Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of lipid metabolites

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Lee, Jong Sam, Srijan K. Pinnamaneni, Su Ju Eo, In Ho Cho, Jae Hwan Pyo, Chang Keun Kim, Andrew J. Sinclair, Mark A. Febbraio, and Matthew J. Watt. Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of lipid metabolites. J Appl Physiol 100: 1467–1474, 2006. First published December 15, 2005; doi:10.1152/japplphysiol.01438.2005.—Consumption of a Western diet rich in saturated fats is associated with obesity and insulin resistance. Saturated fatty acids are also associated with the development of insulin resistance (1, 23, 36, 51); however, the effects of markedly altering the type of fatty acid is also critical. Epidemiological evidence suggests that the consumption of a Western diet that is high in saturated fats closely correlates with the development of insulin resistance in humans (26, 33). Direct examination in muscle cells in vitro indicates that saturated fatty acids cause insulin resistance whereas unsaturated fatty acids exert a protective effect or even improve insulin sensitivity (9, 29). Saturated fats are less readily oxidized (14, 24) and accumulate as DAG and ceramide in vitro, whereas mono- and polyunsaturated fatty acids accumulate as IMTG or free fatty acids (FFA) (14, 29, 41), thus providing a link between fatty acid subtype and insulin resistance. Numerous dietary studies in rodents and humans indicate that saturated fat significantly worsens insulin resistance, whereas monounsaturated and polyunsaturated fatty acids have a less pronounced effect or even improve insulin sensitivity (for review, see Ref. 37).

The biochemical and molecular processes linking saturated fats to insulin resistance remain unresolved but may relate to altered membrane phospholipid fatty acid composition and membrane fluidity and stability (43), changes in lipogenic gene transcription (10), the type of fatty acids within TAG (28), and direct interference with insulin signaling (8, 21, 41, 45, 51). Lipid metabolites that interfere with insulin signal transduction accumulate in tissues of insulin-resistant animals (23, 36, 40, 47, 51); however, the effects of markedly altering the types of dietary fatty acids on the accumulation of these lipid metabolites in whole animals are unknown. The primary aim of the present study was to examine the role of diets rich in either

diacylglycerol; ceramide; L6 myotube; stearoyl CoA desaturase 1

THE EXCESSIVE ACCUMULATION of triacylglycerol (TAG) in obesity is associated with the development of insulin resistance and Type 2 diabetes. Skeletal muscle is a major site for insulin-stimulated glucose disposal (4), and the accumulation of TAG within lipid droplets in skeletal muscle is positively correlated to the severity of insulin resistance (13, 22, 32). However, insulin sensitivity and intramyocellular TAG (IMTG) increase after endurance exercise training (6, 15, 18), and TAG storage protects Chinese hamster ovary cells from lipotoxicity (25), indicating that IMTG per se does not cause insulin resistance but may be a proxy for other lipid metabolites that directly interfere with insulin signaling. Indeed, skeletal muscle insulin resistance is mediated by intramyocellular accumulation of fatty acyl-CoA metabolites, such as diacylglycerols (DAG) and ceramides, which are elevated in insulin-resistant states (1, 23, 36, 51) and directly interfere with insulin signal transduction (8, 21, 41, 45, 51).

Whereas increased availability of fatty acids has been linked to skeletal muscle insulin resistance (3, 5), it appears that the type of fatty acid is also critical. Epidemiological evidence suggests that the consumption of a Western diet that is high in saturated fats closely correlates with the development of insulin resistance in humans (26, 33). Direct examination in muscle cells in vitro indicates that saturated fatty acids cause insulin resistance whereas unsaturated fatty acids exert a protective effect or even improve insulin sensitivity (9, 29). Saturated fats are less readily oxidized (14, 24) and accumulate as DAG and ceramide in vitro, whereas mono- and polyunsaturated fats accumulate as IMTG or free fatty acids (FFA) (14, 29, 41), thus providing a link between fatty acid subtype and insulin resistance. Numerous dietary studies in rodents and humans indicate that saturated fat significantly worsens insulin resistance, whereas monounsaturated and polyunsaturated fatty acids have a less pronounced effect or even improve insulin sensitivity (for review, see Ref. 37).

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saturated or polyunsaturated fatty acids on skeletal muscle fatty acid metabolite accumulation and whole-body insulin sensitivity. We hypothesized that a diet high in saturated fatty acids would induce insulin resistance and increase the contents of skeletal muscle DAG and ceramide, whereas a diet high in polyunsaturated fatty acids would not affect DAG and ceramide contents or impact on insulin sensitivity. To confirm the biochemical responses to fatty acids, independent of potential changes in metabolic or hormonal fluxes, we also incubated rat L6 myotubes, an in vitro skeletal muscle model, with saturated and unsaturated fatty acids and hypothesized that the in vivo effects would persist in vitro.

Stearoyl CoA desaturase 1 (SCD1) is an endoplasmic reticulum-bound enzyme that converts saturated fatty acids (primarily 16:0; 18:0) to monounsaturated fatty acids. Global SCD1 deficiency produces a lean, obesity-resistant, insulin-sensitive phenotype (31); however, preventing the ability to desaturate fatty acids should lead to insulin resistance because saturated fats accumulate as DAG and ceramide. Although polyunsaturated fatty acids reduce and saturated fatty acids increase SCD1 expression in liver (30), the role of dietary fatty acids on SCD1 expression and activity in skeletal muscle is unknown, despite the importance of skeletal muscle in fuel metabolism and insulin resistance. Accordingly, the second aim of this study was to investigate the role of dietary fatty acids on skeletal muscle SCD1 gene and protein expression in vivo.

METHODS

Animal Experiments

Twenty-four male Sprague-Dawley rats (initial body mass 95–110 g) were obtained from Samtako, Bio Korea (Kyong-Ki, Korea) and housed four per cage in an environmentally controlled laboratory (temperature 22 ± 1°C and relative humidity 55 ± 2%) with a 12:12-h light-dark cycle (light, 0700 to 1900). In the week before any experimental intervention, animals were given ad libitum access to standard rodent chow (Samtako, Bio Korea). Food intake was not determined in this study because the polyunsaturated fatty acid diet was too “liquid” to obtain accurate measures of the food remaining in cages. The Animal Ethics Committee of Korea National Sport University approved all procedures performed in this study.

After 1 wk, rats were divided into one of three experimental diet groups. Control rats (Con; n = 8) were fed standard rat chow (61.8% carbohydrate, 15.7% fat, 22.5% protein expressed as percentage of total caloric intake) purchased from Samtako (Bio Korea). The other groups consumed high-fat diets (24.3% carbohydrate; 52.8% fat; 22.9% protein) that consisted primarily of saturated fatty acids (Sat; n = 8) derived from lard and coconut oil or polyunsaturated fatty acids (PUFA; n = 8) derived from safflower oil (ICN Biomedicals). The main fatty acid in the PUFA diet was linoleic acid (18:2, n-6). Animals were provided with food and water ad libitum throughout the experimental period. Animals were maintained on their diets for 8 wk before testing.

Oral Glucose Tolerance Test

After the 8-wk experimental period, rats were overnight fasted and weighed and underwent an oral glucose tolerance test (OGTT). Glucose (20% wt/vol solution) was administered by oral gavage at a dose of 2.0 g/kg body mass, and blood was obtained from a tail vein in unrestrained conscious rats before glucose administration and at 15, 30, 60, and 120 min thereafter. At least 2 days after OGTT and after an overnight fast (10 h), rats were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg body mass) and the vastus lateralis muscle was rapidly dissected out, immediately frozen in liquid nitrogen, and stored at −80°C until analyses. A blood sample was drawn from the femoral artery, and rats were killed by heart removal.

Analyses

Measurement of skeletal muscle lipid metabolites. IMTG content was analyzed as previously described (49). Freeze-dried muscle was powdered and cleaned of all visible connective tissue and blood under magnification. Lipid was extracted by a Folch extraction, the TAG was saponified in an ethanol-KOH solution at 60°C, and glycerol content was determined fluorometrically. DAG and ceramide were extracted and quantified according to the methods of Preis et al. (35) with minor modifications. Briefly, lipids were extracted from freeze-dried, powdered muscle by using chloroform-methanol-PBS + 0.2% SDS (1:2.0.8 vol/vol/vol). DAG kinase and 32P-ATP (15 mCi/mmol cold ATP) were added to extracts, and the reaction was stopped with chloroform-methanol (2:1). Samples were spotted onto thin-layer chromatography (TLC) plates and developed to two-thirds of the total plate length in a solvent consisting of chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10). Bands corresponding to DAG and ceramide were identified against standards after phospho-imaging, dried, scraped from the TLC plate, and counted in a liquid scintillation analyzer (Tri-Carb 2500TR, Packard, Canberra, Australia).

Determination of TAG fatty acid composition. Freeze-dried muscle (~100 mg) was extracted with chloroform-methanol (2:1 by volume) containing 10 mg/l of butylated hydroxytoluene. After storage of the samples overnight at 4°C, each sample was filtered, rinsed with an additional volume of extracting solvent, and partitioned against 0.9% NaCl. This was evaporated under nitrogen gas, and the lipids were reconstituted in chloroform. Samples were spotted onto TLC plates and developed in a solvent consisting of petroleum ether-diethyl ether-acetic acid (85:15:3 vol/vol/vol), and the TAG fraction was identified against a standard (Nu-Chek, 18.5A) and scraped into a vial. Methyl esters of fatty acids of the TAG extract were prepared by transmethylation using 5% H2SO4 in methanol. The fatty acid methyl esters were separated by capillary gas liquid chromatography using a 50 m × 0.32 mm (ID) fused silica, bonded-phase column (BPX70, SGE, Melbourne, Australia) with helium as carrier gas at a flow rate of 43 cm/s. The column oven was maintained at 125°C for 3 min and increased at a rate of 8°C/min to 220°C and this temperature was maintained for the duration of the run. Fatty acids were identified by comparison with standard mixtures of fatty acid methyl esters and the results are expressed as percent of the total TAG fraction.

Evaluation of mRNA content by RT-PCR. Approximately 20 mg of muscle were extracted for total RNA by a modification of the acid guanidium thiocyanate-phenol chloroform extraction method. The total RNA was quantified by spectrophotometry (Hitachi U 2000 spectrophotometer), and 100 ng of each total RNA sample were reverse transcribed. Control samples were also analyzed where all the above reagents are added to RNA samples except the MultiScribe reverse transcriptase. The reverse transcription reactions were performed by use a GeneAmp PCR system 2400 (Perkin-Elmer, Wellesley, MA) with conditions at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Two milliliters of 0.5 mmol/l EDTA (pH 8.0) were added to each sample and stored at −20°C until further analysis. Real-time PCR was employed to quantitate rat SCD1 mRNA. Rat probe and primers were designed (Primer Express version 1.0 Applied Biosystems) from the rat gene sequence accessed from Gen-Bank/EMBL (NM_139192). The sequences of the primers and probe were as follows: forward primer, 5'-CAC GCC GAC CCT CAC AA-3'; reverse primer, 5'-TCT TTG ACA GCC GGG TTG TT-3'; Taqman probe, 5'-TTC TTC TCT CAC GTG GGT 'TGG CTG CTT-3'. Ribosomal 18S was used as a constitutive housekeeping control.
Dietary Fats, Muscle Lipids, and Insulin Resistance

SCD1 Is Increased by a Saturated Fatty Acid Diet

SCD1 mRNA was unaffected by a diet high in polysaturated fatty acids and tended (P = 0.11) to increase with saturated fatty acid feeding (Fig. 4A). We examined SCD1 protein expression by Western blot and report increased SCD1 in Sat compared with Con and PUFA (Fig. 4B). An index of SCD1 enzyme activity, which was calculated as the relative amount of product (16:1) to the corresponding substrate (16:0),

<table>
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<th>Con</th>
<th>Sat</th>
<th>PUFA</th>
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<tr>
<td>FFA, μmol/l</td>
<td>354±29</td>
<td>460±23*</td>
<td>448±25*</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>3.99±0.16</td>
<td>4.61±0.15</td>
<td>4.26±0.10</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>27±5</td>
<td>76±24*</td>
<td>13±3†</td>
</tr>
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Values are means ± SE; n = 8. Con, chow diet; Sat, saturated fatty acid high-fat diet; PUFA, polysaturated fatty acid high-fat diet. *Different from Con, †different from Sat, P < 0.05.
was increased ($P < 0.05$) in Sat compared with Con and reduced ($P < 0.05$) in PUFA (Fig. 4D).

**Effect of Palmitate and Linoleate on Fatty Acid Metabolite Content and Glucose Uptake in L6 Myotubes**

To enable biochemical studies to be conducted in the absence of possible confounding factors, such as alterations in circulating metabolites and hormones, we performed experiments in L6 myotubes. Although the metabolic action of these cells to insulin is blunted and direct comparisons with in vivo studies should be interpreted with caution, L6 myotubes nevertheless possess the proteins required for fatty acid and glucose uptake and metabolism. Glucose uptake was determined in L6 myotubes after 5 h treatment with 0.5 mM palmitate (saturated) or linoleate (polyunsaturated) fatty acids. Palmitate decreased insulin-stimulated glucose uptake, whereas linoleate pretreatment did not affect glucose uptake (Fig. 5A). The addition of palmitate to culture media resulted in significant DAG (5-fold) and ceramide (2-fold) accumulation (Fig. 5, C and D). TAG content determined by fluorometric analysis after 5-h incubation revealed a small increase with palmitate (Fig. 5B). TAG was increased by linoleate treatment (Fig. 5B), whereas DAG and ceramide content were unchanged (Fig. 5, C and D). These data indicate that palmitate-induced ceramide and DAG accumulation are associated with insulin resistance in skeletal muscle cell culture (8, 29, 41). They also indicate that linoleate promotes synthesis of TAG and does not affect insulin-stimulated glucose uptake.

**DISCUSSION**

The present study demonstrates that a diet high in saturated fat induces muscle DAG accumulation and saturation of the TAG pool and is associated with whole-body insulin resistance. Conversely, animals fed a diet high in n-6 polyunsaturated fat retained insulin sensitivity despite small increases in muscle DAG, which may result from enhanced TAG storage. Thus fatty acid oversupply per se is not the major determinant of fat-induced insulin resistance, but rather dietary fat composition (42, 43).

An important caveat of the present study relates to the interpretation of insulin resistance. The OGTT and HOMA-IR assessments do not allow for assessment of tissue-specific...
effects on insulin-stimulated glucose uptake. Although skeletal muscle accounts for >80% of insulin-mediated glucose disposal (4) and is likely to represent events occurring at the whole-body level, we cannot definitively conclude that the observed whole-body changes are due to altered skeletal muscle function. In this regard, it appears that glucose intolerance may only become apparent when insulin resistance occurs in numerous tissues (50). Thus the present data relating to insulin resistance should be interpreted with these considerations in mind.

DAGs and ceramides antagonize insulin signaling in vitro, and this is the first study to directly examine the effects of dietary fatty acid composition on the accumulation of these lipid metabolites in vivo. DAGs are increased in fatty acid-induced insulin resistance (19, 51) and are proposed to interfere with insulin signaling via novel PKC activation (51). In the present study, DAG was elevated in Sat and occurred concomitantly with insulin resistance, observations that are consistent with the present and previous studies in muscle cell culture that ascribe a direct role for saturated, but not unsaturated, fatty acids in this process (7, 9, 14, 29). However, our in vivo finding that DAGs were moderately elevated in PUFA rats, where insulin action was improved, indicates that total DAG accumulation may not be essential for insulin resistance and that other factors such as a critical threshold of intracellular DAG, cellular localization of DAG, or the molecular DAG species may be important mediators of insulin resistance. Collectively, these data indicate that an abundance of dietary saturated fatty acids induce insulin resistance, possibly via a marked increase in DAG content, whereas smaller increases in DAG were not associated with insulin resistance in animals fed an isocaloric diet rich in polyunsaturated fatty acids.

Ceramide is a second messenger in the sphingomyelin signaling pathway and is produced via the hydrolysis of sphingomyelin and from de novo synthesis (palmitoyl-CoA and serine). Ceramides antagonize insulin signaling (8, 41, 45), and ceramide accumulation correlates with the development of skeletal muscle insulin resistance in vivo (1, 16, 44, 47). In contrast with our hypothesis, dietary fatty acid composition did not affect muscle ceramide content. This was unexpected because long-chain saturated fatty acids are thought to exclusively act as the substrate for ceramide de novo synthesis (46); however, a recent study that infused a lipid emulsion primarily composed of linoleate (18:2) induced ceramide generation and insulin resistance in humans (44). A dissociation between ceramide content and insulin sensitivity has been observed previously (19, 23, 51) and, consistent with the present findings, indicates that ceramides may not induce insulin resistance in vivo.

IMTG content positively correlates with insulin resistance (13, 22, 32), which has led to the assumption that excess IMTG storage is undesirable. However, in some circumstances, such
as after endurance exercise training, increased TAG is associated with enhanced insulin sensitivity (6, 15, 18, 44). In the present study, IMTG was increased in rats fed a Sat diet and, to a greater extent, in rats on the PUFA diet. These data are consistent with our studies in L6 myotubes demonstrating marked increases in IMTG with linoleate compared with palmitate treatment. Unsaturated fats promote TAG accumulation in various cell types (e.g., cardiac, pancreatic, Chinese hamster ovary cells) (see Ref. 25), and this and previous studies in skeletal muscle culture (29) have demonstrated increased TAG storage after incubation with unsaturated fats. Thus unsaturated fatty acids may protect cells from “lipotoxicity” by promoting uptake of fatty acids into an inert triglyceride lipid pool, rather than bioactive lipids such as DAGs and ceramides (25). The observations in PUFA rats of elevated muscle TAG and reduced DAG content, lower basal plasma insulin, and enhanced insulin sensitivity compared with Sat supports this possibility. The reason(s) for IMTG accumulation was not addressed in this study but may relate to the preference of DAG acyltransferase (converts DAG to TAG) for unsaturated rather than saturated fatty acids (12, 39).

The fatty acid profile of different body tissues at least partially reflects the fatty acid composition of the diet. In this study we compared the fatty acid profile of TAG between groups. The 8-wk Sat diet increased the degree of saturation within skeletal muscle TAG, which was due to increases in short (12:0, 14:0)- and long (18:0)-chain fatty acids. Whether an increase in TAG saturation contributes to insulin resistance in unknown. It is possible that the excess stearate (18:0) within this pool is hydrolyzed and provides a substrate for DAG and ceramide production, thereby affecting insulin signaling. In contrast with Sat, PUFA increased the degree of unsaturation within IMTG, which was primarily due to increased linoleate (18:2). Increased unsaturation of the muscle membrane fatty acids is associated with improved insulin sensitivity (43), and the lipid-lowering agent bezafibrate reduces the saturation in muscle TAGs and improves insulin sensitivity (28). Here, we show that a greater degree of desaturation in TAG is associated with improved insulin sensitivity, and, conversely, an increase in saturation correlates with insulin resistance.

SCD1 converts SATs to monounsaturated fatty acids and impacts on metabolic function. Our current understanding of the biological role of SCD1 derives from studies in liver and others conducted in SCD1-null (ab/lb, SC1-1/-) mice (11, 31) that demonstrate SCD1 deficiency to induce a leaner phenotype and resistance to diet-induced obesity (11, 31). There are very limited data describing SCD1 regulation in skeletal muscle, a tissue that displays marked structural, functional, and metabolic differences than liver. We show that SCD1 gene and protein expression is not different between chow-fed and polyunsaturated fat-fed rats, which is not consistent with the findings in liver that polyunsaturated fatty acids oppose the induction of sterol regulatory-element binding protein 1c and inhibit SCD1 gene expression (10, 48). In contrast, SCD1 protein expression was elevated in insulin-resistant Sat rats. Regarding the known function of SCD1, these data indicate that rather than being a causative factor in obesity and insulin resistance, increasing skeletal muscle SCD1 may act as a protective mechanism intended to reduce the incorporation of saturated fatty acids into bioactive lipid species such as DAGs and ceramides. Studies that directly test this hypothesis warrant further investigation.

Chronic high-fat feeding induces insulin resistance in rodents; however, the role of specific dietary fats is not well characterized. Previous reports indicate that insulin resistance (20, 43) or glucose intolerance (27) occurs in rats fed a diet high in n-6 PUFA, whereas rats fed diets high in n-3 and with low n-6-to-n-3 ratios maintain normal insulin sensitivity. In this study, we observed an ~20% improvement in HOMA-IR in rats fed a PUFA diet consisting mainly of n-6 fatty acids.
(n-6:n-3 PUFA of >75:1), an effect observed previously in Type 2 diabetes patients (17). The discrepancies between ours and previous rodent studies (20, 27, 43), although difficult to pinpoint, may be due to the duration of treatment (8 wk in the present study vs. 3–4 wk). With respect to the time-course effects, PUFAs upregulate genes involved in fat oxidation and decrease genes involved in lipid synthesis and storage (38). Although the transcriptional response is rapid, the time required for a phenotype change may take weeks to manifest and thus explain the observed differences between studies. A more likely explanation is that the chow-fed animals were mildly insulin resistant. These animals gained weight at a similar rate to the PUFAs, suggesting increased food intake. It is also possible that the small amount of saturated fats in the chow diet (~10% of total calories) resulted in slight insulin resistance in the controls. These possibilities remain to be determined. Despite these differences, it appears that an n-6 PUFAs diet is more desirable than a Sat diet, but that n-3 PUFAs substitution for n-6 is advantageous for insulin action. Thus although a diet supplemented with n-3 PUFAs enhances insulin action (34, 42, 43), our data suggest that a diet rich in n-6 PUFAs is not detrimental to insulin action as previously described and may even result in modest improvements in insulin action.

In conclusion, the results of the present study demonstrate that a diet high in saturated fatty acids induces insulin resistance whereas polyunsaturated fatty acids modestly enhance insulin sensitivity. The insulin resistance in the Sat rich diet was associated with elevated DAG content and increased saturation of muscle TAG. These effects were partially ameliorated with polyunsaturated fatty acids, possibly via increased incorporation into TAG.

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