Enhanced Stimulation of Diacylglycerol and Lipid Synthesis by Insulin in Denervated Muscle

Altered Protein Kinase C Activity and Possible Link to Insulin Resistance

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Denervated muscle is generally regarded as insulin resistant because the ability of insulin to stimulate glucose transport and glycogen synthesis is impaired. Previous studies indicate that insulin resistance in these muscles is likely due to a defect at a postreceptor site in the signaling pathway. Because glucose transport into cells has been reported to be linked to changes in diacylglycerol (DAG) and protein kinase C (PKC), we investigated the effect of denervation on the content and synthesis of DAG and the activity and distribution of PKC in the soleus muscle. The DAG content in muscles denervated for 24 h was 40% greater than in control muscles. This was associated with a two- to threefold increase in the percentage of total PKC activity that was membrane associated, with no significant change in total PKC activity, suggesting an increase in PKC activity in vivo. Studies of glucose disposition confirmed that the stimulation of glycogen synthesis by insulin and, to a lesser extent, 2-deoxyglucose uptake were impaired by denervation. However, the stimulation by insulin of glucose incorporation into DAG and other lipids was two- to threefold greater in denervated than in control muscles, and conversion of glucose to lactate and pyruvate and glucose oxidation to CO_2 were unchanged. The results reveal a dichotomy in the effects of denervation on various actions of insulin, with both insulin resistance and hyperresponsiveness occurring in different pathways of glucose metabolism. They also reveal a potential mechanism for the elevation of muscle DAG after denervation. The results do not support a direct link between DAG-PKC and glucose transport. Based on these findings, a working hypothesis that links enhanced DAG-PKC signaling to insulin resistance is proposed. ***Diabetes*** **40:1707–11, 1991**

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It has been known for >30 yr that denervation of skeletal muscle causes insulin resistance (1). Burant et al. (2) observed that neither insulin binding to its receptor nor its ability to stimulate autophosphorylation of the receptor and receptor tyrosine kinase activity are depressed in muscle 24 h after denervation. Therefore, it was concluded that denervation alters an undefined step in signal transduction distal to the insulin receptor. Because diacylglycerol (DAG) and protein kinase C (PKC) have been implicated as modulators of glucose transport into cells by some investigators (3,4), we examined the content and synthesis of DAG and PKC activity in the soleus muscles of rats 24 h after sciatic nerve section and in muscles of control rats. The results indicate that muscle DAG content is increased by denervation and that this is associated with a translocation of PKC activity from the cytosol to a membrane fraction. In agreement with previous reports, glycogen synthesis and glucose transport were diminished in the denervated soleus (5,6). However, the stimulation by insulin of glucose incorporation into muscle DAG and other lipids was two- to threefold higher, suggesting that the denervated muscle may be regarded as insulin resistant or insulin hyperresponsive, depending on the event being studied. These results also indicate that DAG content and PKC activity are increased in the soleus muscle 24 h after denervation. Furthermore, the increase in DAG may be due, at least in part, to an increase in its synthesis de novo. The possible link between DAG synthesis and content, PKC activity, and insulin resistance is discussed.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing 50–75 g (Charles River Breeding Laboratories, Wilmington, MA) were anesthetized with pentobarbital sodium (3–4 mg/100 g

body wt i.p), and the soleus muscle was denervated by sciatic nerve section 24 h before an experiment. Control muscles were obtained from sham-operated limbs. The rats received no food but had free access to water during the 20–24 h before being killed. Soleus muscles were isolated as described by Maizels et al. (7). They were preincubated for 30 min in 3 ml Krebs-Henseleit solution (KHS) containing 6 mM glucose and then in KHS without glucose for 2 min. Afterwards, they were incubated for 10 or 15 min in KHS containing 6 mM glucose and other additions as described in RESULTS. All incubations were carried out at 37°C in a medium gassed with 95% O_2/5% CO_2.

Glucose transport was assessed from the uptake of 2-[1,2-^{3}H]deoxy-D-glucose (2-DG) (NEN, Boston, MA) during 15 min of incubation in KHS containing 0.3 μCi/ml tracer, 6 mM glucose and insulin (purified pork; Lilly, Indianapolis, IN) as indicated. D-[1-^{14}C] mannitol (0.09 μCi/ml; NEN) was added as a marker to correct for [^{3}H]2-DG in the extracellular space. To measure the disposition of glucose, muscles were preincubated individually in tubes and then transferred to flasks containing KHS supplemented with 0.5 mCi/mmol [U-^{14}C]glucose and 0.1% bovine serum albumin (essentially fatty acid free; Sigma, St. Louis, MO). After incubation, the muscles were rapidly removed and frozen in liquid N_2. The incubation flask was then fitted with a center-well stopper containing phenylethylamine, the medium was acidified with H_2SO_4, and CO_2 was collected. After CO_2 collection, the acidified medium was analyzed for ^{14}C-containing anions (predominantly lactate + pyruvate) as described previously (8). A portion of the frozen muscle was used for lipid analysis and the remainder for quantifying [^{14}C]glucose incorporation into glycogen (9).

For lipid analysis, a 10- to 30-mg portion of frozen muscle was quickly weighed, and the lipids were extracted and DAG mass was measured as described by Preiss et al. (10). The incorporation of ^{14}C from glucose into DAG was determined by a modification of this assay. Briefly, two equal sample portions (from ~10 mg of muscle each) were incubated with nonradioactive ATP, one with and the other without DAG kinase. The lipids in the reaction mixtures were then reextracted and chromatographed on thin-layer chromatography (TLC) plates as for the assay of DAG content. After autoradiography, the various radioactive lipid bands on the TLC plate were excised and counted by liquid-scintillation counting. Phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) were identified on the basis of comigration with authentic standards on the TLC plate. The difference in the radioactivity of [^{14}C]PA in the samples incubated with and without DAG kinase corresponds to the radioactivity in DAG.

PKC was determined by a modification of the method of Niles and Loewy (11). Briefly, muscles were homogenized in 0.4 ml homogenizing buffer containing 250 mM sucrose, 20 mM Tris (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 50 μM leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 20 mM dithiothreitol (DTT). The homogenate was then centrifuged at 100,000 × *g* for 1 h at 4°C, and the cytosol was transferred to a tube kept on ice, whereas the pellet was resuspended in 0.45 ml homogenizing buffer containing 5% Triton X-100. The resuspended pellet fraction was then centrifuged at 14,000 × *g* for 5 min at 4°C, and the pellet was discarded. The supernatant from this spin constitutes the membrane extract. A portion (50–100 μl) of the cytosol or membrane extract was then applied into a 1-ml DEAE cellulose column and washed (11). PKC was eluted with 2 ml washing buffer supplemented with 100 mM NaCl. The reaction mixture for PKC analysis consisted of 15 μl of the column eluate and 35 μl of a mixture containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 μM [γ-^{32}P]ATP (2 μCi), 50 μg histone type IIIS, and with or without 1 mM $CaCl_2$ and 15 μg PS plus 0.3 μg diolein that had been sonicated in water. The reaction was carried out for 10 min at 30°C, after which 40 μl of the incubation mixture was spotted onto a piece of Whatman 3-MM filter paper. The filter paper was then washed extensively in 10% trichloroacetic acid plus 10 mM $Na_4P_2O_7$, dried, and the radioactivity on it was determined by Cerenkov counting. PKC activity was calculated from the difference in protein kinase activity in the presence and absence of $CaCl_2$ and PS plus diolein.

TABLE 1
Effect of 24-h denervation on diacylglycerol (DAG) mass and protein kinase C (PKC) distribution in rat soleus muscle

	DAG mass (pmol/mg muscle)	PKC activity: 100,000 × *g* pellet (% of total)	PKC activity: Total pmol phosphate · min^{-1} · mg^{-1} muscle
Control	86 ± 6	15 ± 5	1.5 ± 0.5
Denervated	121 ± 8*	38 ± 10*	1.6 ± 0.6

Values are means ± SE for 9 (DAG mass) and 5 (PKC activity) muscles taken directly from rats.
*$P < 0.05$ vs. control.

RESULTS

To assess whether DAG-PKC signaling is altered by denervation, DAG content and PKC distribution were compared in soleus muscle from 24-h denervated and sham-operated rats. Denervation caused a 40% increase in the amount of DAG (Table 1). In addition, it caused a two- to threefold increase in the percentage of total PKC that was membrane associated (100,000 × *g* pellet), a phenomenon typically associated with PKC activation. Total PKC activity was unchanged.

Glucose transport and disposition in 24-h denervated and sham-operated muscles are shown in Table 2. In keeping with earlier studies (5), we found that denervation inhibited (>90%) the ability of a supraphysiological concentration of insulin to stimulate glycogen synthesis (Table 2). It also diminished both basal and insulin-stimulated 2-DG uptake, suggesting that glucose transport was impaired. The decrease in insulin-stimulated 2-DG uptake was modest compared with the inhibition of glycogen synthesis; however, after denervation (48–72

TABLE 2
Effects of 24-h denervation on glucose disposition and transport in incubated rat soleus

Treatment	Glucose disposition (pmol glucose-carbon · mg^{-1} muscle · 15 min^{-1})				2-Deoxyglucose uptake (pmol · mg^{-1} muscle · 15 min^{-1})
	Glycogen	Lipids	Lactate + pyruvate	CO_2	
No insulin					
Control	153 ± 27	71 ± 11	2060 ± 550	297 ± 59	690 ± 45
Denervated	94 ± 30	108 ± 20	2525 ± 362	192 ± 46	330 ± 45*
Insulin (10 mU/ml)					
Control	2032 ± 272	276 ± 30	3448 ± 693	431 ± 80	2295 ± 135
Denervated	195 ± 66	506 ± 50	3581 ± 701	382 ± 64	1740 ± 195*

Values are means ± SE of 8–12 experiments. Soleus muscles from sham-operated control and bilaterally denervated rats were incubated in presence of 6 mM [U-^{14}C]glucose or 2-[1,2-^{3}H]deoxyglucose for 15 min as described in METHODS. Uptake of 2-deoxyglucose was measured in separate muscles.
*$P < 0.05$ vs. control by paired analysis.

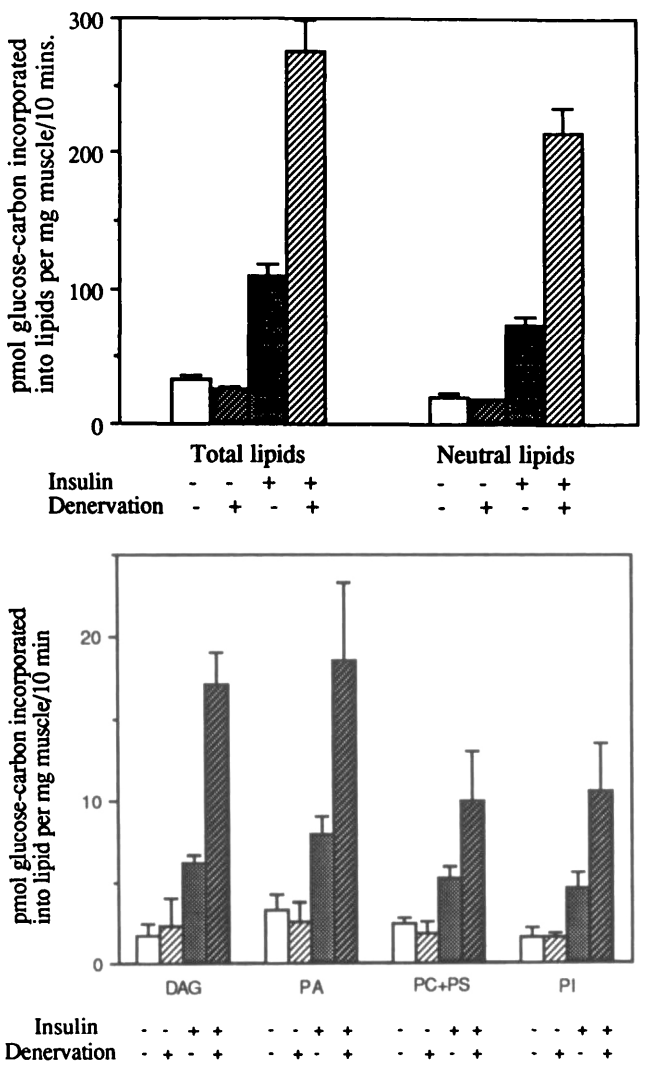

FIG. 1. Effect of denervation and insulin on incorporation of glucose-carbons into diacylglycerol (DAG) and other lipids. Soleus muscles from bilaterally denervated rats were incubated for 10 min in the presence of 6 mM [U-^{14}C]glucose and, where indicated, 10 mU/ml insulin. Lipids were extracted and separated by thin-layer chromatography as described in METHODS. Values are means ± SE of 3–5 observations. PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

h), the inhibition of glucose transport was more marked and a decrease in GLUT4 protein, not seen at 24 h, was observed (unpublished observations).

In contrast to its impaired stimulation of glycogen synthesis, insulin induced a greater stimulation of glucose-carbon incorporation into total lipids in the denervated than the control soleus (Fig. 1). Moreover, denervation had no effect on the stimulation by insulin of either glucose oxidation to CO_2 or its conversion to lactate and pyruvate. To determine into which lipids glucose incorporation was enhanced, lipid subclasses in the muscle extract were separated by TLC and analyzed. The stimulation by insulin of [U-^{14}C]glucose-carbon incorporation into total neutral lipids, DAG, PA, PC+PS, and PI were all substantially greater in the denervated muscles (Fig. 1). Thus, in control muscles, insulin induced a 2- to 3-fold stimulation of the incorporation of glucose-carbon into all of these species, whereas in denervated muscles, it induced a 5- to 10-fold stimulation. In the absence of insulin, no significant differences were observed between control and denervated muscles.

DISCUSSION

The results indicate that several alterations in the DAG-PKC signaling system occur in the soleus muscle during the first 24 h after it is denervated. These include *1*) a 40% increase in DAG content, *2*) a twofold increase in the stimulation by insulin of glucose-carbon incorporation into DAG and other lipids, and *3*) translocation of PKC from the cytosol to a membrane fraction with no change in the total PKC activity, a finding generally associated with activation of the enzyme. To our knowledge, this is the first demonstration of a hyperresponsive insulin effect and an altered postreceptor signaling event in denervated muscle.

The increase in the DAG content of the soleus after denervation is consistent with recent reports (12,13). A sustained increase of DAG mass in these muscles could reflect an increase in phospholipase C–mediated phospholipid hydrolysis, inhibition of DAG kinase or lipase, or an increase in DAG synthesis, de novo. Our data suggest that enhanced de novo synthesis could be a significant contributor, because in the prèsence of insulin, glucose

incorporation into DAG was > twofold greater in the 24-h denervated soleus than in sham-operated control muscles. The observation that glucose incorporation into PA and PI were also increased suggests that a step before PA formation is involved. One possible explanation is that insulin increases the activity of glycerolphosphate acyltransferase (14), a rate-limiting enzyme in lipid synthesis, to a greater extent in denervated than in control muscles. Another explanation is that the concentration of cytosolic long-chain fatty acyl CoA is increased in denervated muscle, due to the absence of contractile activity. In such a setting, insulin, by increasing glycolysis, could hypothetically increase the concentration of glycerol-3-phosphate and secondarily increase PA synthesis by mass action. The observation that fatty acid oxidation is diminished in the denervated muscle (15) at the time that de novo lipid synthesis is increased (Table 2; Fig. 1) is consistent with the notion that cytosolic fatty acyl CoA levels are increased.

The enhanced stimulation by insulin of glucose incorporation into DAG and other lipids of denervated muscle reported here represent the first demonstration of insulin hyperresponsiveness in this model. Denervated muscle is considered insulin-resistant, based on the decreased ability of insulin to stimulate glycogen synthesis and, to a lesser extent, glucose transport (5,6). That insulin-stimulated glucose incorporation into lipid is increased 24 h after denervation and that the conversion of [^{14}C]glucose to $^{14}CO_2$ and lactate + pyruvate is unchanged suggest that the changes in insulin sensitivity occur selectively, depending on the process being examined.

The translocation of PKC isozymes from the cytosol to a membrane fraction is associated with increases in their activity in vivo (16). In this study, the percentage of total PKC activity in the 100,000 × *g* pellet (membrane fraction) was increased nearly two- to threefold in the soleus 24 h after denervation. Because DAG activates PKC by a mechanism involving translocation, it seems likely that the increase in the content of tissue DAG contributed to the redistribution of PKC in the denervated soleus. Our results do not support a direct link between DAG content or PKC activity and the rate of glucose transport in the muscle (17). However, it remains possible that specific PKC isozymes are involved in a manner not revealed by our study.

The nature of the putative link between DAG-PKC signaling and insulin resistance in muscle is worth further investigation. Potential mechanisms include the phosphorylation and inhibition of insulin receptor tyrosine kinase (18) and glycogen synthase (19) by PKC, which has been shown in other tissues. Whether PKC has similar effects on these enzymes and whether it affects the translocation and activity of glucose transporters in skeletal muscle remain to be determined.

In addition to denervation, muscle is the site of insulin resistance in conditions associated with persistent hyperinsulinemia, hyperglycemia, high levels of plasma free fatty acids (20–23), and inactivity (24,25). Where studied, the levels of muscle DAG (12,13,26,27) and triglyceride (23) are increased in these conditions. Based on the findings of this study, we speculate that DAG synthesis de novo from glycerol-3-phosphate and long-chain fatty acyl CoA would also be increased and that this leads to alterations in the activity of one or more PKC isozymes. If so, the working hypothesis presented here may be directly relevant to other models of insulin resistance in skeletal muscle.

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