Role of free fatty acids on insulin resistance

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Abstract

Free fatty acids are essential nutritional components and recent studies identified them as signaling molecules in various physiological processes. It has now been shown that high levels of free fatty acids, particularly saturated fatty acids, may be associated with insulin resistance in obese patients with type 2 diabetes mellitus. Insulin resistance is important in clinical since it is related to various diseases including type 2 diabetes mellitus, dyslipidemia, and abnormalities at cardiovascular level. Recent studies have proposed different molecular mechanisms by which these lipids may alter the signaling pathway of insulin. The purpose of this review is to highlight recent advances in the study of the effect of free fatty acids as modulators of insulin response.

KEY WORDS: Fatty acids. Insulin resistance. Insulin receptor. Insulin receptor substrate.

Introduction

Insulin resistance is a systemic condition where cells cease to respond to this hormone. Under this conditions, highly important metabolic functions of insulin mainly on hepatic, muscular and adipose tissues, such as glucose uptake and synthesis of glycogen, lipids and proteins, among other effects, are altered1,2. It is closely linked with metabolic syndrome, obesity and with the development of type 2 diabetes mellitus (DM2). According to the World Health Organization and the International Federation of Diabetes most recent statistics, more than 1500 million people in the world suffer from obesity or overweight, whereas diabetes affects more than 387 million, a figure that by the year 2035 will be reaching nearly 600 million if appropriate measures are not taken (http://www.who.int/en/index.html; http://www.idf.org).

To date, several factors that can induce insulin resistance have been identified, with increased free fatty acid (FFA) plasma concentration standing out, followed by an altered use of lipids by the muscle tissue3. This phenomenon, which is common in obese patients, has been observed in clinical trials and has been corroborated in molecular investigations.

The mechanisms implicated in insulin resistance by the action of fatty acids (FA) include alterations in the kinase PI3K pathway, production and accumulation of ceramides, protein kinase C (PKC) activation, generation of reactive oxygen species (ROS), endoplasmic reticulum (ER) stress and membrane stiffening.

Insulin actions

Insulin is the body’s main anabolic hormone; it controls crucial energetic functions, such as glucose, lipid and protein metabolism. One of its most important functions is the regulation of glucose homeostasis, since it enables its uptake and storage in muscle and adipose tissue, and favors its storage and inhibits its
production in the liver. In addition, insulin promotes cell division and growth by means of its mitogenic effects.

Insulin activates specific membrane surface receptors known as insulin receptors, which belong to the family of receptors with tyrosine kinase intrinsic activity. After insulin is bound, the receptor auto-phosphorylates and, in turn, phosphorylates and recruits adaptor proteins, such as the insulin receptor substrate (IRS). IRS-1 and IRS-2 are the most common substrates and intermediaries in insulin signal propagation initial stage, which organize molecular complexes and trigger intracellular signaling cascades. Among the different PI3K pathway-activated kinases is Akt, which plays a highly important role in insulin metabolic functions, mainly in glucose uptake by muscle and adipose tissues. Akt participates in the glucose-transporting protein-4 (GLUT-4) translocation from intracellular vesicles to the cell surface in order to increase glucose uptake. Insulin also participates in gluconeogenesis inhibition and favors the synthesis of glycogen, lipids, and protein in hepatic, adipose, and muscle tissues, respectively (Fig. 1).

In turn, insulin mitogenic actions are carried out through the adaptor protein Shc, which activates the mitogen-activated kinases/Ras (MAPKs/Ras) pathway (Fig. 1).

**Fatty acids and insulin resistance**

Insulin resistance molecular causes are diverse: it involves defects in the binding of insulin to its receptor and alterations after this bond. These alterations include a decrease in the number of insulin receptors and in kinases PI3K and Akt activity, GLUT4 transporter expression and function defects, and increased phosphorylation in serine/threonine residues of proteins such as the insulin receptor or IRS, with the latter mechanism being key in the development of insulin resistance. This can cause alterations in the association of the receptor and its substrate with other proteins, a decrease in the state of phosphorylation in both proteins’ tyrosines, and a decrease in their activation and degradation. These insulin resistance mechanisms have been associated with high FFA plasma concentrations.

FA are highly amphipathic carboxylic acids consisting of 4 to 26 carbon atom-long hydrocarbon chains, which have no branches. The term FFA refers to non-esterified FAs, which stem directly from triglyceride and phospholipid metabolism. Depending on the presence or not of double bonds, they are classified in two large groups: those containing only single bonds are known as saturated fatty acids (SFA),
where hydrogen atoms are arranged in the trans position; and FAs with double bonds, which are called unsaturated and can be found both in cis and trans configuration, although the latter rarely occurs naturally. Unsaturated FAs only containing one double bond in their structure are called monounsaturated, whereas those containing two or more are called polyunsaturated.

FAs play important biological roles in living beings: they act as an important source of energy for most tissues of the body, including the heart, skeletal muscle and the liver. In addition, they possess a wide range of biological functions, such as being a structural part of the cell membrane (being essential to its fluidity and functionality) and acting as signaling molecules. FAs are used as the main energy source during periods of fasting or when there is no sufficient glucose. However, when FFA values are abnormally high in the body, as it occurs in obesity, a lipotoxicity state is induced, which leads to activation of different cell responses associated with this toxicity, including oxidative stress, ER stress, apoptosis and inflammation.

FFAs identified as the main causative agents of these responses include SFAs. The first evidence that FFA elevation plays an important role in the development of insulin resistance in muscle tissues was suggested by Randle et al. in 1963, based on observations that a high FFA plasma concentration is commonly associated with diabetes and other carbohydrate metabolism disorders. To date, different molecular mechanisms by means of which FFAs induce insulin resistance have been characterized, and which are next described.

**Fatty acid-induced insulin resistance: PKC participation**

FFAs, which often are elevated in obese individuals, play a highly important role in the association between obesity and insulin resistance. Although the mechanisms by means of which FFAs induce insulin resistance have been partially identified, diverse evidences suggest the participation of PKC, which is activated through different mechanisms.

Currently, dietary lipid excess or obesity are known to elicit an excessive increase of these acids in the bloodstream, which exceeds the storing and oxidation capacity, causing for FFA and their metabolism intermediaries (such as linoleic acid, diacylglycerol [DAG], phosphatidic acid, lysophosphatidic acid and ceramides) to act as insulin resistance important inducers. In particular, DAG is a potent activator of classic (PKCa, PKCb, PKCδ and PKCγ) and new PKC isoforms (PKCδ, PKCe, PKCη and PKCθ). In contrast with PKC acute activation in response to specific stimuli, several studies have reported chronic activation of one or more PKC isoforms in cells or tissues where DAG values are elevated in the long term. Thus, PKC classic and new isoforms activated this way participate in insulin signaling negative regulation, especially by IRS-1 phosphorylation in serine residues.

Elevation of DAG values due to plasma FFA increase has been shown to promote PKCβII and PKCδ activation in skeletal muscle of humans. Activation of both isoforms has been associated with insulin receptor and IRS phosphorylation, which entails a decrease in the phosphorylation status in both proteins’ tyrosines, thus affecting their signaling (Fig. 2).

In hepatic tissue of animals with a fat-rich diet, insulin resistance has been associated with an increase on DAG values that promotes the translocation of PKCe to the plasmatic membrane, where it interacts with insulin receptor catalytic domain. This interaction promotes a decrease in the receptor activity, which affects phosphorylation in IRS-2 tyrosines and Akt2 activity. Consequently, the capability of insulin to activate glycogen synthesis and to inhibit gluconeogenesis is altered. Interestingly, increased DAG values and their association with PKCe activation were the best insulin resistance markers in hepatic tissue of obese humans.

On the other hand, in C2C12 cells, high concentrations of palmitic acid favored DAG increase and the resulting activation of PKAβ, which once activated phosphorylates IRS-1 in serine 307, thus blocking insulin signaling. In this same cell type, another site in IRS-1 that is phosphorylated by PKCθ was identified, serine 1101, by arachidonic acid action. In a recent study, Szendroedi et al. demonstrated that insulin resistance acute induction by lipid infusion in healthy and lean individuals is related to a transient increase in cytosolic content of DAG in skeletal muscle, which was temporally associated with PKCθ activation, an increase in serine 1101 and phosphorylation inhibition in IRS-1 tyrosine, as well as insulin-induced Akt12 inactivation. Increased DAG content in muscle tissue, PKCθ activation and insulin resistance were also observed in healthy obese individuals and in obese patients with DM2.
On the other hand, Pereira et al. demonstrated in vivo that, in hepatic cells, FFA-induced insulin resistance is developed by oxidative stress, which is generated by NADPH oxidase (NOX) through a PKCδ-dependent mechanism. Oxidative stress leads to IκB (I KKβ) and JNK kinases activation, which mediate in IRS-1 and IRS-2 serines, with the resulting alteration of insulin signaling (Fig. 3).

From the results obtained by several investigation groups, it is suggested that the main regulation target in insulin signaling by PKC is IRS. However, in a study carried out by Wang et al., palmitic acid, through PKCθ activation, induces PDK1 phosphorylation in serine 504/505, which alters its enzymatic activity and affects subsequent Akt activation (Fig. 2). In addition, FAs were shown to attenuate insulin receptor transcriptional regulator (HGMA1) activity through a mechanism that involves PKCc activation by palmitoylation. This mechanism reduces the receptor expression and, significantly, sensitivity to insulin.

**Ceramides and insulin resistance**

Other intermediaries of FA metabolism involved in insulin resistance pathogenesis are ceramides, which can accumulate in cells by two main routes: cell membrane sphingomyelin hydrolysis, by action of the sphingomyelinase enzyme, or by its de novo synthesis from long chain SFAs, such as palmitic acid, which implies a multiple-step biosynthetic route that occurs in the ER. So far, different factors, such as tumor necrosis factor alpha (TNF-α), endotoxins and diverse stress stimuli, have been found to activate sphingomyelinase, which leads to the generation of ceramides.

Ceramides’ accumulation, mainly associated with palmitic acid increased concentration, has been shown to be able to affect insulin-induced Akt2 activation. This appears to be due to a direct effect on Akt2 activation, rather than by interfering in the signaling of proteins that are activated before this kinase. Firstly, ceramides have been described to
promote protein phosphatase 2 (PP2A) activation. PP2A is a serine/threonine cytoplasmic phosphatase that is expressed ubiquitously, and plays an important role in the regulation of diverse cellular processes, including metabolic enzymes, hormone receptors, kinase cascades and cell growth regulation. Under physiological conditions, insulin inhibits PP2a. PP2A has been shown to dephosphorylate Akt threonine 308, which is one of the two residues that, together with serine 473, are phosphorylated when the enzyme is active\(^2\) (Fig. 2). In addition, ceramides prevent Akt recruitment by activating atypical \(\beta\) PKC (PKC\(\beta\)), which phosphorylates threonine 34 that is found in Akt PH domain, thus preventing its binding to PI3K and inhibiting its translocation and subsequent activation in response to insulin\(^{30,31}\) (Fig. 2).

Currently, there is controversy about the role played by ceramides in insulin resistance, owing to differences in the obtained results: in some studies, a direct correlation has been found between ceramides accumulation and insulin resistance, but others suggest that SFAs promote insulin resistance by an increase in DAG concentrations, with no significant changes in the ceramides content\(^{32,33}\).

**Fatty acids, oxidative stress and insulin resistance**

Oxidative stress can be defined as a state of unbalance between the production and elimination of ROS, whose overproduction by an increase in FFA plasma concentrations promotes an oxidative stress state that has been associated with mitochondrial damage and with different pathophysiological processes, such as insulin resistance, obesity and diabetes\(^34\). In the case of obesity, FFAs released by adipose tissue that has been damaged as a consequence of its expansion and of the development of hypoxia and cell death, are metabolized by muscle and hepatic tissues, where ROS overproduction and oxidative stress development has been identified\(^35\). In healthy subjects, FFA infusion causes oxidative stress and insulin resistance increase that can be reverted with antioxidants\(^36\). In turn, FFA increase in obesity elicits an increase in the
It should be observed that, in a FFA-excess chronic state, there is pancreatic beta cell dysfunction. In this context, prolonged exposure of the latter to FFA causes, on one hand, an increase in basal insulin release values and, on the other, an inhibition of glucose-induced secretion. In addition, FFAs inhibit insulin gene expression and induce apoptosis in these cells. Alteration in beta cell functions has been suggested to likely be due to ROS production and to oxidative stress state, both in animal models and in studies carried out in humans.

The role of fatty acids in endoplasmic reticulum stress

New evidences establish that ER dysfunction contributes to the development of metabolic conditions, such as obesity and DM2. ER plays a determinant role in Ca²⁺ homeostasis and participates in membrane and secretion proteins maturation and expression. In cell stress conditions that increase ER demand and entail an overload of its functional capacity, a series of alterations known as "endoplasmic reticulum stress" is generated, including a decrease in the transportation of proteins to the Golgi apparatus, unfolding protein expression and calcium depletion of this reservoir. Under these conditions, the ER activates a compensatory mechanism called "unfolding protein response" (UFR), which tries to reestablish ER functions homeostasis. In case this response is insufficient, apoptosis is then unleashed.

UPR triggers three stress sensor enzymes’ activation: 1) double stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK); 2) inositol-requiring endoribonuclease enzyme-1 (IRE-1); and 3) transcription factor 6 precursor (ATF6). Under normal conditions, these proteins are associated, through their luminal domain, with chaperon BIP/GRP78 (binding immunoglobulin protein/78 kDa glucose-regulated protein), thus remaining inactive. Under stress conditions, BIP/GRP78 is separated from sensor enzymes to promote correct protein folding. In addition to BIP separation, all three sensor proteins are activated, which favor the expression of proteins that help with the overload of proteins and enzymes associated with protein degradation in the reticulum.

On the other hand, IRE-1 activation induces its interaction with TRAF protein, a mechanism that favors IKKβ and JNK kinases activation, which in turn can phosphorylate IRS1 in serine residues, thus blocking...
insulin signaling would be the reason why palmitic acid induces insulin resistance in endothelial-origin cells through a biphasic mechanism, which involves an initial increase followed by a sustained reduction of SERCA protein values. Notwithstanding, palmitic acid produced a sustained inhibition of the ATPase activity of the pump. Interestingly, insulin resistance appeared before there was a SERCA expression reduction. PERK and JNK kinases activation by palmitic acid suggests that the mechanism by means of which the acid alters insulin signaling involves ER stress. SERCA overexpression reverted the palmitic acid effect on insulin resistance, indicating that the reduction of its expression and activity would be the reason why palmitic acid induces a state of resistance in endothelial cells. The treatment of diabetic people with rosiglitazone, an antidiabetic drug, increased SERCA expression, thus restoring the pump expression reduction observed in diabetic patients with altered hyperglycemia. The use of the chemical chaperon TUDCA restores SERCA pump expression and activity in obese mice. This way, the decrease in SERCA expression promotes the development of ER stress, with JNK ensuing activation, which desensitizes insulin signal, thus generating a state of insulin resistance and contributing to chronic metabolic deterioration.

**Membrane stiffness and insulin resistance**

Different reports have demonstrated that dietary FA types determine the type of FA available for the composition of cell membranes. Membranes containing phospholipids synthesized from FFA possess a
different and less fluid structure than those incorporating polyunsaturated (or essential) FA\textsuperscript{74,75}. Membrane fluidity degree, as determined by the ratio of polyunsaturated FAs and SFAs, influences on insulin-dependent glucose and GLUT4 transporters uptake and in insulin binding to its receptor effectiveness\textsuperscript{75-77}. Different experimental studies concur in pointing out that the composition of FA in muscle cell membrane phospholipids importantly influences on insulin sensitivity\textsuperscript{75,77,78}.

Beyond cell membrane alterations generated by FFA uptake by phospholipids, changes in the ER and mitochondrial membranes lipid composition that affect these organelles’ function have also been observed. In the case of the ER, membrane fluidity is highly important to its fusogenic activity and plasticity. Borradaile et al.\textsuperscript{79} demonstrated that incubation with FFA causes ER stress, an effect that was associated with a higher proportion of triglycerides with FFA in their membranes. In comparison with triglycerides in control cells, and this may be associated with a decrease in ER membrane fluidity. On the other hand, cholesterol pathophysiological concentrations have been shown to decrease membrane fluidity, as well as SERCA pump activity, an effect not occurring when there are high FA concentrations present in the ER membrane. These data suggest that, if ER membrane fluidity is decreased, reticulum stress will eventually develop, which in turn might generate a state of insulin resistance\textsuperscript{80} (Fig. 3).

In the mitochondrial membrane, FA composition and phospholipid saturation degree are important to its fluidity, its permeability and, therefore, to its appropriate function\textsuperscript{81}. Consumption of a fat rich diet has been suggested to be able to influence on the mitochondrial membrane composition, which has been associated with alterations that entail insulin resistance and DM2\textsuperscript{82,83}. However, recent data by Hoeks et al.\textsuperscript{84} suggest that, although in mice with a fat-rich diet changes are promoted in the mitochondrial membrane lipid composition, no alterations are observed in mitochondrial functions, such as FA oxidation, in spite of insulin resistance being induced. In fact, other studies have suggested that mitochondrial capacity in skeletal muscle is found to be increased in rodents on a fat-rich diet\textsuperscript{85-87}. These findings underscore the need for further studies clarifying the importance of mitochondrial membrane composition in mitochondrial functions and its association with the development of insulin resistance.

### Toll-like receptor-mediated insulin resistance

Increases in the glucose, FFA and pro-inflammatory cytokines secretion values in DM2 have been shown to have important implications for the immune system\textsuperscript{88,89}. Studies in animal models and in humans have suggested a close link between DM2 and changes in innate immune system response\textsuperscript{90}. In this sense, Toll-like receptors (TLR), a family of transmembrane receptors that belong to the innate immune system and that recognize pathogen-associated molecular patterns, have an important participation in the pathogenesis of insulin resistance, inflammation and DM2\textsuperscript{88,89,91}. In particular, TLR-4 have elevated expression and signaling in rodents and humans with obesity and insulin resistance, especially in insulin target tissues\textsuperscript{91-93}. Although the mechanisms by means of which TLR-4 signaling is increased have not been fully defined. A possible explanation is that lipopolysaccharide values, TLR-4 natural ligand, are elevated in individuals with obesity and DM2\textsuperscript{94,95}. Interestingly, FFAs have been reported to be TLR-4 agonists as well, which suggests a possible role of these receptors in obesity-induced chronic inflammation\textsuperscript{96}. TLR-4-mediated signaling activation promotes the generation of pro-inflammatory cytokines through upregulation of several transcriptional factors, such as NF\textsuperscript{κB} and AP-1\textsuperscript{97} (Fig. 4). This way, TLR-4 deficiency in KO mice improves insulin sensitivity and significantly attenuates inflammation in states of diet-induced obesity\textsuperscript{93,98,99}. Recently, Jia et al.\textsuperscript{100} demonstrated that hepatic TLR-4-deficient mice (Tir4L\textsuperscript{−/−}) show an increase in insulin sensitivity and an improvement in glucose tolerance and hepatic steatosis, in spite of the development of obesity due to a fat-rich diet. Furthermore, these KO mice showed an inflammatory response decrease in adipose tissue and in circulating inflammatory markers\textsuperscript{100}. These data reinforce the idea that the immune system plays a crucial role in the development of insulin resistance and DM2.

### Conclusions

Circulating FFA concentrations increase, particularly saturated FA, in pathologic conditions such as obesity, induces insulin resistance through different mechanisms described in the present review. It is otherwise interesting that, beyond the classic mechanisms identified in insulin resistance, such as IRS serine residues phosphorylation by action of different kinases such as
PKC, KNK and IKKβ, recent evidence suggests that SFAs can regulate proteins that are activated downstream to IRS, at the level of PDK1 and Akt, as well as inflammatory cytokines expression. This indicates the complexity of the mechanisms involved in insulin resistance, the complexity of the mechanisms involved in insulin resistance, the multiplicity of regulation spots and why is it so difficult to establish therapeutic measures; however, adequate study and understanding of such mechanisms can provide a better view on them and broaden the therapeutic routes in the face of this disorder.

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Conflicts of interests

The authors declare not having any conflicts of interests.

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Rvation of PKC θ
urated, fatty acids induce insulin resistance: role of intramuscular accum

dulators of the NLRP3 inflammasome in obesity/type


