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Balancing Mitochondrial Redox Signaling: A Key Point in Metabolic Regulation

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Abstract

Mitochondrial reactive oxygen species (mROS) have emerged as signaling molecules in physiology primarily as a result of studies of uncoupling mechanisms in mitochondrial respiration. The discovery that this mechanism negatively regulates mROS generation in many cell types has drawn the attention of the scientific community to the pathological consequences of excess mROS production. From reports of the energetic fluxes in cells grown under normal conditions, the hypothesis that mROS are an integrated physiological signal of the metabolic status of the cell has emerged. Here, we consider recent studies that support this point of view in two key nutrient sensors of the body, beta cells and the hypothalamus, which are the main coordinators of endocrine and nervous controls of energy metabolism and adipose tissue, which is of paramount importance in controlling body weight and, therefore, the development of obesity and type 2 diabetes. In this context, finely balanced mROS production may be at the core of proper metabolic maintenance, and unbalanced mROS production, which is largely documented, might be an important trigger of metabolic disorders. *Antioxid. Redox Signal.* 14, 519–530.

Brief Overview of Mitochondrial Reactive Oxygen Species

Beside other important sources such as NADPH oxidases or the endoplasmic reticulum, for instance (45, 90), the mitochondria respiratory chain and dehydrogenases represent one of the main sources of reactive oxygen species (ROS) in cells. Here we only deal with this mitochondrial compartment as ROS producing organelles in regard to energy sensing within the cell. Estimates from isolated mitochondria suggest that 1% or less of the O_2 consumed is incompletely metabolized and leads to superoxide generation when it accepts an electron from the chain (16, 95, 104). The current view is that mitochondrial superoxide anions are an obligatory by-product of respiratory chain function and that their generation is intimately linked with energetic and oxidative phosphorylation in cells. Several sites of superoxide anion production have been described in mammalian mitochondria, and these have recently been reviewed (16). Among the sites that produce ROS, complex I and III have been shown to possess high productive capacities (10, 25). They contain large amounts of ubisemiquinone (18) that permits the univalent reduction of a small part of molecular oxygen to superoxide (complex I produces superoxide in the matrix, whereas complex III produces it in the matrix and intermembrane space) (47, 104). For signaling, the superoxide can itself be the signal in the matrix, or it can be dismutated by manganese superoxide dismutase (SOD) and converted into hydrogen peroxide (H_2O_2). This latter compound becomes diffusible and/or is transported by channel proteins, such as aquaporins, as recently suggested (13, 73). It can therefore act in both mitochondrial and cytosolic compartments. Part of the H_2O_2 released can also be converted to H_2O through catalase and/or be inactivated by a variety of other enzymatic and nonenzymatic antioxidant systems acting directly or indirectly, such as glutathione peroxidases, peroxiredoxins, thioredoxins, glutathione reductases, glucose-6-phosphate dehydrogenase (NADPH regeneration), and vitamins (67). Apart from these secondary scavenging systems, the respiratory chain itself modulates mitochondrial ROS (mROS) production through specific proteins. The uncoupling proteins (UCP1–5) have been classically viewed as channels that dissipate the proton electrochemical gradient, thereby uncoupling respiration from ATP synthesis and producing heat in the cell. Although this is true for UCP1 in thermogenic tissues, this mechanism is still controversial, and many other functions have been observed or suggested (8, 15, 35, 69, 76). Of particular interest for mROS production is the UCP2

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homolog, which does not normally catalyze the basal proton flux but does so to a moderate extent (mild uncoupling) when activated, as it is the case by superoxide, ensuring an antioxidant effect (22, 34, 35, 69, 76). The role of this protein and its physiological and pathological consequences will be further discussed in the text. Another important factor in regulating mROS levels is the nicotinamide nucleotide transhydrogenase (Nnt), a protein located in the inner membrane of eukaryotes (52). It functions as a redox-driven proton pump, catalyzing the reduction of NADP+ by NADH. Nnt has been proposed to indirectly detoxify H_2O_2 from the matrix through its conversion into water by the glutathione cycle; this latter step consumes NADPH for the regeneration of reduced glutathione (GSH) (6, 38, 82). Wherever they are present, mROS, and particularly H_2O_2, can act on potential redox-sensitive targets (33, 37). mROS signaling is often mediated by proteins that have a cysteine with thiol(ate) groups in their active sites, which might react in a reversible manner with H_2O_2 (37, 56, 67). These data support the existence of specific responses through reversible oxidation-reduction reactions.

Figure 1 summarizes the main steps in mitochondrial superoxide formation originating from the respiratory chain and its derivative H_2O_2 production.

In the context of normal nutrient supply and thereby the transient rise of metabolite fluxes, a short-lived and moderate increase of mROS originating from the respiratory chain (superoxide anion and secondarily H_2O_2) has been identified in some cells and tissues both *in vitro* and *in vivo* (11, 14, 27, 53, 61, 62, 77). This transient production occurs under physiological conditions with no identified damage (11, 61, 62). One of the most elegant demonstrations of transient mROS production was achieved in rat islets, using time-lapse imaging of hydroethidine (HEt), which specifically reacts with O_2^- (14). In that study, normal islets exhibited a rapid increase in ROS levels, as shown by HEt oxidation when the glucose concentration rose from 2 to 10 mM. The images in which the fluorescence of the HEt probe was merged with that of a mitochondrial marker showed the mitochondrial origin of the superoxide anion. Further, the suppression of this glucose-increased mROS production (*e.g.*, through the use of mitochondrial drugs targeting the electron chain transfer, antioxidants, or the overexpression of UCP2) systematically disturbs the normal function of the cell (53, 61, 62, 77), providing evidence that these mitochondrial oxidants also function physiologically as fuel-sensing signaling molecules that regulate metabolism. These points will be discussed in the following paragraphs with regard to two key nutrient sensors of the body, beta cells and the hypothalamus, in which the role of mROS has been recently described, and adipose tissue, which appears to intimately depend on mROS signaling.

mROS as an Integrated Signal for Nutrient Sensing in the Hypothalamus

The brain control of energy status is particularly important in energy maintenance because it is strongly involved in controlling food intake and energy expenditure as well as, through sympathetic (splanchnic) and parasympathetic (vagal) outputs, and glucose and lipid metabolism (17, 99, 109). This control predominantly involves the hypothalamus, which has been suggested to have a central role in the pathogenesis of type 2 diabetes (T2D) (74, 96, 97). The hypothalamus integrates peripheral signals delivered by neural inputs from various organs and by the blood, including metabolites (mainly glucose and fatty acids) and hormones (mainly leptin, insulin, and ghrelin) (1), and it generates the appropriate integrative responses. In particular, specialized cells, such as glucosensing neurons, whose firing rates vary in response to changes in extracellular glucose concentration, have been described (3, 75, 83). Two populations of glucosensing neurons can be defined as local glucose levels rise: excited ones (in which the electrical activity is increased, gluco-excited (GE) neurons) and inhibited ones (which exhibit decreased activity, gluco-inhibited neurons) (3, 36). Such neurons have mainly been characterized in the ventromedial hypothalamus (ventro-median nucleus) and arcuate nuclei. GE neurons share similarities with beta cells, such as the consensual ATP production and the mechanism by which their K_{ATP} channels operate, but studies highlighting this mechanism are not sufficient to explain the response of GE neurons, and some studies show that ATP-independent mechanisms might operate (3, 36). Pioneering studies have identified the necessary redox signaling through mROS production when nutrient fluxes vary in many cells (77). These data lead us to propose that mROS is particularly important in nutrient-sensing mechanisms.

In the hypothalamus where GE neurons are present (the ventral part, especially in the arcuate nucleus), we have shown that a transient increase in glucose concentration can very rapidly trigger an increase in mROS production. This increase occurs both in *ex vivo* hypothalamic slices (61) and *in vivo* in the ventral part of the hypothalamus 1 min after a glucose load (27), which coincides with the timing of the physiological response, that is, neuronal activation and a subsequent peak of insulin. This mROS production is reversed by various antioxidants or a mitochondrial uncoupler; the effect of the latter compound indicates the respiratory chain origin of the mROS (61). In these conditions, both the increase in the firing rate in the arcuate nucleus and the subsequent insulin secretion are abolished, showing that the responses are dependent on mROS. Moreover, direct hypothalamic mROS generation by the respiratory chain inhibitors antimycin and rotenone mimics the effect of glucose. A possible explanation for this finding is that an increase in mROS levels, rather than just the ATP/ADP ratio, constitutes a signal that mediates the stimulatory effect of glucose on hypothalamic neurons. Brain complex I has been suggested as a main source of mROS from the respiratory chain (2), and the production of mROS increases as reduced NADH increases, which predominantly occurs when glucose levels rise. Therefore, the mROS signaling in this context is consistent with the NADH mechanism in mediating glucose signaling, as suggested earlier (111). In this case, mROS elevation can be considered as a signal integrating increases in both reduced NADH (when glucose rises) and the electrochemical proton gradient, the latter reflecting both the phosphate potential (ATP/ADP, Pi ratio) and the uncoupling status of the mitochondria. This proposal is consistent with previous studies that describe expression of UCP2 in cortex and recently in many hypothalamic areas (the suprachiasmatic, paraventricular, dorsomedial, ventromedial, and arcuate nuclei) (28, 91). An increase in brain UCP2 expression correlates with the survival of cortical neurons in conditions of oxygen and glucose deprivation, a situation that exacerbates pathological

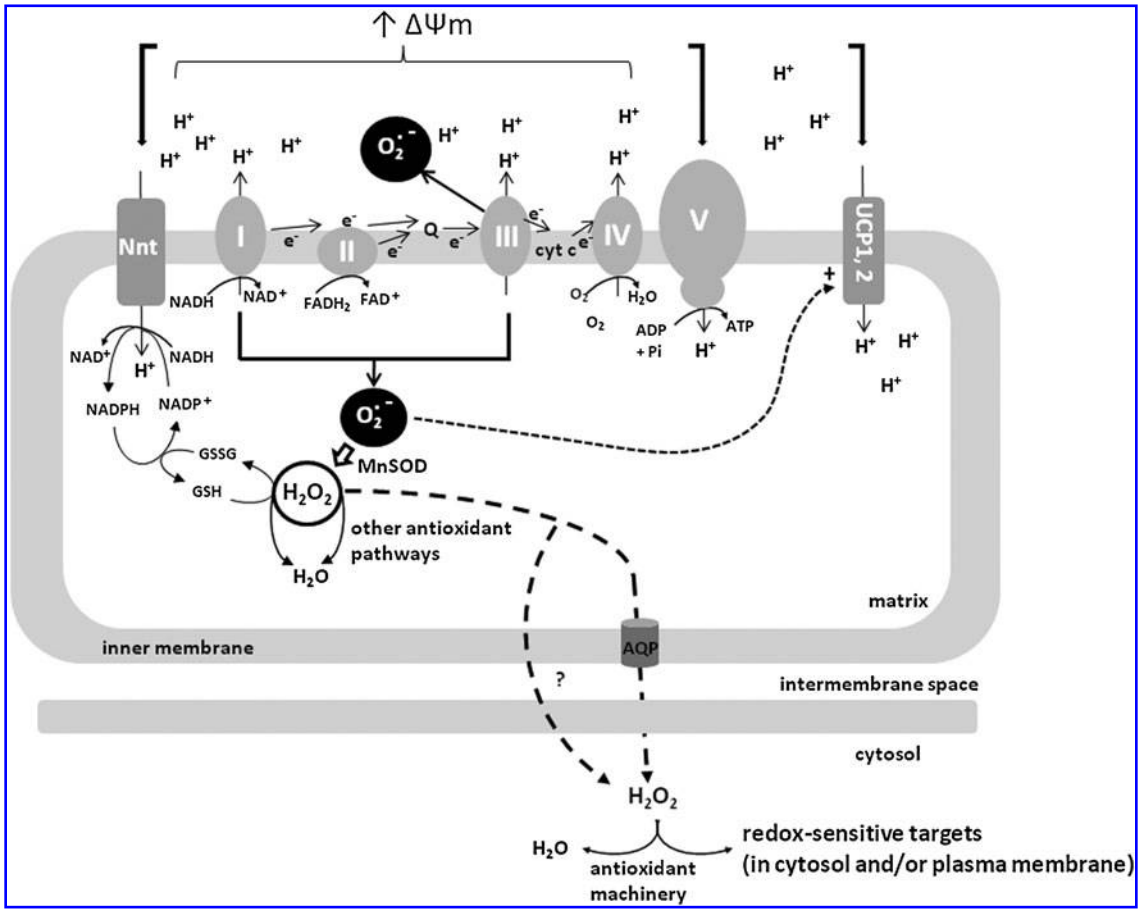

FIG. 1. Schematic illustrations of mitochondrial events promoting mitochondrial reactive oxygen species signaling. Energetic substrates are transported in the matrix and generate reduced molecules (NADH and $FADH_2$) after their oxidation. Electrons are generated from NADH (complex I) and $FADH_2$ (complex II) and pass through the mitochondrial electron transport chain (four complexes, I to IV; Coenzyme Q (Q) and cytochrome c, electron carriers), where they finally reduce O_2 to H_2O. This is associated with the pumping of protons across the inner membrane that establishes an electrochemical gradient ($\Delta\Psi m$). This gradient drives the synthesis of ATP from ADP and inorganic phosphate by complex V, protons being transported back to the matrix. UCPs also consume the proton gradient and lower ATP synthesis (proton leak). In brown adipocytes, UCP1 actively dissipates the proton gradient that leads to heat. The UCP2 homolog is only considered as a mild uncoupler. Increased oxidation of the reduced equivalents NADH and $FADH_2$ accelerates respiration and electron transport. In the respiratory chain, mainly complexes I and III can also leak electron to oxygen, leading to superoxide anions generation (O_2^{-}). The O_2^{-} generates H_2O_2 after dismutation by manganese-dependent superoxide dismutase. H_2O_2 might be quenched in the matrix or in the cytosol by several antioxidant systems, including both enzymes (catalase, glutathione peroxidase, peroxiredoxin, and thioredoxin, for instance) and scavengers (glutathione and vitamins). The nicotinamide nucleotide transhydrogenase participates indirectly to the reduction of H_2O_2 to H_2O: it functions as a redox-driven proton pump, catalyzing the reduction of NADP+ by NADH. The regeneration of glutathione consumes the NADPH. For signaling, H_2O_2 might react in a reversible manner with specific targets (mostly proteins handling a cysteine residue) both in the matrix and/or in the cytosol, maybe crossing the inner membrane through aquaporin protein. cyt c, cytochrome c; H_2O_2, hydrogen peroxide; MnSOD, manganese-dependent superoxide dismutase; Nnt, nicotinamide nucleotide transhydrogenase; Pi, inorganic phosphate; UCPs, uncoupling proteins.

mROS formation in neurons (69). In this study, UCP2 has been shown to lower the level of mROS, a function that has been attributed to a mild mitochondrial uncoupling, which has been verified by measurement of the mitochondrial membrane potential. Low doses of the mitochondrial uncoupler DNP (2,4-dinitrophenol) are also protective. Overexpression of UCP2 *per se* does not alter the membrane potential. The net mROS formation in suspended mitochondria is not different in transgenic UCP2-overexpressing mice *versus* the wild type, indicating that there is no net change in mROS generation due to UCP2 overexpression in the absence of UCP2 activators. Oxygraphic study has shown an increased respiration only after the addition of palmitic acid, indicating an increase in the leakage of protons across the membrane after activation of UCP2, although mitochondrial membrane depolarization is limited. This study, largely confirmed by the ischemic condition of the brain (30, 71), has indicated a protective role for UCP2 through a mild mitochondrial uncoupling activation, mitigated ROS production, and activation of cellular redox signaling.

Other results on pro-opiomelanocortin (POMC) neurons, a particular type of hypothalamic GE neurons, support the

importance of mROS signaling in conjunction with UCP2 activity (81). Thus, the mechanism for obesity-induced loss of glucose sensing in arcuate POMC neurons (an anorexigenic population) involves UCP2, which negatively regulates glucose sensing in POMC neurons. In particular, genetic deletion of UCP2 or treatment with genipin, a UCP2 inhibitor, prevents obesity-induced loss of glucose sensing. In this case, UCP2 scavenging of mROS impairs glucose sensing in glucose-excited neurons and has a pathogenic role in the development of T2D. This mROS signaling might be extended to other fuel nutrients, at least lipids because acute hypertriglyceridemia in normal rats has been shown to trigger a rapid increase in the mitochondrial respiration in the ventral hypothalamus, together with a transient production of ROS (11). Inhibition of fatty acid-CoA mitochondrial uptake (by etomoxir) prevents the hypertriglyceridemia-induced ROS production, indicating the mitochondrial origin of the ROS. The hypothalamic inhibition of this hypertriglyceridemia-induced mROS production by antioxidants fully abolishes the satiety effect of lipids, showing that mROS production is required to restrain food intake during hypertriglyceridemia (11). Moreover, the fasting state was shown, using mitochondrial respiratory studies, to be associated with both high uncoupling in the hypothalamus and an increase in UCP2 protein levels (11). As a consequence, this mitochondrial uncoupling status disrupts the hypertriglyceridemia-induced mROS production, indicating that brain nutrient sensing is modulated according to the energy status (11). Altogether, these findings expand the role of mROS as a real gauge of energy status.

Recently, hypothalamic mROS signaling has been shown to be modulated by hormones. In the arcuate neuropeptide Y (NPY)/agouti-related peptide (AgRP) orexigenic neurons, the orexigenic gut-derived hormone ghrelin exerts its effects through the modulation of mitochondrial respiration and mROS production (5). In this study, ghrelin decreased the mitochondrial membrane potential in normal mice and increased neuronal activation, as assessed by c-fos expression, and the firing rate of NPY neurons, effects that are completely abolished in UCP2$^{-/-}$ mice, in which mROS is still produced (5). Moreover in this mechanism, it was proposed that ghrelin activates AMPK signaling, which requires UCP2 activation to promote feeding in NPY neurons. This mechanism suggests possible causal links between AMPK signaling and mROS production in this population of neurons. Currently, only indirect links have been reported, and no specific thiol residue of AMPK that is redox sensitive has been identified. AMPK is known to be activated by stimuli that increase the cellular AMP/ATP ratio (low glucose and high ghrelin, as occur in the fasting state). H_2O_2-caused activation and phosphorylation of AMPK have recently been discussed, and studies suggest that the target for ROS may not be AMPK itself but one or more components of the respiratory chain, leading to a secondary effect on AMPK *via* increases in the AMP:ATP ratio (49). Whatever the link with AMPK, these results highlight that mitochondria of orexigenic NPY/AgRP neurons need an uncoupled status to promote a normal response. Conversely, WT POMC neurons show a higher mROS production with saline than with ghrelin and need coupled mitochondria for their activation. In the fasting state, ghrelin indirectly hyperpolarizes POMC neurons by activating inhibitory NPY/AgRP (GABAergic) inputs. The hyperpolarization leaves POMC neurons less active, leading to a drop in the respiration rate and mROS production. Finally, mROS scavenging by antioxidant treatment reverses these results in neurons from UCP2$^{-/-}$ mice. Thus, the anorexigenic POMC neurons have been proposed to function inversely to the NPY/AgRP neurons. They can respond to a rise in glucose levels through an mROS increase only under fed conditions, with low ghrelin and well-coupled mitochondria. Although the mechanisms underlying the different mROS production responses in NPY and POMC neurons are clear regarding ghrelin (the POMC population has only 8% of ghrelin receptors), a comprehensive view of the differential effects of nutrient fluxes (lipids *vs.* glucose) according to negative or positive energy balance on these two neuronal populations remains hypothetical (53).

Together, these data from gene invalidation as well as pharmacological approaches demonstrate the master role of brain mROS signaling with regard to the control of both food intake and metabolism. This master component of brain energy sensing would be crucial in the development of both obesity and T2D. Indeed, in the obese and insulin-resistant Zücker rat, a prediabetic model, mROS signaling is altered (27). This model exhibits a hypothalamic hypersensitivity to glucose represented by an enhanced electrical activity in the arcuate nucleus and insulin secretion at low glucose levels (4, 27). These abnormal responses were associated with increased hypothalamic ROS levels at low glucose concentrations, a constitutive oxidized environment at both the cellular and mitochondrial levels, and an overexpression of several subunits of the respiratory chain, together with a dysfunction in mitochondrial respiration (27). In this study, no difference in the number of mitochondria or in uncoupling respiration was observed between the hypothalamus of obese *versus* control rats. Thus, a mechanism other than UCP2 decrease might explain excess ROS production. Recovery of the redox status through glutathione intracerebroventricular infusion fully reverses the hypothalamic hypersensitivity to glucose. Excessive mROS production might be a primary and causal link with the overoxidation of the redox environment, but further experiments are needed to test the order of emergency of these disorders. Nevertheless, hypersensitivity to glucose partially explains the elevated parasympathetic tone generally present in the obese and insulin-resistant state that consequently contributes to the development of hyperinsulinemia in the obese Zücker rat, and subsequently, to T2D onset.

mROS as an Integrated Signal for Glucose Sensing in Pancreatic β-Cells

Blood glucose homeostasis is primarily maintained by the adequate release of insulin by the pancreatic β cell, which operates as a glucosensor. Most of the glucose-derived pyruvate enters the mitochondrial metabolism, and one of the consensus signaling pathways identified is that ATP generation promotes the closure of the K_{ATP} channel, which depolarizes the membrane and then triggers Ca2+ influx by opening voltage-gated Ca2+ channels (92). Numerous amplifying signals have been identified, notably those coming from mitochondrial metabolism (50, 65). mROS production under glucose fluxes has mostly been studied in hyperglycemic diabetics, and the normal signaling process has not received much study. One of the first studies to highlight the dynamic production of mROS in isolated islets in response to

glucose stimulation was performed by Bindokas *et al.* (14). Using time-lapse imaging of HEt oxidation for superoxide recognition, they demonstrated the obligatory increased production of ROS due to mitochondrial metabolism under glucose stimulation in normal rats without demonstrating a causal link between mROS generation and glucose-stimulated insulin secretion (GSIS). Collins *et al.* demonstrated in mouse isolated islets and in a β-cell line that insulin secretion could be stimulated by elevated H_2O_2 levels and that scavenging this H_2O_2 production, which occurs when glucose rises, blunted GSIS (85). Only oxidative stressors (4-hydroxy nonenal, methylglyoxal) can decrease GSIS, in association with an increase in a battery of endogenous antioxidant enzymes (85). Previous studies have investigated the role of H_2O_2 in insulin secretion, and most have concluded that it alters mitochondrial activation and insulin secretion (58, 66, 94). One of the main differences that might explain this discrepancy is the concentration of H_2O_2 used, which was 50 μM or more in these latest studies, rather than the 1–4 μM range used by Collins *et al.* By analogy with the requirement for mROS in the brain's nutrient sensing, we have recently studied and demonstrated that mROS are also required for GSIS (62). In freshly isolated rat islets, GSIS was blocked by antioxidants in a dose-dependent manner, which highly correlated with mROS levels. Further, insulin release was mimicked by mitochondrial complex blockers (rotenone and antimycin). Both phenomena were observed independently of changes in ATP and NADH levels. No extra-mROS were detected in islets co-treated with glucose and carbonyl cyanide m-chlorophenyl hydrazone (an uncoupling compound that increases respiration and diminishes mROS generation). Therefore, the ROS production during the time course of glucose metabolism is solely due to mitochondria. Finally, we showed that this mROS signaling acted on the mobilization of calcium of extracellular origin (62). Altogether, these results strongly suggest that mROS are robust stimulators of insulin secretion. However, other works that investigated the effects of glucose on mROS formation in primary rat β cells (68) or the MIN6 cell line (54), which display higher metabolic responsiveness to glucose (68), did not reveal an increased ROS production in response to a glucose rise. Rather ROS levels were suppressed, especially at lower glucose concentrations (0–5 mM). However, with 10 mM glucose a small subset of β cells has a 10-fold higher HEt-fluorescence intensities (for superoxide anion detection), indicating heterogeneity in the population of cells. The authors propose this heterogeneity of the β-cell population, in which both glycolytic and mitochondrial glucose metabolism differ, would explain the difference in ROS production (68). Apart from the differences in models (fresh isolated islets [including all endocrine cells], FACS-sorted primary β cells, β-cell lines, or cultured islets), these discrepancies regarding mROS production could also be related to the different basal glucose concentrations used (0 or 2.5 *vs*. 5.5 mM). The basal glucose concentration certainly greatly modifies the basal levels of NADH, NADPH, and riboflavin as well as the redox environment and possibly UCP2 activity that alter the subsequent mitochondrial responses. Moreover, the fact that ROS suppression is predominantly observed at low glucose concentrations (below physiological concentrations) might reflect a recovery of the cells from a glucoprivic condition, which has been shown to increase mROS formation, at least in the brain and in neurons (79, 98).

In a study by Bindokas (14), a major difference in superoxide islet regulation was present between control lean animals and Zücker diabetic fatty (ZDF) rats because glucose stimulation triggered a large increase in superoxides in islets of control rats but not in the islets of ZDF rats (their basal mROS production being abnormally elevated), associated with a decreased secretion. This result suggests that one of the defects would be in the mechanism regulating the increase in ROS when the glucose level is raised; this increase is too small in ZDF islets. The amplitude of balanced mROS signaling is then diminished in ZDF rats, which leads to the decreased secretion of insulin. Although antioxidant defenses were not estimated in this work, it is consistent to think that their increase could prevent the necessary reduced-oxidized amplitude required for the activation of targets involved in insulin secretion. One interesting feature of β cells is their reportedly low levels of expression of antioxidant defenses, such as SOD (59), catalase, and glutathione peroxidases (only 1% of the levels in the liver) (63, 100). This feature supports the view that a low-H_2O_2-inactivating enzyme apparatus (but one that is still sufficient to quench a small and transient mROS increase) allows for the proper balance of mROS production necessary to trigger the signaling for insulin secretion. The existence of such an enzyme apparatus is consistent with the fact that overexpression of antioxidant defenses ameliorates the progression of hyperglycemia in established diabetes (110). In most studies, the antioxidant machinery appears to be one of the key components in regulating mROS signals, which clearly needs to be properly balanced to produce its physiological effects. An important factor implied in GSH regeneration and in regulation of mROS levels is the nicotinamide nucleotide transhydrogenase (Nnt). Nnt knockdown and mutations have been shown to impair insulin secretion in response to glucose; this dysfunction is linked to a great enhancement of superoxide production and a decrease in the ATP/ADP ratio, although glucose utilization was higher, suggesting that the uncoupling mechanism is more efficient (38). However, UCP2 does not appear to be the cause of these disorders (80) and the mechanisms involved remain unidentified. Recently, the Goto-Kakizaki/Paris rat model of T2D has highlighted the intricate pathways between the antioxidant machinery and the final mROS signal needed for proper insulin secretion (60). This model of spontaneous T2D is characterized by hyperglycemia and defective GSIS. After the onset of diabetes, although peri-islets exhibit an oxidative environment, the islets themselves appear protected against oxidative damage. They maintain a basal ROS level similar or even lower than that of control islets (60). This protection against oxidative damage appears to be due to both elevated glutathione content (reduced form) and overexpression of a large set of antioxidant proteins. In this model, the most likely mechanism by which ROS production is blunted in diabetic islets is *via* their raised antioxidant defenses and UCP2 expression. Both phenomena might directly decrease the Goto-Kakizaki/Paris β-cell function when exposed to high glucose through insufficient mROS and ATP generation.

Likewise, the role of UCP2 as a negative regulator of insulin secretion, suggested to be a mild uncoupler mostly involved in lowering ATP production (23, 112), can also be considered as a pathway allowing the termination of the mROS signaling after a glucose load in normal conditions through feedback regulation. A recent study found that overexpression of UCP2

both in a transgenic mouse line and in β cells did not alter GSIS, the elevated ATP/ADP ratio, glucose oxidation, mitochondrial membrane potential, or oxygen consumption (87). In this study, only increased UCP2 levels decreased cytokine-induced ROS production, supporting UCP2 as an antioxidant; however, how this works remains unclear. However, another recent study reveals apparently paradoxical roles for UCP2 in β-cell function: according to their genetic background (either UCP2$^{-/-}$ mice of congenic B6 lines or 129/B6 mixed background), GSIS was either diminished, as already described and largely accepted, or increased in isolated β cells, respectively (84). In the 129/UCP2$^{-/-}$ lines, decreased glucose-stimulated H_2O_2 production was observed compared with 129/UCP2+/+ islets. The blood levels of GSH and oxidized glutathione, which are fine markers of redox metabolism, differed markedly between the two genetic backgrounds as did the GSH/oxidized glutathione ratio. In this context, previous results on physiological ROS production or pathological oxidative stress in such models are difficult to interpret (84). Therefore, UCP2 function clearly needs to be reexamined for its role in pancreatic β cells and GSIS and in the pathogenesis of diabetes. Acute manipulation of its activity and comparison of various models would help our understanding of its exact function.

Regarding K_{ATP} channel signaling, it should be underlined that sulfonylurea (glibenclamide), like glucose-level elevation, has been shown to stimulate NADPH oxidase and ROS production in β cells through PKC-dependent activation (103). One of the therapeutic effects of sulfonylurea occurs through the enhanced production of ROS (although of non-mitochondrial origin) it enables, only when the beta-cell defect is still moderate. Recently, the genetic deletion of K_{ATP} channel activity (Sur1$^{-/-}$ β cells) was shown to protect islets against oxidative stress, rendering them less prone to apoptosis induced by high H_2O_2 levels than wild-type β cells (42). This protective effect was attributed to upregulation of the antioxidant enzymes, and the reduced sensitivity of Sur1$^{-/-}$ cells was mimicked by treatment with the sulfonylureas tolbutamide and gliclazide. These results, although under oxidative stress conditions, suggest a putative role of mROS in regulating K_{ATP} channel activity under physiological conditions. Brain H_2O_2-sensitive K_{ATP} channels are well characterized and promote a H_2O_2-dependent modulation of neurotransmitter release, and the H_2O_2 production has been recently showed to be of mitochondrial origin (7, 9). This mechanism implicates sulfonylurea receptor 1 (SUR1), which is an isoform operating in pancreatic β cells. However, Krippeit-Drews and colleagues (58) have shown that exogenous H_2O_2 (using a 1 mM concentration, a 100-fold that used in Collins' study) can lead to the opening of K_{ATP} channels in β cells and thereby inhibit insulin release. This result appears in contradiction with the findings showing that exogenous H_2O_2 (only 1–4 μM) or H_2O_2 derived from glucose metabolism stimulates insulin secretion (62, 85). However, even if the different concentrations explain the opposite results, this finding suggests that K_{ATP} would not be the target of mROS for insulin release under physiological conditions. Other channels (for instance, transient receptor potential or some L-type Ca channels) could be potential targets of mROS under physiological conditions, as we previously discussed and as suggested by others studies (48, 55, 57, 88, 101). Although further experiments will be needed to clarify the mROS-sensitive mechanisms involved in GSIS, these recent studies strongly support a role for mROS as a key integrative component of the mitochondrial energy status for monitoring the physiological response.

mROS as an Integrated Signal to Control Adipogenesis?

Long considered only to be the main energy store of the body, white adipose tissue now appears to be an endocrine organ able to interconnect all physiological functions to this energy store (105). Representing around 10% of total body weight in adults, it can reach >50% in obese people. For these reasons, its quantitative and qualitative relevance is considerable, regardless of the individual. Another evolving view on this tissue corresponds to its cell heterogeneity. Indeed, up until now, most groups have focused their research on white adipocytes, the specialized cells of adipose tissue. The white adipocyte displays a unique structure with a single, large droplet associated with low proportional cytoplasm content and very few mitochondria, especially when compared with its counterpart in brown adipose tissue, the brown adipocyte. In fact, reports increasingly describe the great heterogeneity of the cell population of white adipose tissue. Indeed, adipocytes represent <50% of the cells present in adipose tissue. The other cell fraction corresponds to the stromal fraction that contains endothelial, hematopoietic, and progenitor cells, such as adipose-derived stromal cells (ASCs), which were recently described as multipotent (43). These ASC can give rise to new differentiated adipocytes all throughout life, including in humans, and can participate in the enlargement associated with obesity.

The debate about the effects of ROS on adipocytes was largely concentrated on H_2O_2 mimicking the effect of insulin and being associated with insulin action on glucose transport, suggesting that cellular H_2O_2 generation was integral to insulin signaling (44). Insulin-stimulated H_2O_2 triggers insulin signaling, at least in part through the oxidative inhibition of protein tyrosine phosphatases that negatively regulate the insulin action pathway *via* the de-phosphorylation of various tyrosine residues of the insulin receptor and its substrate proteins (44). The resulting activation of insulin signaling promotes the release of insulin-stimulated H_2O_2 that corresponds to a positive retro-control that enhances insulin's effect. It was proposed that Nox4, a member of the NADPH oxidase family, largely mediates this insulin-stimulated generation of H_2O_2 (44).

In contrast to the insulin-stimulated generation of H_2O_2, and because white adipocytes display a low mitochondrial content, the physiological importance of mROS as a respiratory-chain byproduct was long neglected. In 2003, Wilson-Fritch and colleagues demonstrated that mitochondrial biogenesis is inherent to adipose differentiation *per se* and is influenced by the actions of adipogenic factors, including insulin and thiazolidinediones, *in vitro* as well as *in vivo* (106, 107). This view is currently evolving (31), but building upon this seminal study and using different respiratory chain inhibitors in the presence or absence of antioxidants, we demonstrated that moderate mROS generation inhibits the proliferation of adipocyte progenitors (21). Moreover, we illustrated that adipocyte differentiation is very sensitive to mROS because transient exposure to such stimuli at confluence influences the yield of adipocyte differentiation as

assessed 7 days later. The inhibition of adipogenesis would be mediated *via* the increase in the protein CHOP10, a member of CCAAT-enhancer-binding proteins family that can have a dominant-negative effect on all other members of the family (19). When investigating the putative physiological relevance of our *in vitro* data, we also demonstrated that adipose tissue in obese animals is associated with an increase in GSH as well as many other antioxidant defenses (39). *In vitro*, when these changes were mimicked by the treatment of preadipocytes with an antioxidant, they strongly promoted adipogenesis consistently with our previous *in vitro* data. Altogether, these results led us speculate that obesity is associated with an increase in antioxidant defenses that could, in turn, promote cell proliferation and differentiation through the modulation of redox-sensitive transcription factors. This supplementary adipogenesis further increases the antioxidant defenses that promote and amplify the onset of obesity. Thus, a proadipogenic loop corresponding to a deleterious cycle is established (Fig. 2). This management of redox metabolism is site specific (39) and more adaptive in subcutaneous fat than in internal fat. This pattern is consistent with the protective role of subcutaneous fat (102). Another indirect argument can be found to support the idea that mROS can, as a cell metabolism sensor, control the insulin sensitivity of adipocytes and their endocrine functions. Indeed, inhibition of UCP2 by genipin reduces the insulin-stimulated glucose uptake in 3T3-L1 adipocytes (113). This finding is also consistent with the effects of hyperglycemia on adipocytes, which are associated with a decrease in insulin sensitivity and the stimulation of inflammatory cytokines (64). Indeed, the effects of hyperglycemia can be mimicked by stimulating mROS generation or, in contrast, blunted by any manipulations that decrease mROS generation. However, the downregulation of glutathione-S-transferase in adipose tissue leads to increased protein carbonylation, ROS production, and mitochondrial dysfunction and contribute to the development of insulin resistance and T2D (29). More spectacularly, Villarroya's group demonstrated that the modulation of mROS levels by mitochondrial activity, specifically as a consequence of the action of UCP2, controls adiponectin gene expression (26). This control provides a key physiological mechanism by which the energetic status of adipose tissue conditions systemic insulin sensitivity *via* mROS signaling and its effect on the balance of insulin-sensitizing or inflammatory cytokines (26, 64). These conclusions are very consistent with a recent report showing that mitochondrial superoxide anion levels are increased in four models of insulin resistance, and that either pharmacologic or genetic strategies that override or increase mitochondrial superoxide anion levels reverse or trigger, respectively, the onset of insulin-resistance both *in vivo* and *in vitro* (51). Once again, this implicates mitochondrial superoxide as a metabolic sensor that links excess energy and the intracellular metabolism of muscles with the control of insulin action. In the context of hyperglycemia or dyslipidemia, this impairment could be enhanced by mitochondrial dysfunction (40).

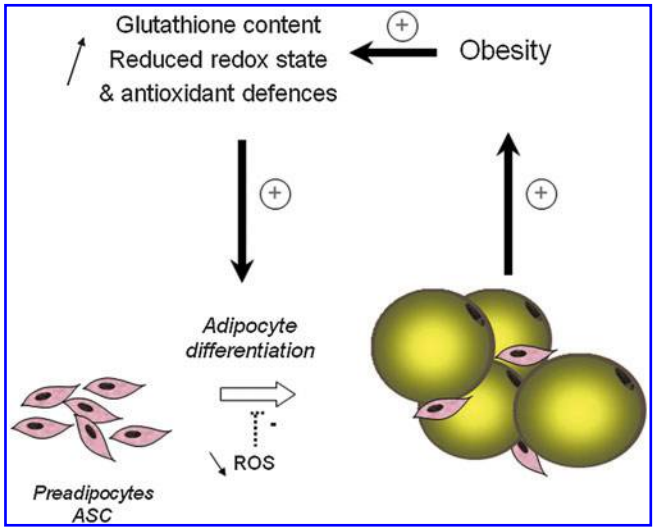

FIG. 2. Deleterious redox cycle in obesity. The adipocyte differentiation and hypertrophia lead to obesity and are associated with an increase in antioxidant defenses and an increase of the glutathione content and its reduced form. These elements promote in turn adipocyte differentiation and hypertrophia by preventing the antiadipogenic mitochondrial reactive oxygen species signal. This in turn promotes further obesity, and a deleterious cycle takes place. ASC, adipose-derived stromal cell; ROS, reactive oxygen species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

More recently, another mitochondrial source of superoxide anion, the p66Shc protein, was documented in the insulin signaling pathway. It is noteworthy that such generation depends on the electron transfer of the respiratory chain but is not intimately linked to mROS generation from complexes I and III. p66Shc was the first mammalian gene whose mutation was demonstrated to increase resistance to oxidative stress and to prolong lifespan (72). It appears that p66Shc translocates to the mitochondria of stress-challenged cells where it catalyzes the formation of H_2O_2 in the mitochondrial intermembrane space through an inherent ROS-producing activity and decreases expression of manganese-dependent SOD (78). This ROS formation might trigger the initiation of the mitochondrial apoptosis pathway (41). This pathway is activated by insulin specifically in adipocytes and regulates insulin signaling through multiple mechanisms. In contrast, the deletion of p66 (Shc) resulted in reduced triglyceride accumulation in adipocytes and decreased *in vivo* fat mass (12). These findings suggest that p66(Shc)-generated ROS regulates the effect of insulin on energy metabolism in mice and that such intracellular oxidative stress accelerates aging by favoring fat deposition and fat-related disorders. However, a recent report described that p66 deficiency in ob/ob mice increased inhibitory phosphorylation of IRS-1 and enhanced the activity of S6K, resulting in the improvement of insulin resistance in obese mice (89). The authors propose an explanation for the discrepancy featuring a pro- or antiadipogenic role for this enzyme according to the insulinemia and nutrient availability corresponding to a normal or diabetic context. They also emphasized that when p66shc KO mice are fed a high-fat diet, they display better glucose control, in spite of lower plasma insulin levels, than wild-type mice.

At the intracellular level, it was demonstrated that mild oxidative conditions enhance the activation of the insulin receptor subunit, suggesting that optimal insulin responsiveness involves a "redox priming" of the subunit (44). Thus, the cross-talk between insulin and ROS signaling could be initiated by the insulin-mediated generation of low levels of

endogenous H_2O_2. This conclusion, consistent with the pro-insulin effect induced by H_2O_2, is quite different from the previous conclusion that oxidative stress from the mitochondrial respiratory chain inhibits adipogenesis. One explanation for these different results is that mitochondrial dysfunctions are not systematically linked to the inhibition of adipogenesis (31) and that triglyceride storage is not only dependent on lipogenesis and free fatty acid uptake but also on lipid oxidation. We can propose that, according to the alteration of electron transfer through the different complexes of the respiratory chain, the oxidation processes could be impaired enough to result in lipid accumulation as a dominant element.

In addition to the role of respiratory-chain-generated mROS on adipocyte function, we questioned whether mROS production may change ASC plasticity because these cells can behave as endothelial-like cells (86). Indeed, ROS have been shown to play major and positive roles in blood vessel growth as well as *in vivo* preconditioning protection (70). We showed that transient and moderate stimulation of mROS generation in ASC before their injection into ischemic hind limbs strongly improved revascularization and the number of ASC-derived CD31-positive cells in the ischemic area (19). mROS generation increased the secretion of the pro-angiogenic and antiapoptotic factors VEGF and HGF and greatly protected ASCs against oxidative-stress-induced cell death (20). These data lead us to propose a provocative scheme for adipose tissue development, similar to tumor growth. When adipocytes become hypertrophic, hypoxia occurs and superoxide anions are generated by the respiratory chain (19, 24, 46, 108). This signal inhibits the adipogenic differentiation of ASCs and promotes their angiogenic potential. This, in turn, triggers neoangiogenesis, which provides nutrients and oxygen to the enlarging tissue, increasing oxygen tension, decreasing hypoxia, and promoting adipogenesis. In this way, adipose tissue can enlarge through successive waves of adipogenesis and angiogenesis.

Moreover, all these phenomena, *in vitro* as well as *in vivo*, could participate in the site-specific adverse effects (lipodystrophia) of drugs able to interact with mitochondria, such as nucleotide reverse transcriptase inhibitors used in HIV-treatment (32, 93).

Altogether, these data provide significant clues to consider mROS a basic fundamental and physiological signal for adipocyte functioning, including its endocrine function and development that are closely linked to its own energetic status. According to the importance of such a link, it is reasonable to propose that it could also participate in any of the dysfunctions associated with impaired mitochondria in adipose tissue that are regularly described in diabetic or obese patients (31). Conversely, any improvement in mitochondrial function and electron transfer would be associated with an improvement in the disease *via*, at least in part, the recovery of physiological mROS signaling (107).

Conclusion

Mitochondria are important energetic organelles that have been shown in recent studies to generate ROS according to nutrient, hormonal, and/or O_2 fluxes and to mediate normal responses. They represent a central crossroads of metabolic pathways, and the genesis of mROS signals, occurring in the respiratory chain, provides the cell with precise information because mROS production reflects both the input and need of energy. Signaling through mROS has now been identified in numerous metabolic studies and in many cell types, shedding light on the overall importance of mROS throughout the body and their crucial role in the control of energy status. The delineation of the physiological signaling that mROS enable *versus* the deleterious effects that they trigger remains blurred and merits further investigation. Much remains to be explored in terms of the physiological mechanism of the signal, its novel targets, and the primary events leading to the progression and establishment of metabolic disorders in which general oxidative stress plays a major role.

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Abbreviations Used

AgRP = agouti-related peptide
ASCs = adipose-derived stromal cells
GE neurons = gluco-excited neurons
GSH = reduced glutathione
GSIS = glucose-stimulated insulin secretion
H_2O_2 = hydrogen peroxide
HEt = hydroethidine
mROS = mitochondrial reactive oxygen species
NPY = neuropeptide Y
POMC = pro-opiomelanocortin
SOD = superoxide dismutase
T2D = type 2 diabetes
UCP2 = uncoupling protein 2
ZDF = Zücker diabetic fatty

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