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Lipid-Induced Insulin Resistance in Human Muscle Is Associated With Changes in Diacylglycerol, Protein Kinase C, and IκB-α

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The possibility that lipid-induced insulin resistance in human muscle is related to alterations in diacylglycerol (DAG)/protein kinase C (PKC) signaling was investigated in normal volunteers during euglycemic-hyperinsulinemic clamping in which plasma free fatty acid (FFA) levels were increased by a lipid/heparin infusion. In keeping with previous reports, rates of insulin-stimulated glucose disappearance (G_{Rd}) were normal after 2 h but were reduced by 43% (from 52.7 ± 8.2 to 30.0 ± 5.3 μmol · kg^{-1} · min^{-1}, $P < 0.05$) after 6 h of lipid infusion. No changes in PKC activity or DAG mass were seen in muscle biopsy samples after 2 h of lipid infusion; however, at ~6 h, PKC activity and DAG mass were increased approximately fourfold, as were the abundance of membrane-associated PKC-βII and -δ. A threefold increase in membrane-associated PKC-βII was also observed at ~2 h but was not statistically significant ($P = 0.058$). Ceramide mass was not changed at either time point. To evaluate whether the fatty acid–induced insulin activation of PKC was associated with a change in the IkB kinase (IKK)/nuclear factor (NF)-κB pathway, we determined the abundance in muscle of IκB-α, an inhibitor of NF-κB that is degraded after its phosphorylation by IKK. In parallel with the changes in DAG/PKC, no change in IκB-α mass was observed after 2 h of lipid infusion, but at ~6 h, IκB-α was diminished by 70%. In summary, the results indicated that the insulin resistance observed in human muscle when plasma FFA levels were elevated during euglycemic-hyperinsulinemic clamping was associated with increases in DAG mass and membrane-associated PKC-βII and -δ and a decrease in IκB-α. Whether acute FFA-induced insulin resistance in human skeletal muscle is caused by the activation of these specific PKC isoforms and the IKK-β/IκB/NFκB pathway remains to be established. *Diabetes* 51:2005–2011, 2002

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Received for publication 6 March 2002 and accepted in revised form 6 May 2002. Posted on the World Wide Web at http://www.diabetes.org/diabetes/rapidpubs.shtml on 7 June 2002.

DAG, diacylglycerol; FFA, free fatty acid; G_{Ra}, total body glucose appearance; G_{Rd}, total body glucose disappearance; IKK, IκB kinase; IMCL-TG, intramyocellular triglyceride; NF, neclear factor; NIH, National Institutes of Health; PI, phosphatidylinositol; PKC, protein kinase C.

Work by several laboratories over the past 10 years has provided evidence that elevated plasma free fatty acid (FFA) levels are responsible for much of the insulin resistance present in obese subjects. The evidence can be summarized as follows: *1*) most obese people have elevated plasma levels of FFA (1,2); *2*) acute elevations of plasma FFA produce insulin resistance dose-dependently in diabetic and nondiabetic individuals (3–6); and *3*) chronically elevated plasma FFA levels cause insulin resistance, as demonstrated by the finding that lowering elevated plasma FFA levels overnight (from ~600 to ~300 μmol/l) normalized insulin sensitivity in obese nondiabetic subjects and significantly improved it in obese diabetic patients (7). The mechanisms by which elevated levels of FFA produce insulin resistance are not well understood. However, the observation that acutely increasing plasma FFAs decreased insulin-stimulated glucose uptake, glycogen synthesis (8), and phosphatidylinositol (PI) 3-kinase activity in skeletal muscle (9) suggested that FFAs interfered with insulin signaling.

Interestingly, it takes 2–4 h for insulin resistance to develop after an acute elevation in plasma FFA and an equally long time for insulin resistance to disappear after plasma FFA levels return to normal (3). The long (2–4 h) delay suggested an indirect rather than direct effect of FFAs. In support of this notion, acute increases in plasma FFAs have been shown to cause increases in intramyocellular triglyceride (IMCL-TG) content in soleus muscle of healthy volunteers. The increase in IMCL-TG occurred several hours after the elevation of plasma FFA levels and coincided with the development of insulin resistance (10), suggesting that an insulin resistance–causing signal was generated during the synthesis (or the breakdown) of IMCL-TG. We hypothesize that diacylglycerol (DAG) may be such a signal because an increase in its synthesis would coincide with that of triglycerides (11) and because it is a well-known allosteric activator of protein kinase C (PKC) (12–14), an enzyme that has been linked to insulin resistance in muscle in a wide variety of rodent models (11,15,16), including rats infused with lipid (17) and massively obese humans (18,19). To evaluate this possibility, DAG mass and PKC activity and distribution were assayed

TABLE 1
Study subjects

	Insulin	Insulin + lipid	*P*
Sex	6 men	6 men	NS
Age (years)	30.3 ± 3.8	40.7 ± 3.4	NS
Height (cm)	178.3 ± 2.9	183.8 ± 3.4	NS
Weight (kg)	74.0 ± 3.5	85.6 ± 5.7	NS
Lean body mass (kg)	61.7 ± 1.4	66.0 ± 2.3	NS
BMI (kg/m^2)	23.1 ± 13	25.3 ± 1.4	NS

Data are means ± SE.

in skeletal muscle biopsies obtained from normal human volunteers in whom insulin resistance was produced by raising plasma FFA levels during a euglycemic-hyperinsulinemic clamp. Because PKC is known to activate NF-κB (20,21) and because NF-κB has recently been linked to fatty acid–induced impairment of insulin action in muscle in rodents (22,23), we have also examined the possibility that the development of insulin resistance in these individuals was related to alterations in the IKK/IκB/NFκB pathway. In addition, the accumulation of ceramide, another lipid metabolite linked to insulin resistance, was evaluated (24).

RESEARCH DESIGN AND METHODS

Subjects. Twelve healthy men participated in these studies. Each subject's age, weight, height, and body composition are shown in Table 1. The two groups differed modestly (and nonsignificantly) with respect to age and BMI; however, far greater differences altered neither the time of onset nor the magnitude of FFA-induced insulin resistance (G.B., unpublished observations). None of the participants had a family history of diabetes or other endocrine disorders or were taking medications. Their body weights were stable for at least 2 months, and their diets contained a minimum of 250 g/day of carbohydrate for at least 2 days before the studies. Informed written consent was obtained from all subjects after explanation of the nature, purpose, and potential risks of these studies. The study protocol was approved by the Institutional Review Board of Temple University Hospital.

Experimental design. All subjects were admitted to the Temple University Hospital General Clinical Research Center the day before the studies. At 6:00 P.M. they ingested a meal of 14 kcal/kg body wt consisting of 53% carbohydrate, 15% protein, and 32% fat. The studies began at 8:00 A.M. the following day, with the subjects reclining in bed. A short polyethylene catheter was inserted into an antecubital vein for infusion of isotopes. Another catheter was placed in a contralateral forearm vein for blood sampling. This arm was wrapped with a heating blanket (~70°C) to arterialize venous blood. The following studies were performed:

Study 1 was a 6-h euglycemic-hyperinsulinemic clamp during which plasma FFA levels decreased to very low levels.

Study 2 was a 6-h euglycemic-hyperinsulinemic clamp with simultaneous intravenous infusion of lipid plus heparin. Plasma FFA levels rose because lipolysis of the infused fat exceeded insulin-mediated antilipolysis.

Muscle biopsies were performed before, during (~2 h), and at the end (~6 h) of the clamps.

Materials. The PKC assay kit was purchased from Amersham Life Science (Arlington Heights, IL). PKC antibodies (polyclonal) were purchased from Santa Cruz technologies (Santa Cruz, CA). Unless otherwise specified, all other reagents were purchased from either Sigma Chemical (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

Euglycemic-hyperinsulinemic clamping with and without lipid/heparin. Regular human insulin (Humulin R; Elli Lilly, Indianapolis, IN) was infused intravenously at a rate of 7 pmol · kg^{-1} · min^{-1} for 6 h and plasma glucose concentrations were clamped at ~5 mmol by a feedback-controlled variable glucose infusion (studies 1 and 2). Glycerol (2.14 g/100 ml) was co-infused with insulin in study 1 to match the glycerol content of the Liposyn II infused in study 2. In study 2, euglycemic-hyperinsulinemic clamping was performed as described above. In addition, Liposyn II (Abbott Laboratories, North Chicago, IL), a 20% triglyceride emulsion (10% safflower and 10% soybean oil) containing 2.14 g/100 ml glycerol plus heparin (0.4 units · kg^{-1} · min^{-1}) was infused at a rate of 1.5 ml/min for 6 h. Serial measurements of rates of glucose turnover and substrate and hormone analyses were obtained before and during the clamps.

Muscle biopsies. Biopsies were obtained from the lateral aspect of the vastus lateralis muscle ~15 cm above the patella from all subjects as described (3). The excised muscle (~150 mg) was dropped immediately into isopentane kept at its freezing point (−160°C) by liquid nitrogen. The frozen muscle was stored at −80°C until it was aliquotted for measurement of PKC bioactivity and isoform distribution, DAG and ceramide mass, and IκB-α abundance.

Glucose turnover. Glucose turnover was determined with 3-[^{3}H]glucose, which was infused intravenously for 8 h, starting with a bolus of 40 μCi followed by a continuous infusion of 0.4 μCi/min. This produced steady-state tracer specific activities within 120 min. Glucose was isolated from blood for determination of 3-[^{3}H]glucose specific activity as described (25). Rates of total body glucose appearance (G_{Ra}) and disappearance (G_{Rd}) were calculated using Steele's equation for non–steady-state conditions (26). Rates of endogenous glucose production were obtained by subtracting rates of glucose infused to maintain euglycemia from G_{Ra}.

Body composition. Body composition was determined by bioelectrical impedance analysis (27).

Substrate and hormone analyses. Plasma glucose was measured with a glucose analyzer (YSI, Yellow Springs, OH). Insulin was determined by radioimmunoassay using an antiserum with minimal (0.2%) cross-reactivity with proinsulin (Linco, St. Charles, MO). Plasma FFA concentrations were determined with a kit from Wako Pure Chemical (Richmond, VA).

Western analysis of PKC and IκB-α. PKC western analysis was performed as described elsewhere (19) with some modifications. In brief, frozen muscle tissue was homogenized in 20 mmol/l Tris, pH 7.4, containing 10 mmol/l EDTA, 2 mmol/l EGTA, 100 mmol/l β-glycerophosphate, 0.05 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 0.1 mg/ml aprotinin. Samples were centrifuged in a Beckman air fuge at 160,000*g* for 20 min to separate the cytosolic fraction. The pellet was resuspended with the same homogenization buffer with 0.2% Triton added, sonicated (Heat systems sonicator, microtip), and left on ice for 1 h. The homogenates were then recentrifuged and the resultant supernatant labeled as the membrane (particulate) fraction. Samples were loaded at a protein concentration of 50 μg/lane onto an 8 and 12% SDS-polyacrylamide gel for PKC and IκB-α, respectively. PKC was determined in the cytosolic and membrane fractions, whereas IκB-α was determined in the cytosolic fraction only. Proteins were electrotransferred onto a polyvinylidene fluoride microporous membrane and isoform-specific polyclonal PKC or IκB-α antibodies (Santa Cruz, CA) at a dilution of 1:1,000 were added to the membranes. Membranes were subjected to enhanced chemiluminescence reagent (Western blot chemiluminescence reagent; Dupont). Multiple autoradiographs (to establish linearity) were quantitated using the National Institutes of Health (NIH) image analysis software (free distribution by NIH, Bethesda, MD). PKC-βI and -βII both ran as doublets when blotted with their respective antibodies. For purposes of comparison and because the two bands sometimes merged, both bands were included in the densitometric analysis.

PKC enzyme assay. PKC activity was determined as described elsewhere (18) using the PKC enzyme assay system (Amersham Life Science). Skeletal muscle was homogenized and the cytosolic and particulate fractions separated as described above. Samples were incubated at 37°C for 15 min. The phosphorylated peptide was separated using binding paper disks, and disks were counted for 10 min using Beckman LS 6500 (Beckman Instruments, Fullerton, CA).

DAG and ceramide content. The measurement of DAG content was determined as described (28). In brief, lipids were extracted from muscle biopsies using chloroform:methanol:PBS + 0.2% SDS (1:2:0.8). Diacylglycerol kinase and [γ-^{32}P]ATP (15 μCi/μmol cold ATP) were added to extracts, and the reaction was stopped using chloroform:methanol (2:1). Samples were run on thin-layer chromatography plates in chloroform:acetone:methanol:acetic acid:water (100:40:20:20:10). The DAG and ceramide bands were counted in a Beckman LS 6500 (Beckman Instruments).

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed using the SAS program (SAS Institute, Cary, NC). ANOVA with repeated measures was used to determine the differences in G_{Rd}, PKC activity and distribution, IκB-α abundance, and DAG and ceramide content across time points. A Student's *t* test and paired nonparametric test (Wilcoxon's signed-rank test) for each time point was then performed if the overall comparison was statistically significant. Because of our finding that PKC measurements in human muscle tend to be more variable than in rodents (S.I. and N.R., unpublished data), all measurements, including a few that were >2 SD from the group mean, were included in the statistical analysis.

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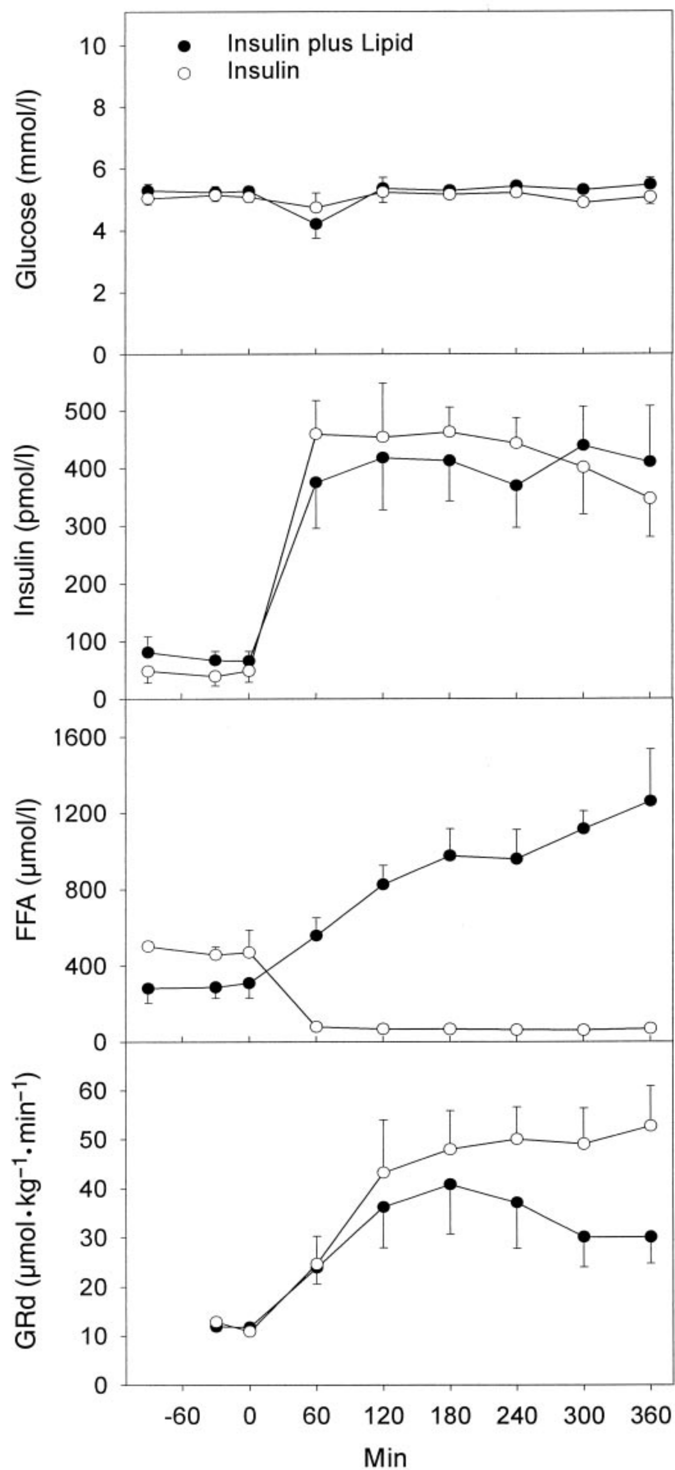

FIG. 1. Plasma glucose, insulin, and free fatty acid (FFA) levels and rates of glucose disappearance during euglycemic-hyperinsulinemic clamping with (●, $n = 6$) and without (○, $n = 6$) lipid/heparin infusion. Results are shown as the mean ± SE. Insulin, insulin infusion; insulin + L/H, insulin plus lipid/heparin infusion.

RESULTS

Glucose, insulin, FFA, and G_{Rd}. Basal glucose concentrations were 5.1 ± 0.1 and 5.3 ± 0.3 mmol/l in the insulin (study 1) and the insulin plus lipid (study 2) groups, respectively (Fig. 1). Mean clamp glucose concentrations were 5.3 ± 0.2 and 5.1 ± 0.2 mmol/l in the two groups. Basal insulin levels were 49 ± 20 and 66 ± 17 pmol/l, and mean insulin levels during the clamps were 428 ± 19 and 403 ± 11 pmol/l in the insulin and insulin plus lipid groups, respectively.

Plasma FFA concentrations decreased from 470 ± 117 μmol/l (at 0 min) to 66 ± 15 μmol/l (at 360 min, $P < 0.001$) in the insulin group. In the insulin plus lipid group, plasma FFA increased from 308 ± 77 μmol/l (at 0 min) to 1,259 ± 274 μmol/l (at 360 min, $P < 0.001$).

G_{Rd} rose from 10.9 ± 0.9 μmol · kg^{-1} · min^{-1} (at 0 min) to 52.7 ± 8.2 μmol · kg^{-1} · min^{-1} (at 360 min) in the insulin group and from 11.8 ± 0.9 to 30.0 ± 5.3 μmol · kg^{-1} · min^{-1} in the insulin plus lipid group. The difference between the two groups of 43% ($P < 0.05$) at 360 min was similar to that in previous reports (3,5,8). Likewise, in agreement with earlier studies (3–5,8), no significant difference was observed between the groups at ~2 h.

To investigate whether the lipid-induced insulin resistance was associated with alterations in DAG, ceramide, and PKC, we have assessed DAG and ceramide content, total PKC activity, and the distribution of specific PKC isoforms in muscle biopsies taken at 0, ~2, and ~6 h.

Total PKC activity. No change in total PKC activity was observed in either the insulin plus lipid or the insulin alone groups after ~2 h (Fig. 2*a* and *b*). However, after ~6 h, significant increases in PKC activity in the membrane (~4-fold) and cytosolic (~3-fold) fractions were observed in the insulin plus lipid group, suggesting that increases in total PKC protein and translocation to the cell membrane had occurred.

PKC distribution of specific isoforms. To determine which PKC isoforms were responsible for the increased PKC activity in the membrane and cytosolic fractions of the subjects infused with lipid, Western analysis was performed using isoform-specific antibodies. As shown in Figs. 2*c* and *d* and Fig. 3, a significant (~6-fold) increase in membrane PKC-δ protein compared with the value at 0 h was observed in subjects infused with insulin plus lipid at ~6 h, whereas no increase was observed in the group infused with insulin alone. As with PKC activity, no change in abundance in either fraction was observed at ~2 h.

A similar increase (~8-fold) of membrane PKC-βII was observed in the insulin plus lipid group at ~6 h (Fig. 2*e* and *f* and Fig. 3). However, in contrast to PKC-δ, PKC-βII content in the cytosol was increased ~4-fold. Thus, the increase in membrane PKC-βII protein at ~6 h was attributable to an increase in its synthesis as well as translocation. Interestingly, membrane-associated PKC-βII abundance was increased ~3-fold at ~2 h compared with 0 time in the lipid-infused group, although the difference did not achieve statistical significance ($P = 0.058$). The doublets seen in PKC-βII and -βI (Fig. 3) may reflect different phosphorylation states of the proteins.

No significant alterations in PKC-ϵ, -θ, or -ζ protein content occurred in the cytosolic or membrane fractions of either group (Table 2 and Fig. 3). PKC-βI in the membrane appeared to show the same trend as PKC-βII; however, due to the small number of muscles biopsy samples available ($n = 3$), the observed increase in its content was not statistically significant (Table 2).

DAG and ceramide content. Infusion of insulin plus lipids (but not insulin) resulted in a >3-fold increase in

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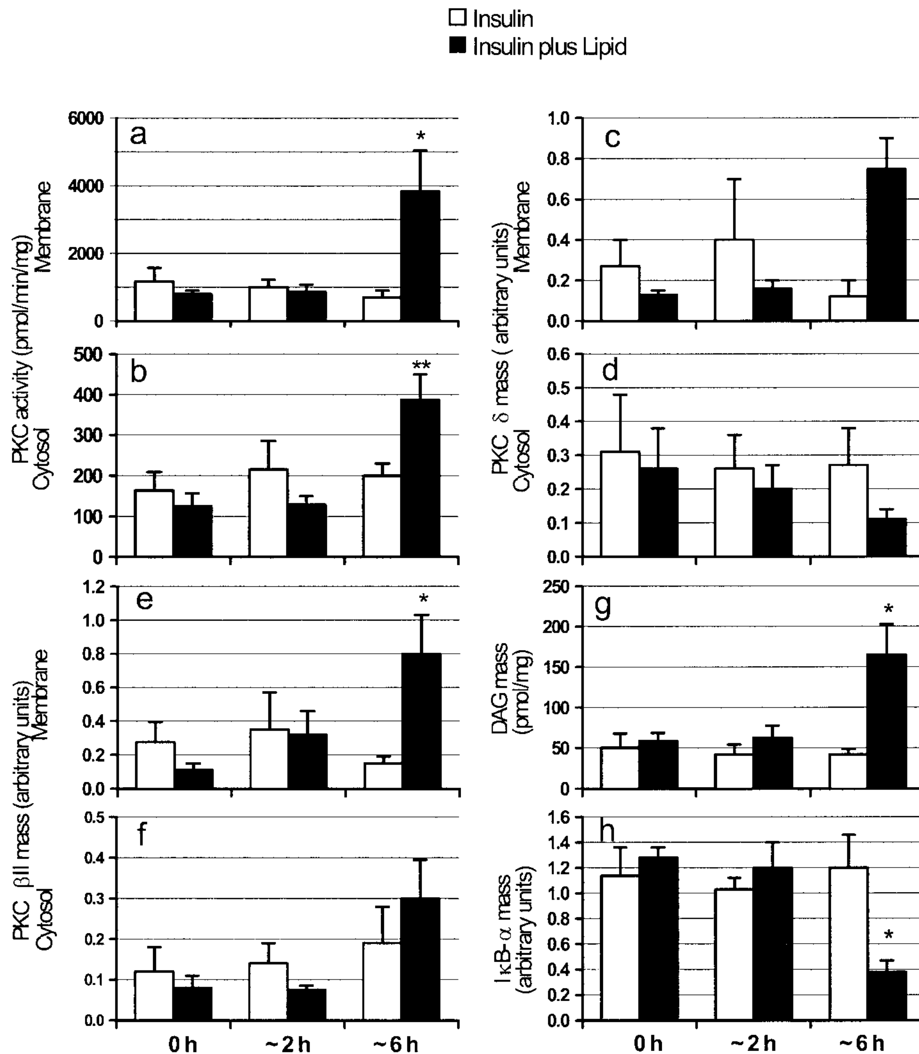

FIG. 2. ***a*** **and** ***b*****: Effect of lipid infusion during a euglycemic-hyperinsulinemic clamp on PKC activity in the membrane (*a*) and cytosolic (*b*) fractions of skeletal muscle at 0, ~2, and ~6 h. Results are means ± SE of six observations. *$P < 0.05$ compared with all other observations, and **$P < 0.03$ compared with values in the same individual at 0 and ~2 h. *c* and *d*: Effect of lipid infusion during a euglycemic-hyperinsulinemic clamp on the distribution of PKC-δ in the membrane (*c*) and cytosolic (*d*) fractions of skeletal muscle at 0, ~2, and ~6 h.. Muscle biopsies were homogenized, and 50 μg protein/lane was loaded on an 8% SDS-polyacrylamide gel. Blots were probed with PKC-δ polyclonal antibodies. Data were normalized to actin and are presented as means of densitometric units ± SE with $n = 6$ for all observations. *$P < 0.003$ compared with biopsies taken at 0 and ~2 h from the same lipid-infused subjects and at ~6 h from the glycerol-infused subjects. *e* and *f*: Effect of lipid infusion during a euglycemic-hyperinsulinemic clamp on the distribution of PKC-βII in membrane (*e*) and cytosolic (*f*) fractions of skeletal muscle. See legend to panels *c* and *d* for details. *$P < 0.02$ vs. values in biopsies taken at 0 and ~2 h from the same individuals and ~6 h from individuals infused with glycerol. *g*: Effect of lipid infusion during a euglycemic-hyperinsulinemic clamp on DAG (*g*) and ceramide (*h*) mass. Lipids were extracted from ~10 mg muscle from biopsies taken at 0, ~2, and ~6 h from subjects infused with either glycerol or lipid. Extracts were run on a TLC plate, incorporation of P^{32} into DAG, and ceramide was determined. Results are means ± SE ($n = 5$) for all observations. *$P < 0.05$ vs. glycerol group at ~6 h and values from the same subjects at 0 and 2 h. *h*: Effect of lipid infusion during a euglycemic-hyperinsulinemic clamp on the degradation of IκB-α. Muscle biopsies were homogenized, and 50 μg protein/lane protein of the cytosolic fraction was loaded on a 12% SDS-polyacrylamide gel. Blots were probed with IκB-α polyclonal antibodies. Data are presented as means ± SE ($n = 6$). *$P < 0.02$ compared with biopsies taken at ~6 h from the glycerol-infused subjects and the lipid-infused subjects at the other time points.**

DAG mass in muscle biopsied at ~6 h but not at 2 h (Fig. 2*g*). In contrast, no significant increase in ceramide was observed at any time (not shown).

IκB-α degradation. The abundance of IκB-α was significantly decreased (~3-fold) in muscle biopsied from subjects infused with insulin plus lipids for ~6 h compared with its preclamp value and compared with the value in insulin alone group (Fig. 2*h* and Fig. 3). No change was seen at 2 h.

DISCUSSION

Raising plasma FFA levels during a euglycemic-hyperinsulinemic clamp causes insulin resistance (diminished rate of G_{Rd}) in human muscle within 3–6 h (6). The results of the present study demonstrate that the development of insulin resistance in this setting was associated temporally with *1*) a fourfold increase in total membrane-associated PKC activity, *2*) translocation of the PKC-β and -δ isoforms from the cytosol to the cell membrane, *3*) a threefold increase in DAG mass, and *4*) a 70% decrease in the abundance of IκB-α, an inhibitor of NFκB. In addition, no change in the content of ceramide, a molecule implicated in the pathogenesis of fatty acid–induced insulin resistance in cultured muscle cells (24), was found.

The increase in PKC activity was presumably related to the increase in the concentration of DAG, a potent allo-

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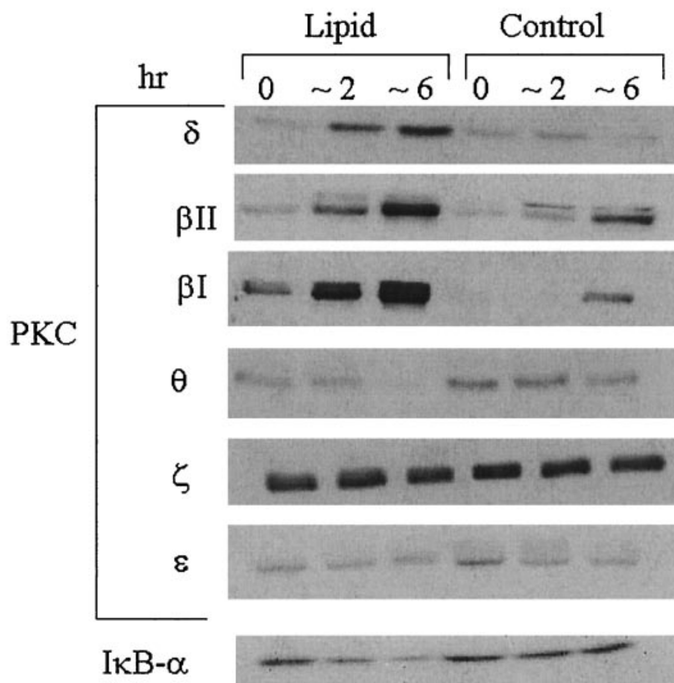

FIG. 3. Western blots showing increases in membrane-associated PKC-βII and -δ and a decrease in cystolic IκB-α in subjects infused with lipid during a euglycemic-hyperinsulinemic clamp. For comparison, blots of membrane-associated PKC-βI, -θ, -ζ, and -ϵ are also shown. See text and legend to Fig. 2 for details.

steric activator of both conventional and novel PKC isoforms (12–14). It was associated with translocation of the PKC isoforms-βII and -δ (but not PKC-ϵ or -θ) to the membrane fraction, which is generally considered a sign of PKC activation (12). In contrast, in a study in rats, in which lipid was infused during euglycemic-hyperinsulinemic clamping, Griffin et al. (17) reported translocation of PKC-θ. PKC activity and DAG mass were not measured in their study nor was the timing of PKC-θ translocation relative to the development of insulin resistance investigated. Whether the reported differences in PKC isoform responses to lipid infusions, i.e., PKC-β and -δ in humans (this study) and -θ in rats (17), reflect species differences remains to be established. This question aside, these studies together clearly suggest that the insulin resistance caused by fat infusion during a euglycemic-hyperinsulinemic clamp is associated with activation of one or more PKC isoforms. The data do not allow us to state with certainty that changes in PKC antedate the insulin resistance, although a distinct trend for PKC-βII translocation to occur before the other events (Fig. 2*e* and *f* and Fig. 3) is at least suggestive. DAG content was not altered at 2 h; however, an early increase in de novo synthesized DAG could have been missed. Future studies with a larger number of subjects and at times between 2 and 6 h could resolve this question.

A linkage between DAG-associated PKC activation and insulin resistance in skeletal muscle had been suggested by early studies in denervated rat muscle (16). In keeping with this, increases in DAG content and altered PKC activity and distribution have been observed in a wide variety of insulin-resistant states in rodents including fat-feeding, obesity, glucose infusion, inactivity, and type 2 diabetes (11,15,29,30). Furthermore, improvement in insulin sensitivity after exercise in the fat-fed rat is associated with a restoration of PKC distribution toward a control pattern (30). More recently, alterations in PKC activity and distribution have been found in obese, insulin-resistant humans, both with (31) and without (19) diabetes. In general, alterations in PKC-θ and -ϵ have been described in the rat, whereas in humans alterations in both PKC-β (19 and this study) and PKC-θ (31) have been reported. The significance of these differences in the isoform(s) affected remains to be established.

Increases in intramuscular triglycerides and DAG (10) presumably occur during a lipid infusion because the increased FFA uptake exceeds its oxidation. Presumably, observed increases in DAG (and PKC) would have been less pronounced without the glucose and insulin infusions (15), because the high rate of glucose uptake during the clamp increases the intramuscular concentration of malonyl-CoA (15), an inhibitor of carnitine palmitoyl transferase-1. This would decrease the oxidation of FFA by inhibiting their transfer from the cytosol into mitochondria and secondarily increase their esterification. In addition, a high rate of glucose uptake would increase the concentration of α-glycerophosphate.

Ceramide is another FFA metabolite linked to insulin resistance. Increased levels of ceramide were first described in insulin-resistant muscle of the Zucker rat by Turinsky et al. (32). Since then, several studies have shown that C2-ceramide can inhibit insulin-stimulated glucose transport, glycogen synthesis, and Akt activation

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TABLE 2
Distribution of PKC isoforms in the membrane and cytosolic fractions

	Glycerol			Lipid		
	0 h	−2 h	−6 h	0 h	−2 h	−6 h
Membrane						
βI	0.03 ± 0.03	0.05 ± 0.02	0.086 ± 0.05	0.05 ± 0.02	0.17 ± 0.03	0.52 ± 0.3
ε	0.27 ± 0.13	0.84 ± 0.75	0.16 ± 0.06	0.24 ± 0.11	0.55 ± 0.43	0.23 ± 0.05
θ	0.44 ± 0.18	0.98 ± 0.86	0.14 ± 0.04	0.23 ± 0.11	0.45 ± 0.29	0.20 ± 0.05
ζ	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.10 ± 0.001	0.10 ± 0.01	0.11 ± 0.01
Cytosol						
βI	0.02 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.06 ± 0.02	0.08 ± 0.02	0.07 ± 0.04
ε	0.34 ± 0.19	0.21 ± 0.08	0.22 ± 0.09	0.25 ± 0.11	0.18 ± 0.08	0.02 ± 0.01
θ	0.29 ± 0.11	0.14 ± 0.05	0.14 ± 0.06	0.15 ± 0.06	0.18 ± 0.06	0.13 ± 0.01
ζ	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.003	0.04 ± 0.003	0.05 ± 0.001

Data are means ± SE. n = 6 for PKC-θ and ε, and n = 3 of densitometric units for PKC-βI and -ε. See legend to Fig. 2*C* and *D* for details

in adipocytes. In addition, exogenous palmitate has been shown to inhibit insulin-stimulated glycogen synthesis and Akt activation in C2C12 myotubes by a mechanism dependent on increased de novo synthesis of ceramide (24). In the present study, we found no increase in ceramide mass in skeletal muscle of lipid-infused men, suggesting that ceramide did not contribute to their insulin resistance. On the other hand, because ceramide synthesis was not assessed, the possibility that a small de novo synthesized ceramide pool might have played a role cannot be ruled out.

Activation of PKC could lead to insulin resistance by several mechanisms. PKC has been shown to serine/threonine phosphorylate both the insulin receptor (19,33–35) and IRS-1 (36,37), leading to impaired insulin signaling. In keeping with this possibility, inhibition of insulin-stimulated PI3-kinase activation and decreased IRS tyrosyl phosphorylation have been shown to accompany the increase in membrane-associated PKC-θ in rats infused with lipid during euglycemic-hyperinsulinemic clamping (17).

Another possibility is that activation of PKC caused insulin resistance by increasing oxidative stress and by activating IKK and/or the NFκB pathway. It has been demonstrated that FFA and hyperglycemia can increase oxidative stress and activate NFκB in endothelium and that these effects are mediated by PKC (38–41). Activation of NFκB can be initiated by the phosphorylation of the inhibitor IκB and its subsequent release from NFκB (42). Activation of PKC can set these events in motion by directly phosphorylating IκB (42) or by causing the generation of reactive oxygen species that can secondarily activate IκB-kinase (IKK-β) (Fig. 4). In fact, phosphorylation by IKK-β is considered the main pathway by which IκB-α is released from NFκB and subsequently subjected to ubiquitination and proteosomal degradation. The result is a decrease in IκB-α mass and movement of NFκB from the cytosol to the nucleus. That such a series of events occurred in the present study is strongly suggested by the marked decrease in IκB-α mass in the subjects infused with lipid.

Two recent studies have implicated IKK and, by inference, IκB and NFκB in the pathogenesis of insulin resistance in rodent skeletal muscle. Kim et al. (22) have shown in rats that the inhibition of insulin-stimulated PI3-kinase activation caused by infusing lipid can be prevented by a high dose of salicylate (an inhibitor of IKK-β). Inhibiting IKK-β would stabilize IκB and prevent activation of NFκB. The same group has found that lipid infusion during a euglycemic-hyperinsulinemic clamp does not cause insulin resistance in IKK-β knockout mice, whereas overexpressing IKK-β increases insulin resistance (22,23). The results of the present study offer additional support for a role of the IKK-β/IκB-α/NFκB pathway in the pathogenesis of fatty acid–induced insulin resistance in human muscle, although as already noted, activation of PKC could cause insulin resistance by direct effects on IRS and other molecules in the insulin-signaling cascade. Whether IKK-β is altered in this situation, and if so, whether it too has direct effects on insulin signaling remains to be determined. A hypothetical scheme that depicts how the metabolic events initiated by infusing lipid/heparin during euglycemic-hyperinsulinemia could lead to changes in IκB and NFκB and insulin resistance is presented in Fig. 4.

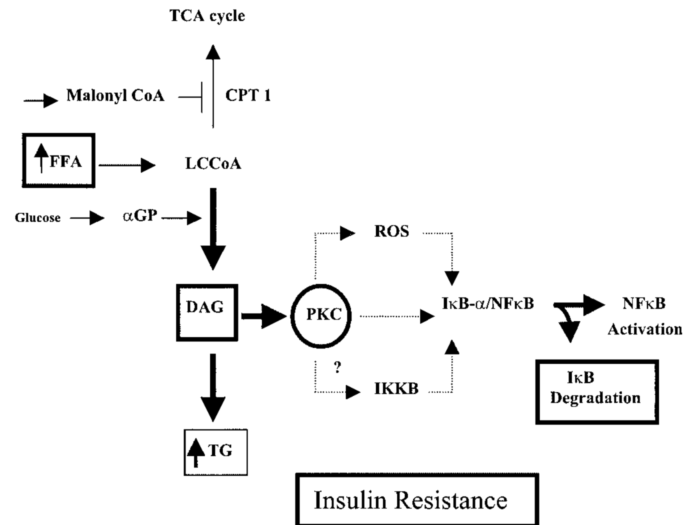

FIG. 4. Hypothetical schema to explain changes in DAG, PKC, and IκB-α associated with insulin resistance in muscle of humans infused with lipid during a euglycemic-hyperinsulinemic clamp. Key initiating events are an increase in plasma FFA, which elevates the cytosolic concentration of long-chain fatty acyl CoA (LCCoA), and an increased uptake into muscle of glucose. The latter enhances glycerolipid (TG, DAG) synthesis both by generating α-glycerophosphate and by enhancing the synthesis of malonyl CoA, which inhibits fatty acid oxidation by mitochondria. It is assumed that activation of PKC sets in motion changes in IκB-α and NFκB by one or more of the indicated mechanisms; however, direct proof that it does so in human muscle is lacking (see text). The precise events that lead to insulin resistance remain to be determined. IKKB, IκB kinase.

Lastly, the IKK-β/IκB-α/NFκB pathway is a major pro-inflammatory pathway (20), and inflammatory processes are now recognized to play a pivotal role in the pathogenesis of coronary artery disease (43). Hence, activation of the IKK-β/IκB-α/NFκB pathway by FFA may explain at least some of the increased prevalence of coronary artery disease in obese patients with type 2 diabetes, because almost all of these patients have increased plasma FFA levels and are insulin resistant.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants R01-AG-07988 (to G.B.) and P01-HL-55854/JDF F 99 6004 (to N.R.), the General Clinical Research Center branch of the National Center for Research Resources (Grant RR-349), and a grant from the Juvenile Diabetes Foundation (JDF 1-2000-319) (to N.R.). In addition, S.I.I. was the recipient of a mentor-based training award from the American Diabetes Association and a traineeship from the NHLBI (HL 07224-25) during the course of these studies.

The authors thank the nurses of the General Clinical Research Center for help with the studies and for excellent patient care, and Constance Harris Crews for typing the manuscript. They also gratefully acknowledge the advice of Dr. John Keaney in carrying out the IκB-α studies.

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