

Skeletal muscle insulin resistance: role of mitochondria and other ROS sources

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Abstract

At present, obesity is one of the most important public health problems in the world because it causes several diseases and reduces life expectancy. Although it is well known that insulin resistance plays a pivotal role in the development of type 2 diabetes mellitus (the more frequent disease in obese people) the link between obesity and insulin resistance is yet a matter of debate. One of the most deleterious effects of obesity is the deposition of lipids in non-adipose tissues when the capacity of adipose tissue is overwhelmed. During the last decade, reduced mitochondrial function has been considered as an important contributor to 'toxic' lipid metabolite accumulation and consequent insulin resistance. More recent reports suggest that mitochondrial dysfunction is not an early event in the development of insulin resistance, but rather a complication of the hyperlipidemia-induced reactive oxygen species (ROS) production in skeletal muscle, which might promote mitochondrial alterations, lipid accumulation and inhibition of insulin action. Here, we review the literature dealing with the mitochondria-centered mechanisms proposed to explain the onset of obesity-linked IR in skeletal muscle. We conclude that the different pathways leading to insulin resistance may act synergistically because ROS production by mitochondria and other sources can result in mitochondrial dysfunction, which in turn can further increase ROS production leading to the establishment of a harmful positive feedback loop.

Key Words

- ▶ insulin resistance
- ▶ obesity
- ▶ diabetes
- ▶ exercise
- ▶ mitochondria
- ▶ oxidative stress
- ▶ ROS production
- ▶ ROS sources

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Introduction

In recent years, the observation that insulin resistance (IR) and type 2 diabetes mellitus (T2DM) are growing dramatically all over the world has stimulated the research on such metabolic disorders and their possible therapy. At present, it is well established that the high incidence of IR is in great part due to the global epidemic of obesity (Amati *et al.* 2009), a medical condition with a chronic imbalance between energy intake and energy expenditure, described as excess body weight in the form of fat. Epidemiological studies show that the mean weight

of the population is increasing and that the prevalence of overweight and obesity in adults and children is rapidly rising, especially in urban populations.

It is well known that there are several factors contributing to the increased risk of developing obesity including inactivity, overeating, higher age, smoking cigarettes, ethnicity and inherited predisposition to obesity.

It is also well established that skeletal muscle plays a central role in the whole body IR (Zierath *et al.* 2000).

However, the mechanisms underlying the relationship between obesity and skeletal muscle IR are yet a matter of debate. The prevailing theory is based on the inability of adipose tissue to store the excess energy, which results in an elevated outflow of free fatty acids (FFA) from fat depots at other tissues including skeletal muscle (Sethi & Vidal-Puig 2007). The excessive lipid content within this tissue causes metabolic dysregulation, including IR. The theory also suggests that mitochondria are key players in the IR development, but there are different views about the mechanisms by which mitochondria contribute to IR pathogenesis. It has been proposed that a decrease in mitochondrial fatty acid oxidation caused by mitochondrial dysfunction and/or reduced mitochondrial content leads to the accumulation of increased levels of intracellular fatty acyl-CoA and diacylglycerol, which interfere with the insulin signaling (Lowell & Shulman 2005). It has also been proposed that the development of IR in skeletal muscle is due to an enhancement in mitochondrial oxidant production in response to excess fuel relative to demand, which results, through different pathways, in decreased insulin signaling and glucose transport (Rindler *et al.* 2013).

It is worth noting that even though in any case a pivotal role in the obesity-linked IR is assigned to mitochondria (Lark *et al.* 2012), recent researches propose the involvement of other cellular organelles and enzymes (Newsholme *et al.* 2007).

The purpose of this review is to examine the literature examining how the mitochondria might act as a contributory factor to skeletal muscle IR describing possible cellular and molecular mechanisms. Another not secondary purpose of the review is to find a way, if any, to reconcile the different views on the role of mitochondria in IR development.

Obesity

In the 21st century, obesity is one of the most important public health problems in the world. It is a chronic disease in the same sense as hypertension and atherosclerosis. The etiology of obesity is the imbalance between the energy ingested with food and the energy expended. The excess energy is stored in fat cells that enlarge and/or increase in number. Although the fact that overweight people likely die young is long known, only recently the mechanisms by which obesity causes ill-health and reduces life expectancy have been understood. The disease that is increased by the greatest degree in obese people, compared with lean ones,

is T2DM. This condition usually develops in middle age and is associated with increased liability to cardiovascular disorders, which may lead to damage to the eyes, kidneys, peripheral nerves and circulation.

T2DM is characterized by excessively high plasma glucose concentrations, brought on by an imbalance between glucose and its main regulator, insulin. Although the primary defect in the pathogenesis of T2DM is not known, the manifestation of this progressive metabolic disorder is probably due to a combination of genetic and environmental factors (Leahy 2005). Common environmental influences include a high-fat high-calorie diet, lack of physical activity and obesity. Genetic predisposition can lead to defects in β -cell function, glucose sensing, insulin signaling and appetite regulation.

Although identifying the mechanisms underlying T2DM is difficult, at present, it is well established that a pivotal role in its development is played by IR.

The mature adipocyte contains a single large fat droplet, which occupies most of the cell's volume and is surrounded by a thin rim of cytoplasm that lies between the droplet and the plasma membrane. The primary function of the adipocyte is to store energy in the form of triglycerides (TG) during periods of energy surplus and to mobilize these stored lipids as fatty acids when energy is required (Otto & Lane 2005). Fatty acids are released into the blood stream and supply the peripheral tissues, especially skeletal muscle and the liver, as an energy-rich fuel.

Adipose tissue does not only store energy but also acts as an active endocrine and paracrine organ that plays a major role in the control of metabolism through secretion of a large number of biologically active molecules (Matsuzawa *et al.* 1999), collectively known as adipocytokines or adipokines, including plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor α (TNF- α), resistin, leptin and adiponectin (Vettor *et al.* 2005).

The increase in adipose tissue mass in obesity is the result of both an increase in the number (hyperplasia) and size (hypertrophy) of fat-laden adipocytes (Otto & Lane 2005). Although the hypertrophy of adipocytes is rapidly reduced by food deprivation, i.e. fasting, hyperplasia is resistant to change. Therefore, once an individual has experienced the obese state, the 'new' adipocytes acquired persist and are quickly refilled if energy intake exceeds expenditure.

It has been proposed that hyperplasia and hypertrophy of fat cells represent the pathological lesion of obesity (de Ferranti & Mozaffarian 2008). Enlarged fat cells produce the clinical problems associated with

obesity either because of the mass of the extra fat or because of the increased secretion of free fatty acids and numerous peptides from enlarged fat cells. Indeed, the dysregulated production of the adipocytokines participates in the pathogenesis of obesity-associated metabolic syndrome, even though the mechanisms by which fat accumulation leads to such a dysregulation have not yet been elucidated. Increased production of PAI-1 and TNF- α from accumulated fat contributes to the development of thrombosis (Shimomura *et al.* 1996) and IR (Hotamisligil *et al.* 1993), respectively. In contrast, adiponectin exerts insulin-sensitizing (Berg *et al.* 2001) and anti-atherogenic effects (Okamoto *et al.* 2002), and therefore, a decrease in plasma adiponectin is associated with IR and atherosclerosis. Adiponectin is believed to activate the 5'-AMP activated protein kinase (AMPK), which seems to play a role in insulin-independent glucose uptake by the muscle (Yamauchi *et al.* 2002).

Insulin resistance

IR is a reduction of the responses of peripheral target tissues including muscle, adipose tissue and liver to a physiological concentration of insulin, so that IR is characterized by reduced tissue insulin sensitivity and reduced glucose, lipid and protein metabolism.

IR and functional impairment of islet β cells are the main pathological causes and hallmarks of T2DM. IR occurs before the islet β cell damage, so that at least initially the majority of diabetes patients are non-insulin dependent and capable of producing insulin, but are only deficient in their cellular response. When pancreatic β -cells are no longer able to compensate for IR by adequately increasing insulin production, impaired glucose tolerance appears, characterized by excessive postprandial hyperglycemia (Gerich 2003). IR in skeletal muscle and abnormal pancreatic β -cell function are the earliest detectable defects preceding hyperglycemia even 10 years before diabetes is diagnosed. Multiple factors contribute to IR, and it is widely accepted that obesity, reduced physical activity and genetic alterations are the main risk factors for IR. However, the precise molecular mechanism of IR remains largely unknown.

Skeletal muscle, by virtue of its mass and high rate of insulin-stimulated glucose transport, represents an important tissue in the development of IR (Caro *et al.* 1989). Thus, skeletal muscle IR represents a major defect in the maintenance of normal levels of glycemia (Zierath *et al.* 2000) and is often accompanied by a variety

of metabolic and cardiovascular abnormalities, including hypertension, dyslipidemia, T2DM and atherosclerosis (DeFronzo & Ferrannini 1991). Because of the importance of skeletal muscle in the development of IR, many researchers focused on this tissue, and the results of their works seem to indicate that the impaired glucose uptake that characterizes skeletal muscle IR results from impaired insulin receptor signaling (Goodyear *et al.* 1995, Bjornholm *et al.* 1997, Cusi *et al.* 2000).

Insulin action

In normal conditions, the insulin actions are initiated by its interaction with specific receptors on the cell surface of many cell types including muscle cells, adipocytes, hepatocytes and neurons of the CNS (White & Kahn 1994). The insulin receptor spans the plasma membrane and, upon binding with insulin, transmits inside the cell a signal, which is amplified and triggers a variety of intracellular responses promoting energy storage and inhibiting mobilization of energy reserves. Indeed, insulin activates glucose uptake by muscle cells and adipocytes, and promotes glycogen and fat synthesis in hepatic cells.

The binding of insulin to the α -subunits of its receptor stimulates the tyrosine activity of the receptor β subunit. This tyrosine kinase phosphorylates several intracellular proteins leading to the activation of different signaling pathways. Although the extracellular signal-regulated kinase (ERK) pathway is mainly involved in growth, the activation of phosphatidylinositol 3-kinase (PI3K) is involved in the metabolic actions of insulin (White 2002). Such actions are mediated by the insulin receptor-catalyzed phosphorylation of the insulin receptor substrates 1 and 2 (IRS1, IRS2). The tyrosine-phosphorylated IRS proteins then interact with and activate PI3K, a critical player in insulin signaling particularly with regard to glucose homeostasis (Cheatham *et al.* 1994). PI3K appears to facilitate the translocation to the plasma membrane of the insulin-responsive glucose transporter (GLUT4), which mostly resides within intracellular storage sites. The process plays a crucial role in insulin-mediated glucose transport into the skeletal muscle (Zisman *et al.* 2000), but the link between PI3K and glucose transport is not completely known. It is likely that the mechanism is mediated by the recruitment and activation of 3-phosphoinositide-dependent kinases (PDK), and subsequent PDK-dependent phosphorylation of a serine/threonine kinase, protein kinase B (PKB/AKT) (Kohn *et al.* 1996). Although AKT has several substrates, including atypical protein kinase C (PKC) isoforms and glycogen synthase kinase 3 (GSK3),

another substrate of 160kDa (AS160), has emerged as an additional molecule important in the activation of glucose transport in muscle. AS160 is a protein that in activated form prevents GLUT4 translocation to the membrane. It is phosphorylated and inactivated by AKT so that, by AS160 inhibition, insulin promotes the GLUT4 translocation from inner vesicles, promoting fusion to the plasma membrane and consequently glucose uptake (Sakamoto & Holman 2008).

Some works have provided evidence that an atypical protein kinase C (PKC ζ) plays a role in insulin signaling and glucose transport activation. PKC ζ is activated by insulin in a PI3K-dependent manner in L6 muscle cells (Bandyopadhyay *et al.* 1997). The use of PKC ζ inhibitors and inactive mutants has demonstrated that this kinase is required for full insulin stimulation of glucose transport. Moreover, insulin induces the phosphorylation of PKC ζ by PDK1 and the relocalization of PKC ζ to GLUT4 vesicles in rat adipocytes (Bandyopadhyay *et al.* 1999). These studies indicate that there may be a requirement for the activation of both PKB and PKC ζ for GLUT4 translocation and subsequent glucose transport stimulation. However, a complete understanding of the mechanisms by which PKC ζ interacts with other components of the signaling pathway remains to be achieved.

It is worth noting that to propagate the tyrosine kinase signal, as well as signaling through downstream protein and phospholipid kinases, insulin binding also initiates the deactivation of members of the family of the protein tyrosine phosphatases, such as protein tyrosine phosphatase 1B (PTP1B) and phosphatase and tensin homolog (PTEN), that under resting cellular conditions, have an activity, which exceeds kinase activity and inhibit insulin signaling (Elchebly *et al.* 1999, Wijesekara *et al.* 2005) (Fig. 1, upper panel).

Impairment of insulin signaling in insulin resistance

Subjects with obesity (Goodyear *et al.* 1995) and subjects with T2DM (Bjornholm *et al.* 1997) exhibit reduced IRS-1 tyrosine phosphorylation and reduced PI3K activity compared with their respective controls. The reduction in tyrosine phosphorylation of IRS-1 and IRS-2 has been related to their increased serine/threonine phosphorylation (Paz *et al.* 1997). Proposed IRS serine/threonine kinases include inhibitor kappa B kinase (IKK) (Gao *et al.* 2002), c-Jun amino-terminal kinases (JNK) (Aguirre *et al.* 2000) and mammalian target of rapamycin (mTOR) (Li *et al.* 1999). As a result of IRS serine/threonine

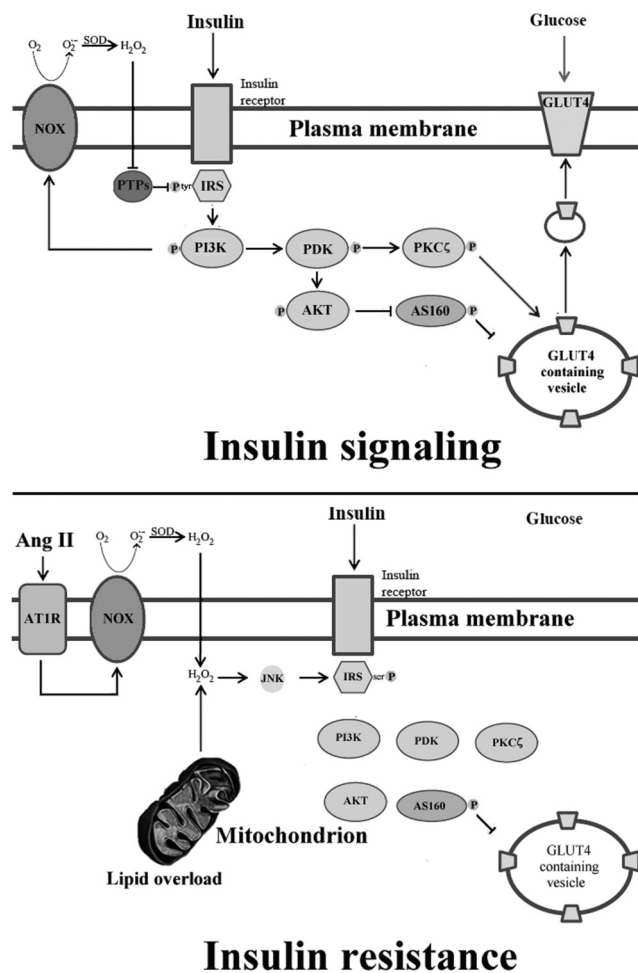


Figure 1 Schematic representation of insulin-stimulated glucose uptake in skeletal muscle and its inhibition in obese or high-fat diet-fed animals. GLUT4, glucose transporter type 4; NOX, NADPH oxidase; SOD, superoxide dismutase; Ang II, angiotensin II; AT1R, Ang II type 1 receptor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PTPs, protein tyrosine phosphatases; PKC ζ , atypical protein kinase C; PDK, phosphoinositide-dependent kinase; Akt, protein kinase B; AS160, 160 kDa protein; JNK, c-Jun amino-terminal kinases. Reproduced, with permission, from Rindler PM, Crewe CL, Fernandes J, Kinter M & Szweda LI (2013) Redox regulation of insulin sensitivity due to enhanced fatty acid utilization in the mitochondria. *American Journal of Physiology* volume 305, pages H634–H643. Copyright The American Physiological Society (APS). All rights reserved.

phosphorylation, PI3K levels are reduced with subsequent alteration of downstream effectors, i.e. decreased activity of Akt (Kim *et al.* 1999) and atypical PKC (Kim *et al.* 2003), and decreased glucose uptake, presumably due to reduced GLUT4 activity/translocation (Shulman 2000) (Fig. 1, lower panel).

It is worth noting that although considerable evidence supports the model describing a major role for IRS in IR, results inconsistent with such a model still remain,

including the observation that the origin of IR can occur independently of IRS (Hoehn *et al.* 2008).

The prevalent theory on impaired insulin signaling in obesity links IR to the increase of circulating FFA. Indeed, according to this theory, one of the most deleterious effects of obesity is the deposition of lipids in non-adipose tissues. In obesity, when the capacity of visceral and subcutaneous adipose tissue is overwhelmed, circulating levels of fatty acids are markedly increased. In these conditions, other tissues are used for lipid accumulation (e.g. liver, muscle, pancreas and heart) (Sethi & Vidal-Puig 2007). As these organs are less capable to storing lipids than adipocytes and more adversely affected, lipotoxicity may be the result culminating, in IR of muscles, liver and pancreas (Shulman 2000).

This hypothesis was supported by findings from human studies involving lipid infusion. Such a procedure increases intramyocellular lipid content (Bachmann *et al.* 2001, Brechtel *et al.* 2001) and inhibits insulin-stimulated glucose uptake in subjects who are healthy (Boden *et al.* 1991) and in subjects with T2DM (Boden & Chen 1995). Thus, it was shown that the inhibition of insulin-stimulated glucose uptake inversely correlated with the increased FFA levels produced by the lipid infusion (Belfort *et al.* 2005).

It is worth noting that, although lipid infusion is a well-documented method of producing skeletal muscle IR, it has become increasingly evident that lipid oversupply does not act alone in this process.

Because the mitochondria are the primary cellular site for fatty acid oxidation and utilization, during the last decade, reduced mitochondrial function has been considered as an important contributor to 'toxic' lipid metabolite accumulation and consequent IR.

More recently, Fisher-Wellman and coworkers (Fisher-Wellman *et al.* 2014) reported that lower oxidative capacity is not a requirement for the early phases of development of IR in obesity, leaving open, however, the possibility that when mitochondrial capacity is diminished it might contribute to reduced insulin sensitivity. They also found that mitochondrial H_2O_2 emission was increased, supporting a previous report implicating oxidative stress in the pathogenesis of IR (Anderson *et al.* 2009).

At present, it is considered unlikely that IR is explained by a single cause. Rather, it is more likely that IR may develop as a result of multiple complementary mechanisms.

Mitochondrial dysfunction in insulin resistance

For a long time, mitochondria were mainly considered for their role in the energy production, being the sites of the oxidative phosphorylation, which couples the electron transfer from respiratory substrates to oxygen with the ATP synthesis. In such process, food-derived substances are oxidized by transfer of electrons to electron carriers such as NAD^+ , FMN and FAD, which, in a series of subsequent steps, transfer electrons to the several redox centers, and finally to oxygen. Respiratory chain components involved in the electron transfer are in great part organized in four protein complexes inserted in the inner mitochondrial membrane (Navarro & Boveris 2007). Complexes I and II transfer electrons to the lipid-soluble carrier ubiquinone, from which the electrons pass through Complex III, cytochrome c (another mobile carrier) and Complex IV, to the oxygen. The fall in electron potential energy is used to pump protons from the mitochondrial matrix to intermembrane space, thus setting up a proton-motive force (Mitchell & Moyle 1965). Such a force drives protons back into the matrix through the mitochondrial ATP synthase leading to ATP synthesis (Walker *et al.* 1995).

Mitochondria are not only key actors in global energy modulation, but also are required for a wide range of functions fundamental for the cell. It is therefore not surprising that mitochondrial dysfunction is associated with a large proportion of diseases, such as neurodegenerative disorders, cardiomyopathies, metabolic syndrome, cancer and obesity (Nunnari & Suomalainen 2012).

Mitochondrial dysfunction was first described in the context of glucose intolerance ~40 years ago (Yamada *et al.* 1975), but a role for mitochondria in IR emerged only from the late 1990s when studies in humans suggested the existence of mitochondrial dysfunction in obese and insulin-resistant patients (Kelley *et al.* 1999, Simoneau *et al.* 1999). From these reports the idea arose that a decrease in tissues of mitochondrial oxidative capacity, due to loss in mitochondrial content and/or function, results in insufficient lipid oxidation with the effect to exacerbate lipid excess and lead to IR.

Most of the human studies looked at the potential differences in skeletal muscle mitochondria abundance and function between insulin-sensitive, insulin-resistant and type 2 diabetes patients. Skeletal muscle mitochondria

from type 2 diabetes patients exhibited lower activity of the respiratory chain (Kelley *et al.* 2002, Ritov *et al.* 2010), most probably arising from a reduction in the expression of genes encoding mitochondrial enzyme subunits (Mootha *et al.* 2003, Patti *et al.* 2003). Heilbronn and coworkers (Heilbronn *et al.* 2007) and Lefort and coworkers (Lefort *et al.* 2010) found a decrease in several markers of mitochondrial metabolism in IR subjects. Similar results were also obtained in overweight children (Fleischman *et al.* 2009) and in patients with genetic defects in insulin receptor signaling (Sleigh *et al.* 2011).

Peluso and coworkers (Peluso *et al.* 2002) analyzed skeletal muscle of obese IR individuals and of lean controls and found that mitochondrial carnitine acylcarnitine translocase was specifically decreased at translational and trasductional level, suggesting that this decrease may contribute to the elevated muscle concentrations of triglycerides, diacylglycerol and fatty acyl-coenzyme A characteristic of IR muscle. Further support to the link between intramyocellular lipids and mitochondrial function was found in insulin-resistant subjects, where lower ATP synthase was found in concomitance with elevated intramyocellular lipids compared to control subjects (Petersen *et al.* 2004). However, other studies observed an *in vivo* impairment in mitochondrial function in diabetic patients (Szendroedi *et al.* 2007) without accumulation of intramyocellular lipids (Schrauwen-Hinderling *et al.* 2007, Phielix *et al.* 2008). Ritov and coworkers (Ritov *et al.* 2005) compared volunteers with type 2 diabetes, obese sedentary nondiabetic volunteers and lean volunteers and found a selective decrease in the number and activity of subsarcolemmal mitochondria and suggested that this deficit may contribute to the pathogenesis of muscle insulin resistance in type 2 diabetes because of the potential importance of subsarcolemmal mitochondria for signal transduction and substrate transport. Boushel and coworkers (Boushel *et al.* 2007) and Holloway and coworkers (Holloway *et al.* 2007) claimed that blunting of mitochondrial respiration in type 2 diabetic patients or obese women, respectively, can be attributed to lower mitochondrial content, whereas mitochondrial function was normal. Young, lean, insulin-resistant offspring of type 2 diabetic patients exhibited lower rates of muscle mitochondrial substrate oxidation compared to insulin-sensitive control subjects, and the authors hypothesized that insulin resistance in skeletal muscle of insulin-resistant offspring is associated with dysregulation of intramyocellular fatty acid metabolism, possibly because of an inherited defect in the activity of

mitochondrial oxidative phosphorylation (Befroy *et al.* 2007). Because high-fat diet can result in obesity and T2DM, which are characterized by reduced mitochondrial mass and function, it was possible to hypothesize that high-fat diet is able to affect the expression of genes involved in mitochondrial biogenesis. To test this hypothesis, Sparks and coworkers (Sparks *et al.* 2005) studied skeletal muscles from insulin-sensitive healthy men and mice fed an isoenergetic high-fat diet for 3 days and 3 weeks, respectively. They found a downregulation of genes involved in oxidative phosphorylation and mitochondrial biogenesis and suggested that these changes that mimic those observed in diabetes and insulin resistance (Kelley *et al.* 2002, Mootha *et al.* 2003, Patti *et al.* 2003, Heilbronn *et al.* 2007, Lefort *et al.* 2010, Ritov *et al.* 2010), if sustained, might result in mitochondrial dysfunction in the prediabetic/insulin-resistant state. However, longer-term (28 days) overfeeding studies on non-obese sedentary men failed to support such an idea because the levels of proteins regulating mitochondrial biogenesis or determining oxidative capacity, which were increased at day 3, returned to basal at day 28 (Samocho-Bonet *et al.* 2012).

All the previously mentioned human studies consistently show that *in vivo* mitochondrial function is reduced in insulin-resistant subjects and/or type 2 diabetic patients. This mitochondrial impairment could contribute to the onset of insulin resistance by favoring lipid accumulation and because decreased ATP is available for membrane transports and signal transduction pathways. Although the previously cited studies all converge on the idea that mitochondrial dysfunction is a key feature of insulin-resistant muscle in men, some studies found no such correlation (De Feyter *et al.* 2008a,b, Karakelides *et al.* 2010, Samjoo *et al.* 2013).

Taken together, studies on men are mainly consistent with the association between insulin resistance and mitochondrial impairment, although in general, they do not discriminate between cause and effect. In addition, in human studies, it is difficult to control parameters such as energy intake, quality of the diet and level of activity.

Many studies have also been carried out in animal models of obesity and diabetes, mainly rats and mice. The results obtained in these animal models are mostly in agreement with those obtained in human studies. The most used protocol to induce insulin resistance is overfeeding with high-fat/high-sugar diets in rats (Chanseume *et al.* 2006, Gomes *et al.* 2012, Crescenzo *et al.* 2013, Warren *et al.* 2014) or

in mice (de Wilde *et al.* 2008, Shelley *et al.* 2009, Yuzefovych *et al.* 2013), however, models of genetic obesity were also used (Holmström *et al.* 2012). However, other observations report discrepant results, showing that high-fat feeding in rats was associated with no variation (Atgié *et al.* 1993, De Feyter *et al.* 2008a,b) or even a higher mitochondrial capacity (Hancock *et al.* 2008), and IR could be related to incomplete intramitochondrial β -oxidation (Koves *et al.* 2008). Several studies show that insulin resistance arises when mitochondrial function is unaffected or even improved (Turner *et al.* 2007, Hoeks *et al.* 2008, Ara *et al.* 2011, Boudina *et al.* 2012), or conversely that impaired mitochondrial functioning alone does not cause insulin resistance (Wredenberg *et al.* 2006). These latter findings data seem to indicate that, at least in rodents, consumption of high-fat diet is not always accompanied by mitochondrial dysfunction, but rather leads to improved mitochondrial oxidative capacity and/or biogenesis (Gómez-Pérez *et al.* 2012, Wessels *et al.* 2015), even in the presence of IR. The duration of high-fat feeding also seems to differently affect mitochondrial function in skeletal muscle. In fact, oxidative phosphorylation was found increased after short-term (2–3 weeks) dietary treatment, but decreased after long-term treatment (Chanseume *et al.* 2007, Laurent *et al.* 2007, Bonnard *et al.* 2008).

An intriguing hypothesis proposed by several authors is that insulin resistance develops when mitochondrial dysfunction and the following inability to oxidize fatty acids takes place in conjunction with an increase in lipid flux to skeletal muscle (Chow *et al.* 2010). The above hypothesis is supported by the observation that the exposure to saturated fatty acids of isolated mitochondria (Abdul-Ghani *et al.* 2008) or cells (Hirabara *et al.* 2010, Jheng *et al.* 2012, Yang *et al.* 2012, Teodoro *et al.* 2014) clearly elicits mitochondrial dysfunction, which in the cells is accompanied by IR. However, in more complex systems, such as in men and experimental animal models, the link between plasma NEFA delivery to skeletal muscle, mitochondrial impairment and insulin resistance is less strong. Acute elevation in plasma NEFA by fasting in men was found to induce mitochondrial dysfunction in skeletal muscle in the absence of insulin resistance (Hoeks *et al.* 2010), whereas acute infusion of FFA+insulin in rats blunted insulin signaling in skeletal muscle without any effect on mitochondrial function (Barazzoni *et al.* 2012). Taken together, the previously mentioned results are in line with the idea that mitochondrial dysfunction may

arise in consequence of muscular fat accumulation and could in turn elicit insulin resistance.

The cellular capacity of lipid oxidation is affected by mitochondrial number, mitochondrial activity and mitochondrial degree of coupling between oxidation of fuels and ATP synthesis. Moreover, substrate oxidation rate is strictly linked to ATP turnover (Boveris *et al.* 2000), so that, in resting skeletal muscle, changes in mitochondrial degree of coupling, but not changes in organelle number and/or activity, would affect the amount of oxidized fuels, even if ATP turnover does not vary. In fact, the mitochondrial coupling efficiency determines how many calories are used to perform work (ATP) or to produce heat. To our knowledge, few data on the regulation of mitochondrial degree of coupling in skeletal muscle in response to obesity-induced insulin resistance are available. An increased degree of coupling in skeletal muscle mitochondria has been found after short-term dietary treatment with lipid-rich diet, when insulin resistance is not yet elicited (Crescenzo *et al.* 2014, 2015). Therefore, these results could be consistent with a role for mitochondrial impairment in the onset of insulin resistance. When mitochondria are more coupled, less fuels are oxidized to synthesize the same amount of ATP. As during high-fat feeding there is an increased lipid supply to skeletal muscle (Crescenzo *et al.* 2015), lipid supply would exceed lipid burning, leading to ectopic fat deposition. Interestingly, after 7 weeks of lipid-rich diet, insulin resistance develops, but the modification of mitochondrial degree of coupling disappears (Lionetti *et al.* 2007), probably due to high-fat-induced alterations in lipid composition of the mitochondrial membranes, an important determinant of mitochondrial efficiency (Jastroch *et al.* 2010). An increased mitochondrial degree of coupling has also been found when a lipid-rich, fructose-rich diet was given to rats for 2 weeks, a period that also elicits insulin resistance (Crescenzo *et al.* 2015). By comparing the two sets of results, it can be hypothesized that, at least in this model of insulin resistance, the increased mitochondrial degree of coupling could contribute to elicit insulin resistance. Accordingly, the amount of skeletal muscle ceramides, which are responsible for the impairment of insulin signaling (Coen & Goodpaster 2012), is increased with lipid-rich diet and is even higher with lipid-rich, fructose-rich diet (Crescenzo *et al.* 2015), probably reaching a threshold level that partly blocks the insulin transduction pathway.

Oxidative stress in insulin resistance

The oxygen utilization by aerobic organisms results in the production of chemical species possessing one or more unpaired electrons, called free radicals, which can initiate chain reactions eventually leading to cell structural and functional alterations (Valko *et al.* 2007). Indeed, various processes occurring in mitochondria and other cellular sites, including cytosol, endoplasmic reticulum, peroxisomes and lysosomes (Venditti *et al.* 2015, Di Meo *et al.* 2016), lead to partial oxygen reduction and formation of free radicals and other reactive oxygen species (ROS). ROS include superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$). Of these, $\bullet OH$ is the most reactive and can be generated by the reaction of H_2O_2 with iron (or copper) ions (the Fenton reaction). $\bullet OH$ can attack polyunsaturated fatty acids, causing a loss of biomembrane integrity, proteins and enzymes, damaging functional properties and nucleic acids, giving rise to mutations and ultimately cell senescence or death (Halliwell & Gutteridge 2015).

In most (if not all) mammalian cells, another free radical, containing nitrogen and named nitric oxide ($NO\bullet$), is synthesized from L-arginine by NO synthases (NOS) (Knowles & Moncada 1994). $NO\bullet$ is relatively unreactive, but may be converted to a number of more reactive derivatives, known collectively as reactive nitrogen species (RNS). Thus, reacting with superoxide, $NO\bullet$ produces peroxynitrite ($ONOO^-$), a highly reactive oxidant, able to damage many biological molecules and decompose releasing small amounts of $\bullet OH$ (Radi *et al.* 2002).

To neutralize the oxidative effects of ROS and RNS, aerobic organisms have evolved a system of biochemical defences (Yu 1994, Davies 2000). An oxidant generation that exceeds the antioxidant capacity of cells results in oxidative stress development (Sies 1997), a deleterious process that has been related to many pathological conditions (Valko *et al.* 2007, Reed 2011, He & Zuo 2015) and seems to also be involved in the etiology of IR, primarily in skeletal muscle tissue, and the subsequent development of T2DM (Henriksen *et al.* 2011). It has also been suggested that oxidant overproduction arises from (i) the mitochondrial electron transport chain in conditions of energy surplus and (ii) the cellular NADPH oxidase activated via angiotensin II (AT1) receptors (Henriksen *et al.* 2011).

ROS have been traditionally regarded as toxic by-products of metabolism, which, if occurring in a massive extent, may cause organ dysfunction. However,

it is now believed that, when moderately produced, they are essential for several physiological processes leading to adaptive cellular responses. This idea is supported by the observation that, at the cellular level, ROS regulate growth, apoptosis and other signaling, whereas at the system level, they contribute to complex functions, including blood pressure regulation, cognitive and immune functions (Brieger *et al.* 2012). It has also been shown that although accumulation of oxidative damage results in organism death (Muller *et al.* 2007), several longevity-promoting interventions increase generation of ROS that activate stress responses that are beneficial to the organism and extend life span (Ristow & Schmeisser 2011). Like ROS, RNS play a dual role as they can be either harmful or beneficial to living systems. Nitric oxide, early identified as a signaling molecule in blood vessel modulation (Ignarro *et al.* 1987, Bogdan 2001) and now known as a regulator of important physiological processes (Bogdan 2001), can cause cellular toxicity either damaging metabolic enzymes or generating peroxynitrite by reaction with superoxide (Pacher *et al.* 2007).

Similarly, mitochondrial ROS production is thought to both play a major role in tissue oxidative damage and dysfunction and provide protection against excessive tissue dysfunction (Venditti & Di Meo 2006), even though it is apparent that in various conditions remarkable contribution to tissue oxidative stress and protection is provided by other sources of ROS and RNS (Di Meo *et al.* 2016). The issue is complicated by the strong interaction existing among the various cellular sources of ROS (Camões *et al.* 2009, Vannuvel *et al.* 2013), which makes it difficult to establish what source of reactive species plays a prominent role in different physiological and pathological conditions. However, it is likely that the relevance of the role played in cellular damage and signal transduction is dependent on the type of reactive species produced and localization of their source and their targets (Finkel & Holbrook 2000).

Although ROS, such as $\bullet OH$, may cause irreversible, non-specific damage to target macromolecules, H_2O_2 and/or $O_2^{\bullet-}$ are the best candidates as signaling molecules. Both species are formed by enzymes and their concentrations are enzymatically controlled. However, precise identification of ROS involved in a signaling event is not easy. Usually, one ROS gives rise to other species and their effects on a target molecule may not be very different from each other.

The complexity of the role played by the reactive species in the physiological and pathological conditions

is apparent examining the action of such species in glucose uptake.

Reactive species and insulin signaling

Oxidants have been consistently implicated in the development of IR and subsequent T2DM. In fact, although the observation that the cellular models of IR are characterized by persistently elevated ROS levels (Houstis *et al.* 2006) suggests that ROS contribute to IR, there are other data that suggest a completely opposite view. At present, it is well established that H_2O_2 is able to exert stimulatory or inhibitory effects on insulin signaling, depending on the concentration of H_2O_2 and/or the site of production relative to various components of insulin signaling pathway(s) (Rindler *et al.* 2013). However, it is unclear as to why in certain cellular and animal models, increased ROS levels are associated with IR and scavenging the oxidants improves metabolic homeostasis, whereas in other models, increased ROS levels are associated with improved insulin sensitivity and antioxidants make things worse. Possible explanations of the discordant results include differences in sources and rates of ROS generation, changes in the function of oxidants depending on the disease stage and differences in the various metabolic models used. Furthermore, because redox homeostasis presumably has a narrow biological window, too high or too low levels of oxidants might produce similar pathological effects.

NOX-derived H_2O_2

The membrane-bound NADPH oxidases constitute the only enzyme family with the sole function to produce ROS. A NADPH oxidase generating $O_2^{\bullet-}$ was first identified in phagocytes, which showed bactericidal activity (Babior 1999). The phagocyte NADPH oxidase is composed of two membrane proteins P22PHOX and GP91PHOX (cytochrome b558 heavy chain, later designated as NOX2, which is the catalytic subunit of the enzyme), three cytosolic proteins P67PHOX, P47PHOX and P40PHOX and a small GTP-binding protein RAC (Chanock *et al.* 1994). In resting cells, the enzyme is dormant, and its components are distributed between the cytosol and plasma membrane. Bacterial infection induces translocation of the cytosolic components to the phagosome membrane where they associate with cytochrome b558 and give rise to the catalytically active NADPH oxidase (Chanock *et al.* 1994).

The presence of NOX2 homologs was firstly suggested by the observation that $O_2^{\bullet-}$ is produced in a NADPH-dependent manner in no phagocytic cells, in which NOX2 is not expressed (Suh *et al.* 1999). To date, five NOX isoforms (NOX1, NOX2, NOX3, NOX4 and NOX5) and two related enzymes (DUOX1 and DUOX2), which have different regulation and specific subcellular localization and generate distinct ROS, have been reported (Katsuyama 2010). NOX1, NOX2 and NOX3 generate $O_2^{\bullet-}$. NOX4, NOX5, DUOX1 and DUOX2 produce H_2O_2 .

Recent studies have shown that skeletal muscle expresses NOX including the catalytic NOX2, NOX4, DUOX1 and DUOX2 enzymes in various subcellular compartments (Sakellariou *et al.* 2014). In particular, NOX2 and NOX4 appear to be present on the sarcolemma (Javesghani *et al.* 2002), whereas NOX4, but not NOX2, is present in skeletal muscle mitochondria (Sakellariou *et al.* 2013).

Skeletal muscle generates ROS both under resting conditions and during exercise. However, although the contribution of mitochondria to ROS generation is well established (Di Meo & Venditti 2001), a potential role for NOX remains uncertain. NOX2-derived ROS are proposed to play a role in Ca^{2+} release from the sarcoplasmic reticulum, a key signaling step in muscle contraction (Hidalgo *et al.* 2006). Furthermore, both NOX2 and NOX4 are involved in the proliferation of skeletal muscle precursor cells (Mofarrahi *et al.* 2008).

In the 1970s, substantial evidence was obtained that exogenously added H_2O_2 could mimic the insulin signaling activity (Czech *et al.* 1974a) and oxidation of key fat cell sulfhydryls in response to insulin receptor interaction plays a role in mediating the glucose transport activation (Czech *et al.* 1974b). A few years later, insulin was shown to activate a plasma membrane enzyme system with the properties of an NADPH oxidase resulting in the downstream production of H_2O_2 (May & de Haen 1979), which plays a role in facilitating normal signal transduction by insulin.

Beneficial effect of ROS

In skeletal muscle, insulin receptor binding increases the activity of NOX localized on the plasma membrane, by PI3K activation, thus leading to enhanced H_2O_2 concentration proximal to the receptor (Espinosa *et al.* 2009).

NOX-derived H_2O_2 is able to enhance as well as reduce insulin sensitivity. Indeed, NOX4-deficient mice display greater IR than wild-type mice when fed a high-fat diet (Li *et al.* 2012). Further support for a NOX role in

the enhancement of insulin sensitivity comes from the observation that mice lacking one of the key enzymes involved in ROS elimination, glutathione peroxidase 1 (GPX1), resist to high-fat diet-induced whole body IR (Loh *et al.* 2009). The dependence of the increased insulin sensitivity in GPX1 (-/-) cells on ROS is demonstrated by the observation that it is decreased by the antioxidant N-acetylcysteine to that of control mice. The NOX involvement is demonstrated by the finding that AKT phosphorylation stimulated by insulin is higher in the GPX1 (-/-) cells and is suppressed by treatment with diphenylene iodonium chloride, a NOX inhibitor (Loh *et al.* 2009). Furthermore, insulin-stimulated PTEN oxidation is increased in GPX1 (-/-) cells and is suppressed by NOX inhibition.

It has been reported that the protein tyrosine phosphatases PTP-1B and PTEN, known to negatively regulate insulin signaling pathway (Elchebly *et al.* 1999, Wijesekara *et al.* 2005), are transiently oxidized and inactivated by H₂O₂ (Tonks 2006). Therefore, the data from GPX1 (-/-) mice support the ability of H₂O₂, produced by NOX to stimulate the insulin signaling cascade by inhibiting protein tyrosine phosphatases. Moreover, analysis of individuals with selenoprotein deficiency suggests that these observations may potentially extend to humans as well (Schoenmakers *et al.* 2010).

Harmful effect of ROS

Harmful effect of NADPH-produced ROS on insulin sensitivity is found after exposure of cultured cardiomyocytes to high glucose, which leads to NADPH oxidase (NOX2) activation, resulting from RAC1 activation and P47PHOX translocation and ROS production (Balteau *et al.* 2011). Hyperglycemia also impairs insulin signaling that is rescued by treatment with gp91ds-tat, a specific inhibitor of NOX2 activation suggesting that such an activation and ROS production are responsible for IR.

Although the etiology of skeletal muscle IR is multifactorial, there is clear evidence that one contributor is overactivity of the renin-angiotensin system (RAS).

The RAS is known for its role in the regulation of several physiological processes, including blood pressure, cardiac mass and contractility, and fluid balance (Schmieder *et al.* 2007). Angiotensinogen (Agt) is cleaved by the enzymes renin and angiotensin-converting enzyme (ACE) successively, to form angiotensin II (Ang II), the main bioactive peptide of this system. RAS overactivity is linked with the multifactorial etiology of hypertension.

Ang II exerts its physiological actions, primarily via two G-protein-coupled receptors, Ang II type 1 receptor (AT1R) and type 2 receptor (AT2R) (Schmieder *et al.* 2007). Its hypertensive effect is mediated through AT1R so that AT1R blockers and ACE inhibitors are commonly used for antihypertensive therapy (Schmieder *et al.* 2007).

Several clinical trials have shown that treatment of hypertensive patients with the previously mentioned drugs result in lower risk for T2DM when compared with other antihypertensive agents (Kim *et al.* 2006). Moreover, AT1R blockade ameliorates IR and glucose intolerance in several rodent models of obesity (Chu *et al.* 2006). These effects agree with previous observations showing that systemic delivery of Ang II in animal models is associated with defects in vascular delivery of glucose and insulin to skeletal muscle (Richey *et al.* 1999) and with impairment of the glucose transport system in this tissue (Ogihara *et al.* 2002).

Studies on cultured L6 myocytes indicated that Ang II inhibited the insulin-PI3K signaling pathway stimulating serine phosphorylation of IRS-1, one of the major substrates of the insulin receptor kinase (Wei *et al.* 2006). IRS-1 contains multiple tyrosine phosphorylation motifs that serve as docking sites for SH2 domains that mediate the metabolic and growth-promoting functions of insulin (Folli *et al.* 1997). However, IRS-1 also contains over 30 potential serine/threonine phosphorylation sites, whose phosphorylation inhibits insulin stimulation of tyrosine phosphorylation, resulting in the inhibition of downstream insulin signaling molecules (Andreozzi *et al.* 2004). Ang II induces serine phosphorylation at both sites and inhibits downstream signaling, including Akt phosphorylation, GLUT-4 translocation to the sarcolemma and NO[•] production in the endothelium (Folli *et al.* 1997, Andreozzi *et al.* 2004).

The Ang II effects seem to depend on the capacity of Ang II, acting through the AT1R, to generate ROS in various cell types including skeletal muscle (Shiuchi *et al.* 2004, Blendea *et al.* 2005). Indeed, skeletal muscle superoxide production, as assessed by ethidium fluorescence, was shown to be significantly enhanced in rats infused with Ang II for 2 weeks, and the observed increase was greatly attenuated by AT1R blockade with valsartan (Shiuchi *et al.* 2004). Moreover, increased superoxide content and IR were found in soleus muscle with local elevated Ang II levels (Blendea *et al.* 2005). Valsartan, angiotensin II receptor blocker, and tempol, a superoxide dismutase mimetic, reduced superoxide production and improved insulin sensitivity (Blendea *et al.* 2005),

suggesting that Ang II impairs insulin sensitivity at least in part through the generation of ROS. However, it was not known whether Ang II-induced ROS production in skeletal muscle was mainly through NADPH oxidase and consequently contributed to IR in skeletal muscle.

The studies on cultured L6 myocytes also provided the first evidence for the involvement of NADPH in Ang II-induced IR (Wei *et al.* 2006). Indeed, they demonstrated that Ang II increased NADPH oxidase activity, thereby increasing ROS generation and impairing insulin-induced IRS tyrosine phosphorylation, AKT activation and GLUT4 translocation to plasma membranes. AT1R block by losartan or NADPH oxidase inhibition by apocynin suppressed ROS production and restored insulin-stimulated IRS1, AKT activation and GLUT4 translocation. Furthermore, knockdown of *P47phox* reduced NADPH oxidase activity and improved insulin-mediated AKT phosphorylation and GLUT4 translocation in the presence of Ang II. However, the study did not clarify the intermediate steps linking ROS to IR in skeletal muscle.

ROS activate multiple transcription factors, including NF- κ B (Kamata *et al.* 2002), whose activation is involved in high-fat diet-induced liver IR in mice (Cai *et al.* 2005). To determine the role of ROS-mediated NF- κ B activation in blunting insulin effects on skeletal muscle, a study employing transgenic hypertensive (Ren-2) rats and Ang II-treated myotubes was performed (Wei *et al.* 2008). The results of the study suggested that Ang II-induced ROS generation via NADPH oxidase activates NF- κ B, which in turn contributes to skeletal muscle IR.

Obesity seems to play a role in the stimulation of RAS and then in the onset of IR. In fact, obesity is associated with overactivation of both systemic and adipose RAS in humans and animals (Kalupahana & Moustaid-Moussa 2011). In humans, obesity is associated with increases in plasma angiotensinogen (Engeli *et al.* 2005), renin (Uckaya *et al.* 1999), ACE and Ang II (Engeli *et al.* 2005). The increase in plasma renin levels in obesity is likely secondary to the increased sympathetic tone present in obese individuals (Troisi *et al.* 1991). Adipose tissue renin, ACE and AT1 expression are also increased in obesity (Gorzelnjak *et al.* 2002). Most, but not all studies show that adipose Agt expression is also higher in obese humans (Goossens *et al.* 2007). Moreover, weight loss leads to reductions in plasma Agt, renin, ACE and adipose Agt levels (Kalupahana & Moustaid-Moussa 2011). Animal studies show that, similar to humans, both systemic and adipose RAS components are overexpressed in most diet-induced obese rodent models (Boustany *et al.* 2004).

Mitochondria-derived H₂O₂

It has now been about 50 years since it was showed that electron transfer along mitochondrial respiratory chain leads to ROS formation (Jensen 1966, Loschen *et al.* 1974). The primary reactive species generated within mitochondria by univalent auto-oxidation of electron carriers is O₂^{•-} (Turrens & Boveris 1980), which is converted by enzymes called superoxide dismutases (SOD) to H₂O₂, which can be reduced by Fe²⁺ ion to •OH radical (Fenton reaction). Although the main sites involved in mitochondrial ROS production are believed to be localized at Complexes I and III, succinate-dependent ROS production by Complex II from rat skeletal muscle and glycerol 3-phosphate-dependent production by Complex II from several rat tissues have also been reported (Venditti *et al.* 2013).

To date, the relative importance of each mitochondrial site to ROS production is still controversial, but their localization is important for establishing ROS effects as it determines if O₂^{•-} is produced in the mitochondrial matrix or in the intermembrane space. Thus, both generators of Complex I and Complex III release O₂^{•-} into the matrix where it can damage mitochondrial DNA, whereas Complex III generator also releases O₂^{•-} into the intermembrane space, where it has easier access to the cytosol (Brand 2010).

Mitochondrial ROS production in insulin resistance

Mitochondria-derived oxidative stress is fairly well established as a mechanism underlying the pathological complications associated with diabetes (Brownlee 2001). However, its role as a primary factor in the development of IR (and subsequent overt diabetes), although long suspected, has been based largely on indirect evidence (Evans *et al.* 2002, 2005). The first direct evidence indicating a causal role was reported by Houstis and coworkers (Houstis *et al.* 2006) who utilized two distinct means of inducing IR in 3T3-L1 adipocytes, TNF α and dexamethasone treatment. In both models, IR induction resulted in elevated ROS production prior to any detectable decline in insulin sensitivity, fulfilling the criterion that the establishment of a 'causal' mechanism within any process requires that causal stimulus must precede the effect. Furthermore, using different mitochondrial- and non-mitochondrial-targeted approaches, scavenging either O₂^{•-} or H₂O₂, insulin sensitivity was partially restored in all cases, fulfilling the criterion that removal

or inhibition of the causal stimulus must attenuate or prevent the effect (Houstis *et al.* 2006).

Subsequent studies, using various animal models and mitochondrial-targeted approaches, provided strong experimental evidence of a link between mitochondrial-derived ROS and IR *in vivo*. Several studies were performed modifying the antioxidant potential of muscle mitochondria either by treating animals with mitochondrial-targeted antioxidants or increasing their expression. Although $O_2^{\bullet-}$ dismutation to H_2O_2 can occur spontaneously, the radical is rapidly converted to H_2O_2 by mitochondrial SOD. Complex I-derived $O_2^{\bullet-}$ is released into the matrix (Muller *et al.* 2004) and is eliminated in such a compartment by a form of SOD containing manganese in the active site (MnSOD) (Fridovich 1995). Part of the $O_2^{\bullet-}$ released in the intermembrane space by Complex III generator is eliminated by a different SOD isozyme, containing copper and zinc (Cu and ZnSOD) (Okado-Matsumoto & Fridovich 2001). In turn, H_2O_2 is scavenged by catalase (CAT), peroxiredoxin (PRDX) or GPX. Thus, MnSOD overexpression was found to improve insulin-dependent skeletal muscle glucose uptake and whole body insulin sensitivity in high-fat-fed rodents (Hoehn *et al.* 2009, Boden *et al.* 2012). Furthermore, evidence was provided that mitochondrial superoxide production is a common feature of many different models of IR and, in particular, IR was rapidly reversible upon exposure to agents that act as mitochondrial uncouplers, respiratory chain inhibitors or mitochondrial MnSOD mimetics (Hoehn *et al.* 2009).

Moreover, overexpression of peroxiredoxin 3 (PRDX3), a PRDX isoform located in mitochondria, produced significantly lower amount of H_2O_2 in skeletal muscle, reduced blood glucose levels and protected against IR induced by high-fat diet (Chen *et al.* 2008). Support for a role of mitochondrial H_2O_2 in IR impairment was provided by the observation that attenuating the increase in mitochondrial H_2O_2 release due to high-fat diet either by treating rats with the mitochondrial-targeted antioxidant SS31 (a small, cell permeable peptide) or by genetically engineering the overexpression of catalase in mitochondria of mouse muscle, completely preserved insulin sensitivity (Anderson *et al.* 2009). Similarly, it was showed that mice with targeted overexpression of the human catalase gene to mitochondria (MCAT) were protected from age-induced decrease in muscle mitochondrial function, energy metabolism and lipid-induced muscle IR (Lee *et al.* 2010).

The above reports provide strong evidence that mitochondrial-derived ROS are a signal for modulating insulin sensitivity. However, they do not clarify which species mediates the development of IR. The observation that MnSOD mimetics (Chen *et al.* 2008) and MnSOD overexpression (Hoehn *et al.* 2009, Boden *et al.* 2012) are able to limit the development of IR and to improve glucose tolerance when administered to high-fat-fed mice suggested a role for $O_2^{\bullet-}$ in the negative regulation of insulin sensitivity (Hoehn *et al.* 2009). However, in normal cell, the steady-state concentration of $O_2^{\bullet-}$ is maintained at very low levels by endogenous SODs, which increase the rate of its dismutation to that of a diffusion-controlled process (Forman & Fridovich 1973, Liochev & Fridovich 2007). Furthermore, recent observation that SOD2 overexpression does not alleviate muscle insulin resistance alone and does not exert additive effect when combined with increased H_2O_2 scavenging suggest that treatments designed to enhance H_2O_2 removal provide greater protection against IR than those that target $O_2^{\bullet-}$ (Lark *et al.* 2015). On the other hand, there are other reasons suggesting the primary role of H_2O_2 in insulin signaling inhibition. The mechanism by which ROS initiate cellular signaling is thought to involve modifications of thiol groups of cysteine residues of target protein. $O_2^{\bullet-}$ is able to oxidize thiols to thiyl radical, which can initiate a chain reaction, but the rate constants for this reaction are quite slow, probably no more than $10^3 M^{-1} s^{-1}$ (Winterbourn & Metodiewa 1995), which is insignificant in comparison to the rate constant at which $O_2^{\bullet-}$ is reduced to H_2O_2 by MnSOD ($>10^9 M^{-1} s^{-1}$) (Forman & Fridovich 1973). Conversely, thiol group oxidation by H_2O_2 forms reactive sulfenic acid ($-SOH$) that, in turn, can form disulfide bonds with nearby cysteines ($-S-S-$) or undergo further oxidation to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid. These modifications result in changes in protein structure and function, which are in part reversible by reducing systems such as thioredoxin and peroxiredoxins (Roos & Messens 2011). Furthermore, both thermodynamic and kinetic considerations suggest that among possible oxidation states of cysteine, formation of sulfenic acid derivatives or disulfides can be relevant as thiol redox switches in signaling (Forman *et al.* 2010).

Thus, in terms of a signaling role, H_2O_2 is considered to fulfill the requirements of a second messenger, whereas $O_2^{\bullet-}$ is more likely to function simply as a precursor of H_2O_2 rather than as a primary second messenger (Forman *et al.* 2010).

Impairment of insulin signaling

Although the mechanisms by which mitochondrial H_2O_2 production impairs insulin signaling are not fully characterized, the link between ROS production and IR has been ascribed to alterations in various intracellular signaling pathways. Among the several proteins proposed as potential effectors, there are various kinases such as PKCs, IKK β , JNK and p38 MAP kinase. These kinases have been postulated to catalyze the phosphorylation of serine residues in IRS-1 inhibiting its activity and directing it for degradation by the proteasome (Paz *et al.* 1997, Zhande *et al.* 2002). Such effects culminate with a reduction in the phosphorylation of tyrosine residues of IRS-1 by insulin, blocking its downstream signal transduction (Tirosh *et al.* 1999, Bloch-Damti & Bashan 2005, Evans *et al.* 2005).

In particular, c-Jun NH2-terminal kinase 1 (JNK1) appears to be a major factor in IR development because of its capacity to inhibit IRS activity via phosphorylation of Ser³⁰⁷, a post-translational modification preventing the interaction between insulin receptor and IRS (Aguirre *et al.* 2000, 2002).

It is worth noting that, contrary to the results of cell-based experiments, subsequent study performed on mice showed that IRS1 ser³⁰⁷ is required to maintain normal insulin signaling (Copps *et al.* 2010).

However, whatever the serine phosphorylated may be, there is evidence that activation of the JNK pathway is important for maintaining IR (Kaneto *et al.* 2004) and that the increases in body mass and blood glucose levels and the loss of insulin sensitivity induced by high-fat diet are decreased in JNK1-knockout mice relative to wild-type control animals (Hirosumi *et al.* 2002).

A direct link between mitochondrial H_2O_2 production and JNK1 activation has not yet been demonstrated, but an inverse relationship between protein tyrosine phosphatase PTP-1B activity and JNK1 activation has been shown by treating H4IIEC hepatocytes with increasing concentrations of H_2O_2 (Iwakami *et al.* 2011). With relatively low concentrations of H_2O_2 ($\leq 5.0 \mu M$), PTP-1B activity was inhibited and insulin-stimulated phosphorylation IRS and Akt was enhanced. With higher H_2O_2 concentrations ($\geq 5 \mu M$), JNK1 was activated, whereas insulin-stimulated IRS and Akt phosphorylation was reduced. These data clearly show that insulin sensitivity is modulated by changes in H_2O_2 concentration. Moreover, the observation that suppression of β -oxidation (Koves *et al.* 2008) or overexpression of mitochondrial antioxidant enzymes that remove H_2O_2 (Chen *et al.* 2008,

Anderson *et al.* 2009) decreases the loss of insulin sensitivity induced by high-fat diet, suggests that the increase in mitochondrial H_2O_2 generation contributes to obesity-linked IR. If so, mitochondrion functions as a refined sensor of substrate availability changes, able to adjust metabolism to optimize use and clearance of available substrates.

Another possibility is that elevated H_2O_2 release may directly target a key component of the glucose uptake process itself. An idea requiring further investigation is that elevated mitochondrial H_2O_2 release may contribute to high-fat diet-induced IR leading to the dissociation of hexokinase (HK) from mitochondria.

It has been shown that glucose phosphorylation, an integral step for insulin-stimulated glucose uptake, is functionally impaired in high-fat diet-fed rats (Furler *et al.* 1997, Halseth *et al.* 2000) and T2DM patients (Bonadonna *et al.* 1996). Glucose phosphorylation is catalyzed by HK, and gene and protein expression of HKII, the predominant isoform found in skeletal muscle, are increased in response to insulin (Vogt *et al.* 2000) but depressed in patients with T2DM (Kruszynska *et al.* 1998, Pendergrass *et al.* 1998). HKI and HKII can also bind to the mitochondrial outer membrane through interactions with mitochondrial porin in skeletal muscle (Anflous-Pharayra *et al.* 2007). Interestingly, overexpression of HKII in insulin-resistant mice fed a high-fat diet does not improve insulin-stimulated glucose uptake (Fueger *et al.* 2004). One possibility to explain this observation is that glucose phosphorylation is not solely a function of HKII content, but also dependent upon the subcellular localization of HK on mitochondria. When bound to mitochondria in skeletal muscle, HK displays greater sensitivity for ATP derived from mitochondria than exogenous ATP (Viitanen *et al.* 1984), suggesting that HK association with mitochondria provides a bioenergetic advantage to glucose phosphorylation. In both rodent (Chen-Zion *et al.* 1992) and (Vogt *et al.* 1998) human striated muscle, HKII association with the mitochondrial outer membrane is promoted by insulin via Akt phosphorylation of HKII (Pastorino *et al.* 2005) and glycogen synthase kinase-3b (GSK-3b) inhibition (Cross *et al.* 1995), a basally active negative regulator of glycogen synthesis. The inhibition of GSK-3b decreases the phosphorylation tone on VDAC, increasing the binding affinity between VDAC and HK (Pastorino *et al.* 2005). GSK-3b activity is increased during nutrient overload (Eldar-Finkelman *et al.* 1999) and oxidative stress (Dokken *et al.* 2008), and muscle-specific overexpression of GSK-3b is casually linked to IR

(Pearce *et al.* 2004). In addition, exogenous H₂O₂ has been shown to dissociate HKII from mitochondria in cultured cardiomyocytes (Wu *et al.* 2011a,b), providing a potential direct link between mitochondrial/cellular redox control and HK association with mitochondria.

Mechanisms of H₂O₂ generation

There are several mechanisms by which obesity may lead to an increase in mitochondrial O₂^{•-} and H₂O₂ production. A first mechanism invokes the inverse relationship between the rates of electron flow and electron leak along the respiratory chain.

The rate of the electron flow and tetravalent reduction of O₂ is modified in response to cellular energy need, which is prevalently expressed by cytosolic ADP concentration. When the cellular demand for energy is high, ATP breakdown to ADP and phosphate increases and, with the greater ADP availability, respiration rate also increases causing ATP regeneration. Respirations in different mitochondrial metabolic states were defined as State 4 or basal respiration and State 3 or active respiration (Chance & Williams 1956). In *in vitro* experiments, State 4 occurs in the presence of respiratory substrates but not of ADP and is characterized by rate of O₂ consumption lower than that observed in State 3, which occurs in the presence of ADP.

The energy released during State 4 is used to restore the proton gradient dissipated because of the presence of mechanisms that uncouple oxidative phosphorylation from ATP synthesis, inducing the leak of protons back in the mitochondrial matrix. During State 3, the fast energy release, linked to the fast dissipation of the proton gradient of the inner mitochondrial membrane, is turned toward the ATP synthesis. The presence of ADP also decreases the reduction degree of the components of the respiratory chain so that in the State 3 each component of the chain is more oxidized than that in the State 4. As a consequence, the rate of electron leak and univalent reduction of O₂ is higher during State 4 than during State 3. This is why ROS production rate by respiratory chain is controlled by mass action and, at the same O₂ concentration, it increases when electron flow slows down increasing the concentration of electron carrier in the reduced form (R[•]):

$$d\left[O_2^{\bullet-}\right]/dt = k \cdot [O_2] \cdot [R^{\bullet}]$$

Although it is unlikely that cells *in vivo* are ever truly engaged in state 4 respiration, near state 4 conditions likely occur during periods of nutrient overload combined

with minimal ATP demand (that is, high caloric intake combined with a sedentary lifestyle).

These conditions would be expected to elevate the reducing pressure within the respiratory chain, accelerate mitochondrial O₂^{•-} generation and H₂O₂ release and trigger an oxidative shift in the redox environment. In support of this notion, high dietary fat intake generates an increase in partially oxidized lipid intermediates indicative of mitochondrial overload (Koves *et al.* 2008) and decreases the GSH/GSSG ratio in muscle, indicative of a shift in the intracellular redox environment to a more oxidized state (Anderson *et al.* 2009). This appears to be mediated by a remarkable increase in the mitochondrial H₂O₂ release, which in turn can be due to increased H₂O₂ production and/or decreased H₂O₂ scavenging in response to the lipid overload (Anderson *et al.* 2009). Treatment of high-fat-fed rodents with SS31 (mitochondrial-targeted small antioxidant peptide), as well as the transgenic expression of the human catalase gene within muscle mitochondria (mCAT), completely blocked the development of IR, as well as the associated increase in H₂O₂ release capacity and oxidative shift in the redox environment. Transgenic mice fed a standard chow diet also exhibited improved skeletal muscle insulin sensitivity compared with chow-fed WT mice (Anderson *et al.* 2009).

On the whole, these findings suggest that the degree of insulin sensitivity within a skeletal muscle cell may rely on the degree of reduction or oxidation within the intracellular redox environment. In this context, mitochondrial H₂O₂ release and the resulting oxidative shift in the cellular redox environment during nutrient overload is viewed as a metabolic feedback sensor to decrease insulin sensitivity (Fisher-Wellman & Neuffer 2012).

Another mechanism invokes the metabolic shift toward an increased fat consumption occurring in skeletal muscle when fat acid circulating levels increase during obesity (Muoio & Neuffer 2012) and high-fat feeding (Randle *et al.* 1988, Muoio & Neuffer 2012). Regardless of the energy intake or energy requirement, mitochondrial ROS generation is higher during fatty acid oxidation than that during glycolytic metabolite pyruvate oxidation (Anderson *et al.* 2007, Seifert *et al.* 2010). Actually, mitochondrial oxidation of both substrates results in the production of acetyl-CoA that reduces NAD⁺ and FAD⁺ in the Krebs cycle. However, unlike what happens in pyruvate oxidation, the first stage in fatty acid oxidation is the electron transfer from FADH₂, formed by acyl-CoA dehydrogenases, to the electron transport flavoprotein. Electrons are then transferred to the ubiquinone pool located downstream of complex I, the point where electrons coming from NADH enter the electron transport chain.

In this situation, the reverse electron flow from coenzyme Q (Ernster & Lee 1967) would bring complex I to a more reduced state, thus enhancing the probability that electrons catalyzing univalent reduction of O₂ are released (Fisher-Wellman & Neuffer 2012). In fact, it has been shown that skeletal muscle mitochondria and permeabilized fibers exhibit rates of H₂O₂ generation significantly higher in the presence of palmitoylcarnitine than that in the presence of pyruvate (Anderson *et al.* 2007, Seifert *et al.* 2010).

Increased dependence on β -oxidation also results in the accumulation of several fatty acid derivatives some of which have been found to interact with the mitochondrial inner membrane and interfere with electron transport thus stimulating mitochondrial ROS generation (Schonfeld & Wojtczak 2008, Seifert *et al.* 2010).

The observation that suppression of β -oxidation (Koves *et al.* 2008) as well as selective scavenging of mitochondrial H₂O₂ (Chen *et al.* 2008, Anderson *et al.* 2009) preserves insulin sensitivity of skeletal muscle in models of diet-induced obesity, suggests that high dietary fat increases ROS production by muscle mitochondria.

Ang II-induced impairment of insulin signaling in skeletal muscle cells (Wei *et al.* 2006, 2008) and in isolated skeletal muscles (Diamond-Stanic & Henriksen 2010) is thought to be due to NADPH-oxidase-derived ROS. However, in a model of IR associated with excessive RAS activation, impaired State 3 mitochondrial respiration was observed in mouse skeletal muscle (Takada *et al.* 2013). Moreover, in mouse skeletal muscles, chronic Ang II infusion caused glucose intolerance and mitochondrial abnormalities together with increased mitochondrial ROS generation (Mitsuishi *et al.* 2009).

There is also evidence that deletion of Sirt3, which is primarily located in mitochondria (Verdin *et al.* 2010), impairs insulin signaling in cultured myoblasts, leading to a decrease in tyrosine phosphorylation of IRS-1 (Jing *et al.* 2011). SIRT3 has a strong impact on mitochondrial antioxidant capacity. Indeed, it deacetylates and activates several enzymes critical in maintaining cellular ROS levels such as SOD2, so that the catalytic activity of the enzyme is diminished when Sirt3 is deleted (Qiu *et al.* 2010). SIRT3 also stimulates the activity of mitochondrial isocitrate dehydrogenase, IDH2, (Someya *et al.* 2010), which, via energy-dependent transhydrogenation (Vogel *et al.* 1999) promotes NADH-supported NADP⁺ reduction to NADPH, which in turn provides the reducing equivalents for conversion of oxidized to reduced glutathione. Based on these results, the possibility that angiotensin II-induced IR

is due to mitochondrial ROS generation causally linked to Sirt3 dysregulation has been investigated (Macconi *et al.* 2015). Thus, it has been found that angiotensin II-promoted IR in skeletal muscle cells is associated with increase in mitochondrial O₂^{•-} production, which in turn, leads to decreased Sirt3 expression and activity. The idea that Ang II-induced IR through SIRT3 dysfunction has been supported by the observation that acetyl-L-carnitine, a modulator of mitochondrial metabolism, restores SIRT3 levels, MnSOD activity, rescuing skeletal muscle cells from mitochondrial superoxide-driven IR (Macconi *et al.* 2015).

The relative role of NADPH oxidase and mitochondria in ROS generation in Ang II-treated skeletal muscle cells remains unknown. However, there is evidence of cross-talk between NADPH oxidase and mitochondria in regulating ROS generation and that NADPH oxidase-derived ROS can trigger mitochondrial ROS formation and vice versa (Dikalov 2011). It is, therefore, conceivable that Ang II-induced NADPH oxidase activation can concur to trigger mitochondrial changes in L6 myotubes leading to IR.

Nitric oxide synthase

Nitric oxide synthases (NOS) are enzymes catalyzing the conversion of L-arginine into L-citrulline and nitric oxide (NO[•]) (Radi *et al.* 2002), a free radical relatively unreactive which, however, can be converted into a number of more reactive derivatives. To date, three isoforms of nitric oxide synthase have been identified. Two isoforms, neuronal NOS (nNOS; type I NOS) and endothelial NOS (eNOS; type III NOS), are expressed constitutively and regulated by the interaction of Ca²⁺ with calmodulin (Nathan & Xie 1994). The other isoform, inducible-NOS (iNOS; type II NOS), is induced in response to infection, inflammation or trauma and is not regulated by Ca²⁺ because it forms a complex with calmodulin at very low concentrations of Ca²⁺ (Nathan & Xie 1994).

Recently, iNOS has emerged as an important player in IR, suggesting that RNS can be involved in IR development. High-fat-fed mice (Perreault & Marette 2001) and rats (Carvalho-Filho *et al.* 2005) all have significantly greater skeletal muscle iNOS expression compared with their respective controls without an associated increase in eNOS or nNOS. Moreover, the increased expression of iNOS was associated with increased RNS generation and impaired insulin sensitivity in skeletal muscle (Carvalho-Filho *et al.* 2005). It was also found that high-fat-fed

mice with a specific disruption in the iNOS gene, which canceled the enzyme activity, were protected from the decrements in both skeletal muscle insulin signaling and insulin-mediated glucose transport observed in high-fat-fed wild-type mice (Perreault & Marette 2001). The authors proposed that the production of NO[•] by iNOS was responsible for inducing IR in the wild-type animals. Involvement of NO[•] is supported by the observation that soleus muscle exposed to NO[•] donors had decreased insulin-stimulated glucose uptake and glycogen synthesis, effects that were associated with reduced insulin-stimulated phosphorylation of IR, IRS-1 and AKT (Carvalho-Filho *et al.* 2005). However, the previously mentioned result does not allow to exclude the possibility that the effects were due to NO[•] derivatives. Indeed, NO[•] readily reacts with superoxide to form peroxynitrite, which has been reported to modulate cellular functions through the nitration of tyrosine residues, even though the tyrosine nitration actually depends on reactions between ONOO⁻ and CO₂ leading to tyrosine residue radical and nitrogen dioxide (NO₂[•]), which couple to form the nitrated product (Radi 2013). In fact, subsequent work showed that peroxynitrite treatment fully reproduced the tyrosine nitration and inhibitory effect of iNOS induction on both insulin-stimulated glucose uptake and PI3K activity (Pilon *et al.* 2010). However, significant elevation in skeletal muscle iNOS protein content was not found in obese individuals despite evidence for IR, showing that skeletal muscle iNOS is not necessarily increased in all states of IR (Kraus *et al.* 2012).

Mitochondrial dysfunction and oxidant production

The two theories centered on mitochondrial function propose different mechanisms to explain the onset of obesity-linked IR in skeletal muscle. In the former, a decrease in mitochondrial fatty acid oxidation, caused by mitochondrial dysfunction and/or reduced mitochondrial content is thought to cause an accumulation of increased levels of intracellular fatty acyl-CoA and diacylglycerol. These molecules activate novel protein kinase C, which in turn activates a serine kinase cascade leading to increased serine phosphorylation of IRS-1 and IR (Lowell & Shulman 2005). In the latter, enhancement in mitochondrial oxidant production in response to excess fuel relative to demand is viewed as an underlying cause of the development of IR in skeletal muscle (Rindler *et al.* 2013). Indeed, tissue exposure to ROS results in the stimulation

of various kinases, including p38MAPK and JNK1 that is associated with decreased insulin signaling and glucose transport.

We are inclined to think that on the basis of available data, it is possible to propose an integrative model placing ROS production by mitochondria and other cellular sources as the primary consequence of the increase in circulating levels of fatty acids. The enhanced oxidant production, in turn, should cause mitochondrial dysfunction and both should lead to IR. This idea is based on a series of observations. Normally, the rate of mitochondrial ROS generation is rather low and results in minimal damage, because mitochondria have a highly efficient antioxidant defence system able to scavenge a large number of the ROS produced (Venditti *et al.* 2013). However, when in several circumstances, high rates of ROS production occur, a substantial part of oxidants may escape the scavenging systems. In these conditions mitochondrial DNA, proteins and lipids exposed to high levels of ROS undergo structural alteration and damage, which can result in the impairment of important mitochondrial functions. It is likely that mitochondrial oxidative damage and dysfunction are due to [•]OH radicals produced within mitochondria. These oxyradicals are highly reactive, short-lived species and are expected to cause damage at or near the site of formation. Therefore, they may inactivate components of the respiratory chain, enzymes of the Krebs cycle and other mitochondrial proteins, leading to mitochondrial dysfunction. Moreover, even though it is extremely unlikely that [•]OH radicals can be released by mitochondria, oxidative damage to components of cytoplasm and other cellular structures can result from mitochondrial leakage of other ROS, such as H₂O₂ that is able to readily cross mitochondrial membranes and reach such structures where, in the presence of Fe²⁺ ligands, it can generate [•]OH radical.

It is conceivable that increased oxidative stress in skeletal muscle may also impair mitochondrial function in T2DM. This idea is supported by the observation that mitochondrial function and structure are impaired in skeletal muscle from rodents placed on high-fat diet and with the altered oxidative stress markers (Bonnard *et al.* 2008).

It is worth noting that the ROS generation in the cell is also able to strengthen ROS mitochondrial production leading to enhanced damage and dysfunction of the organelles through several mechanisms (Fig. 2).

A process by which ROS production becomes self-amplifying is named ROS-induced ROS release (RIRR) (Zorov *et al.* 2000). RIRR is generated by circuits requiring

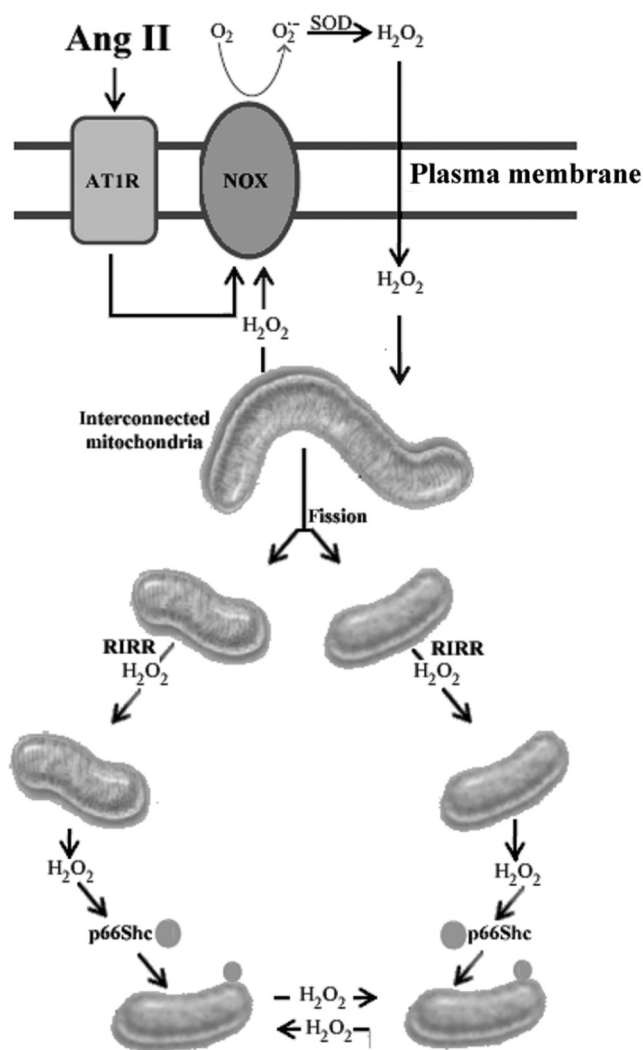


Figure 2
Schematic representation of self-amplifying ROS production in skeletal muscle from obese animals that is supposed to start by Ang II-linked NOX activation. NOX, NADPH oxidase; Ang II, angiotensin II; AT1R, Ang II type 1 receptor; RIRR, ROS-induced ROS release; p66shc, 66-kDa isoform of growth factor adaptor Shc. Adapted, under the terms of the Creative Commons Attribution 4.0 International license, from Di Meo S, Reed TT, Venditti P & Victor MV (2016) Role of ROS and RNS sources in physiological and pathological conditions. *Oxidative Medicine and Cellular Longevity*, volume 2016, article ID 1245049.

mitochondrial membrane channels, including the inner membrane anion channel (IMAC) (Garlid & Beavis 1986) and the mitochondrial permeability transition (MPT) pore (Vercesi *et al.* 1997), a protein complex that facilitates the traffic of water and small molecules between the mitochondrial matrix and cytoplasm.

A condition that leads to RIRR is the exposure to high oxidative stress resulting in an increase in ROS that reaches a threshold level that triggers the opening of MPT pore. Under oxidative stress, mitochondrial Ca^{2+}

overload takes place, which depresses mitochondrial function (McCormack *et al.* 1990) and triggers several processes, including MPT pore opening (Zorov *et al.* 2000). This, in turn, causes collapse of mitochondrial membrane potential and transient increase in ROS generation (Zorov *et al.* 2000). Such increase can be due to a respiratory chain block caused by MPT induction. In addition to ROS effects on mitochondria where the RIRR is originated, the ROS release into cytosol, which seems to occur through IMAC (Aon *et al.* 2003), can lead to RIRR activation in the neighboring mitochondria. Thus, ROS trafficking between mitochondria constitutes a positive feedback mechanism for enhanced ROS production potentially leading to significant mitochondrial and cellular injury.

A link between MPT pore opening and mitochondrial dysfunction and IR was demonstrated by inhibiting *in vivo* and *in vitro* the protein cyclophilin D (CypD) that regulates MTP pore opening by directly binding to pore constituent proteins (Taddeo *et al.* 2014). Indeed, mice lacking CypD were protected from high-fat diet-induced glucose intolerance due to increased glucose uptake in skeletal muscle. Furthermore, in *in vitro* models of skeletal muscle IR, it was observed that pharmacological inhibition of MTP pore opening with the CypD inhibitor cyclosporine A prevented IR at the level of insulin-stimulated GLUT4 translocation to the plasma membrane (Taddeo *et al.* 2014).

A phenomenon similar to RIRR can also depend on NO^{\bullet} because NO^{\bullet} can readily diffuse from mitochondria to cytosol, as well as from cytosol to mitochondria, a process called mitochondria cytosol NO^{\bullet} cross-talk (Moncada & Erusalimsky 2002). Within mitochondria, NO^{\bullet} is able to act as an inducer of permeability transition (Balakirev *et al.* 1997), through a direct effect on the MPT pore and an indirect effect secondary to oxidative phosphorylation inhibition (Vieira & Kroemer 2003).

There are reasons to think that ROS, released by mitochondria, can interact not only with other mitochondria but also with other sources of ROS. It is now apparent that there is a substantial interplay between ROS sources, so that activation of one can lead to the activation of the others, resulting in RIRR that further increases ROS production and oxidative stress.

Several examples of the interplay between mitochondrial and NOX-derived ROS have been reported (Daiber 2010). On the whole, they suggest the presence of a feedforward cycle in which NADPH oxidases increase mitochondrial ROS that further activate cytoplasmic NADPH oxidases and increase cellular ROS production.

An example is provided by the Ang II-induced increase in H₂O₂ production by mitochondria isolated from vascular smooth muscle cells (Doughan *et al.* 2008). This increase was inhibited by pre-incubation of intact cells with apocynin, a NADPH oxidase inhibitor, and by depletion of P22PHOX subunit of NADPH oxidase. Ang II also induced mitochondrial dysfunction, as documented by increase in state 4 and decrease in state 3 respiration that was attenuated by apocynin suggesting that Ang II induces mitochondrial H₂O₂ production and mitochondrial dysfunction by activating the endothelial cell NADPH oxidase.

Mitochondrial ROS production can also lead to and be increased by a process, the mitochondrial fission that is involved in IR. As it is known, mitochondrial shape can be modified through two finely regulated processes of fusion and fission, resulting in elongated, interconnected mitochondrial networks and fragmented, discontinuous mitochondria, respectively (Frazier *et al.* 2006). In mammals, mitochondrial fission is mediated by the protein dynamin-related protein 1 (DRP1) and the fission protein 1 (FIS1) (Nunnari *et al.* 2002, Liesa *et al.* 2009), whereas fusion is mediated by the dynamin-like proteins mitofusins 1 and 2 (MFN1 and MFN2) and the optic atrophy gene 1 (OPA1) (Cipolat *et al.* 2004, Palmer *et al.* 2011). The proteins that mediate mitochondrial dynamics are highly regulated and consequently integrated into cellular signaling pathways.

The study of specific changes in mitochondrial shape showed that the loss of fusion or fission activity results in dysfunctional mitochondria suggesting that morphology and function of mitochondria are closely linked (Nunnari & Suomalainen 2012). The importance of mitochondrial fusion can be explained by the need for exchange of intermembrane space and matrix contents between mitochondria, so that defects and transient stresses may be partially buffered. Conversely, mitochondrial fission should create organelles of the appropriate size for transport along actin or microtubule networks (Nunnari & Suomalainen 2012).

The fission process and ROS metabolism are strongly linked. On the one hand, ROS are able to influence fusion and fission rates via differential modulation of mitochondrial fission–fusion proteins (Wu *et al.* 2011a,b) altering the mitochondrial morphology as demonstrated by the observation that acute exposure to H₂O₂ of mouse myocytes induces slow fragmentation of the mitochondrial reticulum (Fan *et al.* 2010). On the other hand, mitochondrial fragmentation leads to reduced respiratory capacity and enhanced ROS production

(Yu *et al.* 2008). The changes in mitochondrial network structure seem to provide an example of RIRR because ROS play a role in mitochondrial fission and increase ROS generation from restructured mitochondria (Handy & Loscalzo 2012).

Nitric oxide appears to play opposite roles in mitochondrial fission–fusion. Indeed, it increases mitochondrial fragmentation and cell death in neurodegenerative diseases by its effects on dynamin-related protein-1 (DRP1), which promotes mitochondrial fission (Cho *et al.* 2009). Conversely, in myogenesis, NO• promotes the fusion of mitochondria into an elongated network by inhibiting Drp1-mediated fission (De Palma *et al.* 2010).

Clear evidence is also available indicating a link between oxidant-induced mitochondrial fission process and IR and impairment of glucose metabolism. In fact, excess palmitate (PA) was found to induce mitochondrial fragmentation and increase fission-related proteins DRP1 and FIS1 in differentiated C2C12 muscle cells. The fragmentation was associated with increased oxidative stress, depolarization, loss of ATP production and reduced insulin-stimulated glucose uptake. Moreover, genetic and pharmacological inhibition of DRP1 attenuated PA-induced mitochondrial fragmentation and IR in C2C12 cells (Jheng *et al.* 2012). Smaller and shorter mitochondria and increased mitochondrial fission machinery were also found in skeletal muscle of mice with genetic and diet-induced obesity, and inhibition of mitochondrial fission improved the muscle insulin signaling and systemic insulin sensitivity of obese mice. ROS involvement in PA-induced mitochondrial fragmentation was demonstrated by the observation that PA significantly increased intracellular ROS level, whereas the co-treatment with α -tocopherol, a ROS scavenger, attenuated ROS generation and PA-induced mitochondrial fragmentation. These findings, showing a relationship between ROS-linked mitochondrial fission and mitochondrial dysfunction and IR in skeletal muscle, suggest that the disruption by ROS of mitochondrial dynamics may underlie the pathogenesis of muscle IR in obesity and T2DM (Jheng *et al.* 2012).

The notion of alterations in mitochondrial dynamics in insulin-resistant states is supported by other reports. Thus, mitochondrial size is reduced in skeletal muscle of obese and diabetic patients compared to that in lean subjects (Kelley *et al.* 2002). This alteration in mitochondrial morphology is associated with a repression of the mitochondrial fusion protein MFN2 as demonstrated by the finding that skeletal muscles of both obese and non-obese diabetic patients show a reduced expression

of MFN2 level compared to control subjects (Bach *et al.* 2005). It has also been found that MFN2 deficiency in mouse soleus muscles increases H₂O₂ concentration, produces mitochondrial dysfunction, activates JNK and blocks insulin-stimulated AKT phosphorylation leading to muscle IR (Sebastián *et al.* 2012). A major conclusion of the study is that the increase in H₂O₂ production is able to induce a decrease in MFN2 level that contributes to impair insulin signaling and insulin sensitivity in skeletal muscle.

Excess oxidants can also augment mitochondrial ROS production by upregulating the expression of the lifespan regulator, the 66-kDa isoform of growth factor adaptor Shc (P66SHC) protein, which has been implicated in the development of aging and aging-related diseases (Migliaccio *et al.* 1999). Indeed, mice lacking the P66SHC have reduced H₂O₂ generation, are long lived and resistant to a number of age-related pathologies mostly associated with tissue oxidative damage (Migliaccio *et al.* 1999). The protein seems to reside mainly in the cytosol (Giorgio *et al.* 2005), with only a marginal fraction localized in the mitochondrial intermembrane space (Nemoto *et al.* 2006). Thioredoxin (TRX) 1 and glutathione keep the protein in the inactive reduced state. However, stress factors, including ROS, can increase the expression of the protein that is activated by thiol oxidation, which causes a dimer–tetramer transition (Gertz *et al.* 2008). Activated P66SHC translocates to the mitochondrial intermembrane space where it associates with cytochrome c producing H₂O₂, which can trigger MPT pore opening (Giorgio *et al.* 2005). At present, it is known that P66SHC is able to increase intracellular ROS levels by exploiting other mechanisms, including the activation of membrane-bound NADPH oxidases (Tomilov *et al.* 2010) and, possibly, by downregulation of antioxidant enzyme synthesis (Wu *et al.* 2006).

It is now known that P66SHC-generated H₂O₂ is also involved in the regulation of receptor-activated signal transduction pathways. Indeed, insulin activates the redox enzyme activity of p66Shc specifically in adipocytes and that P66SHC-generated ROS regulate insulin signaling through multiple mechanisms, including AKT phosphorylation, FOXO localization and regulation of selected insulin target genes (Berniakovich *et al.* 2008). It has also been reported that inactivation of P66SHC in mice improves glucose tolerance and insulin sensitivity (Tomilov *et al.* 2010) and confers resistance to hyperglycemia-induced endothelial dysfunction (Camici *et al.* 2008). Furthermore, studies on HeLa cells and murine embryonic fibroblasts (MEFs) have shown that depletion of P66SHC enhanced glucose

uptake, and redirects glucose carbon toward anabolic metabolism. This change in metabolism was mediated by the mammalian target of rapamycin (mTOR) because inhibition of mTOR with rapamycin reversed the glycolytic phenotype caused by P66SHC deficiency (Soliman *et al.* 2014).

Unfortunately, there is little information about a possible involvement of the protein on skeletal muscle IR.

However, examining the role of P66SHC in glucose transport regulation in L6 myoblasts with either reduced or increased levels of the p66^{Shc} protein, it was shown that low levels P66SHC increased glucose uptake, whereas high levels reduced it. Furthermore, P66SHC was found to regulate glucose transport by controlling, via MAPK, the integrity of the actin cytoskeleton and by modulating cellular levels of GLUT1 and GLUT3 transporter proteins, which are mainly expressed in L6 myoblasts.

In conclusion, theoretical and experimental evidence appears to indicate that mitochondrial dysfunction is not an early event in the IR development, but rather a complication of the hyperlipidemia-induced ROS production in skeletal muscle, which might promote mitochondrial alterations, lipid accumulation and inhibition of insulin action. On the other hand, the pathways leading to IR may act synergistically because mitochondrial dysfunction can further increase ROS production leading to the establishment of a harmful positive feedback loop.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

All the authors contributed equally to the preparation of the manuscript.

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