Ceramide Content Is Increased in Skeletal Muscle From Obese Insulin-Resistant Humans

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Increased intramyocellular lipid concentrations are thought to play a role in insulin resistance, but the precise nature of the lipid species that produce insulin resistance in human muscle are unknown. Ceramides, either generated via activation of sphingomyelinase or produced by de novo synthesis, induce insulin resistance in cultured cells by inhibitory effects on insulin signaling. The present study was undertaken to determine whether ceramides or other sphingolipids are increased in muscle from obese insulin-resistant subjects and to assess whether ceramide plays a role in the insulin resistance of Akt in human muscle. Lean insulin-sensitive and obese insulin-resistant subjects (n = 10 each) received euglycemic-hyperinsulinemic clamps with muscle biopsies basally and after 30, 45, or 60 min of insulin infusion. The rate of glucose infusion required to maintain euglycemia (reflecting glucose uptake) was reduced by >50%, as expected, in the obese subjects at each time point (P < 0.01). Under basal conditions, total muscle ceramide content was increased nearly twofold in the obese subjects (46 ± 9 vs. 25 ± 2 pmol/2 mg muscle, P < 0.05). All species of ceramides were increased similarly in the obese subjects; in contrast, no other sphingolipid was increased. Stimulation of Akt phosphorylation by insulin in the obese subjects was significantly reduced after 30 min (0.96 ± 0.11 vs. 1.84 ± 0.38 arbitrary units) or 45–60 min (0.68 ± 0.17 vs. 1.52 ± 0.26) of insulin infusion (P < 0.05 for both). Muscle ceramide content was significantly correlated with the plasma free fatty acid concentration (r = 0.51, P < 0.05). We conclude that obesity is associated with increased intramyocellular ceramide content. This twofold increase in ceramide may be involved in the decrease in Akt phosphorylation observed after insulin infusion and could theoretically play a role in the reduced ability of insulin to stimulate glucose uptake in skeletal muscle from obese subjects.
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Abundant evidence suggests that increased lipid availability plays a role in the molecular mechanisms responsible for insulin resistance in skeletal muscle. It has long been recognized that increased plasma triglycerides and free fatty acids (FFAs) are associated with insulin resistance in vivo in humans (1), and recent biochemical and noninvasive imaging studies show that the intramyocellular content of lipids is increased (2–6). Incubation of cultured muscle cells with fatty acids decreases glucose uptake and glycogen synthesis and inhibits insulin receptor signaling through Akt (7). In vivo, lipid infusions decrease skeletal muscle insulin-stimulated glucose metabolism and insulin receptor signaling (8–12). In particular, inhibition of insulin receptor substrate (IRS)-1–associated phosphatidylinositol (PI) 3-kinase activity appears to mediate, at least in part, the insulin resistance associated with infusion of a triglyceride emulsion (13,14).

The increase in intramyocellular lipid in insulin-resistant humans is manifested as an increase in triacylglycerol content (2–6). However, triacylglycerol itself may not be the lipid species that is the proximal cause of the insulin resistance. Rather, some other less abundant lipid species are thought to be involved. Candidates include diacylglycerol (13,15,16), fatty acyl CoA species, and ceramides. Ceramides are a family of sphingolipids that differ in the fatty acyl moiety and are known to induce insulin resistance in cultured cells (7,17–20). In particular, in C2C12 muscle cells, a cell-permeable ceramide induces insulin resistance and inhibits insulin-induced Akt serine phosphorylation and activation (7). In this system, ceramide mimicked the deleterious effects on insulin signaling produced by incubation of the cells with palmitate, a precursor in the de novo synthesis of ceramide (7). Moreover, ceramide can be generated by hydrolysis of sphingomyelin, catalyzed by sphingomyelinase (21). Activation of sphingomyelinase and generation of ceramide is believed to be involved in the mechanism of action of tumor necrosis factor (TNF)-α, an inflammatory cytokine that may play a role in insulin resistance (22–24), interleukin-1 and γ-interferon, and a variety of cell stressors (21). The involvement of ceramides in the etiology of insulin resistance in human skeletal muscle is unknown. The present study was undertaken to determine if ceramide content of muscle from obese insulin-resistant human subjects is increased in concert with a reduction in insulin-stimulated Akt phosphorylation. The results of the study show that

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FFA, free fatty acid; IRS, insulin receptor substrate; PI, phosphatidylinositol; TNF, tumor necrosis factor.

ceramide content is increased nearly twofold in muscle from insulin-resistant obese subjects in whom insulin stimulation of Akt Ser^{473} phosphorylation is abnormal.

RESEARCH DESIGN AND METHODS

Subjects were placed into one of two study groups based on BMI. Individuals with a BMI <27 kg/m^2 were categorized as lean control subjects ($n = 10$). This group did not have a family history (first-degree relative) of type 2 diabetes. Subjects with a BMI of 27–33 kg/m^2 were categorized as obese nondiabetic ($n = 10$). Two of the obese subjects had a single first-degree relative with type 2 diabetes. All subjects had a normal 75-g oral glucose tolerance test and were judged to be in good health based on physical examination, medical history, routine blood work, urinalysis, and electrocardiogram. All subjects were free of medications and were not participating in any form of regular exercise. Subjects were instructed to maintain their normal diet and abstain from exercise for 3 days before the study. The purpose, nature, and potential risks of the study were explained to all subjects, and written consent was obtained before participation. All studies were reviewed and approved by the Institutional Review Boards at the University of Texas Health Science Center as San Antonio and Georgia Tech University. All studies were performed at the Audie L. Murphy Memorial Veterans Hospital, General Clinical Research Center, San Antonio, Texas, in the morning after a 10- to 12-h overnight fast.

Hyperinsulinemic-euglycemic clamp. A hyperinsulinemic-euglycemic clamp with biopsies of the vastus lateralis muscle was performed as described (25). An antecubital vein was cannulated for the infusion of insulin and 20% glucose. A hand vein was cannulated in a retrograde fashion, and the hand was placed in a heated box (55°C) for sampling of arterialized blood. Blood was drawn every 10 min for the last 30 min of the rest period for the determination of basal plasma insulin and plasma glucose concentrations. After 60 min bed rest, a percutaneous muscle biopsy was obtained with a Bergstrom cannula from the vastus lateralis muscle under local anesthesia. Muscle specimens (75–200 mg) were immediately blotted free of blood, frozen in liquid nitrogen, and stored under liquid nitrogen until processing. Subjects rested for 60 min after the basal biopsy. At time $t = 0$, a constant infusion of insulin [40 mU/(m^2 · min)] was started. The plasma glucose concentration was measured every 5 min by a glucose analyzer (Beckman Instruments, Fullerton, CA), and an infusion of 20% glucose was adjusted to maintain euglycemia. Blood was obtained every 10 min during the insulin clamp for measurement of plasma insulin concentrations. A second percutaneous muscle biopsy was obtained from the other leg at 30, 45, or 60 min after insulin infusion began. Insulin infusion was terminated after the last biopsy. All subjects had at least one muscle biopsy basally. Some of the subjects received multiple insulin infusions and a second biopsy; if so, the infusions were conducted on separate days. The rate of infusion of glucose required to maintain euglycemia was taken as a measure of insulin-stimulated glucose uptake. This measure underestimates the true rate of glucose uptake to the extent that endogenous glucose production is incompletely suppressed by insulin.

Immunoblot analysis of Akt Ser^{473} phosphorylation. Frozen muscle specimens were homogenized as described (26,27), and protein concentration was determined by the Lowry method (28). Akt activation was assessed using immunoblot analysis of 100 μg total muscle protein by determining the phosphorylation of Ser^{473} using a specific antiphospho-Akt antibody. All blots were stripped and reprobed for protein expression, and protein phosphorylation was expressed relative to protein levels.

Muscle sphingolipid analysis. Muscle sphingolipid content was determined as described (29). Tissues were homogenized in the smallest volume possible of PBS such that the amount of tissue per unit volume is the same for all samples. Approximately 2 × 2 mg of protein per sample is transferred to a 13 × 100-mm screw-cap glass test tube for sphingolipid extraction via established methods (25).

A 20-μl aliquot of the reconstituted sample was added to a final volume of 1 ml of a methanol:acetic acid (99:1) solution containing 5 mmol/l ammonium acetate. This solution was infused into the ion source at ~5–10 μl/min, and individual sphingolipids were identified via precursor ion scans (25). Optimal ionization and collision conditions for individual molecular species in each class of sphingolipids were determined.

Crude sphingolipid extracts were loaded onto a high-performance liquid chromatography instrument directly coupled with the ion source of a tandem mass spectrometer (MS/MS). Free sphingoid bases and complex sphingolipids were separated by either reverse- or normal-phase chromatography, respectively (25). The former used shorter columns (2.1 × 50 mm) and higher flow rates (1 ml/min), whereas the latter used an amino column that was both shorter (2.1 × 50 mm) and had higher flow rates (1.5 ml/min.). Quantitation was determined by comparing the peak areas generated by the internal standards with those of the endogenous species.

TABLE 1
Subject characteristics

	Lean	Obese
n	10	10
Sex (M/F)	6/4	5/5
Age (years)	29.8 ± 2.2	32.0 ± 3.1
Weight (kg)	72.2 ± 3.2	90.9 ± 2.5*
BMI (kg/m^2)	23.6 ± 0.8	31.0 ± 0.5*
Lean body mass (%)	75 ± 2	67 ± 2†
Fasting plasma glucose (mg/dl)	89 ± 1	94 ± 3†
Fasting plasma insulin (μU/ml)	4.9 ± 0.8	9.6 ± 1.6†

Data are means ± SE. *$P < 0.001$, †$P < 0.05$ vs. lean group.

Other analyses. Plasma insulin concentrations were determined by radioimmunoassay (Diagnostics Products, Los Angeles, CA). Body composition was determined by bioimpedance (RJL Bio-106 Spectrum Body Composition Analyzer; RJL Systems, Detroit, MI).

Materials. Anti–phospho-Akt antibody was purchased from Upstate Biotechnology (Waltham, MA). Polyclonal anti-Akt was purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit horseradish peroxidase conjugate secondary antibody and the enhanced chemiluminescence reagent system were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Bio-Rad (Hercules, CA).

Statistical analysis. A two-way ANOVA was used to determine differences in Akt phosphorylation and sphingolipid content between the two groups, with a posteriori comparisons (StatView; SAS Institute, Cary, NC). Correlation analysis was performed using Pearson's product moment correlation.

RESULTS

The groups were well matched with respect to age, sex, and ethnicity. BMI was used to place individuals into either the lean control or obese nondiabetic group. Body weight, BMI, percent lean body mass, fasting plasma glucose, and fasting plasma insulin levels were significantly elevated in the obese nondiabetic group when compared with the lean control subjects (Table 1). All subjects had normal glucose tolerance. Fasting plasma insulin concentrations were significantly greater in the obese subjects (Table 1), as were area under the curve for insulin concentrations during the oral glucose tolerance test ($P < 0.001$).

Hyperinsulinemic-euglycemic clamp. Plasma insulin and FFA concentrations during the euglycemic clamp are given in Table 2. Plasma insulin concentrations were significantly greater in the obese subjects basally and at every time point during the insulin infusion. Likewise, fasting plasma FFA concentrations were increased in the obese subjects. Moreover, plasma FFA concentrations were less inhibited by insulin infusion in the obese group. The glucose infusion rates required to maintain euglycemia during the insulin infusions are shown in Fig. 1. Because the insulin concentrations achieved during the insulin infusions were greater in the obese subjects (presumably due to decreased insulin clearance because the insulin infusion rates were identical in the two groups), glucose infusion rates were also expressed relative to the insulin concentration. The glucose infusion rate, expressed per kilogram of fat-free mass, was lower in obese nondiabetic subjects at all time points ($P < 0.01$).

Sphingolipid concentrations in vastus lateralis muscle. Sphingolipid content of skeletal muscle was determined using high-performance liquid chromatography electrospray ionization tandem mass spectrometry. The results of the analysis for total ceramide content are

FIG. 1. Glucose infusion rates required to maintain euglycemia in lean (□) and obese (■) subjects. Infusion rates shown are means ± SE for the last 15 min of each insulin infusion. The number of lean subjects studied was 4, 6, and 8, and the number of obese subjects was 9, 4, and 4 at 30, 45, and 60 min, respectively. *$P < 0.05$ vs. lean control subjects. LBM, lean body mass.

shown in Fig. 2. The results at 45 and 60 min did not differ in either group; therefore, data from these time points were pooled for statistical comparisons. The results for the individual ceramide species are also given in Table 3. Under basal conditions, total muscle ceramides were increased by nearly twofold in the obese subjects ($P < 0.05$), and ANOVA showed the obese subjects to have greater ceramide content overall ($P < 0.05$). Although total ceramides decreased somewhat in the obese subjects during the insulin infusion, this effect was not statistically significant. Of the total ceramides present, C24, C24:1, and C18 ceramides were the most abundant. A small amount of C16 dihydroceramide was also detected and did not differ between the two groups. No individual species was overrepresented in the obese subjects; rather, the increase was uniform. Insulin infusion did not affect the composition of ceramides (Table 3). In addition, neither obesity nor insulin infusion affected the content of other sphingolipids assayed (Table 4).

Correlation analysis was performed to determine the relationship between total ceramide content and the glucose infusion rate required to maintain euglycemia during the insulin infusions as well as basal plasma FFA concentrations. When data from all subjects were pooled, there was no significant correlation of ceramide with either

FIG. 2. Total ceramide content of skeletal muscle from lean (□) and obese (■) subjects. Data are given as picomoles ceramide per 2 mg muscle wet weight and are shown as means ± SE. Total ceramide was taken to be the sum of all ceramide species, not including dihydroceramide. For each group, 45- and 60-min values did not differ and were pooled for statistical comparison. The number of lean subjects studied were 8, 3, and 8, and the number of obese subjects were 9, 6, and 4 at the basal state, 30, and 45–60 min, respectively. *$P < 0.05$ vs. lean control subjects.

TABLE 2
Plasma insulin and FFA concentrations during euglycemic-hyperinsulinemic clamps

	Time (min)									
	−30	−20	−10	0	10	20	30	40	50	60
Plasma insulin (μU/ml)										
Lean	4 ± 1	4 ± 1	4 ± 1	4 ± 1	57 ± 3	47 ± 2	47 ± 2	48 ± 2	47 ± 3	53 ± 4
Obese	9 ± 1*	8 ± 1*	9 ± 1*	9 ± 1*	77 ± 6*	70 ± 4*	65 ± 4*	60 ± 4*	64 ± 5*	69 ± 4*
Plasma FFAs (mmol/l)										
Lean	0.65 ± .07	0.63 ± .07	0.59 ± 0.06	0.61 ± 0.07	0.59 ± 0.06	0.36 ± 0.04	0.23 ± 0.02	0.17 ± 0.04	0.16 ± 0.04	0.14 ± 0.04
Obese	0.88 ± 0.66*	0.88 ± 0.07*	0.85 ± 0.07*	0.86 ± 0.07*	0.79 ± 0.08*	0.68 ± 0.07*	0.49 ± 0.06*	0.50 ± 0.09*	0.50 ± 0.10*	0.50 ± 0.10*

Data are means ± SE. Plasma insulin and FFA concentrations achieved during euglycemic clamps are shown. *$P < 0.01$ vs. lean subjects.

TABLE 3
Muscle ceramide concentrations

	n	Ceramide							
		C16	C16 DHC	C18	C20	C22	C24:1	C24:0	Total
Lean									
Basal	8	2.07 ± 0.26	1.27 ± 0.36	7.81 ± 0.84	0.23 ± 0.08	2.95 ± 0.36	5.06 ± 0.58	5.7 ± 0.58	25.1 ± 2.1
30 min	3	2.66 ± 0.90	4.59 ± 2.15	7.84 ± 0.38	0.91 ± 0.21	4.64 ± 0.65	6.03 ± 1.05	6.03 ± 1.05	29.3 ± 4.2
45–60 min	8	1.51 ± 0.18	1.16 ± 0.22	7.74 ± 1.94	0.28 ± 0.10	2.51 ± 0.48	4.81 ± 0.82	4.94 ± 1.09	22.9 ± 4.6
Obese									
Basal	9	8.53 ± 4.71*	3.03 ± 1.45*	12.8 ± 1.85*	1.39 ± 0.72*	4.71 ± 0.66*	7.79 ± 0.95*	8.02 ± 0.93*	46.3 ± 8.8*
30 min	6	3.02 ± 1.07	1.29 ± 0.35	10.98 ± 1.47	0.90 ± 0.37	4.21 ± 0.63	6.69 ± 0.72	8.79 ± 2.16	35.9 ± 5.6
45–60 min	7	2.17 ± 0.61	1.35 ± 0.44	8.64 ± 1.97	0.37 ± 0.11	3.78 ± 0.81	6.34 ± 1.90	6.52 ± 1.53	29.2 ± 5.8

Data are means ± SE. Ceramide content of biopsies of vastus lateralis muscle was assayed as described in the text. Units are given in picomoles per 2 mg muscle. *$P < 0.05$ vs. lean control subjects.

variable. However, exclusion of one outlying subject in the obese group revealed a significant correlation between ceramide and the fasting plasma FFA concentration ($r = 0.51$, $P < 0.05$, Fig. 3).

Akt phosphorylation. The effect of insulin infusion on Akt Ser^{473} phosphorylation is given in Table 5 and illustrated in Fig. 4. Muscle biopsies of the vastus lateralis were performed basally and after 30, 45, or 60 min of insulin infusion, and Akt Ser^{473} phosphorylation and Akt protein content were quantified by immunoblot analysis. Figure 4*A* shows a representative comparison of a lean and obese subject who received biopsies basally and after 60 min of insulin infusion. Average data for the blots are shown in Fig. 4*B*. Because the plasma insulin concentrations during the insulin infusions were ~40% higher in the obese subjects, Akt phosphorylation was expressed relative to the protein content and plasma insulin concentration (Fig. 4*B*). Akt Ser^{473} phosphorylation was significantly reduced in the obese subjects after either 30 or 45–60 min of insulin infusion (Fig. 4*B*). Because each study had a basal time point, values from all basal biopsies were pooled. As for ceramides, data from the 45- and 60-min time points did not differ from each other and so they were pooled for analysis. Table 5 shows Akt Ser^{473} phosphorylation data expressed relative to Akt protein content, without regard to insulin concentration during the clamp. Results of these analyses showed that, under basal conditions, Akt Ser^{473} phosphorylation was slightly, but not significantly, decreased in the obese subjects. After 30 min of insulin infusion, however, Akt phosphorylation was stimulated in both groups of subjects (Table 5). In lean insulin-sensitive subjects, Akt phosphorylation remained elevated for 45–60 min of insulin infusion. In contrast, Akt phosphorylation after 45–60 min of insulin infusion in the obese subjects decreased significantly ($P < 0.05$) compared with the 30-min value for that group and was also significantly decreased compared with the lean control group (Table 5, $P < 0.05$). There were no differences in Akt protein expression between the two groups (Table 5). Correlation analysis between total ceramide content under basal conditions and insulin stimulation of Akt Ser^{473} phosphorylation revealed a modest negative correlation ($r = -0.45$, $P = 0.09$).

DISCUSSION

Insulin resistance characterizes skeletal muscle in obesity and type 2 diabetes. Increasing evidence supports the notion that in insulin-resistant muscle, abnormalities in lipid use lead to increased myocellular lipid content (30). The increase in lipid content is believed to result in decreases in insulin receptor signaling and insulin resistance (13,31). Although the increase in lipid content is manifested as an increase in triacylglycerol (2–6), it is likely that it is not triacylglycerol itself but some other less abundant lipid species that are the proximal cause of insulin resistance. One candidate for such a lipid species is ceramide. Ceramides are a family of sphingolipids that differ in the fatty acyl moiety and are known to induce insulin resistance and apoptosis in cultured cells (7,17–20). As an intermediate in the sphingomyelin pathway, ceramide can be generated by hydrolysis of sphingomyelin or can be produced by de novo synthesis (32). In cultured C2C12 cells, a cell-permeable ceramide analog decreased insulin-stimulated glucose uptake, glycogen synthesis, and Akt serine phosphorylation, but not insulin receptor and IRS-1 tyrosine phosphorylation and IRS-1–associated PI 3-kinase activity (7). Because these abnormalities mir-

TABLE 4
Sphingolipid content of skeletal muscle

	n	Sphingomyelin	Sphingosine	Sphinganine	Sphingosine 1-phosphate	Sphinganine 1-phosphate
Lean						
Basal	8	440 ± 44	124 ± 21	3.1 ± 0.5	93 ± 15	6.0 ± 0.9
Insulin	8	359 ± 31	139 ± 22	2.4 ± 0.5	93 ± 9	6.6 ± 0.7
Obese						
Basal	9	514 ± 66	136 ± 17	4.2 ± 0.9	107 ± 14	9.1 ± 2.1
Insulin	9	400 ± 61	136 ± 22	3.2 ± 0.4	99 ± 16	6.1 ± 1.0

Data are means ± SE in units of picomoles per 2 mg muscle wet weight. Sphingomyelin is presented as total content, including all fatty acid species.

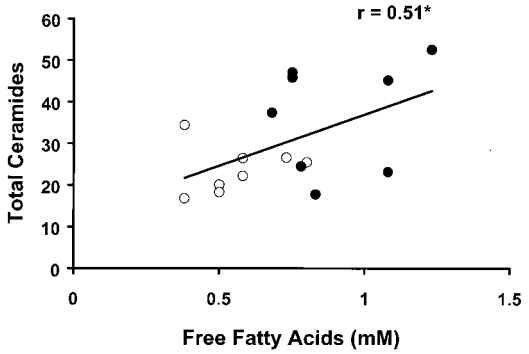

FIG. 3. Correlation between fasting plasma FFA concentration and total ceramide content of muscle under basal conditions. ○, Lean subjects; ●, obese subjects. The regression shown is for all subjects pooled. *$P < 0.05$.

rored those produced by incubation of the cells with palmitate, de novo synthesis of ceramide was invoked as the mechanism of palmitate-induced insulin resistance (7). Recent studies have confirmed the role of ceramide in inhibition of Akt and downstream signaling, but not IRS-1 function, in 3T3-L1 adipocytes (33) or L6 muscle cells (34). However, the results from another study indicate that ceramides may also inhibit insulin receptor signaling upstream of Akt, at the level of IRS-1 (19). Moreover, infusion of a triglyceride emulsion into rodents inhibited IRS-1–associated PI 3-kinase activity, possibly via Ser^{307} phosphorylation of IRS-1 (13), and this may also occur in human muscle (14). These latter abnormalities were unaccompanied by increased muscle ceramide concentrations; rather, increases in fatty acyl CoA and diacylglycerol were invoked as potential explanations (13). Whether ceramide plays a role in insulin resistance in human muscle is unknown.

Skeletal muscle from insulin-resistant subjects displays a wide variety of abnormalities in insulin receptor signaling, including decreased tyrosine phosphorylation of the insulin receptor and IRS-1, diminished IRS-1–associated PI 3-kinase activity, and impaired phosphorylation of Akt on Thr^{308} and Ser^{473} (26,27). Moreover, infusion of a triglyceride emulsion induces insulin resistance (27) and decreases IRS-1–associated PI 3-kinase activity during an insulin infusion (31). Although various lipid species have been proposed to be relevant to insulin resistance (13,15,16), little data are available in human muscle. The present study was undertaken, in part, to determine whether ceramide levels are increased in skeletal muscle from obese insulin-resistant subjects. We used a highly sensitive high-performance liquid chromatography electrospray ionization tandem mass spectrometry technique to analyze the content of individual ceramide species and other sphingolipids (29). The major new finding of this study is that ceramide concentrations are increased nearly twofold in muscle from the obese subjects. Furthermore, the results show that ceramide concentrations were increased in the obese subjects regardless of the nature of the fatty acyl moiety. This twofold increase was similar to the increase in ceramide produced by incubation of C2C12 cells with palmitate, a maneuver that resulted in insulin resistance (decreased insulin-stimulated glycogen synthesis) and decreased insulin-stimulated Akt phosphorylation in these cells (7). The increase in ceramide content of the obese muscle was specific because there were no differences in muscle content of sphingomyelin or the sphingoid bases. Because of the lack of increase in dihydroceramide, sphingosine, and sphinganine, the present data provide no evidence that the increase in ceramide is due to de novo synthesis. However, the increase in plasma FFA concentration observed in this study and its correlation with the muscle ceramide content suggest that increased substrate

TABLE 5
Effect of insulin infusion on Akt phosphorylation and protein expression

		Time (min)	
	Basal	30	45–60
Phospho-Akt/protein (arbitrary units)			
Lean	0.12 ± 0.05	0.83 ± 0.18	0.70 ± 0.12
Obese	0.06 ± 0.03	0.71 ± 0.08	0.40 ± 0.10*†
Akt protein (arbitrary density units)			
Lean	3.88 ± 0.68	3.00 ± 0.72	4.22 ± 0.68
Obese	4.46 ± 0.74	5.34 ± 1.0	4.38 ± 0.57

Data are means ± SE. For phospho-Akt/protein, data are presented as the ratio of the density of the anti–phospho-Ser^{473} blot to that of the anti–Akt protein expression blot. Akt protein is presented as arbitrary density units. *$P < 0.05$ vs. lean group; †$P < 0.05$ vs. 30-min time point for the obese group.

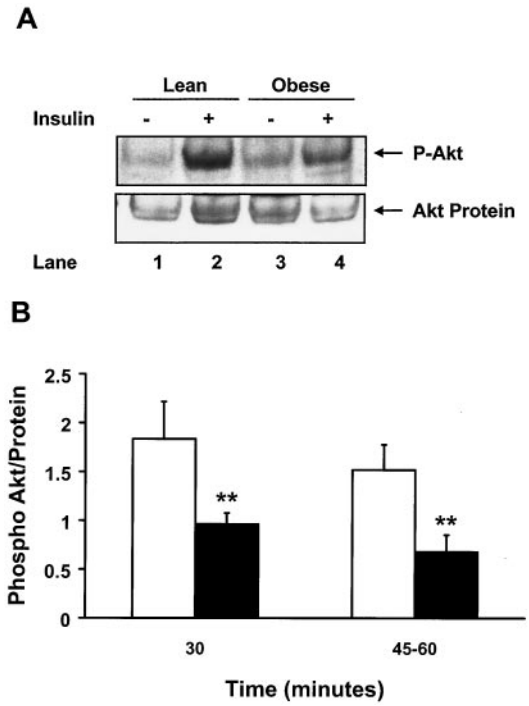

FIG. 4. Insulin stimulation of Akt Ser^{473} phosphorylation in muscle of lean and obese subjects. Muscle proteins were resolved by SDS-PAGE, and the proteins were transferred electrophoretically to nitrocellulose membranes. Membranes were probed with anti–phospho-Ser^{473} antibody. The membranes were stripped and reprobed for Akt protein. *A*: Insulin stimulation of Akt Ser^{473} phosphorylation in representative lean (*lanes 1* and *2*) and obese (*lanes 3* and *4*) subjects infused with insulin for 60 min. Biopsies were taken basally and at the end of 60 min. *B*: Mean data ± SE for lean (□) and obese (■) subjects from quantification of digitally scanned immunoblots. Data are presented as the ratio of phospho-Akt signal to the Akt protein expression level, corrected for the plasma insulin concentration achieved during the 30-, 45-, and 60-min insulin infusions. **$P < 0.01$ vs. lean control subjects.

for ceramide synthesis is available. On the other hand, the sphingomyelin content of obese muscle was somewhat higher than that in lean control subjects, and these data suggest that the likely source of ceramide elevation is via turnover of sphingomyelin. Such a situation would be consistent with an increased response of muscle to inflammatory cytokines such as TNF-α, which is known to be increased in obesity. However, statistical analysis taking into account the variability in the measured amounts does not make this conclusion unequivocal. Additional studies will be required to determine whether the increased ceramide content in skeletal muscle of obese subjects is due to de novo synthesis or sphingomyelinase activity.

As expected, during insulin infusion, the obese subjects exhibited reduced insulin-stimulated glucose metabolism, as assessed by a decreased rate of glucose infusion required to maintain euglycemia. Because all insulin infusions were ≤60 min in duration, it is possible that a portion of the decrease in the glucose infusion rate in the obese subjects may have been due to impaired suppression of endogenous glucose output. However, that could not have accounted for all of the decrease in the glucose infusion rate, and, in addition, there is ample additional evidence that obese subjects are insulin resistant (26). As a marker of an effect of ceramide on insulin receptor signaling, we assessed insulin stimulation of phosphorylation of Akt Ser^{473}. The results showed that there was a modest reduction in basal Akt phosphorylation and a dramatic impairment of insulin stimulation of Akt Ser^{473} phosphorylation. This is similar to the findings of Krook et al. (35) in patients with type 2 diabetes and is consistent with the decrease in upstream signaling observed in obese subjects (26,27). Taken together, the pattern of increased ceramide concentrations and decreased Akt phosphorylation is consistent with the idea that elevated ceramides contribute to insulin resistance in skeletal muscle. However, the situation is clearly more complex in vivo in human muscle than in cultured cells. As noted above, the actions of ceramide on insulin signaling have been reported to occur at the level of Akt as well as further upstream. In the present study, the relationship between ceramide levels and Akt activation was examined, and upstream events were not characterized. However, in previous studies, we characterized upstream insulin receptor signaling events in obese subjects and found reduced insulin stimulation of IRS-1 tyrosine phosphorylation and association of PI 3-kinase activity with IRS-1 (26). Although muscle ceramide levels were not measured in those studies, the subjects were similar, and we could expect that the obese subjects who took part in the present study also had reduced IRS-1 function. The relationship between increased ceramide concentration and IRS-1 function in human muscle remains to be investigated.

Several mechanisms have been described to explain the effect of ceramides to inhibit Akt activity. Earlier studies suggested that TNF-α, acting through ceramide, increased the activity of a membrane-bound proline-directed serine/threonine kinase (36), later identified as c-jun NH_2-terminal kinase (37). In rat FAO hepatoma cells, sphingomyelinase, cell-permeable ceramide, and TNF-α all produced dramatic decreases in IRS-1 tyrosine phosphorylation and association of the p85 regulatory subunit of PI 3-kinase

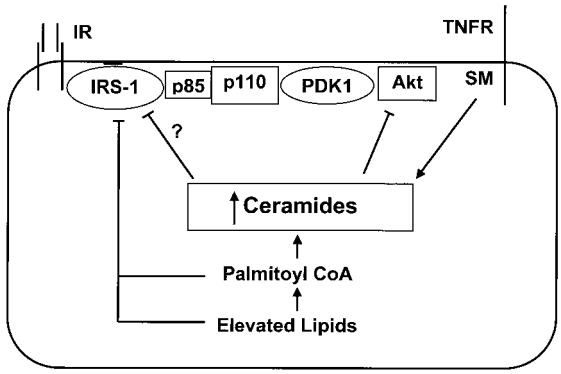

FIG. 5. Model for effects of increased ceramides on insulin signaling. p85 and p110 are regulatory and catalytic subunits of PI 3-kinase. IR, insulin receptor; PDK1, PI phosphate-dependent kinase 1; SM, sphingomyelin; TNFR, TNF receptor.

with IRS-1 (19). More recently, a cell-permeable ceramide analog was used to show that in a neural-derived (HMN1) cell line and in COS-7 cells, ceramide-induced decreases in Akt activity were not due to activation of an Akt phosphatase or direct inhibition of Akt; on the other hand, ceramide had no effect on signaling through IRS-1 and PI 3-kinase (20). More recent studies using brown adipocytes showed that ceramide mediated insulin resistance induced by TNF-α and activated an okadaic acid–inhibitable protein phosphatase (38). Finally, Hajduch et al. (18) also used a cell-permeable ceramide in L6 muscle cells to show that ceramide induced an abnormality in membrane localization of Akt. Thus, most, if not all, studies report that ceramides, whether added exogenously, produced by de novo synthesis from palmitate, or generated by sphingomyelinase activity, inhibit Akt activity. However, the findings differ regarding whether ceramides inhibit insulin signaling through IRS-1. The reasons for the differences are not clear but are likely to involve differences among cell types.

The present results taken together with previous findings suggest the following model for the etiology of abnormalities in insulin signaling in muscle from obese insulin-resistant subjects (Fig. 5). The increase in ceramide content is sufficient to account for decreased insulin stimulation of Akt activity, which could, in theory, itself lead to a reduction in insulin-stimulated glucose uptake. However, muscle from obese subjects also displays marked abnormalities in insulin receptor and IRS-1 tyrosine phosphorylation and decreased association of PI 3-kinase activity with IRS-1 (26,27). The abnormality in insulin receptor function is unlikely to be due to increased ceramides but may be secondary to increases in fatty acyl CoA or diacylglycerol (13,15,16). The abnormality in IRS-1 function may be due in part to the action of ceramides (19), but it also is likely to be due to the influences of other lipid abnormalities (13) and inherited influences (39). The relative contributions of these abnormalities to decreased insulin-stimulated glucose uptake in skeletal muscle remain to be defined.

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REFERENCES

1. Schalch DS, Kipnis DM: Abnormalities in carbohydrate tolerance associated with elevated plasma nonesterified fatty acids. *J Clin Invest* 44:2010–2020, 1965
2. Pan DA, Lillioja S, Kriketos AD, Milner MR, Baur LA, Bogardus C, Jenkins AB, Storlein LH: Skeletal muscle triglyceride levels are inversely correlated with insulin action. *Diabetes* 46:983–988, 1997
3. Goodpaster BH, Thaete FL, Simoneau J-A, Kelley DE: Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivity. *Diabetes* 46:1579–1585, 1997
4. He J, Watkins S, Kelley D: Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* 50:817–823, 2001
5. Phillips DI, Caddy S, Ilic V, Fielding VA, Frayn KA, Borthwick AC, Taylor R: Intramuscular triglyceride and insulin sensitivity: evidence for a relationship in nondiabetic subjects. *Metabolism* 45:947–950, 1996
6. Szcepaniak LS, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD, Stein DT: Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo. *Am J Physiol* 276:E977–E989, 1999
7. Schmitz-Pfeiffer C, Craig DL, Biden TJ: Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. *J Biol Chem* 274:24202–24210, 1999
8. Felber J-P, Vanotti A: Effects of fat infusion on glucose tolerance and insulin plasma levels. *Med Exp Int J Exp Med* 10:153–156, 1964
9. Pelkonen R, Miettinen TA, Taskinen M-R, Nikkila EA: Effect of acute elevation of plasma glycerol, triglyceride and FFA levels on glucose utilization and plasma insulin. *Diabetes* 17:76–82, 1968
10. Thiebaud D, DeFronzo RA, Jacot E, Golay A, Acheson K, Maeder E, Jequier E, Felber J-P: Effect of long chain triglyceride infusion on glucose metabolism in man. *Metabolism* 31:1128–1136, 1982
11. Ferranini E, Barrett EJ, Bevilacqua S, DeFronzo RA: Effect of fatty acids on glucose production and utilization in man. *J Clin Invest* 72:1737–1747, 1983
12. Boden G, Jadali F, White J, Liang Y, Mozzoli M, Chen X, Coleman E, Smith C: Effect of fat on insulin-stimulated carbohydrate metabolism in normal men. *J Clin Invest* 88:960–966, 1991
13. Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI: Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103:253–259, 1999
14. Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI: Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 97:2859–2865, 1996
15. Itani SI, Ruderman NB, Schmieder F, Boden G: Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IκB-α. *Diabetes* 51:2005–2011, 2002
16. Ellis BA, Poynten A, Lowy AJ, Furler SM, Chisholm DJ, Kraegen EW, Cooney GJ: Long-chain acyl-CoA esters as indicators of lipid metabolism and insulin sensitivity in rat and human muscle. *Am J Physiol* 279:E554–E560, 2000
17. Cazzolli R, Carpenter L, Biden TJ, Schmitz-Peiffer C: A role for protein phosphatase-2a–like activity, but not atypical protein kinase Cζ, in the inhibition of protein kinase B/Akt and glycogen synthesis by palmitate. *Diabetes* 50:2210–2218, 2001
18. Hajduch E, Balendran A, Batty IH, Litherland GJ, Blair AS, Downes CP, Hundal HS: Ceramide impairs the insulin-dependent membrane recruitment of protein kinase B leading to a loss in downstream signalling in L6 skeletal muscle cells. *Diabetologia* 44:173–183, 2001
19. Kanety H, Hemi R, Papa MZ, Karasik A: Sphingomyelinase and ceramide suppress insulin-induced tyrosine phosphorylation of the insulin receptor substrate-1. *J Biol Chem* 271:9895–9897, 1996
20. Zhou H, Summers S, Birnbaum MJ, Pittman RN: Inhibition of Akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis. *J Biol Chem* 273:16568–16575, 1998
21. Hannun YA, Obeid LM: The ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J Biol Chem* 277:25847–25850, 2002
22. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87–91, 1993
23. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995
24. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271:665–668, 1996
25. DeFronzo RA, Tobin J, Andres R: Glucose clamp technique: a method for quantifying insulin secretion and insulin resistance. *Am J Physiol* 237: E214–E223, 1979
26. Cusi K, Maezono K, Osman A, Pendergrass M, Patti M, Pratipanawatr T, DeFronzo R, Kahn C, Mandarino L: Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J Clin Invest* 105:311–320, 2000
27. Krook A, Bjornholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Wallberg-Henriksson H, Zierath JR: Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes* 49:284–292, 2000
28. Lowry OH, Rosebrough NJ, Fan AL, Randall R: J: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275, 1951
29. Sullards MC, Merrill AH Jr: Analysis of sphingosine 1-phosphate, ceramides, and other bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Science STKE* 67:1–11, 2001
30. Colberg S, Simoneau J-A, Thaete FL, Kelley DE: Impaired FFA utilization by skeletal muscle in women with visceral obesity. *J Clin Invest* 95:1846–1853, 1995
31. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim J, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI: Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277:50230–50236, 2002
32. Merrill AH: De novo sphingolipid biosynthesis: a necessary, but dangerous, pathway. *J Biol Chem* 277:25843–25846, 2002
33. Summers SA, Garza LA, Zhou H, Birnbaum M: Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. *Mol Cell Biol* 18:5457–5464, 1998
34. Chavez JA, Knotts TA, Wang L, Li G, Dobrowsky RT, Florant GL, Summers SA: A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. *J Biol Chem* 278:10297–10303, 2003
35. Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H: Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. *Diabetes* 47:1281–1286, 1998
36. Liu J, Mathias S, Yang Z, Kolesnick RN: Renaturation and tumor necrosis factor-α stimulation of a 97-kDa ceramide-activated protein kinase. *J Biol Chem* 269:3047–3052, 1994
37. Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN: Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature* 380:75–79, 1996
38. Teruel T, Hernandez R, Lorenzo M: Ceramide mediates insulin resistance by tumor necrosis factor-α in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. *Diabetes* 50:2563–2571, 2001
39. Pratipanawatr W, Pratipanawatr T, Cusi K, Berria R, Adams JM, Jenkinson CP, Maezono K, DeFronzo RA, Mandarino LJ: Skeletal muscle insulin resistance in normoglycemic subjects with a strong family history of type 2 diabetes is associated with decreased insulin-stimulated insulin receptor substrate-1 tyrosine phosphorylation. *Diabetes* 50:2572–2578, 2001