Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: Evidence for PKC activation and oxidative stress-activated signaling pathways

Rafik Ragheb^{a,b,c,d,e,*}, Gamila M.L. Shanab^a, Amina M. Medhat^a, Dina M. Seoudi^a, K. Adeli^{c,e}, and I.G. Fantus^{b,d,e,*}

^a University of Ain Shams, Department of Biochemistry, Faculty of Science, Cairo, Egypt

^b University Health Network, Toronto, Ontario, Canada M5G 2C4

^c Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

^d Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5

^e University of Toronto, Department of Laboratory Medicine and Pathobiology, Toronto, Ontario, Canada M5G 1L5

Abstract

In the present study, we examined the effects of free fatty acids (FFAs) on insulin sensitivity and signaling cascades in the C2C12 skeletal muscle cell culture system. Our data clearly manifested that the inhibitory effects of PKC on insulin signaling may at least in part be explained by the serine/threonine phosphorylation of IRS-1. Both oleate and palmitate treatment were able to increase the Serine³⁰⁷ phosphorylation of IRS-1. IRS-1 Serine³⁰⁷ phosphorylation is inducible which causes the inhibition of IRS-1 tyrosine phosphorylation by either I κ B-kinase (IKK) or c-jun N-terminal kinase (JNK) as seen in our proteomic kinases screen. Furthermore, our proteomic data have also manifested that the two FFAs activate the IKK α/β , the stress kinases S6 kinase p70 (p70SK), stress-activated protein kinase (SAPK), JNK, as well as p38 MAP kinase (p38MAPK). On the other hand, the antioxidant, Taurine at 10 mM concentrations was capable of reversing the oleate-induced insulin resistance in myocytes as manifested from the glucose uptake data. Our current data point out the importance of FFA-induced insulin resistance via multiple signaling mechanisms.

Keywords

Free fatty acid (FFA); Insulin resistance (IR); Skeletal muscle cell (C2C12); Protein kinase C (PKC); Insulin receptor substrate-1 (IRS-1)

*Corresponding authors. Addresses: Division of Biochemistry, Faculty of Science, Ain Shams University, Abbasia Sq., Cairo 11566, Egypt. Fax: +20 2 26839622 (R. Ragheb); 294-Williamson Rd., Markham ON, Canada L6E 1X1 (I.G. Fantus). rafik.ragheb@utoronto.ca (R. Ragheb), fantus@mshri.on.ca (I.G. Fantus).

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Introduction

Insulin resistance is one of the features of the metabolic syndrome associated with obesity [1]. Elevation of plasma FFA has been shown to impair insulin action, and to be a risk factor for the development of type-2 diabetes [2]. Skeletal muscle is the most important target of insulin action for glucose disposal and the development of insulin resistance [3–7].

It is well documented that lipids reduce insulin sensitivity and glucose uptake in skeletal muscle cells [8]. The first theory was postulated by Randle et al., glucose–fatty acid cycle [9]. However, lipids may also reduce insulin sensitivity through other different mechanisms that affect the insulin signaling through the activation of PKC isoenzymes [9–11]. Lipids may also cause attenuation of the insulin signaling independent of PKC as shown in different studies [10–13]. Schmitz-Peiffer et al. reported that the activation of protein phosphatase 2A (PP2A); a serine/threonine phosphatase, can reduce PKB/Akt activation in skeletal muscle cells [10]. The current study was performed in order (1) to investigate in detail the mechanisms of FFA-induced insulin resistance using oleate and palmitate, the most predominant FFA in the circulation, (2) to elucidate the molecular mechanisms involved in the induction of insulin resistance in the C2C12 muscle model by focusing our attention on oleate. A number of reports have manifested how oleic acid induces and contributes to the induction of insulin resistance in other models such as hepatocytes, adipocytes, and pancreatic β cells with no clear understanding of the exact mechanism or the causality of its occurrence at the molecular level muscle model.

Materials and methods

Materials

Dulbecco's modification Eagle's medium 1 $\times$. MOD (DMEM) was from Gibco–BRL – Life Technologies, Inc. (Grand Island, USA). Fetal bovine serum (FBS) and horse serum were from Life Technologies, Inc. (Gaithersburg, MD). 4-(2-Hydroxyethyl) 1-piperazinethane sulfonic acid (Hepes), (1 μ ci) deoxyglucose H^3 , and 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (Bis-I), were from Sigma Chemical Co., USA. Fatty acid free bovine serum albumin, oleic acid, and palmitate were from Sigma Chemical Co., USA. PKB antibodies and phosphospecific-Threonine³⁰⁸ PKB, phospho-Serine⁴⁷³ PKB, IRS-1 (total), Serine³⁰⁷ IRS-1 were from Upstate Biotechnology (Lake Placid, NY).

Cell culture

C2C12 myoblasts were maintained in DMEM, pH 7.4 (4.5 g/l glucose, L-glutamine, 110 mg/L sodium pyruvate, pyridoxine hydrochloride, $NaHCO_3$). Cells were then seeded at a density of 3×10^4 cell/cm² into 10 mm dishes and differentiated to myotubes [10].

Lipid preincubation

Lipid-containing media were prepared by conjugation of 1 mM oleate or palmitate with BSA (4%), using the method modified and described by Svedberg et al. [14].

Glucose uptake/phosphorylation assays

Lipid-pretreated myotubes were incubated in the SF-DMEM in the absence or presence of 100 nM insulin for 30 min. Cells were treated with 2-D-[H³]glucose [1 µCi/µl] for 10 min. Glucose uptake was determined using β-counter [10].

Chemiluminescent immunoblot analysis

The treated cells were lysed in a lysis buffer and the total cell lysates were subjected either directly or after immunoprecipitation against a target protein to chemiluminescent immunoblotting for total PKB/Akt; also their phosphorylated membranes were then incubated in an enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech) for 60 s and exposed to Kodak Hyperfilm. Films were developed and quantitative analysis was performed using an Imaging Densitometer [15].

Proteomic screen and Kinetworks™ KPKS-1.1 (protein kinase screen) and KPSS-4.0 (phosphoprotein screen)

Myotubes pretreated with oleate or palmitate (1 mM) and untreated controls were lysed in 0.5 ml of lysis buffer. The lysate was subjected to ultracentrifugation for 30 min at 100,000g or more using a Beckman Table Top TL-100 ultracentrifuge. The resulting supernatant fraction was removed and submitted to Kinexus Bioinformatics Corporation (Vancouver, Canada) for analysis using Kinetworks™ KPKS-1.0. However, only 350 µg of protein was used for Kinetworks KPSS-4.0 [16].

Statistical methods

All results were expressed as means ± SE. Statistical calculations were performed using Student's *t*-test. Data analysis were performed using the computer program Sigma Plot Version 8.02 software and were considered significant at $p < 0.05$.

Results

Effect of oleate and palmitate preincubation on insulin-stimulated glucose uptake

Fig. 1A and B shows the effect of FFAs on glucose uptake. Insulin-stimulated glucose uptake was measured in cells pretreated for 16 h with either oleate or palmitate in the presence or absence of 100 nM insulin for 30 min. The glucose uptake exhibited approximately half fold increase over basal control ($p < 0.05$) in control cells treated with 100 nM insulin. Oleate at 1 mM caused a significant change in glucose uptake compared to both basal and insulinstimulated controls, although palmitate at the 1 mM concentration caused a significant reduction in glucose uptake after insulin ($p < 0.05$).

Phosphorylation of PKB/Akt

Fig. 1C shows that C2C12 myotubes had been pretreated with or without FFA(s) for 16 h and with or without insulin stimulation for 10 min. Following electrophoretic transfer to nitrocellulose membranes, the proteins were immunoblotted with anti-phospho-Serine⁴⁷³, anti-phospho-Threonine³⁰⁸, or anti-Akt antibody for the total protein (Mass). The Serine⁴⁷³ or Threonine³⁰⁸ were clearly reduced in cells treated with palmitate in comparison with

control or oleate. The reduction in phospho-PKB (Serine⁴⁷³ or Threonine³⁰⁸) was statistically significant ($p < 0.05$) in cells treated with palmitate. However, the trend seen in the reduction of phospho-PKB (Serine⁴⁷³ or Threonine³⁰⁸) by about 26% and 28%, respectively, in cells treated with oleate it was not statistically significant. There was no significant difference in the PKB/Akt protein levels of the control in comparison with cells treated with oleate or palmitate.

PKC isoenzymes and related proteins activation after FFAs treatment

Fig. 2A and B represents the phosphosite screen using the proteomics approach. It clearly shows a significant increase in the phosphorylation of PKC alpha (122%), alpha/beta (631%), delta (351%), and epsilon (698%) isoforms in cells treated with oleate. However, cells treated with palmitate have shown a slight increase in the phosphorylation of PKC alpha (80%), alpha/beta (71%), delta (51%), and epsilon (1.8%). Consistent with these findings, using the kinase screen, Fig. 2B, clearly shows an upregulation of the expression level of Inhibitor NF κ B kinase alpha/beta (IKK α/β) seen in both oleate (71%) and palmitate (58%) fatty acid-treated cells, indicating the activation of NF κ B pathway as an effector function for the PKC activation. Oleate activates the stress kinases S6 kinase p70 (p70SK) (742%), stress-activated protein kinase (SAPK) (128%), JNK (128%), and p38 MAP kinase (p38MAPK) (79%), extracellular regulated kinase 1,2, (ERK1) (32%), (ERK2) (11%), MAP kinase 3,6 (MEK3) (283%), (MEK6) (280%), and oncogen Raf-1 (486%). Cells treated with palmitate have also shown activation in the phosphoproteins: the stress kinases S6 kinase p70 (p70SK) (318%), stress-activated protein kinase (SAPK) (665%), JNK (665%), and p38 MAP kinase (p38MAPK) (1207%), extracellular regulated kinase 1,2, (ERK1) (42%), (ERK2) (93%), MAP kinase 3,6 (MEK3) (379%), (MEK6) (151%), and oncogen Raf-1 (18%).

In Fig. 2C, the use of PKC chemical inhibitor Bis-I and the measurement of the glucose uptake in cells treated with FFAs and Bis-I at 2 μ M are shown. The combination of both oleate and Bis-I was able to restore the insulin effect on glucose uptake. However, palmitate and Bis-I treated cells were still insulin resistant.

The effector functions of stress kinases activation on Serine³⁰⁷ phosphorylation status of IRS-1 and the effect of antioxidant Taurine

Fig. 3A represents FFA-treated myotubes and the insulin-stimulated IRS-1 Serine³⁰⁷ phosphorylation. The total cell lysates were immunoprecipitated (IP) for IRS-1, and were then immunoblotted (IB) for Serine³⁰⁷ phosphorylation. The results show an upregulation for the expression level of Serine³⁰⁷ IRS-1, which is an indication of the increase of serine phosphorylation in both oleate- and palmitate-treated cells in comparison with their controls.

In Fig. 3B, the use of Taurine, an antioxidant chemical inhibitor, and the measurement of glucose uptake in cells preincubated with oleate (1 mM) and Taurine at 10 mM are shown. The combination of both oleate and Taurine was able to restore the insulin effect on glucose uptake.

Discussion

In the present study, we studied the direct effects of chronic treatment of oleate and palmitate in C2C12 cell line at the functional and signaling levels. Myotubes are characterized by over expression of Glut-4, which is the most sensitive glucose transporter to insulin stimulation [10]. The assessment of glucose uptake showed an approximately half fold increase over basal state in control cells treated with insulin. However, cells treated with either oleate or palmitate showed a statistically significant reduction in insulin-stimulated glucose uptake. At the same time, oleate increased basal glucose uptake in a dose-dependent manner to a level similar to that in control cells stimulated with insulin.

The C2C12 model is a well-established model for studying insulin-signaling events; however, it has a weak response to insulin stimulation. The anabolic responses to insulin stimulation such as the stimulation of the glucose uptake have clearly demonstrated that insulin stimulates glucose uptake only 50% above basal state [17]. Earlier, Kotliar et al. had suggested that an acute insulin stimulation of glucose transport is not solely dependent on the presence of insulin receptor and Glut-4 protein, indicating the presence of some additional proteins [18]. FFA treatment has been well documented to have effects on the expression level of other proteins such as peroxisome proliferator-activated receptor alpha (PPAR α), glucokinase, Glut-1, and Glut-2 glucose transporters [19] which is not the focus of our current study.

Downstream of IRS-1, our data have shown a slight non-significant reduction of 26% in the phosphorylation of Serine⁴⁷³ PKB and of 28% in Threonine³⁰⁸ residues in cells treated with oleate. This would indicate that a partial activation of PI3K may be still enough for the stimulation of PKB, indicating that other mechanisms might be involved in the oleate-induced insulin resistance in muscle cells instead. However, palmitate clearly showed a reduction in the phosphorylation of PKB (46% and 50%, respectively) for both Serine⁴⁷³ and Threonine³⁰⁸ in comparison with the controls. Previous studies show that palmitate impairs PKB activation through the upregulation of PP2A [10]. PKB activation by insulin was found to be normal in lysates obtained from muscle biopsies of normal subjects after intralipid infusion [20]. Its activation by physiologic insulin levels in isolated diabetic muscle was also normal, but PKB activity was reduced at higher insulin concentration [21]. Cazzolli et al. previously showed that the inhibitory effect of PP2A occurs as a result of ceramide formation in the case of palmitate-treated cells [22].

Other mechanisms might be involved in the development of insulin resistance upon treatment with FFAs as we tested the activation of PKC in our model. It was postulated that FFAs activate classical PKC isozymes in pretreated muscle cells that lead to insulin resistance development [10,23,24]. To test this hypothesis in our model, we applied the proteomic approach to study the key protein components involved in the C2C12 insulin resistance model, following FFA chronic treatment. We used the comparative proteomic analysis to screen for different kinases [75], phosphatases [27] and phosphosite proteins [31]. The phosphosite screen clearly showed a significant increase in the phosphorylation of PKC α (122%), PKC β (631%), PKC δ (351%), and PKC ϵ (698%) isoforms in cells treated with oleate but only alpha (80%) and delta (51%) in cells pretreated with palmitate.

Consistent with these findings, using the kinase screen clearly showed an upregulation of the inhibitor NF κ B kinase alpha/beta (IKK α/β) in FFA-treated cells, indicating the activation of the downstream target; the NF κ B pathway as a probable effector for PKC activation. The two FFAs (oleate and palmitate) activated the IKK α/β , the stress kinases S6 kinase p70 (p70SK) (742%) and (318%), stress-activated protein kinase (SAPK) (128%) and (665%), c-jun N-terminal kinase (JNK) (128%) and (665%), and p38 MAP kinase (p38MAPK) (79%) and (1207%). Additional supportive evidence is the amelioration of the glucose uptake in cells treated with both oleate and the Bis-I, but not in cells treated with palmitate. PKC activation has been implicated in several studies of insulin resistance and diabetes and even in the absence of lipid oversupply [11,25,26]. The inhibitory effects of PKC on insulin signaling may at least in part be explained by the more recently identified role of the serine/threonine phosphorylation of IRS-1, which has been implicated in the inhibition of its tyrosine phosphorylation [26,27].

In agreement with the previous notion, we demonstrated specifically that FFA treatment was able to increase the Serine³⁰⁷ phosphorylation for IRS-1 as a mechanism for FFA-induced insulin resistance in skeletal muscle cells [28,29]. Mussig et al. have demonstrated that SH-2 domain-containing tyrosine phosphatase (Shp2) is required for PKC-dependent phosphorylation of Serine³⁰⁷ in IRS-1 [30].

Serine³⁰⁷ in IRS-1 occurs in response to TNF- α as a paralleled surrogate marker for IKK activation [31]. Consequently, IRS-1 Serine³⁰⁷ is inducible leading to the inhibition of IRS-1 function by either IKK or c-jun N-terminal kinase (JNK) [32–34]. Interestingly, we also have seen how Taurine (10 mM) as an antioxidant was capable of reversing glucose uptake dysfunction and restoring insulin sensitivity in oleate-treated cells. The current finding might carry a substantial interest as a potential therapeutic target for muscle insulin resistance and the treatment of type-2 diabetes [32–34].

Conclusion

There is a strong correlation between insulin resistance and the increased lipids in the muscle tissue. Different species of fatty acids have a different pattern of protein expression and phosphorylation in the C2C12 skeletal muscle cells. FFAs induce muscle insulin resistance via a number of pathways such as PKC, NF κ B, and the stress kinases activation and implicate their effect in desensitizing the insulin-signaling cascade.

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Fig. 1.

(A,B) Effect of FFAs on glucose uptake at 1 mM. In the presence or absence of 1 mM oleate or palmitate, myotubes were incubated with or without 100 nM insulin for 30 min. In fresh SF-DMEM, in the presence of deoxyglucose [H^3] labeled (1 $\mu Ci/\mu l$) for 10 min. Results shown are combined means of three independent experiments ($n = 3$), each carried out in duplicates. Two-tailed t -test was conducted from three independent experiments comparing FFA-treated myotubes with their controls, graphs represent means $\pm$ SE. *Statistically significant from basal control ($p < 0.05$). #Statistically significant from insulin-stimulated control ($p < 0.05$). (C) Effect of oleate and palmitate treatment and the phosphorylation status of PKB/Akt. C2C12-differentiated myotubes had been pretreated with or without FFA(s) for 16 h, and with or without insulin stimulation for 10 min. The proteins were immunoblotted with anti-phospho-Serine⁴⁷³ (A), anti-phospho-Threonine³⁰⁸ (B), or anti-Akt antibody for the total (C). Two-tailed t -test was conducted to compare FFA-treated myotubes versus appropriate basal or insulin stimulated, significance difference from STD ($n = 3$ experiments/group) ($p < 0.05$).

The phosphosite and kinases screen using the proteomic approach in myotubes treated with either oleate or palmitate. Lipid-pretreated myotubes with oleate or palmitate (1 mM) and cells with no treatment were lysed. The homogenate, a pool of three independent experiments ($n = 3$), was submitted to Kinexus Bioinformatics Corporation (Vancouver, Canada) for Kinetools TM KPKS-1.0 and Kinetools KPSS-1.0. All the screens performed have used multi-immunoblotting with prevalidated antibodies against both kinases and phosphoproteins, including the PKC isoforms. The relative quantity of a particular band in a lane is expressed as a percentage of the total intensity data in the lane. Any change greater than 25% may be considered significant (A,B). In (C), Bis-I, used at the concentration of (2 μ M), along with preincubation with either oleate or palmitate (1 mM) the glucose uptake was measured, *statistically significant compared to basal control ($p < 0.05$), #statistically significant compared to oleate/Bis-I treated cells ($p < 0.05$).

Fig. 3.

The effector functions of PKC activation on Serine³⁰⁷ phosphorylation status of IRS-1 and glucose transporters expression levels. In (A), after immunoprecipitation procedure against IRS-1 the cell lysates were subjected to chemiluminescent immunoblotting for Serine³⁰⁷ IRS-1 and the mass IRS-1. Two-tailed *t*-test was conducted to compare FFA-treated myotubes versus appropriate basal or insulin stimulated, significance difference from STD compared to basal control ($n = 3$ experiments/group). ($p < 0.05$). (B) Taurine (10 mM), the antioxidant chemical inhibitor, was used to measure the glucose uptake, in cells preincubated with oleate. Taurine was able to restore the insulin effect on glucose uptake. *t*-Test was conducted to compare FFA-treated cells versus their controls, significance difference from STD ($p < 0.05$).