporine (see above). The interaction of palmitoyl-CoA with serine activates a pathway of “de novo” synthesis of ceramine, which in turn activates the production of nitrogen reactive species and probably of ROS, as these molecule types are involved in the activation of apoptotic pathways. However, palmitate also activates ROS production through mechanisms independent of ceramide synthesis. Thus, increases in ROS can be the direct result of fatty acid effects through alterations in mitochondrial respiratory chain and uncoupling [117] or through activation of non-mitochondrial pathways such as NAD(P)H oxidase activities [118].

However, the action of palmitate on apoptosis is not always dependent on ROS [119]. Another potential mechanism of fatty acid effects on apoptosis is their direct interaction with mitochondrial components. Fatty acids bind membranes and possess the potential to alter their permeability, favour uncoupling, and induce opening of the mitochondrial PT pore, thereby activating a cascade of apoptotic events [120, 121]. The relative intensity of these effects appears to be dependent on nature of the fatty acid (length, extent of de-saturation,...) and the diverse mechanisms involved. For example, while both palmitic acid and arachidonic acid open the mitochondrial PT pore, the action of the latter is more direct. In fact, arachidonic acid which originates from cytosolic phospholipase A(2) activity, has been suggested as mediator of TNF α action on apoptosis via the induction of the mitochondrial PT [122].

Lipoapoptotic processes constitutes one example of cross-talk among metabolism, ROS and apoptosis, a process particularly relevant in such disease settings as diabetes, lipoatrophy of adipose tissue or cardiomyopathies, which involve disturbances in lipid metabolism and availability. However, the

apoptotic potential of fatty acids is not always deleterious and may, in fact, be beneficial for long-term health. Epidemiological evidence has established that ingestion of long-chain polyunsaturated omega-3 fatty acids, such as docosahexaenoic acid or eicosapentaenoic acid, abundant in fish oils, has positive effects on many human disorders and diseases, including cardiovascular disease and cancer [123]. Although the action of omega-3 fatty acids is complex and involves a number of integrated signalling pathways, recent studies in cancer cells suggest that docosahexaenoic acid induces cell cycle arrest and apoptosis through mechanisms involving both Bcl2 and regulation of cytochrome c release from mitochondria [124].

4. Apoptosis in skeletal muscle

Skeletal muscle can undergo apoptosis as post-mitotic tissue, both in response to specific physiological stimuli or as part of a pathological process. Although studies on apoptotic pathways in differentiated muscle cells are scarce, several mechanistic are similar to those found in other cell types [125].

Nevertheless, skeletal muscle is a unique tissue in terms of apoptosis, in particular as regards to differentiated myotubes:

- Muscle cells are multi-nucleated, and myonuclei have been reported to undergo apoptosis individually, maintaining cellular integrity and function. It is likely that apoptosis of individual myonuclei, and subsequent degradation of associated sarcoplasmic segments, contributes to the development of muscle fiber atrophy [126, 127].
- Myotubes contain variable mitochondrial content, dependent both on fiber type and the extent of training

Skeletal muscle contains two morphologically and biochemically distinct mitochondrial pools, the subsarcolemmal and intermyofibrillar mitochondria, located beneath the sarcolemma and between the myofibrils, respectively. These populations possess different characteristics, including higher cardiolipin content and more elevated state-3 respiration rates in intermyofibrillar mitochondria [128]. They can therefore respond differentially to pro-apoptotic stimuli.

4.1. Apoptotic processes in muscle cell differentiation, and acquisition of cell death resistance

Acquisition of apoptosis resistance occurs during skeletal muscle cell differentiation. Myoblasts are stem cells stimulated to re-enter the cell cycle. Damaging agents (growth factor withdrawal, DNA damage, oxidative stress, alterations in the cell cycle process or in cellular metabolism) can induce

activation of apoptosis, thereby eliminating the possibility that injured cells initiate differentiation [129]. During early stages of differentiation, myogenic precursor cells withdraw irreversibly from the cell cycle. In fact, cell cycle exit is a prerequisite for induction of the contractile phenotype by myogenic transcription factors, and confers to mature myotubes a resistance to apoptosis.

Myogenesis is promoted by MyoD and homologous helix-loop-helix factors as myogenin, whose induction indicates commitment to differentiation programme. These myogenic transcription factors function to induce the expression of p21, an inhibitor of several cyclin-dependent kinase activities, and thus promote cell cycle exit [130]. Inhibition of cdk activity results in dephosphorylation of the pRb pocket protein [131], leading to repression of E2F transcription factors. This activity, in turn, guarantees cell cycle progress and apoptosis sensitization [132]. Hence, once the post-mitotic state has been established, cells less susceptible to apoptosis can acquire a differentiated phenotype characterized by myosin heavy chain expression. Finally, cells can fuse to form multi-nucleated myotubes. Presumably, such coordination between cell proliferation and apoptosis provides the adult organism with a mechanism to limit the life span of proliferative myogenic precursors during embryogenesis.

Differentiated muscle fibers of “in vivo” skeletal muscle exhibit a strong resistance to apoptosis, as previously described. This resistance could be due to the presence of apoptosis repressors in unusually high concentrations. For example, the anti-apoptotic molecule FLIP (Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein) is strongly expressed in skeletal muscle and is responsible for the resistance of skeletal muscle to Fas-mediated apoptosis in myositis [133]. Similarly, skeletal muscle expresses high levels of ARC (apoptosis repressor with caspase recruitment domain) which selectively interacts with caspase-8 rendering it inoperative. Human ARC expression is restricted to skeletal muscle and cardiac tissue [134] where it acts as an apoptosis inhibitor. ARC not only blocks caspase-dependent events but also caspase-independent events associated with apoptosis, including hypoxia-induced cytochrome c release. Moreover, ARC can prevent necrosis-like cell death via preservation of mitochondrial function [135] and interaction with Bax, a pro-apoptotic member of the Bcl-2 family [136]. Recently, the novel apoptosis repressor APIP (Apaf-1-interacting protein) has been shown to play a role in muscle regulation of early steps of apoptosis. APIP binds to Apaf-1 in competition with procaspase-9 thereby, suppressing events downstream of cytochrome c release. APIP is highly expressed in skeletal muscle and heart where it is responsible for inhibition of ischemia/hypoxia-induced cell death [137]. Skeletal muscle also exhibits high levels of hILP (human IAP-like protein, also called XIAP), which inhibits upstream and downstream caspases. While apoptosis has been implicated in muscle fiber cell death in some

myopathies as well as in other pathological processes (see below), there is consensus that muscle fibers usually do not undergo apoptosis in inflammatory myopathies [138,139]. In fact, the high expression of hILP protein has been proposed as a mechanism explaining the rare incidences of muscle fiber apoptosis in T-cell inflammatory myopathies [140].

4.2. Apoptosis in skeletal muscle pathologies

Despite its resistance to cell death, evidence of muscle cell apoptosis is found in numerous skeletal muscle myopathies [141-143], as well as in physiological processes such as ageing or even exercise. Hindlimb suspension, microgravity, and immobilization of skeletal muscle are established animal models used to induce muscle atrophy. The decrease in muscle mass associated with muscle unloading and immobilization is coincident with both a reduction in myonuclear number per fiber, and increased incidence of DNA fragmentation. However, these apoptotic mechanisms seem to affect individual myonuclei more than whole cells [144, 145]. Exercise and treatment with IGF-1/GH are each thought to play a role in mediating cell survival within skeletal muscle because they attenuate myonuclei apoptosis [146]. A recent study shows how expression of the inhibitor of differentiation-2 protein, a negative regulator of the myogenic transcription factor family, correlates with muscle unloading, suggesting its involvement in mediating apoptosis. Similarly, microgravity up-regulates the expression of p53, thus increasing Bax expression, which is probably involved in apoptosis-related atrophy [147]. Denervation of skeletal muscle results in a progressive atrophy similar to that observed in muscle unloading paradigms. In fact, the atrophy observed during denervation is similar to classical manifestations of apoptosis, although myocytes lose viability long after elimination of any myotrophic influence of the nerve and no large-scale nuclear DNA fragmentation is observed [126]. Furthermore, apoptosis can also result from burn injuries [148]. Therefore, it is quite likely that apoptosis is a common and widespread mechanism for muscle fiber loss, although there remains neuromuscular diseases lacking evidence of apoptosis i.e. myofibrillar myopathy [149].

In skeletal muscle, ageing results in fiber loss (i.e. sarcopenia) together with biochemical and morphological abnormalities. Some have suggested that ROS-induced damage to macromolecules over the life-time of an individual, could play a role in the ageing process, possibly via apoptosis (see below). In fact, apoptosis increased with age in rat skeletal muscle by a procaspase-9 activation-dependent mechanism, indicating mitochondrial control [150].

Muscle damage can also be induced by ischemia followed by reperfusion. While evidence of apoptosis following ischemia/reperfusion in cardiac muscle is plentiful, the findings for skeletal muscle remain controversial. Nevertheless,

the mechanism at work seems to be directly related to ROS production during reperfusion [151].

Excessive or unaccustomed-to levels of exercise can increase DNA fragmentation [152]. In fact, intense exercise causes metabolic changes that may influence mitochondrial function. During exercise ROS production increases due to heightened oxidative phosphorylation, pH decreases owing to proton and lactate accumulation, and increases in Ca^{2+} concentration. All of these factors may alter PT pore activity, thereby leading to MOMP [153]. On the other hand, exercise can induce expression of the heat-shock protein 70kDa within both skeletal and cardiac muscle [154]. This protein is known to inhibit both caspase-dependent and independent apoptosis pathways, acting as a protective mechanism against cell death. Therefore, moderate endurance training could induce the up-regulation of protective anti-apoptotic mechanisms that would cancel apoptosis progress.

Muscular dystrophies are considered examples of fiber loss by necrosis. However, evidence in one study suggests that apoptosis precedes necrosis in dystrophin-deficient muscle from mdx mice [155-157]. Exercise-induced damage is thought to play a role in dystrophies since muscle fibers are subjected to continual stress from contractile activity. Sarcolemmal protein defects cause membrane instability and unbalance in cytosolic Ca^{2+} concentrations. These factors, together with oxidative stress, are possible mediators of early apoptosis in dystrophies [158].

A prominent feature of several cancer types is cachexia. This syndrome is characterized by significant loss of lean body mass and muscular wasting, and appears to be mediated by tumour products and cytokines such as $\text{TNF}\alpha$ [159]. Though different pathways could be involved in such muscle loss, caspases are known to participate in catabolic events associated with cachexia [160].

It should be noted that lipotoxic processes in skeletal muscle resulting from increased lipid accumulation have been reported in diseases such as type II diabetes [161]. Unfortunately, despite extensive research on the role of fatty acid on apoptotic pathways in related cell types, including cardiomyocytes or smooth muscle cells (see below), studies on the relationship between lipotoxicity and apoptotic pathways in skeletal muscle are not available.

4.3. The involvement of mitochondria in skeletal muscle apoptosis

Mitochondria plays a key role in apoptosis regulation, but little is known about its role within skeletal muscle. As is in other cell types, skeletal muscle mitochondria can undergo a permeability transition following Ca^{2+} uptake. Membrane permeabilization due to PT pore opening is thought to be essential in regulating mitochondrially- driven apoptosis within myotubes, while

transient openings of the PT pore in a low conductance mode, would provide the cell with a fast Ca^{2+} release channel, thereby contributing to Ca^{2+} homeostasis [162]. Furthermore, PT pore sensitivity to Ca^{2+} within skeletal muscle has been shown to be dependent on the rate of electron flow through complex I of the respiratory chain [163].

Although proteins released during MOMP are involved in cell death control, studies focused on expression of apoptosis-related proteins in skeletal muscle remain scarce. Both anti-apoptotic (Bcl-W, Bcl X_L) and pro-apoptotic (Bad, Bax) members of the Bcl-2 family are expressed in developing skeletal muscle *in vivo*. They are also expressed in embryonic, fetal, and neonatal muscle stem cells, myoblasts, and myotubes *in vitro*. Moreover, Bcl-2 is essential for several stages of fetal and postnatal myogenesis since Bcl-2 null mice show altered muscle development [164].

As mentioned above, apoptosis may originate through mitochondria-dependent signalling in response to external stimuli, but internal alterations within mitochondria may also induce cell death. Genetic defects that inhibit oxidative phosphorylation cause a decline in mitochondrial energy production and an increase in oxidative stress. This may initiate programmed cell death by affecting PT pore activity [165]. Skeletal muscle from patients suffering mitochondrial diseases involving genetic alterations in mitochondrial DNA shows cytochrome c release to the cytoplasm as well as DNase I activity in

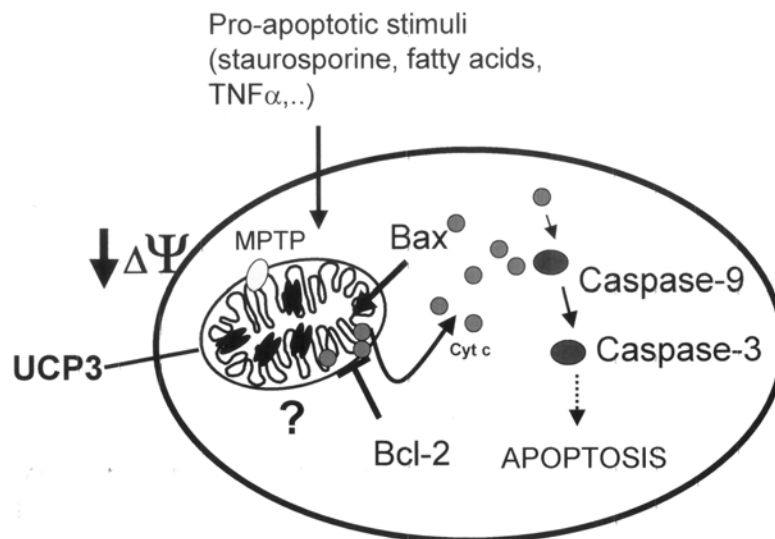


Figure 2. Placement of UCP3 in mitochondria, in the context of apoptotic pathways and regulation of mitochondrial inner membrane potential ($\Delta\Psi$). Lowering $\Delta\Psi$ by UCP3 may have consequences on the permeability transition pore (PT) in mitochondria. Control of ROS production by UCP3 may also influence apoptotic pathways. Potential direct interactions between UCP3 and mitochondrial components of regulation of apoptosis (Bcl-2 or Bax families) remain to be experimentally explored.

nuclei, suggesting the induction of apoptosis [166]. This occurs particularly in the red-ragged fibers present in the tissue of these patients, which show accumulations of abnormal mitochondria. In patients exhibiting encephalomyopathy, caspase-3 activation has been reported in red-ragged fibers but apoptosis is rarely completed in these muscles, probably due to strong sarcoplasmic expression of the apoptosis inhibitor XIAP [167].

Sometimes latent mitochondrial dysfunctions are responsible for apoptosis in skeletal muscle as shown by the occurrence programmed cell death in myopathic mice with collagen VI deficiency [168]. Collagen VI deficiency causes a mitochondrial dysfunction that is reversible by treatment with cyclosporin A, a specific inhibitor of the PT pore. Thus, mitochondrial permeability transition is most likely involved in the origins of human Bethlem myopathy and Ullrich dystrophy, both caused by mutations in collagen VI gene.

5. Mitochondrial uncoupling proteins, ROS and cell death. The biological activity of uncoupling protein-3 in skeletal muscle

There is a widespread consensus that one of the biological activities of mitochondrial UCPs (at least UCP2 and UCP3), in addition to its metabolic effects promoting substrate oxidation, is to influence ROS production in mitochondria. Mild uncoupling, such as that originated by UCP activity, has long been known to reduce ROS production in association with reduction in mitochondrial proton-motive potential. Mitochondrial ROS production is intimately linked to the mitochondrial membrane potential ($\Delta\Psi$) such that hyperpolarization promotes ROS production [169-171]. Since the magnitude of ROS production is largely dependent on - and correlate with- $\Delta\Psi$ even a modest reduction (mild uncoupling), via increased proton conductance across the mitochondrial inner membrane reduces ROS formation [170, 172, 173]. This can be explained on the basis of increased respiration due to the respiratory control, and this phenomenon is quite impressive, for instance, in heart mitochondria in which a 10% decrease in $\Delta\Psi$ leads to a 55% decrease in ROS production as monitored by H_2O_2 production [174]. Consequently both chemical uncouplers [16] and even the mild uncoupling given by UCPs can intensively down-regulate mitochondrial ROS [18,19].

Interestingly, it has been shown that ROS can stimulate UCPs expression and activity [175,176]. Multiple studies have reported an increase of UCP2 expression after oxidative stress [177,178]. Studies from the M.D.Brand group have shown that the superoxide anion directly activates UCP2 from the matrix side of mitochondrial inner membrane [179] and that endogenous matrix superoxide from complex I can activate UCP3 [180]. Further studies revealed that 4-hydroxy-2-nonenal, a product of the interaction of ROS with

fatty acids, acts also as a direct activator of the proton conductance pathway mediated by UCP3 [181], thus providing a novel evidence of cross-talk of ROS and fatty acid-dependent regulation of UCPs biological activity. However, the actual capacity of direct activation of UCP2 by superoxide in spleen or lung mitochondria has been questioned on the basis of studies using UCP2 “knockout” mice [182].

It has also been reported that ubiquinone is an obligatory partner of UCPs activity [183]. This suggests that, together with mild uncoupling, UCPs can exert its effects on ROS production through direct interaction with a key component of a major site of ROS production, the ubiquinone cycle. However, the role of ubiquinone in UCPs activity is still controversial [184, 185].

In relation to UCP2, enhanced ROS production has been observed in multiple “loss-of-function” models, ranging from inhibition of UCP2 activity in mitochondria [17], to blockage of UCP2 expression in cell culture by use of antisense oligonucleotides [186] or to targeted disruption (“knockout”) of the UCP2 gene in mice [18]. The phenotype of UCP2 “knockout” mice is characterized by a resistance to brain injuries, e.g. resistance to some brain infections [18], stroke [187] and cerebral ischemic injury [188]. However, it remains unclear to what extent these protective effects of UCP2 gene disruption are caused by intrinsic enhancement in ROS production in mitochondria lacking UCP2 or are indirect. Several reports indicate that the permanent absence of UCP2 triggers adaptive mechanisms of induction of cellular antioxidant machinery (superoxide dismutase activity, glutathione levels...) and it is believed that this is what, in fact, confers on cells and tissues a physiological resistance to ROS-mediated challenges [189]. Moreover, not every type of neural damage is negatively associated with the abundance of UCP2: e.g. reduced excitotoxic neural death in the brain of neonates [190] is associated with high UCP2 expression and over-expression of UCP2 protects against neurotoxicity [191]. It is unclear whether there is specificity in the role of UCP2 in neural cells, as studies of other cell types have not provided conclusive evidence and, for instance, UCP2 deficiency does promote oxidative stress in liver [192]. UCP2 has been reported to favour cell death in HeLa cells without affecting fibroblasts [193], whereas over-expression of UCP2 promotes apoptosis in cardiomyocytes [194]. Extensive studies have been performed on the role of UCP2 in the pancreatic beta-cell after observation of disturbances in the glucose/insulin ratio in UCP3 “knockout” mice [195]. Experimental manipulations of UCP2 amounts and activity correlate to a role for UCP2 in reducing ROS production in the beta cell. However, adverse effects of superoxide on beta cell glucose sensing appear to be caused by activation of UCP2 [196]. In this sense, lipotoxicity due to excess free fatty acids may affect beta-cells, and it has been reported that pancreatic islets from mice lacking UCP2 resist the toxic effects of palmitate [197].

Although less studied than UCP2, similar observations have been made for UCP3 in relation to ROS production. Targeted disruption of UCP3 in mice leads to enhanced production of ROS in skeletal muscle mitochondria [19]. Biochemical markers of oxidative damage) increased in the skeletal muscle of these mice [198]. However, to date few studies have addressed the role of UCP3 in the control of cell death. We have recently described that induction of UCP3 at physiological levels by cells that usually do not express this protein does not lead to apoptosis, but rather enhances cell sensitivity to apoptotic inducers acting via mitochondrial-dependent pathways [199]. This effect is also observed in skeletal muscle cells, a homologous cell system for UCP3 expression (Y.Camara, C.Duval, F.Villarroya, unpublished observations). These observations are consistent with a recent report indicating that UCP3 up-regulation is among the first events leading to muscle damage in amyotrophic lateral sclerosis [200]. However, a recent study indicates that UCP3 over-expression in ganglionic neural cells serves as a protective mechanism against hyperglycemia-induced apoptosis [201]. This may also indicate that the role of UCP, as in UCP2, may be dependent on the type of cell target and death-inducing challenge.

The effects of UCPs, and particularly of UCP3, on apoptosis may be related to their impact on the bioenergetic status of the cells. Treatments with chemical uncouplers do not lead to apoptosis in and of themselves, but can enhance sensitivity to Fas-induced apoptosis [202]. It therefore remains quite possible that mild reductions in the mitochondrial membrane potential, such as those elicited by UCPs under the physiological regulation of intracellular metabolites, could alter sensitivity to apoptosis. Another possibility is that UCPs alter apoptosis sensitivity by their interaction with other mitochondrial proteins involved in apoptotic machinery. To date, the only interaction reported for UCPs is the capacity of 14.3.3 proteins, which are known anti-apoptotic factors inhibiting Bad [203] and Bax [204], to bind UCP3 [205]. Finally, it cannot be excluded that increases or decreases in UCP3, much like UCP2, elicit adaptive changes down-regulating or up-regulating, respectively, antioxidant machinery components, thereby influencing the capacity of muscle cells to respond to sudden inducers of oxidative stress and cell death. Yet another aspect to be considered is that expression of UCP3 gene is up-regulated in skeletal muscle cells by stimuli related to metabolic stress signals. Such signals include increases in the free fatty acid concentrations [206,207] or other agents related to cellular stress such as TNF α [208] or glucocorticoids [209]. In fact, these agents are known as activators of apoptosis in several cell types [121,210] to include, in some instances, muscle cells. It remains unclear, however, whether the parallel increases in UCP3 gene expression and apoptosis in response to these agents are part of the activation of the program of apoptosis or an attempt to reduce ROS production and counteract oxidative stress.

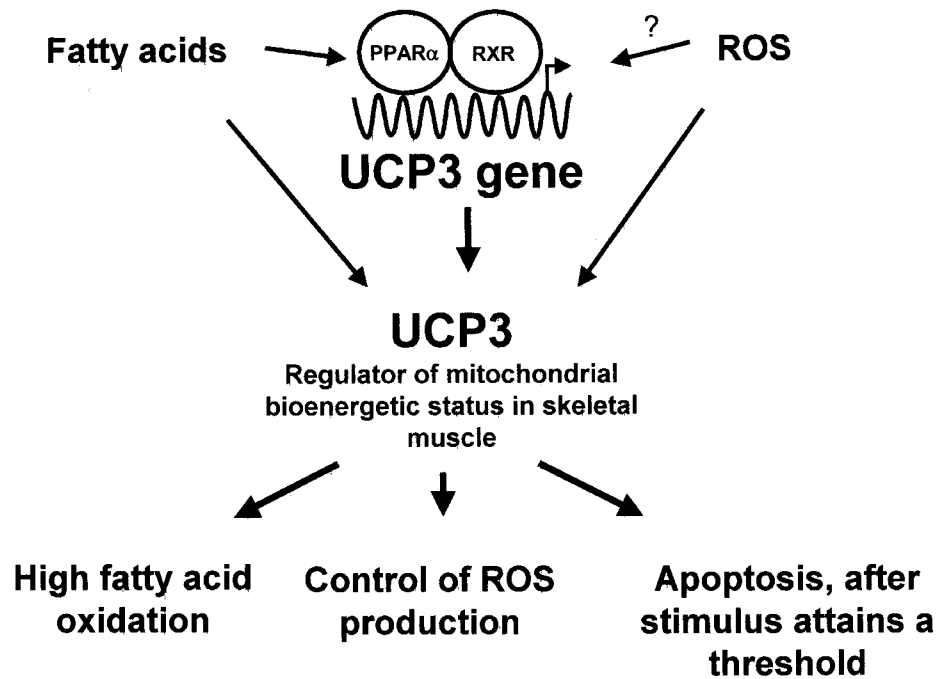


Figure 3. Working model summarizing the role of UCP3 in skeletal muscle biology. (see last part of the chapter for details)

Finally, the role of fatty acids in relation to cross-talk among mitochondrial UCPs activity, ROS production and apoptosis should be discussed. Fatty acids are activators of the proton conductance pathway elicited by UCPs. Moreover, fatty acids induce UCP3 gene transcription by activating heterodimers of peroxisome proliferators-activated receptors (PPAR) and retinoid X receptors (RXR) that interact with the UCP3 gene promoter [211]. Every physiological or pathological situation in which there is a high availability of fatty acids to skeletal muscle, leads to increased UCP3 gene expression [212]. Fatty acids elicit intracellular events closely related to the expected functions of UCP3: they enhance ROS production during oxidation in mitochondria and they also promote apoptosis through ROS-dependent or independent mechanisms (see above). It can therefore be concluded that UCP3 induction could, under the stimulus of fatty acids, confer on mitochondria tendency to oxidize actively these fatty acids without excess ROS production. Perhaps, after a certain toxicity threshold resulting from fatty acid excess, has been attained, high levels of UCP3 can serve to elicit activation of mitochondrial-dependent apoptotic pathways. These would selectively eliminate from the organism that cell (or, in the context of muscle, that fiber or cell region) which can no longer withstand disturbances of such intensity

In summary, UCP3 appears to be at the centre of the cross-talk for pivotal processes controlling skeletal muscle cell biology: mitochondrial bioenergetics, control of ROS production, and apoptosis. The evidences

summarized here indicate that the amounts and activity of this protein may influence these processes. Further research will be required to establish precisely the impact of manipulating UCP3 amounts and activity in muscle cells and to what extent UCP3 could be considered a potential therapeutic target in pathologies involving skeletal muscle.

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