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REVIEW

T. COLL, ET AL. [2006] MED HYPOTHESES RES 3: 739-750.

INFLAMMATION AND FATTY ACID-INDUCED INSULIN RESISTANCE IN SKELETAL MUSCLE CELLS

TERESA COLL, RICARDO RODRÍGUEZ-CALVO, XAVIER PALOMER AND MANUEL VÁZQUEZ-CARRERA*

PHARMACOLOGY UNIT, DEPARTMENT OF PHARMACOLOGY AND THERAPEUTIC CHEMISTRY, FACULTY OF PHARMACY, UNIVERSITY OF BARCELONA, SPAIN

ABSTRACT. FREE FATTY ACIDS are potent signaling molecules that are abnormally elevated in obesity and type 2 diabetes mellitus. Accumulating evidence suggests that free fatty acids may mediate the development of insulin resistance, linking this process with obesity. Skeletal muscle accounts for the majority of insulin-stimulated glucose utilization, and thus is the major site of insulin resistance. In this review, we will discuss concisely the molecular and cellular links between fatty acid-induced insulin resistance and inflammation in skeletal muscle cells. We focus on the effects of palmitate, since this saturated fatty acid, leads to increased intracellular levels of diacylglycerol. This is in contrast to the monounsaturated fatty acid oleate. Diacylglycerol is a potent allosteric activator of the protein kinase C θ isoform, a serine/threonine kinase that has been linked to insulin resistance. This kinase may directly affect the phosphorylation of the insulin receptor substrate 1 (IRS1). Alternatively it may indirectly activate several serine/threonine kinases. These kinases subsequently affect IRS1 phosphorylation and activate the pro-inflammatory transcription factor NF-κB. This leads to enhanced expression of inflammatory cytokines, such as tumor necrosis factor and interleukin-6. Activation of NF-κB by fatty acids may also interfere with the activity of the transcription factor peroxisome proliferator-activated receptor-β/δ, leading to a fall in the expression of its target-genes involved in fatty oxidation. This may reduce fatty acid utilization and oxidation, thus promoting its accumulation in skeletal muscle in the form of triglycerides and diacylglycerol. Drugs targeting the protein kinase C/NF-κB pathway or activating PPARβ/δ may prevent or ameliorate the development of fatty acid-mediated insulin resistance in skeletal muscle.

*ADDRESS ALL CORRESPONDENCE TO: DR. MANUEL VÁZQUEZ-CARRERA, UNITAT DE FARMACOLOGIA, FACULTAT DE FARMÀCIA, DIAGONAL 643, E-08028 BARCELONA, SPAIN.
TELEPHONE: 93-4024531. FAX: 93-4035982. E-MAIL: mvazquezcarrera@ub.edu

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1. INTRODUCTION

Insulin resistance is a common pathophysiological state defined as a failure of the target tissues to respond properly to normal levels of plasma insulin. The first abnormality observed in insulin resistance is a decrease in insulin-stimulated glucose uptake in skeletal muscle and adipose tissue and a fall in the ability of insulin to suppress hepatic glucose production [1,2]. In the initial states of the pathology, euglycemia is maintained because enhanced insulin secretion by pancreatic β-cells compensates for peripheral insulin resistance. However, when this pathology is maintained chronically, hyperinsulinemia exacerbates insulin resistance and contributes to β-cell failure. This leads to the development of impaired glucose tolerance and overt clinical type 2 diabetes [1,2].

Obesity predisposes individuals to the development of insulin resistance. Most patients suffering insulin resistance are obese. Adipose tissue secretes signaling molecules that are involved in the pathogenesis of insulin resistance. Among these molecules, free fatty acids (FFA) may link obesity with insulin resistance. Different evidence supports the idea that elevated FFA levels are responsible for much of the insulin resistance present in type 2 diabetic patients. Thus, several studies have consistently demonstrated that elevated plasma FFA levels produce insulin resistance in diabetic patients and in nondiabetic subjects [3-6]. In addition, elevated plasma FFA levels may lead to diacylglycerol-mediated activation of protein kinase C (PKC) [7,8]. This enzyme has been linked to insulin resistance in a wide variety of rodent models [9-11], including rats infused with lipid [12], and obese humans [13,14]. Furthermore, once the FFA palmitate is activated to palmitoyl-CoA by acyl-CoA synthase, it becomes the precursor of *de novo* synthesis of ceramides, that can attenuate insulin signaling pathways leading to insulin resistance [15]. Finally, strong evidence about the role of lipids in muscle insulin resistance has been obtained by studies with NMR spectroscopy, which show a strong relationship between the accumulation of intramyocellular triglyceride content and insulin resistance [16-18]. In spite of these data, the mechanisms by which elevated FFA levels cause insulin resistance are not well understood.

In addition, accumulating evidence suggests a link between inflammation and type 2 diabetes. Markers of inflammation, including pro-inflammatory cytokines [such as tumor necrosis factor α (TNFα), interleukin (IL)-18 and IL-6] have been reported to have elevated levels in type 2 diabetes [19-25]. TNF-α was the first inflammatory cytokine postulated as a critical mediator of insulin resistance [26,27]. This cytokine is overproduced in the adipose tissue and muscle tissues of obese humans [28-30]. In spite of the potent inhibitory effect of TNF-α on insulin signaling in both adipose tissue and skeletal muscle, the concentrations of TNF-α in the serum of both lean and obese subjects is very low. This suggests that TNF-α secreted by muscle cells and adipocytes acts in an autocrine fashion [29,31]. Another cytokine, IL-6, has the strongest correlation with insulin resistance and type 2 diabetes [19,20,24]. Its plasma levels are increased 2–3 fold in patients with obesity and type 2 diabetes compared with lean control subjects [20]. Until recently, the main source of IL-6 production was thought to be macrophages and peripheral mononuclear cells. However, recent evidence suggests that adipose and skeletal muscle cells are important sites of IL-6 production [32-34].

Skeletal muscle accounts for the majority of insulin-stimulated glucose utilization and is, therefore, the major site of insulin resistance. Deciphering both the mechanisms by which inflammation in skeletal muscle develops during insulin resistance and the contribution of inflammation to this pathological process is crucial to understanding, preventing or treating this pathology. In this review we will discuss concisely the molecular and cellular links between fatty acid-induced insulin resistance and inflammation in skeletal muscle cells.

2. FATTY ACID-INDUCED INSULIN RESISTANCE IN SKELETAL MUSCLE CELLS

Insulin is the main hormone involved in blood

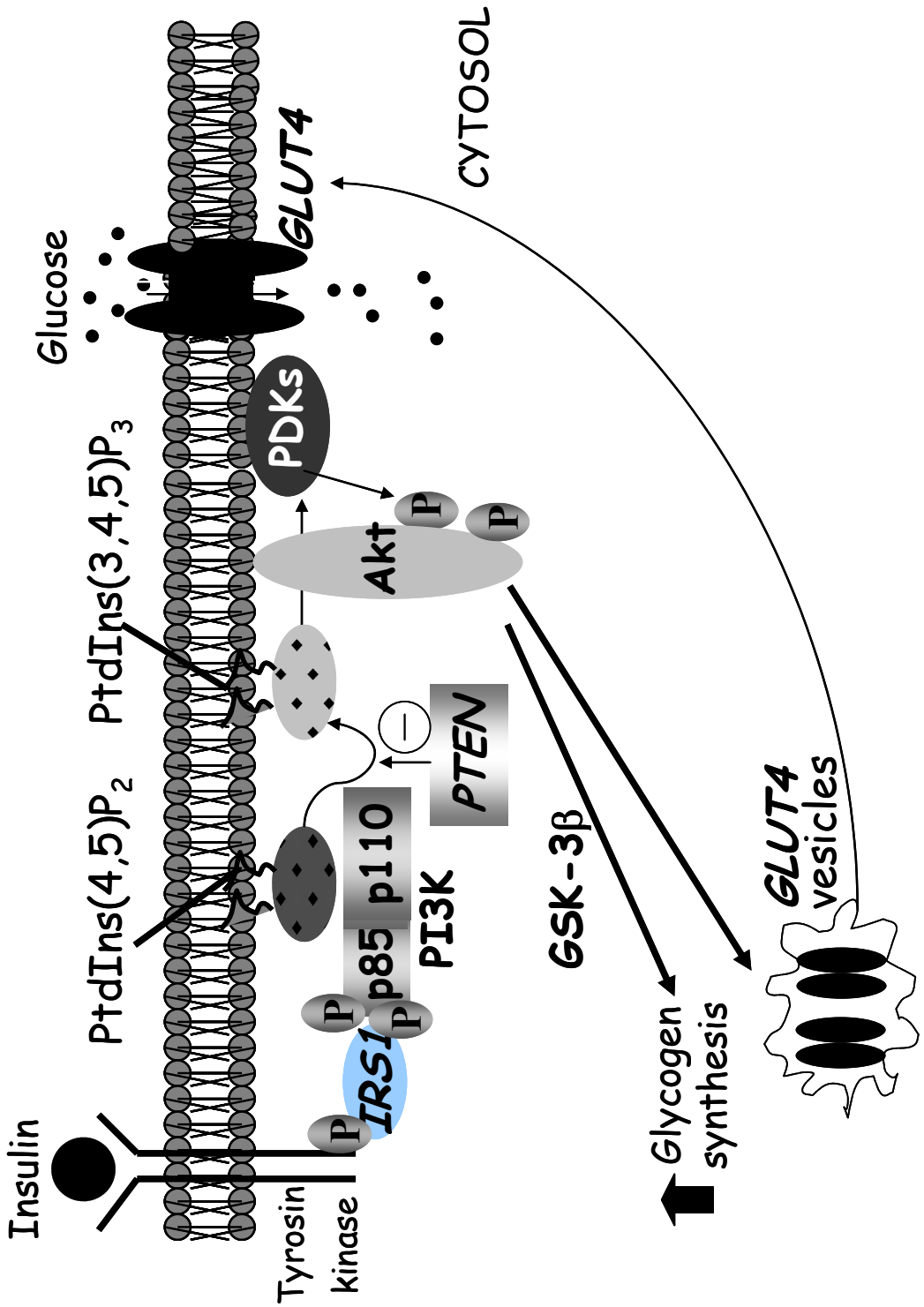

FIGURE 1. SCHEMATIC DIAGRAM SHOWING INSULIN-SIGNALING PATHWAYS.

Abbreviations: PtdIns(3,4,5)P_3, phosphatidylinositol triphosphate; PtdIns(4,5)P_2, phosphatidylinositol diphosphate; IRS, insulin receptor substrate; p85 and p100, subunits of phosphatidylinositol 3-kinase (PI3K); PTEN, phosphatase and tensin homologue deleted on chromosome 10; Akt, protein kinase B; GLUT4, glucose transporter 4; PDK, phosphoinositide-dependent kinase.

glucose uptake and metabolism. Circulating insulin rapidly interacts with its receptor in target tissues, which are mainly: liver, skeletal muscle and adipose tissue. The insulin receptor belongs to the family of cell surface receptors that possess intrinsic tyrosine kinase activity. It phosphorylates downstream targets such as the insulin receptor substrate (IRS) proteins, which mediate most of the biological actions of insulin [35] (FIG. 1). IRS1, the best-studied insulin receptor kinase substrate, is tyrosine phosphorylated (*i.e.*, Tyr^{608} and Tyr^{628} in the rat) in response to insulin generating docking sites. This favors its interaction with Src homology domain-2-containing proteins, including the p85 subunit of phosphatidylinositol 3-kinase (PI3K) [35]. PI3K has been shown to be essential for linking insulin receptor with downstream responses [36]. Accordingly, IRS1 and PI3K connect activated insulin receptor to proteins that regulate glucose metabolism, such as glucose transporters. Activation of PI3K leads to stimulation of Akt or protein kinase B, which contributes to enhanced glucose uptake, glycogen synthesis, and protein synthesis [37,38]. Thus, Akt activation promotes the uptake of glucose in skeletal muscle and adipose tissue by stimulating the translocation of the insulin responsive glucose transporter 4 (GLUT4) from intracellular stores to the plasma membrane, increasing by this way the glucose influx in these tissues.

2.1. MECHANISMS INVOLVED IN FFA-INDUCED CYTOKINE EXPRESSION IN SKELETAL MUSCLE CELLS

FFA concentrations may rise up to 2 mM in human and in rodent plasma [33], being palmitate the most abundant saturated FFA in plasma (palmitate, 20-35%; stearate, approximately 10%). The inhibition of signaling downstream of the insulin receptor is a primary mechanism by which fatty acids lead to insulin resistance. A decrease in IRS1 tyrosine phosphorylation has been associated with a reduction in glucose uptake in cells and tissues in animal models of obesity and in type 2 diabetic patients. The mechanisms of this process are not well established [39]. However, recent evidence suggests that additional phosphorylation sites are involved in the development of insulin resistance. In addition to the tyrosine phosphorylation sites, IRS1 also contains fifty potential serine/threonine phosphorylation sites that regulate its function, leading to enhanced or inhibited insulin effects. For instance, insulin controls the functions of IRS1 by phosphorylating different serine residues. By this mechanism, insulin may enhance or terminate insulin effects [40]. In addition, the factors involved in the development of insulin resistance (FFA, cytokines, cellular stress, etc.) activate serine/threonine kinases (c-Jun N-terminal kinase, JNK; protein kinase C, PKC; mammalian target of rapamycin, mTOR; S6kinase, S6K; IκB kinase, IKK; extracellular signal-related kinase, ERK) that may enhance the phosphorylation of IRS1 on serine residues, thereby attenuating insulin signaling [35,38]. This mechanism is thought to contribute to insulin resistance and the development of type 2 diabetes [37,38]. Exposure of cells to TNF-α also stimulates inhibitory phosphorylation of IRS1 serine residues [41-43]. However, as stated above, the concentrations of TNF-α in the serum of both lean and obese subjects is very low, which suggests that the TNF-α secreted by muscle cells acts in an autocrine fashion [29,31]. Little is known about the mechanisms responsible for the increased expression of TNF-α in skeletal muscle, but elevated plasma FFA levels could be involved. We have recently reported that exposure of skeletal muscle cells to the saturated fatty acid palmitate led to an increase in TNF-α gene expression, and a fall in the expression of GLUT4 [44]. Furthermore, cells exposed to palmitate had low amounts of IRS1, but increased Ser^{307} phosphorylation [44]. These changes, that were observed in skeletal muscle cells incubated with palmitate, may involve the activation of several pathways. Thus, elevated FFA levels presumably increase FFA uptake, exceeding its oxidation, fact that in turns leads to increased intramuscular triglycerides and diacylglycerol, which is a potent allosteric activator of both conventional and novel PKC isoforms. Interestingly, it has been reported that the incubation of skeletal muscle cells with palmitate results in the

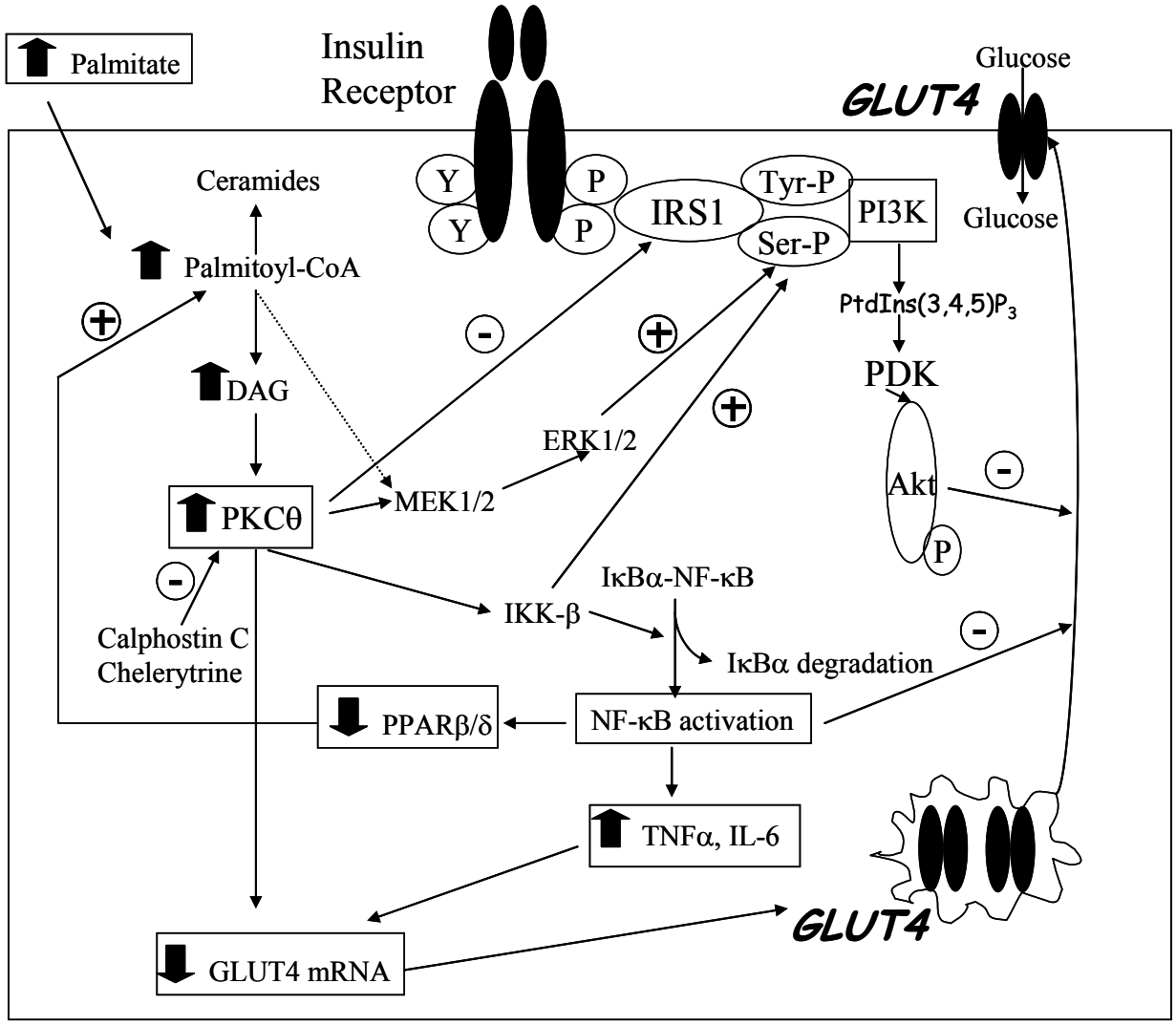

FIGURE 2. SCHEMATIC DIAGRAM SHOWING MECHANISMS INVOLVED IN FATTY ACID-INDUCED INSULIN RESISTANCE IN SKELETAL MUSCLE CELLS. Elevated levels of plasma palmitate presumably increase palmitate uptake, exceeding its oxidation. This in turns leads to increased intramuscular triglycerides and diacylglycerol, which is a potent allosteric activator of the PKCθ isoform. This PKC isoform can either phosphorylate IRS1 or activate several serine/threonine kinases, leading to enhanced serine phosphorylation of IRS1. One of these serine/threonine kinases, IKK-β, activates the transcription factor NF-κB. This enhances the expression of inflammatory cytokines, such as TNF-α. All these events lead to impaired insulin signaling. Abbreviations: PtdIns(3,4,5)P_3, phosphatidylinositol triphosphate; IRS, insulin receptor substrate; p85 and p100, subunits of phosphatidylinositol 3-kinase (PI3K); Akt, protein kinase B; GLUT4, glucose transporter 4; PDK, phosphoinositide-dependent kinase; IKK, IκB kinase; ERK, extracellular signal-related kinase; MEK, mitogen-activated protein kinase-ERK kinase; PKC, protein kinase C; DAG, diacylglycerol; PPAR, peroxisome proliferator-activated receptor.

activation of PKCθ, which is the most abundant PKC isoform in skeletal muscle [12,14]. The activation of PKCθ by palmitate could lead to insulin resistance through several mechanisms. First, this PKC isoform can phosphorylate IRS1 [45], the major mediator of the insulin response in muscle [46], leading to impaired insulin signaling (FIG. 2). In fact, it has been reported that PKCθ knockout mice are protected from fat-induced insulin resistance [47]. Some studies support the in-

volvement of PKC in fatty acid-induced insulin resistance. The phosphorylation of IRS1 on Ser^{307} and the fall in its expression was prevented in palmitate-treated cells coincubated in the presence of chelerythrine, an inhibitor of the PKC catalytic site [48]. These data are consistent with those observed in 3T3-L1 adipocytes, in which fatty acids enhanced IRS-1 phosphorylation, a process that precedes its degradation [49,50]. Moreover, PKC inhibition prevented the fall in insulin-stimulated Akt phosphorylation and completely abolished the changes in TNF-α and GLUT4 expression caused by palmitate. Second, PKCθ has the unique ability among the PKC isoforms to activate NF-κB [12], which has been linked to fatty acid-induced impairment of insulin action in skeletal muscle in rodents [51,52]. NF-κB also regulates the expression of TNF-α (FIG. 2). In resting cells, NF-κB is present in the cytoplasm as an inactive heterodimer, consisting of the p50 and p65 subunits, complexed with an inhibitor protein subunit, IκB. After stimulation, a serine kinase (IκB-kinase, IKK-β) is activated leading to the phosphorylation of IκB. This event converts IκB into a substrate for ubiquitination and subsequent degradation, releasing the NF-κB heterodimer, which then translocates to the nucleus and regulates the expression of proinflammatory genes, such as TNF-α. In humans, Itani et al. [53] reported that lipid infusion during an euglycemic-hyper-insulinemic clamp increased PKC activity and degradation of the mass of the NF-κB inhibitor IκBα. Activation of PKC can lead to the activation of NF-κB by directly phosphorylating IκBα [54] or by causing the generation of reactive oxygen species (ROS) that can secondarily activate IKK-β. In fact, phosphorylation by IKK-β is considered the main pathway by which IκBα is released from NF-κB and subsequently subjected to ubiquination and proteosomal degradation. IKK-β may also be involved in the phosphorylation of IRS1 on Ser^{307} in response to palmitate, since IRS1 is a direct *in vitro* substrate of this kinase [55]. In summary, IKK-β can impact on insulin signaling either by directly phosphorylating IRS1, or by activating the transcription factor NF-κB. Among other factors, NF-κB stimulates the production of inflammatory mediators, including TNF-α and IL-6 [52]. The saturated FFA palmitate activated NF-κB by mediating degradation of IκBα [33,34]. Interestingly, the presence of the PKC inhibitor chelerythrine prevented the fall in the content of IκBα induced by palmitate. This suggests that PKC was involved in NF-κB activation by palmitate in skeletal muscle cells. Furthermore, NF-κB binding sites are present in the TNF-α promoter [56,57], supporting a role for NF-κB in the palmitate-mediated induction of this cytokine. In fact, pretreating cells with NF-κB inhibitors reduced palmitate-mediated induction of TNF-α [44]. In addition to IκBα, changes in IκBβ may also lead to NF-κB activation. It has recently been reported that IκBβ protein abundance was reduced in the vastus lateralis muscle of subjects with type 2 diabetes compared to control subjects. This could provide a explanation for the increased activation of the NF-κB pathway in type 2 diabetes [58]. Interestingly, aerobic exercise training in these patients caused an increase in IκBα and IκBß protein to levels comparable with that of control subjects, suggesting that these abnormalities can be reversed by exercise training.

PKC is also an upstream regulator of the MAPK/ERK cascade [59,60]. We have shown that activation of the MAPK/ERK cascade is involved in the palmitate-induced expression of TNF-α. This suggests that PKC activation leads to the activation of this cascade [44]. However, inhibitors of the MAPK/ERK cascade did not prevent the fall in GLUT4 expression caused by palmitate, indicating that a blockade of upstream targets, *i.e.*, PKC, is required to achieve this objective.

Activation of the PKC/NF-κB pathway is also involved in palmitate-mediated induction of IL-6 expression and secretion in skeletal muscle cells [33,34]. Strong evidence suggests that IL-6 secretion may be partly responsible for the effects of the saturated fatty acid palmitate on insulin resistance in skeletal muscle cells, since exposure of skeletal muscle cells to palmitate caused a fall in the mRNA levels of GLUT4 and insulin-stimulated

glucose uptake. These reductions were prevented in the presence of anti-IL-6 antibody, which neutralizes the biological activity of mouse IL-6 in cell culture [34].

Palmitoyl-CoA is a precursor of sphingolipid synthesis and palmitate treatment may result in enhanced synthesis of ceramides [61], which can attenuate insulin signalling pathways, leading to insulin resistance [62]. We demonstrated that ceramides were not involved in palmitate-induced TNF-α and IL-6 in skeletal muscle cells by using: C_2-ceramide, a cell-permeable ceramide analogue; and the sphingosine analogue ISP1, which inhibits the formation of 3-ketodihydrosphingosine from palmitoyl-CoA and L-serine [34,44].

All these data suggest that the PKC/NF-κB pathway is a crucial link between increased FFA levels and the induction of a pro-inflammatory state during the development of insulin resistance and type 2 diabetes mellitus.

2.2. MECHANISMS INVOLVED IN THE REGULATION OF THE OXIDATIVE CAPACITY IN SKELETAL MUSCLE CELLS

As stated above, lipid accumulation occurs in skeletal muscle in diabetic states, which suggests that there is reduced oxidative capacity [63]. In fact, fatty oxidation rates [64] and fatty acid oxidative enzyme activities [64,65] are up to 50% lower in muscle from obese subjects compared with lean subjects. These reports suggest that impaired fatty acid oxidation precedes muscle lipid accumulation. Fatty acid catabolism in skeletal muscle is mainly regulated by peroxisome proliferator-activated receptors (PPARs), ligand-activated transcription factors that regulate the expression of genes involved in fatty acid uptake and oxidation, lipid metabolism and inflammation [66]. The PPAR family consists of three members: PPARα (NR1C1 according to the unified nomenclature system for the nuclear receptor superfamily), PPARβ/δ (NR1C2) and PPARγ (NR1C3) [67]. PPARα is expressed primarily in tissues that have a high level of fatty acid catabolism such as liver, brown fat, kidney, heart and skeletal muscle [68]. PPARβ/δ is ubiquitously expressed. PPARγ has a restricted pattern of expression, mainly in white and brown adipose tissues, whereas other tissues such as skeletal muscle and heart contain limited amounts. In order to be transcriptionally active, PPARs need to heterodimerize with the 9-*cis*-retinoic acid receptor (RXR) (NR2B). PPAR-RXR heterodimers bind to DNA specific sequences called peroxisome proliferator-response elements (PPREs). These consist of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA), separated by one nucleotide (DR-1). These sequences have been characterized within the promoter regions of PPAR target genes. However, the regulation of gene transcription by PPARs extends beyond their ability to transactivate specific target genes. PPARs are also capable of regulating gene expression independently of binding to DNA. This regulation occurs through a mechanism termed receptor-dependent transrepression [69]. For instance, a physical interaction of PPARα with NF-κB has been reported. This leads to reduced activity of both proteins [70]. In fact, a recent study showed that inactivation of NF-κB enhanced the expression of apoA-I through activation of PPARα [71].

Recent studies have shown that PPARβ/δ plays an important role in controlling fatty acid metabolism in skeletal muscle cells [72-75]. This PPAR subtype induces the expression of the target genes involved in fatty acid utilization and oxidation in myocytes. It has been suggested that PPARα and PPARβ/δ share similar functions in myotubes in terms of fatty acid metabolism. In agreement with this hypothesis, Muoio et al. [76] showed that fatty acid oxidation was not impaired in the skeletal muscle of PPARα$^{-/-}$ mice. This is probably because PPARβ/δ compensates for the lack of PPARα in these mice. Therefore, impaired PPARβ/δ signaling may be implicated in lipid accumulation in skeletal muscle. We have recently reported that the exposure of skeletal muscle cells to palmitate reduces the expression of the PPARβ/δ–target genes involved in fatty acid

utilization. This occurs through a mechanism that may involve NF-κB activation. Such changes were prevented by treatment with a PPARβ/δ activator. Interestingly, palmitate exposure led to increased protein-protein interaction between the p65 subunit of NF-κB and PPARβ/δ [77]. These findings are in concordance with the results reported by Westergaard et al. [78], who showed that PPARβ/δ physically interacts with p65 in psoriatic lesions. Furthermore, they showed a p65-dependent repression of PPARβ/δ, but not of PPARα or PPARγ. Surprisingly, the interaction between p65 and PPARβ/δ was high in skeletal muscle cells that were not exposed to palmitate. This suggests that the levels of endogenous agonists in these cells were low, or that NF-κB regulates the basal activity of PPAR. The latter suggestion is in agreement with a previous study showing that inactivation of basal NF-κB activity enhanced the expression of the PPARα-target gene apoA-I [79]. This is because most of the genes under the control of PPARα are also regulated by PPARβ/δ. It can be hypothesized that activation of NF-κB by elevated plasma FFA levels may result in a reduction in PPAR signaling, leading to a fall in fatty acid utilization and oxidation. This may favor the accumulation of fatty acids in skeletal muscle, in the form of triglycerides and diacylglycerol. Furthermore, accumulation of these lipid species may enhance the inflammatory process in skeletal muscle. Taking into account that insulin resistance correlates closely with intramyocellular lipid levels in skeletal muscle [80-82], the reduction in the activity of NF-κB in diabetic patients by PPARβ/δ agonists may became a therapeutic target for the treatment of this pathology. In support of this hypothesis, it has been reported that activation of PPARβ/δ induces fatty acid β-oxidation in skeletal muscle and attenuates the metabolic syndrome [83], in which insulin resistance plays a pivotal role.

CONCLUSIONS

The treatment and prevention of insulin resistance and type 2 diabetes requires lifestyle changes, including weight reduction, increased physical activity and diet. However, many diabetic patients are not able to control the pathology with lifestyle modifications. In such cases, drugs are needed to manage type 2 diabetes. Drugs targeting the PKC-NF-κB pathway or those activating PPARβ/δ may become an additional pharmacological strategy to treat this pathology, since they may reduce the inflammatory component of type 2 diabetes in skeletal muscle.

ACKNOWLEDGEMENTS

We thank the University of Barcelona's Language Advisory Service for their helpful assistance. Part of the work summarized in this review was supported by grants from the Fundació Privada Catalana de Nutrició i Lípids, Fundación Ramón Areces, Ministerio de Educación y Ciencia, Spain (SAF2003-01232 and SAF2006-01475) and by FEDER funds form the European Union. Ricardo Rodríguez-Calvo was supported by a grant from the Fundación Ramón Areces. Teresa Coll (Formación Personal Investigador) and Xavier Palomer (Programa Juan de la Cierva) were supported by grants from the Ministerio de Educación y Ciencia, Spain.

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RECEIVED ON 5-4-2006.
ACCEPTED ON 7-2-2006.