Palmitate-Induced Activation of the Hexosamine Pathway in Human Myotubes

Increased Expression of Glutamine:Fructose-6-Phosphate Aminotransferase

Cora Weigert, Karsten Klopfer, Christiana Kausch, Katrin Brodbeck, Michael Stumvoll, Hans U. Häring, and Erwin D. Schleicher

The nutrient sensing capacity of the hexosamine biosynthetic pathway (HBP) has been implicated in the development of insulin resistance of skeletal muscle. To study the molecular mechanism of the free fatty acid (FFA)-induced activation of the HBP myotubes obtained from muscle biopsies of metabolically characterized, subjects were stimulated with different fatty acids for 20 h. Incubation with the saturated fatty acids palmitate and stearate (0.5 mmol/l) resulted in a three- to fourfold increase in mRNA expression of glutamine:fructose-6-phosphate aminotransferase (GFAT), the key and rate-limiting enzyme of the hexosamine pathway. Unsaturated fatty acids or 30 mmol/l glucose had little or no effect. Palmitate increased the amount of GFAT protein nearly two-fold, and subsequently, the concentration of UDP-N-acetylglucosamine, the end product of the HBP, was 1.3-fold enhanced in the palmitate-stimulated myotubes. The nonmetabolized fatty acid bromopalmitate had no effect. The DNA binding activity of the transcription factor Sp1, a target down-stream of the HBP, was increased by palmitate and completely lost after enzymatic removal of O-GlcNAC. No correlation was found between the palmitate-induced increase in GFAT protein and the insulin resistance in the respective subjects. The findings reveal a new mechanism for how FFAs induce the activation of the HBP. Diabetes 52:650–656, 2003

Insulin resistance is defined as the inability of insulin to stimulate normal rates of glucose uptake and glycogen synthesis. Skeletal muscle insulin resistance, which is a major characteristic of type 2 diabetes and obesity (1,2), has been correlated to increased availability of free fatty acids (FFAs) (3,4). Previous studies showed that elevation of plasma FFA by acute infusion of triglyceride-heparin emulsions in humans induces insulin resistance (5,6), while lowering plasma FFA decreases insulin resistance (7). In detail, elevated plasma FFAs inhibit insulin-stimulated glucose uptake and reduce insulin-induced glycogen synthase activity (4,5). It has been postulated by Randle et al. (8) that substrate competition between glucose and FFA is involved in the lipid-induced insulin resistance. The increased fatty acid oxidation occurring in the high FFA status decreases glucose oxidation, which then causes decreased glucose transport and/or phosphorylation (8). The biochemical mechanisms involve inhibition of pyruvate dehydrogenase (PDH) by increased concentrations of acetyl-CoA and the inhibition of phosphofructokinase (PFK) by increased citrate levels (8). Previous studies have challenged this substrate competition mechanism, since acute elevations in plasma fatty acids in humans result in decreased levels of intracellular glucose-6-phosphate (5) and glucose (9), indicating no accumulation of glucose-6-phosphate by inhibition of PDH and PFK. Furthermore, recent data suggest a role for JNK kinase-β (9) and/or protein kinase C θ (10) in fatty acid–induced insulin resistance. Taken together, the mechanism(s) responsible for the FFA-induced defects in insulin-stimulated glucose metabolism are incompletely understood (11).

The hexosamine biosynthetic pathway (HBP) is one promising candidate in the development of insulin resistance (12). Glutamine:fructose-6-phosphate aminotransferase (GFAT) as a rate-limiting enzyme of this minor glucose metabolic pathway catalyzes the conversion of fructose-6-phosphate (F-6-P) to glucosamine-6-phosphate (GlcN-6-P) with glutamine as an amino-donor (13). GlcN-6-P is very rapidly further converted and activated to uridine-5'-diphosphate-N-acetylglucosamine (UDP-GlcNAc), serving as essential substrate for protein glycosylation (14). Marshall et al. (15) discovered that increased flux through the HBP caused by ambient high glucose concentrations and insulin results in decreased insulin action on glucose transport in adipocytes. Several subsequent in vivo studies indicate that activation of the HBP by hyperglycemia (12), glucosamine infusions (16,17), overexpression of GFAT in transgenic mice (18), or elevated plasma FFAs (19,20) impaired glucose uptake and glycogen synthesis in skeletal muscle mass (19-21), indicating no accumulation of glucose-6-phosphate by inhibition of PDH and PFK.
muscle. More recently, evidence has been provided for an involvement of the HBP in linking energy intake and energy expenditure (21) and for an upregulated GFAT activity by obesity in vivo (20,22). While elevated glucose levels or glucosamine increase the HBP flux by mass action, the activating effect of FFA on the HBP is not completely understood. In particular it is not known whether this activation is due to post-translational modifications and/or to an increased protein amount of GFAT.

Therefore, it was our aim to study the hypothesis that FFAs activate the HBP in skeletal muscle cells by induction of GFAT expression. Since it has been suggested that the individual composition of elevated FFAs may influence the development of insulin resistance (23–25), we assessed the effect of different fatty acids on the activity of the HBP using myotubes obtained from muscle biopsies of normoglycemic healthy volunteers (26,27). Particularly, we examined whether different FFAs 1) cause an increase in GFAT mRNA and protein expression, 2) influence the activity of the HBP assessed by measuring the end product UDP-GlcNAc, and 3) affect the DNA binding activity of the transcription factor Sp1, one target of the HBP.

RESEARCH DESIGN AND METHODS

Subjects. Skeletal muscle cells derived from a total of 16 normoglycemic nonobese subjects were recruited from a study population of healthy volunteers. Insulin sensitivity was determined with the euglycemic-hyperinsulinemic clamp and expressed as glucose infusion rate (GIR) per lean body mass (LBMI (μmol·kg\(^{-1}\)·min\(^{-1}\)) (26)). Glucose was clamped at the individual fasting glucose concentration (range 4.1 to 5.2 mmol/l). The study was approved by the local ethical committee, and informed written consent had been obtained from all subjects before the biopsy.

Materials. Cell culture media and supplements were from Gibco (Eggenstein, Germany). Oligonucleotides were synthesized by Life Technologies (Karlsruhe, Germany). Klenow enzyme and poly(dI-C)) were from Boehringer (Mannheim, Germany); fatty acids, monoclonal anti-α-sarcosomic actin, and β-N-acetylglucosaminidase (from canavalia ensiformis) were from Sigma (Munich, Germany); and [α\(^{32}\)P]dATP was from Hartmann (Braunschweig, Germany). The production of [\(^{32}\)P]dATP was optimized to obtain sufficient labeled nucleotides with high specific activity (28). Briefly, GFAT antiserum was raised in rabbits by in vitro transcription. Hybridization was performed overnight in 5°C SSC, 0.1% SDS at 68°C. After washing with NET the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG for 1 h at room temperature. Visualization of immunocomplexes was performed by enhanced chemiluminescence as previously described (31).

Electrophoretic mobility shift assay. Nuclear proteins were prepared as described recently (31). Synthetic oligonucleotides containing a high affinity binding site for Sp1 (5′-AGCCGGGAGCCCGCCCCCTTTCCCCCAGGGCTG-3′) were end-labeled with [α\(^{32}\)P]dATP (3,000 Ci/mM) and Klenow enzyme and were incubated with up to 6 μg nuclear protein in 20 μl 7 mM Tris-HCl pH 7.9, 100 mM KCl, 3.6 μmol/l MgCl\(_2\), 0% glycerol on ice for 20 min; 0.05 mg/ml poly[d(I-C)] was added as unspecific competitor. The samples were run on a 5% non-denaturating polyacrylamide gel in a buffer containing 25 mM Tris-HCl pH 8.0, 190 mmol/l glycine, and 1 mM EDTA. Gels were dried and analyzed by autoradiography.

Statistical analysis. Results are expressed as means ± SE of four separate experiments. *P < 0.05; **P < 0.01 vs. control. Representative Northern blots of GFAT and GAPDH are shown in the upper part of the figure. Arrows indicate the main 5-kb transcript of human GFAT (8- and 10-kb transcripts were also weakly visible and regulated in an identical manner, but are not shown) and the 1-kb transcript of GAPDH, respectively.

RESULTS

Phyisiological concentrations of palmitate activate GFAT mRNA expression. To study whether FFAs alter the HBP in myotubes obtained from human skeletal muscle, we first investigated the effect of palmitate on GFAT mRNA expression. As shown in Fig. 1 increasing concentrations of palmitate enhance GFAT mRNA in the myotubes. Stimulation of myotubes with 0.125 mmol/l palmitate was not sufficient to significantly increase GFAT mRNA expression, while 0.25 mmol/l led to an 3.8-fold increase in the amount of GFAT mRNA compared to unstimulated cells. Higher concentrations of palmitate up to 2 mmol/l cause only little further increase (4.8-fold),...
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FIG. 2. Effect of different fatty acids on GFAT mRNA expression. Myotubes were stimulated for 20 h with 0.5 mmol/l of different fatty acids as indicated in the figure. Densities of the 3-kb transcript of GFAT were normalized relative to those of GAPDH mRNA signals. The ratio of the control cells was set as 1. Results are expressed as mean ± SE of four separate experiments. *P < 0.05; **P < 0.01 vs. control. Representative Northern blots of GFAT and GAPDH are shown in the upper part of the figure. Arrows indicate the main 3-kb transcript of human GFAT (8- and 10-kb transcripts were also weakly visible and regulated in an identical manner, but are not shown) and the 1-kb transcript of GAPDH, respectively.

indicating that the major effect of palmitate on GFAT mRNA expression occurred between 0.125 and 0.25 mmol/l palmitate. For further studies 0.5 mmol/l of fatty acids was used, providing a near maximum stimulation of GFAT expression in a physiological range of plasma FFA concentrations.

Weak activation of GFAT mRNA expression by unsaturated fatty acids. To investigate the specificity of the up regulation of GFAT mRNA expression by the saturated hexadecanoic palmitic acid, the saturated octadecanoic acid stearate and the unsaturated fatty acids palmitoyloleate (cis-9-hexadecenoic acid), oleate (cis-9-octadecenoic acid), and linoleate (cis,cis-9,12-octadecadienoic acid) were studied. The saturated fatty acids palmitate and stearate induce GFAT mRNA expression strongly (3.1-fold and 4.2-fold compared with control), while the mono- or diunsaturated fatty acids had no or only a weak effect (1.5-fold induction by linoleate) on GFAT mRNA levels (Fig. 2).

Palmitate increases the expression of GFAT protein and the cellular amount of UDP-GlcNAc in myotubes. Since these data revealed a new mechanism of activation of the hexosamine pathway by FFAs, we studied whether palmitate induces also the amount of GFAT protein and the flux through the hexosamine pathway, determined as cytosolic concentration in UDP-GlcNAc. The less effective fatty acid linoleate and the nonmetabolized fatty acid bromopalmitate were added to evaluate the specificity of the effect of palmitate. Furthermore, we studied the effect of ambient high glucose concentrations (30 mmol/l) on GFAT expression in these cells. In this set of experiments determination of GFAT mRNA by Northern blotting revealed again a strong increase in GFAT mRNA after treatment with palmitate (2.8-fold) with less activation by linoleate and bromopalmitate (Fig. 3A). High glucose concentrations had no effect on GFAT mRNA expression compared with control cells.

To assess whether the saturated fatty acid-induced increase in GFAT mRNA expression was translated into an enhanced GFAT protein production, cellular GFAT protein content was studied by Western blotting. As shown in Fig. 3B, palmitate induced a 1.7-fold increased GFAT protein expression. The less pronounced effect of linoleate and bromopalmitate did not reach significance (1.4-fold and 1.2-fold, respectively), and high glucose concentrations had no effect.

Subsequently, the palmitate-induced increase in the amount of GFAT protein was accompanied by an enhanced concentration of the end product of the hexosamine biosynthetic pathway, UDP-GlcNAc. Determination of this activated UDP-hexosamine revealed a 1.3-fold accumulation after incubation with palmitate (Fig. 3C). Stimulation of myotubes with high glucose concentrations, linoleate, or bromopalmitate had no effect on the cellular amount of UDP-GlcNAc (Fig. 3C).

Since previous data provide evidence for a high glucose plus insulin-induced increase in GFAT activity (22), we studied the effect of insulin alone and high glucose plus insulin on GFAT protein expression. The combination of high glucose (30 mmol/l) and insulin (10 or 100 nmol/l) caused a slight but not significant increase in GFAT protein levels (116 ± 13 and 151 ± 32%, respectively), while 100 nmol/l insulin alone had no effect (109 ± 36%). Similar changes were observed in cellular UDP-GlcNAc content which again failed to reach statistical significance (data not shown).

Effect of palmitate on Sp1 DNA binding activity. Previous studies in endothelial and mesangial cells provide evidence that the DNA binding activity of the transcription factor Sp1 is up regulated by an increased flux through the hexosamine biosynthetic pathway, leading to stimulation of Sp1 driven promoter activity and subsequent enhanced gene transcription (33–35). Therefore, using electrophoretic mobility shift assay we studied the effect of palmitate on Sp1 as a target downstream of the hexosamine pathway in human myotubes obtained from muscle biopsies from different subjects. Treatment with palmitate resulted in enhanced DNA binding activity of Sp1 to a Sp1 consensus sequence (Fig. 4A), indicating an increased activity of this transcription factor. DNA binding activity of nuclear proteins to an Oct-1 binding sequence was not significantly changed (Fig. 4A) as assessed by densitometry (data not shown) indicating specificity of the palmitate effect on Sp1. Since it has been postulated that an increased O-GlcNAc modification of Sp1 generated by an enhanced flux through the hexosamine pathway accounts for the activation of this transcription factor (34), we studied the effect of enzymatic removal of O-GlcNAc on DNA binding activity of Sp1. Incubation of nuclear extracts obtained from palmitate-stimulated myotubes and unstimulated control cells with β-N-acetylglucosaminidase resulted in complete loss of DNA binding activity (Fig. 4B), indicating that the O-GlcNAc modification is necessary for basal Sp1 DNA binding activity. Furthermore, the results...
suggest that the palmitate-induced increase in Sp1 DNA binding activity may be mediated by enhanced O-GlcNAc modification, providing a molecular mechanism by which the increased flux through the hexosamine pathway upregulated Sp1 activity.

No correlation of palmitate-induced GFAT protein expression in myotubes and insulin sensitivity of the donor subjects. Since the palmitate-induced activation of GFAT expression in vitro might contribute to the development of insulin resistance in skeletal muscle, we evaluated in a total number of 16 normoglycemic nonobese subjects the relationship of the palmitate-dependent increase in GFAT protein with the insulin sensitivity of the respective subjects, ranging from a GIR/LBM of 23.4 to 126.5/H18528 mol/H11002 kg/H11002 min/H11002. We found no correlation between the palmitate-induced GFAT protein concentration in the in vitro cultured myotubes and the insulin sensitivity of the respective subjects (Fig. 5).

Upon comparison of basal GFAT protein amount and insulin sensitivity, we found in the myotubes obtained from insulin-resistant subjects (GIR/LBM 29.2/H11006 1.4) a basal GFAT expression (related to basal α-sarcomeric actin expression) of 0.91 ± 0.26 compared with 0.57 ± 0.07
in the insulin sensitive group (GIR/LBM 60.8 ± 6.4). Therefore, subjects with lower insulin sensitivity tended to have higher mean basal GFAT protein expression in cultured myotubes, although this effect did not reach statistical significance.

**DISCUSSION**

In this report we provide evidence for a new mechanism of the fatty acid–induced activation of the HBP in human myotubes: 1) palmitate enhances the mRNA expression of GFAT, the rate-limiting enzyme of this pathway; 2) palmitate increases the amount of GFAT protein; 3) palmitate increases subsequently the concentration of UDP-GlcNAc (the end product of the HBP); and 4) palmitate activates the DNA binding activity of Sp1 (a target downstream of the HBP). The effects were observed at normophysiological concentration of 0.5 mmol/l; in human plasma the concentration of FFAs is <0.7 mmol/l in the postabsorptive state and might rise to >1 mmol/l after a fatty meal or during fasting. The average percentage of saturated C16 and C18 fatty acids is ~40% (36). Thus, the dose-response curve (Fig. 1) is well within the physiological range.

The observed effect depends on the structure of the fatty acid—saturated C16 and C18 fatty acids are the strongest stimulators, while unsaturated C16 and C18 fatty acids had no or only weak effects on GFAT mRNA expression. The induction of GFAT expression by the nonmetabolized bromopalmitate was comparable with linoleate. This indicates that metabolism of saturated fatty acids is necessary for the complete stimulation of GFAT expression or that an FFA receptor–like protein with FFA specificity exists in human myotubes. The molecular mechanism for this up-regulation is currently unknown. Only a few data are published regarding the regulation of GFAT expression—in a breast cancer cell line, EGF stimulated GFAT mRNA expression, while glucose alone had no effect (37), and in mesangial cells GFAT gene activation by angiotensin II was reported (38). Prolonged dietary treatment of pigs with conjugated linoleic acid for 45 days increased besides other genes, the expression of GFAT mRNA (39), thus indicating that the induction of GFAT expression by FFAs could also occur in vivo. However, no data providing a possible mechanism for this gene activation are reported. Specific fatty acid–regulated transcription factors have been identified, including peroxisome proliferator–activated receptor-α, γ, and δ, HNF4α, NfκB, and SREBP1c (36,40), whereby the activation of NfκB might be specific for saturated fatty acids (40). Computer analysis of the 5′-flanking region of human GFAT revealed similarities to binding sites for NfκB. Therefore, studies on human GFAT promoter regulation are necessary to clarify which transcription factors and which signaling pathways might be involved in saturated fatty acid–induced GFAT gene activation.

Culturing the myotubes for 48 h in ambient high glucose concentrations revealed no effect on GFAT mRNA and protein expression, supporting earlier findings where no glucose-induced activation of GFAT mRNA expression was found (37,41). We also observed no high glucose effect on the amount of UDP-GlcNAc, while in other reports a hyperglycemia-induced increase in UDP-GlcNAc concentrations in skeletal muscle has been described (41,42). However, the flux through the HBP is tightly regulated—GFAT activity is inhibited in an autocrine feedback loop by UDP-GlcNAc (13) and GlcN-6-P (43) and is posttranslationally modified by protein kinase A phosphorylation (44). A recently described striated muscle-specific splice variant of GFAT exhibits a markedly increased susceptibility to inhibition by UDP-GlcNAc (45).

This feedback inhibition should also be considered arguing that the palmitate-induced increase in UDP-GlcNAc concentrations is rather low (1.3-fold). For instance, in hindlimb muscles of ob/ob mice, the average increase in UDP-GlcNAc is only 1.67-fold (20) and intralipid-infusion in rats resulted in peripheral insulin resistance without a detectable increase in UDP-GlcNAc levels (46). At first glance, these somewhat controversial data indicate that an increased flux through the HBP is not adequately mirrored by the UDP-GlcNAc concentration. Thus, moderate elevations in UDP-GlcNAc content are sufficient to induce marked peripheral insulin resistance (19,47), but the lack of elevated UDP-GlcNAc concentrations did not predict sustained insulin sensitivity (46). Therefore, it may be more appropriate to assess the activity of the HBP by O-GlcNAc modification of proteins, e.g., Sp1.

One aim of our study was to investigate a possible relationship of the FFA-induced increase in GFAT expression with the insulin sensitivity of the respective subjects. No correlation was found suggesting that no primary defect in the signaling cascade from FFA to GFAT gene expression is responsible for the development of insulin resistance in vivo. However, one could assume that the often-found higher circulating FFA levels in insulin-resistant subjects may promote the activation of the HBP and consequently the progression of the metabolic disorder.

The ability of FFA to induce insulin resistance was demonstrated by several groups in vivo or in vitro. Only few studies investigated the effect of individual FFA on insulin signaling. It appears from these reports that satu-
rated fatty acids might have a pronounced ability to induce insulin resistance compared with unsaturated fatty acids (23–25,48)—an increased availability of saturated fatty acids has been correlated with development of insulin resistance in vivo and in vitro (23–25,48), while replacement of the fat by polyunsaturated fatty acids prevented such an effect (23). However, also unsaturated fatty acids (e.g., oleate) (49) interfere with insulin action when assayed in ex vivo muscle strips.

Together, our data show that 1) there were no significant relationships between insulin sensitivity in vivo and basal or regulated GFAT expression in the myotubes; and 2) while the data show that FFA can upregulate GFAT activity and affect Sp1 binding, there is no evidence that this activation of the hexosamine pathway correlates with the development of insulin resistance in humans. In conclusion, the induction of GFAT mRNA and protein expression preferentially by saturated fatty acids provides a new mechanism for the fat-induced activation of the HBP in a prediabetic metabolic milieu.

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