Fatty Acid-induced Insulin Resistance in L6 Myotubes Is Prevented by Inhibition of Activation and Nuclear Localization of Nuclear Factor κB*

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Recent studies have implicated inhibitor of κB kinase (IKK) in mediating fatty acid (FA)-induced insulin resistance. How IKK causes these effects is unknown. The present study addressed the role of nuclear factor κB (NFκB), the distal target of IKK activity, in FA-induced insulin resistance in L6 myotubes, an in vitro skeletal muscle model. A 6-h exposure of myotubes to the saturated FA palmitate reduced insulin-stimulated glucose uptake by ~30%, phosphatidylinositol-3 kinase and protein kinase B phosphorylation by ~40%, and stimulated inhibitor of κBα degradation and the nuclear translocation of NFκB. On the other hand, the Ω-3 polyunsaturated FA linoleate neither induced insulin resistance nor promoted nuclear localization of NFκB. Supporting the hypothesis that IKK acts through NFκB to cause insulin resistance, the IKK inhibitors acetylsalicylate and parthenolide prevented FA-induced reductions in insulin-stimulated glucose uptake and NFκB nuclear translocation. Most importantly, NFκB SN50, a cell-permeable peptide that inhibits NFκB nuclear translocation downstream of IKK, was sufficient to prevent palmitate-induced reductions in insulin-stimulated glucose uptake. Acetylsalicylate, but not NFκB SN50, prevented FA effects on phosphatidylinositol-3 kinase activity and protein kinase B phosphorylation. We conclude that FAs induce insulin resistance and activates NFκB in L6 cells. Furthermore, inhibition of NFκB activation, indirectly by preventing IKK activation or directly by inhibiting NFκB nuclear translocation, prevents the detrimental effects of palmitate on the metabolic actions of insulin in L6 myotubes.

Insulin resistance is a hallmark of obesity/type 2 diabetes. Although the pathogenesis of insulin resistance is poorly understood, dyslipidemia has been proposed as a candidate mechanism. Supporting this hypothesis are observations that plasma and tissue lipid levels are inversely correlated with insulin sensitivity (1, 2), that reduced availability of lipids improves insulin sensitivity (3–6), that a short-term lipid infusion that induces insulin resistance (6–10) demonstrated that a short-term lipid infusion that induces insulin resistance causes insulin resistance also reduces skeletal muscle insulin resistance (11) and type 2 diabetes (23–27). Indeed, a recent study in humans (11) demonstrated that a short-term lipid infusion that induces insulin resistance in L6 myotubes.


can inhibit insulin signaling (6, 12–15). More recent in vivo and in vitro studies have implicated inhibitor of κB kinase (IKK)1 in mediating the detrimental effects of lipids on insulin action (11, 16–18). Thus, inhibition of IKK activity by salicylates prevents the development of skeletal muscle insulin resistance caused by short-term lipid infusions in rats and mice (17) and improves insulin sensitivity in type 2 diabetes (16), and IKK heterozygote knockout mice are resistant to the development of insulin resistance induced by a high fat diet or a lipid infusion (18). However, the mechanism(s) by which increased IKK activity mediates lipid-induced insulin resistance are poorly understood.

IKK is a serine kinase identified as a proximal element of the pro-inflammatory IKK/κB/NFκB pathway (19, 20). Activated IKK phosphorylates inhibitor of κB (IκB), a cytoplasmic protein that inhibits nuclear translocation of nuclear factor κB (NFκB), a family of transcription factors that function as homo- or heterodimers in the regulation of the expression of pro-inflammatory, immunomodulatory, and anti-apoptotic genes (19, 20). Phosphorylated IκB is ubiquitinated and degraded in the proteasome, thus releasing NFκB for translocation to the nucleus and activation of gene expression. It has been proposed that elevated NFκB activity may play a role in the pathogenesis of a number of diseases, including atherosclerosis, and chronic inflammatory conditions such as inflammatory bowel disease and rheumatoid arthritis (21, 22). It is interesting that recent studies have also postulated a role for long-term low grade inflammation in the pathogenesis of the metabolic or insulin resistance syndrome, a cluster of diseases that include obesity and type 2 diabetes (23–27). Indeed, a recent study in humans (11) demonstrated that a short-term lipid infusion that induces skeletal muscle insulin resistance also reduces skeletal muscle IκB levels, suggesting that NFκB is activated in insulin-resistant states.

The studies discussed above support the hypothesis that lipid-induced insulin resistance is associated with, and may require, the activation of NFκB. However, neither the effects of lipids on NFκB activation nor the role of NFκB activation in fatty acid-induced insulin resistance has been determined. The current study addressed each of these issues in an in vitro skeletal muscle model, the L6 myotube. The data demonstrate a critical role for the IKK/IκB/NFκB pathway in lipid-induced insulin resistance in L6 myotubes. Thus, fatty acids induce insulin resistance and stimulate nuclear translocation of NFκB in a dose- and time-dependent manner consistent with a mech-

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§§ The abbreviations used are: IKK, inhibitor of κB kinase; IκB, inhibitor of κB; NFκB, nuclear factor κB; αMEM, α-minimal essential medium; P32, phosphatidylinositol-3; PKB, protein kinase B; FA, fatty acid; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; 2DG, 2-deoxyglucose; ASA, acetylsalicylate; PC, positive control.
anistic role for NiFbB in lipid-induced insulin resistance. Furthermore, inhibition of nuclear translocation of NiFbB by three independent methods blocks the effects of palmitate on insulin action. One of these inhibitors blocks NiFbB nuclear translocation downstream of IKK activation and IxB degradation, demonstrating that NiFbB activation is an important mechanism of lipid-induced insulin resistance in L6 myotubes.

EXPERIMENTAL PROCEDURES

L6 Cell Culture—A line of insulin-sensitive L6 skeletal muscle cells (28, 29) was used in the present study (a kind gift from Dr. Amira Klip, Hospital for Sick Children, Toronto, ON, Canada). Stock myotubes were prepared and maintained in monolayer culture in a MEM containing 10% fetal bovine serum (v/v), 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO2 at 37 °C until they reached ~40–50% confluence. To obtain fully differentiated myotubes, stock myotubes were reseeded at a density of 4000 cells/cm2 into 6-well plates, in 10% fetal bovine serum-αMEM for 24 h. The medium was then replaced with 2% fetal bovine serum-αMEM and antibiotics. The cells were maintained for ~7–8 days, with a medium change every 24–48 h, before use in experiments.

Experimental Design—L6 myotubes underwent a short-term (0.2–6.0 h) exposure to BSA-conjugated FAs (0.025–0.4 mM) in the absence or presence of inhibitors of the IKK/IxB/NFκB pathway. Where used, IxBα and NFκB inhibitors were added 1 h before FAs. Control cells were exposed to BSA alone. Thereafter, the effects of these exposures on [3H]2-deoxyglucose uptake in the absence or presence of 100 nM insulin, PI3-kinase activity, and PKB phosphorylation in the presence of 100 nM insulin, IκBα levels, and nuclear localization and DNA binding of NFκB were determined.

Fatty Acid Solution Preparations—Stock FA solutions were prepared by conjugating FA with FA-free BSA (Sigma, St. Louis, MO). In brief, sufficient FAs were dissolved in preheated 0.1 M NaOH and dialyzed 1:10 in prewarmed (45–50 °C) αMEM containing 12% (w/v) BSA, to give a final FA concentration of 2.0 mM. This gave a final molar ratio of free FA/BSA of 1.5:1.0, near that observed in human serum (30, 31), and would be expected to give an unbound free FA concentration of ~8 mM (32). Stock FA solutions were filter-sterilized and dialyzed with cell culture media for use in experiments. Control media contained 0.1% NaOH and BSA but no lipid. Where required, the pH of preparations was adjusted to 7.4.

[3H]2-Deoxyglucose Uptake—[2-14C]Deoxyglucose uptake was determined as described previously (33). In brief, after experimental treatments, the myotubes were incubated for 20 min at 37 °C in the absence or presence of 100 nM insulin, rinsed 2× in HEPES-buffered solution (20 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO4, and 1 mM dithiothreitol) and then incubated for 5 min in a HEPES-buffered solution containing 10 μM [3H]2-deoxyglucose (0.5 μCi/ml [3H]-2-deoxyglucose). Glucose uptake was stopped by washing three times with 2.0% NaCl, 0.9% NaCl, and cells were collected in 1.25 ml of 0.05 M NaOH. Cell-associated radioactivity was determined by scintillation counting. Protein concentration was determined by the Bradford method using a kit from Bio-Rad Laboratories (Hercules, CA), and the results were expressed as picomoles of 2-deoxyglucose transported per minute per milligram of protein.

Nuclear Protein Extracts, Electrophoretic Mobility Shift Assays (EMSAs), and Measurement of IxBα and Nuclear NFκB—Nuclear protein extracts were prepared for the analysis of DNA binding of NFκB and nuclear levels of NFκB. In brief, after experimental treatment, cells were pelleted and incubated on ice in a buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.0 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride for 15 min followed by addition of Igepal to a final concentration of 1% (v/v). Cells were vortexed for 10 s, and nuclei were collected by centrifugation (12,000 × g, 30 s). The supernatant containing cytoplasmic proteins was discarded. The pelleted nuclei were washed once with the buffer described above and then resuspended in a buffer containing 20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol, and a protease inhibitor mixture consisting of 0.5 ng/ml pepstatin A and 5 ng/ml each of leupeptin, antipain, soybean trypsin inhibitor, and aprotinin. The nuclei were mixed vigorously on a shaking platform (4 °C, 15 min), centrifuged (12,000 × g, 5 min), and the supernatant containing nuclear protein was snap-frozen on dry ice. Protein concentrations were determined by the Bradford method as described above.

For EMSAs, radiolabeled DNA probes were generated by annealing a template containing the κB-palindromic binding sequence (5‘-CACCAGCGAGGGGATCTCCCTTCCTCC-3’) to a complementary 10-base primer (5‘-AAAGGAGGGG-3’) (34). The overhang was filled in with the Klenow fragment of DNA polymerase I in the presence of [α-32P]dCTP, and 5 mM dATP, dGTP, and dTTP. EMSAs were performed as described by Ballard et al. (34). In brief, 32P-labeled κB probes were incubated with 5 μg of nuclear extract for 15 min at RT in the presence of 1 μg of poly(dI-dC), 5 μg of BSA, 5 μM dithiothreitol, 100 mM KCl, 20 mM HEPES, and 1 mM EDTA. Protein-DNA complexes were resolved by electrophoresis on a 5% polyacrylamide gel under non-denaturing conditions in a Tris-borate/EDTA buffer. After drying the gel, the DNA-protein complexes were visualized by autoradiography. For supershift assays, nuclear extracts were pre-incubated with α-p65 or α-p50 antibodies (Cell Signaling, Beverly, MA) for 30 min before use in EMSAs. Nuclear levels of p65 (using nuclear protein extracts) and IxBα (Cell Signaling) levels (using whole cell extracts) were determined by a standard immunoblotting technique.

PI3-kinase Activity and Akt Immunoblots—For experiments requiring measurement of PI3-kinase activity and PKB phosphorylation, myotubes were exposed to 100 nM insulin for 10 min at 37 °C. Thereafter, media were aspirated, an excess volume of ice-cold phosphate-buffered saline was added and aspirated, and the cells were flash-frozen in liquid nitrogen. Protein extracts were prepared in 200 μl of a buffer containing, as final concentrations, 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. PI3-kinase activity was assayed according to a standard protocol. In brief, immunoprecipitation of active PI3-kinase was achieved by incubating 250 μg of cell protein with 1.5 μg of α-IRS-1 antibody (Upstate Biotechnology) for 2 h followed by addition of protein A-Sepharose for 2.0 h (Amersham Biosciences). The immune complexes were incubated for 10 min at 22 °C with phosphatidylinositol (10 μg; Avanti Polar Lipids, Inc., Alabaster, AL) in the presence of 50 μM [γ-32P]ATP (5 μCi; PerkinElmer Life and Analytical Sciences). The containing phosphatidylinositol-3-phosphate was separated by thin layer chromatography and was quantitated by scraping the phosphatidylinositol-3-phosphate spot from the TLC plate, followed by scintillation counting. Total PKB and phosphorylated PKB (serine-473) were measured with commercially available antibodies (Cell Signaling) using a standard immunoblot protocol.

Materials—α-MEM and penicillin/streptomycin were obtained from Invitrogen. Bovine fetal bovine serum was from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Trypsin/EDTA was from Mediatech (Herndon, VA). The FAs palmitic acid, linoleic acid, and linolenic acid, and BSA, insulin, and aprotinin were obtained from Sigma Chemical. Palmitic acid from NU-CHEK (Elysian, MN) was used for some NFκB experiments to rule out the possibility of nonspecific effects of the fatty acid preparation. Reagents for PAGE were from Bio-Rad. [α-32P]ATP and [3H]2-deoxyglucose were obtained from PerkinElmer Life and Analytical Sciences. NFκB SN50 and mutant NFκB SN50 cell-permeable peptides from ENZO Life Sciences (Cutchogue, NY; and Cambridge, MA). NFκB SN50, containing the nuclear localization signal of the p50 subunit of NFκB and the hydrophobic region of actrosyte fibroblast growth factor, competitively inhibits NFκB nuclear translocation (35). Parthenolide, a sesquiterpene and specific inhibitor of IκB, was obtained from Sigma (36).

Statistical Methods—All results are expressed as means ± S.E. Statistical significance was determined by unpaired or paired t tests where appropriate, using the statistics module of Microsoft Excel (Microsoft Corp., Seattle, WA). Statistical significance was assumed at p < 0.05.

RESULTS

Palmitate and Linoleate, but Not the Ω-3 Polysaturated FA Linolenate, Induce Insulin Resistance and Stimulate the Nuclear Translocation of NFκB in L6 Myotubes—We first determined the effects of fatty acids on insulin sensitivity and NFκB nuclear translocation in L6 myotubes. Three long chain fatty acids were chosen for study: palmitate, a C16:0 saturated fatty acid; linoleate, a C18:2 Ω-6 polyunsaturated fatty acid; and linolenate, a C18:3 Ω-3 polyunsaturated fatty acid. A 6-h incubation of L6 myotubes with 0.4 mM palmitate or linoleate conjugated with 2.4% BSA (1.5:1.0 molar ratio) decreased absolute insulin-stimulated 2DG uptake by ~30% compared with myotubes incubated with 2.4% BSA alone (Fig. 1A), and decreased net insulin-stimulated 2DG uptake by ~80 and ~60%, respectively (Fig. 1B). Unlike palmitate and linoleate, lino-
labeled had no effects on insulin-stimulated 2DG uptake compared with control cells. Similar to their effects on insulin sensitivity, palmitate and linoleate, but not linolenate, stimulated DNA binding of NFκB (Fig. 1C). As expected, palmitate also decreased cytosolic IkBα levels and increased nuclear levels of p65 (Fig. 2, A and B). Furthermore, using supershift assays p65 and p50 were determined to be the NFκB family members comprising the heterodimer binding to DNA (Fig. 2C). Taken together, these data demonstrate that fatty-acid induced insulin resistance and NFκB activation are coincident in L6 myotubes. Furthermore, fatty acids that do not induce insulin resistance do not activate NFκB.

Time and Concentration Dependence of Palmitate-induced Insulin Resistance and NFκB Nuclear Translocation in L6 Myotubes—We next determined the time course of fatty acid-induced insulin resistance in L6 myotubes (Fig. 3). The motivation for these experiments was 2-fold. First, previous in vivo (6–11) studies demonstrate that a 5-h exposure of skeletal muscle to lipid is required for the development of skeletal muscle insulin resistance, but it is unknown whether this is the case in L6 myotubes. Second, we hypothesized that nuclear translocation of NFκB would precede the development of fatty acid-induced insulin resistance. Insulin resistance was present after a 6-h incubation of L6 myotubes with palmitate but was not present at the earlier time-points measured, although a trend toward decreased insulin-stimulated 2DG uptake was evident from 2 h onward (Fig. 3, A and B). It is noteworthy that palmitate-stimulated nuclear translocation of NFκB was evident as early as 1 h after addition of the fatty acid and increased nuclear translocation was present at 4 and 6 h. (Fig. 3C). These data demonstrate that activation of the IKK/IκB/ NFκB pathway occurs before the development of fatty acid-induced insulin resistance in L6 cells.

We next determined the concentration-dependence of fatty acid-induced insulin resistance and NFκB translocation, reasoning that similar concentrations of fatty acids would be required to induce insulin resistance and nuclear translocation of NFκB. L6 myotubes were incubated for 6 h in the presence of increasing concentrations of palmitate before the measurement of glucose uptake and NFκB translocation. Palmitate concentrations of 0.1 mM or below did not significantly decrease absolute or net insulin-stimulated 2DG uptake or activate NFκB compared with controls (Fig. 4, A–C). However, increasing the palmitate concentration to 0.2 or 0.4 mM reduced absolute 2DG uptake in the presence of insulin,
determined as described in the legend to Fig. 1 and under “Experimental Procedures.” A, 2DG uptake in the absence (−) or presence (+) of 100 nM insulin. B, net insulin-stimulated 2DG uptake (+Insulin 2DG uptake less −Insulin 2DG uptake in BSA controls). C, a representative EMSA is shown depicting the palmitate concentration dependence of nuclear localization of NFκB in response to palmitate treatment. Data represent the mean of three independent experiments performed in triplicate (2DG uptake) or in duplicate (NFκB EMSA). *, significant difference (p < 0.05) between the insulin-treated groups and the corresponding non-insulin-treated control group. #, significant difference between the indicated treatment and the corresponding BSA-treated control.

Fig. 3. Time dependence of palmitate-induced decrements in insulin-stimulated 2-deoxyglucose glucose uptake and increases in NFκB nuclear translocation. L6 myotubes were exposed to 0.4 mM BSA-complexed palmitate or BSA alone for 0.2–6.0 h, and then [3H]2DG uptake and NFκB nuclear translocation were determined as described in the legend to Fig. 1 and under “Experimental Procedures.” A, 2DG uptake in the absence (−) or presence (+) of 100 nM insulin. B, net insulin-stimulated 2DG uptake (+Insulin 2DG uptake less −Insulin 2DG uptake in BSA controls). C, a representative EMSA is shown depicting the time-course of nuclear localization of NFκB in response to palmitate treatment. Data represent the mean of three independent experiments performed in triplicate (2DG uptake) or in duplicate (NFκB EMSA). *, significant difference (p < 0.05) between the insulin-treated groups and the corresponding non-insulin-treated control group. #, significant difference between the indicated treatment and the corresponding BSA-treated control.

The IKK Inhibitors Salicylate and Parthenolide Prevent Palmitate-induced Insulin Resistance and Nuclear Translocation of NFκB—Administration of salicylate prevents the development of lipid-induced insulin resistance in rodents (17, 18) and improves insulin sensitivity in type 2 diabetes (16). One mechanism of action is postulated to be inhibition of lipid-induced increases in IKK activity (11, 16–18). Thus, we reasoned that if fatty acid-induced insulin resistance and activation of NFκB in L6 cells requires increased IKK activity, salicylates should prevent these events. To test this hypothesis, we determined the effects of salicylates and a second IKK inhibitor, parthenolide, on palmitate-induced nuclear translocation of NFκB. Exposure of L6 myotubes to 5 mM acetylsalicylate (ASA) prevented palmitate-mediated decreases in insulin-stimulated 2DG uptake. Thus, absolute and net insulin-stimulated 2DG uptake in the presence of palmitate were significantly reduced compared with 2DG uptake in the presence of BSA alone, BSA/ASA, or palmitate/ASA (Fig. 5, A and B). Insulin-stimulated 2DG uptake was similar in the latter three groups. Similar to ASA, parthenolide prevented palmitate-induced decrements in absolute and net insulin-stimulated 2DG uptake (Fig. 6, A and B). It is noteworthy that both ASA (Fig. 5C) and parthenolide (Fig. 6C) completely blocked palmitate-induced nuclear translocation of NFκB. Taken together, these data lend further support to a role for the IKK/IκB/NFκB pathway in mediating lipid-induced insulin resistance and implicating NFκB activation as a required step in this process.

NFκB SN50, a Peptide Inhibitor of Nuclear Translocation of NFκB, Is Sufficient to Prevent Palmitate-induced Insulin Resistance in L6 Myotubes—The data in the previous section demonstrate a requirement for activation of the IKK/IκB/NFκB
pathway in mediating palmitate-induced insulin resistance in L6 myotubes. However, the role of NFκB nuclear translocation, independent of IKK activation and IκB degradation, remains unclear. Thus, we hypothesized that a specific inhibition of nuclear translocation of NFκB would be sufficient to prevent the detrimental effects of palmitate on insulin action. To test this hypothesis, a cell-permeable peptide inhibitor of NFκB nuclear translocation, NFκB SN50, was used. A mutant peptide, NFκB SN50Mut, containing a Lys-Arg to Asn-Gly amino acid substitution in the nuclear localization sequence, was used as control. Co-incubation of L6 cells with NFκB SN50 and palmitate prevented the development of insulin resistance and nuclear translocation of NFκB (Fig. 7). Thus, absolute and net insulin-stimulated 2DG uptake in the presence of palmitate were significantly reduced compared with 2DG uptake in the presence of palmitate + NFκB SN50 or BSA alone (Fig. 7, A and B). The effects of NFκB SN50 were specific, because 2DG uptake in the presence of NFκB SN50Mut was similar to that in the presence of palmitate alone. It is noteworthy that the protective effects of NFκB SN50 were associated with a complete blockade of palmitate-induced nuclear translocation of NFκB, whereas palmitate-induced NFκB nuclear translocation did occur in the presence of NFκB SN50Mut (Fig. 7C) Taken together, these data demonstrate that fatty acid induced insulin resistance in L6 myotubes requires nuclear translocation of NFκB.

Palmitate Decreases Insulin-stimulated PI3-kinase Activity and PKB Phosphorylation; ASA, but Not NFκB SN50, Prevents the Effects of Palmitate—To address the association/dissociation between activation of IKK/IκB/NFκB by fatty acids and changes in upstream/proximal insulin signaling events, we next addressed the effects of palmitate in the absence or presence of IKK/IκB/NFκB pathway inhibitors on insulin-stimulated PI3-kinase activity and PKB phosphorylation (Fig. 8, A and B). A 6-h incubation with 0.4 mM palmitate/2.5% BSA was sufficient to decrease insulin-stimulated PI3-kinase activity and PKB phosphorylation by ~40%. As expected, acetylsalicylate prevented the palmitate-induced decreases in maximal stimulation of PI3-kinase activity and PKB phosphorylation by...
insulin. However, the nuclear localization inhibitor, NFκB-SN50, was unable to prevent the detrimental effects of palmitate on insulin signaling, demonstrating dissociation of fatty acid effects on the insulin signaling pathway and NFκB nuclear translocation in L6 cells.

**DISCUSSION**

The major goal of the current study was to determine the role of NFκB in mediating the detrimental effects of fatty acids on insulin action in an *in vitro* model of rat skeletal muscle, the L6 myotube. The motivation for these studies were recent observations (11, 16–18) implicating elevated IKK activity as a mediator of lipid-induced insulin resistance. However, the target(s) of IKK activity that result in the development of insulin resistance remains unclear. Several novel observations arise from our studies. First, we demonstrate that NFκB activation and nuclear translocation is required for palmitate-induced insulin resistance in L6 myotubes. Second, we demonstrate that three independent inhibitors of activity of the IKK/IκB/NFκB pathway prevent the detrimental effects of palmitate on insulin action. Third, we demonstrate that inhibition of IKK activation (acytalsalicylate), but not NFκB activation (NFκB SN50), prevents palmitate-induced decreases in insulin signaling. Finally, we demonstrate that 1-3 fatty acids neither activate the IKK/IκB/NFκB pathway nor induce insulin resistance in L6 myotubes.

Based on the demonstrations that IκB is a substrate of IKK activity, that IκB levels are decreased by a lipid infusion that induces skeletal muscle insulin resistance in humans (11), and on preliminary reports that overexpression of the IκB super-repressor in liver or fat protects against the detrimental effects of obesity on insulin action (37–39), a reasonable hypothesis is that activation of NFκB is involved in the development of lipid-induced insulin resistance. The current study offers both direct and indirect evidence in support of this hypothesis. Most importantly, we demonstrate that an inhibitor of nuclear translocation of NFκB (NFκB SN50) that acts downstream of IKK and IκB prevents the development of palmitate-induced insulin resistance. Furthermore, we demonstrate that the activation of the IKK/IκB/NFκB pathway occurs substantially before the development of insulin resistance. Thus, IκB levels are reduced by ~90% and nuclear localization of NFκB is evident within 1 h of palmitate treatment, whereas insulin resistance is not evident for between 4 and 6 h after exposure to palmitate. Taken together, these data implicate a role for other cellular mechanisms, in addition to IKK activation, in fatty acid induced insulin resistance in L6 myotubes.

An implication of the current study is that a product of a NFκB-regulated gene is required for the induction of insulin resistance by FA in L6 cells. We attempted to address this possibility by inhibiting gene expression in palmitate-treated cells using cycloheximide (inhibits mRNA translation) and actinomycin D (inhibits mRNA synthesis). It is unfortunate that both reagents substantially increased basal glucose uptake by an unknown mechanism (data not shown), with a consequent loss of insulin stimulation of glucose uptake. Thus, it remains to be determined whether expression of NFκB-regulated genes play a role in the development of insulin resistance in the L6 model.

A number of previous studies have demonstrated that the IKK inhibitor acetylsalicylate prevents the inhibitory effects of cytokines and lipids (17, 18) on insulin activation of the insulin signaling pathway. In agreement with these studies, we demonstrate that acetylsalicylate prevents palmitate-induced decreases in maximal insulin stimulation of PI3-kinase activity and PKB phosphorylation. Furthermore, we demonstrate that prevention of NFκB nuclear localization, independent of IKK activity, has no effect on palmitate-induced decreases in insulin signaling. Taken together, these data suggest that activation of IKK is necessary for mediating the effects of fatty acids on insulin signaling but that NFκB activation is not required. This conclusion is supported by the recent observation demonstrating serine phosphorylation of IRS-1 by IKK in human embryonic kidney 293 cells (40). A further implication of our data is that the effects of fatty acids on insulin action seem to extend beyond fatty acid effects on insulin signaling. This conclusion is based on our observation of dissociation of the effects of NFκB SN50 on palmitate-induced decreases in insulin-stimulated glucose uptake and the effects of NFκB SN50 on palmitate-induced decreases in insulin signaling. Thus, NFκB SN50 prevents the detrimental effects of palmitate on insulin-stimulated glucose uptake but does not alter the effects of palmitate on insulin-stimulated PI3-kinase activity or PKB phosphorylation. These data imply that the contribution of fatty acid-induced activation of NFκB-dependent pathways to the development of insulin resistance in L6 cells is independent of fatty acid effects on the proximal insulin signaling pathway. However, although this hypothesis is intriguing, the relevance of
such mechanisms in in vivo models of lipid-induced insulin resistance remains to be established.

A comment on the model used in this study and the applicability of our observations to in vivo models and human obesity/type 2 diabetes is warranted. It is commonly recognized that investigation of the metabolic actions of insulin in vitro models of skeletal muscle is challenged by the reduced responsiveness of these models to the metabolic effects of insulin. Nonetheless, these models do serve a useful purpose in that they enable biochemical and molecular studies to be conducted that are substantially more difficult to conduct in vivo. The data presented in the current study are consistent with a series of in vivo and in vitro studies demonstrating a mechanistic link between activation of pro-inflammatory pathways and insulin resistance. However, the data also point to a need for in vivo studies to determine the role of NFκB activation per se, independent of IKK/IκB activation, in the pathogenesis of insulin resistance. In this regard, the recent preliminary reports that decreasing NFκB expression by expression of the IκB super-repressor protects against the effects of high fat feeding on insulin action and improves insulin action in the db/db mouse are intriguing (37–39).

It is noteworthy that both palmitate and linoleate, but not linolenate, activate NFκB and induce insulin resistance. Previous in vivo and in vitro studies support the hypothesis that palmitate and linoleate decrease insulin action (2, 14, 41). On the other hand, there is evidence (42–46), albeit mixed (45, 47, 48) to suggest that Ω-3 polyunsaturated fatty acids and their derivatives may have beneficial effects on insulin action. Data from our studies do not demonstrate that linolenate increases insulin sensitivity but clearly demonstrate that this Ω-3 polyunsaturated fatty acid is not detrimental to insulin action in L6 cells. Importantly, we can conclude that at least a partial basis of the inert nature of linolenate with respect to insulin action is an inability to activate the IKK/IκB/NFκB pathway.

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