

Increased Glycogen Synthase Kinase-3 Activity in Diabetes- and Obesity-Prone C57BL/6J Mice

Hagit Eldar-Finkelman, Sandra A. Schreyer, Michi M. Shinohara, Renee C. LeBoeuf, and Edwin G. Krebs

Although the precise mechanisms contributing to insulin resistance and type 2 diabetes are unknown, it is believed that defects in downstream components of the insulin signaling pathway may be involved. In this work, we hypothesize that a serine/threonine kinase, glycogen synthase kinase-3 (GSK-3), may be pertinent in this regard. To test this hypothesis, we examined GSK-3 activity in two inbred mouse strains known to be susceptible (C57BL/6J) or resistant (A/J) to diet-induced obesity and diabetes. Examination of GSK-3 in fat, liver, and muscle tissues of C57BL/6J mice revealed that GSK-3 activity increased twofold in the epididymal fat tissue and remained unchanged in muscle and liver of mice fed a high-fat diet, compared with their low-fat diet-fed counterparts. In contrast, GSK-3 activity did not change in the epididymal fat tissue of A/J mice, regardless of the type of diet they were fed. In addition, both basal and diet-induced GSK-3 activity was higher (2.3- and 3.2-fold, respectively) in the adipose tissue of C57BL/6J mice compared with that in A/J mice. Taken together, our studies suggest an unsuspected link between increased GSK-3 activity and development of insulin resistance and type 2 diabetes in fat tissue of C57BL/6J mice, and implicate GSK-3 as a potential factor contributing to susceptibility of C57BL/6J mice to diet-induced diabetes. *Diabetes* 48:XXX-XXX, 1999

Inulin resistance, characterized by hyperinsulinemia and hyperglycemia, is considered to be one of the earliest changes associated with the onset of type 2 diabetes. Although the precise molecular mechanism underlying insulin resistance is unknown, it is believed that defects in downstream components of the insulin signaling pathway may be one cause. Hence, identification of these defects is likely to make an important contribution to our understanding of the etiology of type 2 diabetes.

From the Division of Women's Health (H.E.-F.), Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; and the Departments of Medicine (S.A.S., R.C.L.) and Pharmacology (M.M.S., E.G.K.), University of Washington, Seattle, Washington.

Address correspondence and reprint requests to Hagit Eldar-Finkelman, Departments of Medicine, Division of Women's Health, Brigham and Women's Hospital, Boston, MA 02115.

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DTT, D,L-dithiothreitol; GS, glycogen synthase; GSK-3, glycogen synthase kinase-3; IRS, insulin receptor substrate; PKB, protein kinase B; PP2A, protein phosphatase 2A.

Insulin mediates a wide variety of cellular processes, including glycogen synthesis, glucose transport, mitogenesis, and protein synthesis (1-3). A key event in insulin action is the phosphorylation of the insulin receptor substrates (IRS-1, IRS-2) on multiple-tyrosine residues, which results in simultaneous activation of several signaling components, including phosphatidylinositol 3-kinase, protein kinase B (PKB), p70 ribosomal protein S6 kinase, and mitogen-activated protein kinase (4-6). Although IRS-1 exhibits insulin-stimulated tyrosine phosphorylation, in unstimulated cells it is predominantly phosphorylated on serine/threonine residues (7,8). The role of the latter type of phosphorylation is not clear, although in some instances it has been shown to mediate negative feedback regulation of insulin signaling (8-11). This suggests that serine/threonine kinases may play a key role in the generation of states associated with insulin resistance.

In this work, we hypothesize that glycogen synthase kinase-3 (GSK-3), a serine/threonine kinase, may be a factor contributing to insulin resistance and type 2 diabetes. The rationale of our hypothesis is based on several lines of evidence. First, GSK-3 is constitutively active in unstimulated cells and is inhibited by insulin (12-14), which characteristics suggest a role for this enzyme in suppressing the insulin signal. Second, the enzyme phosphorylates two important targets of insulin action, glycogen synthase (GS) (15,16) and IRS-1 (11), which results in inhibition of GS and impairment of insulin signaling in intact cells (11,17). Finally, studies performed in patients with type 2 diabetes (or non-insulin-dependent diabetes [NIDDM], as referred to in these studies) showed that GS activity was markedly decreased in these subjects (18,19). Furthermore, decreased activation of PKB, an upstream regulator of GSK-3 (20), by insulin was also detected in these patients (21). In the present work, we hypothesize that increased GSK-3 activity in vivo may be associated with development of insulin resistance and type 2 diabetes. To test this hypothesis, we used inbred mouse strains known to be susceptible (C57BL/6J) or resistant (A/J) to diet-induced obesity and diabetes (22). Diets rich in saturated fat have often been used in rodent feeding studies to produce obesity and insulin resistance, conditions similar to those that occur in type 2 diabetes (22,23). Here, we report that changes in GSK-3 activity occur as a function of the type of diet and the strain of mice.

RESEARCH DESIGN AND METHODS

Animals. Male C57BL/6J and A/J mice, aged 6-8 weeks, were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained in a temperature-controlled (25°C) facility with a strict 12-h light/dark cycle and were given

free access to food and water. For 15 weeks, they were fed rodent diet (Wayne Rodent BLOX 8604; Teklad, Madison, WI) that was low in fat (4% wt/wt) or a high-fat diet (Diet#F1850; Bioserve Industries, Frenchtown, NJ) that contained 35.3% (wt/wt) fat (primarily lard) and 36.6% carbohydrate (primarily sucrose). The high-fat diet has been previously shown to induce obesity and diabetes in C57BL/6J mice (22). Food was removed from the mice 4 h before the collection of blood from the retro-orbital sinus into tubes containing anticoagulant (1 mmol/l EDTA). Blood plasma was obtained and immediately stored at -70°C before analysis. Mice were killed by cervical dislocation.

Tissue extraction. Epididymal fat, liver, and skeletal muscle were removed from mice and immediately homogenized in ice-cold extraction buffer (50 mmol/l β -glycerophosphate, pH 7.3, 1.5 mmol/l EGTA, 1 mmol/l EDTA, 2 mmol/l DTT [D,L-dithiothreitol], 0.2 mmol/l sodium orthovanadate, 1 mmol/l benzamide, 10 mg/ml aprotinin, 20 mg/ml leupeptin, 1 mmol/l NaF, 0.5 mg/ml microcystine [Gibco-BRL, Grand Island, NY], and 2 mg/ml pepstatin A [Sigma, St. Louis, MO]). Triton X100 (1% final concentration) was added to the homogenates, followed by centrifugation at 1000g at 4°C , which resulted in the separation of a fat layer. The resulting supernatants were filtered through glass filters (Schleicher and Schuell, Keene, NH) to remove any residual fat, and the filtrates were centrifuged at 10,000g. The resulting supernatant solutions were used for kinase assays and Western blot analysis.

GSK-3 kinase assays. Aliquots of tissue extracts containing equal amounts of protein (0.5 mg) were incubated at 4°C for 3 h with specific antibody against GSK-3 β (14) prebound to protein A Sepharose (Sigma). The immunoprecipitates were washed twice with 50 mmol/l Tris, pH 7.3, 0.5 mol/l LiCl, and 1 mmol/l DTT and twice with 50 mmol/l Tris, pH 7.3. The kinase reactions were then started by addition of a synthetic phosphopeptide substrate PGS-1 peptide patterned after GS phosphorylation sites (14) at a final concentration of 200 $\mu\text{mol/l}$ to reaction mixtures that also included 50 mmol/l Tris, pH 7.2, 250 $\mu\text{mol/l}$ [γ - ^{32}P]ATP (0.25 mCi/ml), 10 mmol/l MgCl_2 , and 0.5 mg/ml bovine serum albumin in a final volume of 30 μl ; incubation was carried out for 15 min at 30°C . The reaction mixtures were spotted on phosphocellulose paper squares (p81 Whatman), which were washed with 100 mmol/l phosphoric acid, dried, and counted for radioactivity. Assays were performed in duplicate.

Western blot analysis. Aliquots containing equal amounts of protein (50 μg) were subjected to SDS-PAGE and blotted on polyvinylidene fluoride (Millipore, Bedford, MA) membranes. Membranes were incubated with affinity-purified antibodies against GSK-3 β , which were kindly provided by Jackie R. Vandenheede (Katholieke University, Leuven, Belgium), or anti-phospho PKB antibodies (New England Biolabs, Beverly, MA). Blots were developed using an enhanced chemiluminescence method (Du Pont, Wilmington, DE).

Statistical analysis. Values are reported as means \pm SE. Statistical differences were determined by analysis of variance using Systat for the Macintosh. Post hoc analysis of significance was made by Tukey's test for additivity. $P < 0.05$ was accepted as statistically significant.

RESULTS

Animal characteristics. Values of the body weights and blood plasma glucose, insulin, and leptin levels of the two types of mice used in these studies are summarized in Table 1. Consistent with previous reports, the high-fat diet induced a phenotype associated with diabetes and obesity in C57BL/6J mice (22,23). This was evidenced by a 30% increase in body weight, a 56% increase in blood plasma glucose, and a more than threefold increase in circulating insulin levels. Leptin lev-

els increased sixfold upon feeding of the high-fat diet to the C57BL/6J mice. In contrast, the high-fat diet did not induce a diabetic phenotype in A/J mice, which showed only a modest weight increase (4 g) and no significant changes in blood plasma glucose or insulin levels. Although the A/J mice fed the high-fat diet were not obese or diabetic, their leptin levels increased threefold compared with those in their low-fat-fed counterparts (Table 1). Overall, body weights and plasma glucose, insulin, and leptin levels were markedly elevated in the high-fat-fed C57BL/6J mice, compared with the A/J mice. **GSK-3 activity in C57BL/6J mice.** GSK-3 activity was initially examined in fat, liver, and muscle tissues of low- and high-fat diet-fed C57BL/6J mice. As shown in Fig. 1A, GSK-3 activity was significantly elevated (twofold) in the epididymal fat tissue of diabetic high-fat-fed mice, compared with control animals. In contrast, enzyme activity was similar in skeletal muscle regardless of the type of diet consumed. Interestingly, a slight reduction in GSK-3 activity (15%) was found in liver tissue of the high-fat diet-fed animals compared with controls. These studies indicated that GSK-3 activity was specifically increased in the adipose tissue of animals fed a high-fat diet. The expression of GSK-3 protein levels was also examined in these tissues and is presented in Fig. 1B. GSK-3 expression levels were significantly higher in liver and muscle compared with fat tissue; this observation correlated with the sixfold increase in GSK-3 activity detected in these tissues (Fig. 1A). There were no remarkable changes, however, in protein expression levels in tissues from either low- or high-fat diet-fed mice (Fig. 1B). These results suggest that changes in GSK-3 activity occur specifically in the fat tissue of diabetic animals.

GSK-3 activity in A/J mice. The studies described above suggested that increased GSK-3 activity was associated with development of insulin resistance and type 2 diabetes in C57BL/6J mice fed a high-fat diet. To verify that these changes were not simply related to changes in diet per se, we used the A/J mice strain that is resistant to high-fat diet-induced diabetes (22) (Table 1). As shown in Fig. 2, no significant changes in GSK-3 activity or its expression levels were detected in the epididymal fat tissue of A/J mice, regardless of the type of diet they were fed. In addition, GSK-3 activity was always higher in C57BL/6J mice, compared with A/J mice (2.3- and 3.6-fold in basal and high-fat diet conditions, respectively; Figs. 1A and 2A). GSK-3 activity was also compared in muscle and liver of both mice strains. As shown in Fig. 3, while GSK-3 activity was twofold higher in fat tissue of C57BL/6J mice versus A/J mice, the enzyme activity was similar in muscle and liver of both

TABLE 1
Body weight and plasma glucose, insulin, and leptin levels for mice fed low- or high-fat diet for 15 weeks

	Body weight (g)	Plasma		
		Glucose (mg/dl)	Insulin (ng/ml)	Leptin (ng/ml)
C57BL/6J				
Low-fat diet	31.3 \pm 0.3	141 \pm 7	0.74 \pm 0.05	4.5 \pm 0.4
High-fat diet	41.9 \pm 1.2*	221 \pm 16*	2.44 \pm 0.61*	27.6 \pm 2.1*
A/J				
Low-fat diet	27.5 \pm 0.7†	160 \pm 13	0.61 \pm 0.04	6.6 \pm 0.7
High-fat diet	31.6 \pm 0.9‡§	165 \pm 9†	0.74 \pm 0.04§	18.7 \pm 1.8*§

Data are means \pm SE. $n = 7-10$. Mice were fasted for 4 h before analysis. * $P < 0.001$, ‡ $P < 0.05$, low- vs. high-fat fed mice; † $P < 0.05$, § $P < 0.001$, C57BL/6J vs. A/J mice fed same diet. Statistical differences were determined using Tukey's post hoc test after analysis of variance.

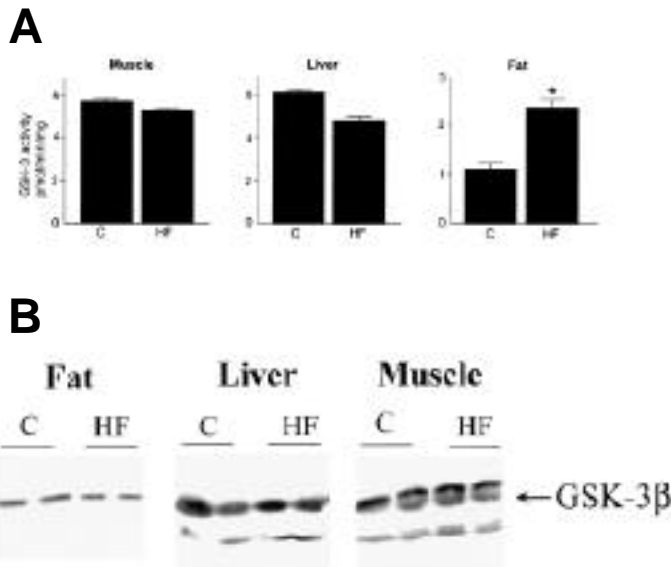


FIG. 1. GSK-3 activity and its expression in fat, liver, and muscle tissue of C57BL/6J mice. **A:** Mice fed with low-fat (C) or high-fat (HF) diet were killed, and epididymal fat, liver, and skeletal muscle tissues were removed and immediately homogenized in extraction buffer, as described in METHODS. After centrifugation, the same amount of protein (0.5 mg) was immunoprecipitated with anti-GSK-3 β antibody, and GSK-3 activity was assayed in the immunoprecipitate complex using synthetic phosphopeptide PGS-1 substrate, as described in METHODS. Results are presented as the amount of phosphate incorporated in 1 min into the PGS-1 peptide and are means \pm SE of 8–9 animals. $*P < 0.001$ C vs. HF. **B:** The same amount of protein from tissue extracts (50 μ g) was subjected to SDS-PAGE, followed by Western blot analysis using specific affinity purified antibodies against GSK-3 β , as described in METHODS. Two representative samples from each tissue and type of diet are presented.

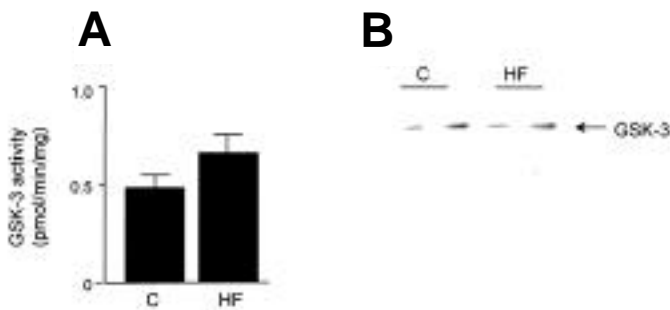


FIG. 2. GSK-3 activity and its expression in epididymal fat tissue of A/J mice. **A:** Mice fed with low-fat (C) or high-fat (HF) diet were killed, and epididymal fat tissue was removed and homogenized in extraction buffer, as described in METHODS. After centrifugation, the same amount of protein lysate (0.5 mg) was immunoprecipitated with anti-GSK-3 β antibody, and GSK-3 activity was assayed in the immunoprecipitate complex using synthetic phosphopeptide PGS-1 substrate, as described in METHODS. Results are presented as the amount of phosphate incorporated in 1 min into the PGS-1 peptide and are means \pm SE of 8–9 animals. **B:** The same amount of protein from tissue extracts (50 μ g) was subjected to SDS-PAGE, followed by Western blot analysis using specific affinity purified antibodies against GSK-3 β , as described in METHODS. Two representative samples from each type of diet are presented.

strains. This emphasizes that GSK-3 activity in the adipose tissue may play a key role in susceptibility of the animal strain to high-fat diet-induced diabetes.

PKB activity in C57BL/6J mice. Phosphorylation of a key regulatory site, serine 9, of GSK-3 was shown to inhibit the

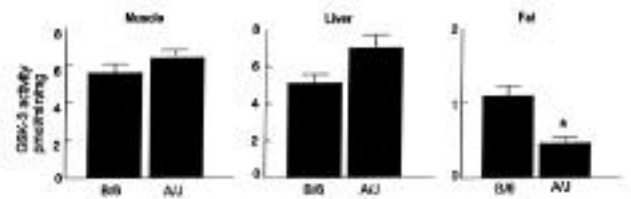


FIG. 3. GSK-3 activity tissue of C57BL/6J mice versus A/J mice. Muscle liver and fat tissue of C57BL/6J (B/6) or A/J mice fed normal (low-fat) diet were removed and homogenized in extraction buffer as described in METHODS. After centrifugation, the same amount of protein lysate (0.5 mg) was immunoprecipitated with anti-GSK-3 β antibody, and GSK-3 activity was assayed in the immunoprecipitate complex using synthetic phosphopeptide PGS-1 substrate, as described in METHODS. Results are presented as the amount of phosphate incorporated in 1 min into the PGS-1 peptide and are means \pm SE of 8–9 animals. $*P < 0.001$ B/6 vs. A/J.

enzyme activity (24). Insulin mediates the phosphorylation of this serine site via activation of PKB (6,20). It was therefore tempting to suspect that decreased PKB activity in the fat tissue of high-fat diet-fed mice may play a role in increased GSK-3 activity. PKB is activated by phosphorylation of two key sites, threonine 308 and serine 473, and the latter was shown to be a critical site generating increased PKB activity when phosphorylated (6). Phosphorylation of PKB on serine 473 was analyzed in extracts from fat tissue of C57BL/6J mice fed high- or low-fat diet, using antibody against this site when phosphorylated. Results indicated that no significant changes in PKB activity are detected in the fat tissue of either diet group (Fig. 4). In addition, our data also indicated that PKB protein level, or its mobility shift on SDS-PAGE, which usually coincides with the enzyme activation (25), was not different between the two diet groups (data not shown).

DISCUSSION

In these studies, we report that increased GSK-3 activity is associated with development of insulin resistance and type 2 diabetes in high-fat-fed C57BL/6J mice. We show that GSK-3 activity was specifically elevated in the epididymal fat tissue of diabetic mice, but did not change appreciably in liver and muscle. In contrast, GSK-3 activity remained essentially constant in epididymal fat tissue of the A/J resistant mice when fed either low- or high-fat diet. These results suggest an unsuspected link between increased GSK-3 activity and the development of insulin resistance and type 2 diabetes in C57BL/6J mice. Our results also indicated that GSK-3 activity is significantly higher (2.3-fold) in adipose tissue of C57BL/6J mice fed a regular (low-fat) diet compared with that of A/J mice on this diet. These differences could also implicate GSK-3 as one of the factors contributing to susceptibility of animals to diet-induced diabetes.

Our studies show that fat, liver, and muscle respond differently during development of insulin resistance. GSK-3 activity was enhanced in fat tissue, suppressed in liver, and unchanged in muscle as a result of the high-fat diet conditions. These observations suggest that GSK-3 functions and/or its regulation differ among the tissues. For example, the increased levels of insulin in the circulation of diabetic mice most probably led to the inhibition of GSK-3 in the liver (Fig. 1A); however, muscle and fat did not respond in the same fashion. Interestingly, recent studies reported similar observations, in which activation of phosphatidylinositol 3-kinase

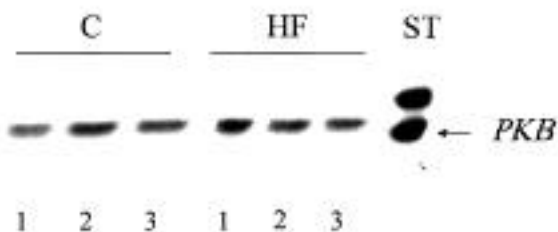


FIG. 4. PKB activity in fat tissue of C57BL/6J mice. Mice fed low-fat (C) or high-fat (HF) diet were killed, and epididymal fat tissue was removed and homogenized in extraction buffer as described in METHODS. An equal amount of protein from tissue extracts (50 μ g) was subjected to SDS-PAGE, followed by Western blot analysis using antiphospho-PKB antibody (New England Biolabs). Three representative samples from each type of diet are presented. ST is a standard of extract from NIH/3T3 cells treated with platelet-derived growth factor (provided by New England Biolabs).

by insulin in high-fat-feeding rats was markedly enhanced in liver but was suppressed in fat and muscle (26). Another example is the regulation of GS by GSK-3, which appears to be dependent on cell type. It was shown that GSK-3 does not play a key role in insulin-induced GS activation in 3T3 L1 adipocytes (27). However, in muscle, GSK-3 activity is important in the regulation of GS (15,28), and it was recently demonstrated that the inhibition of GSK-3 in human muscle cells is essential for activation of GS (29). Our studies also included measurements of GS activity in adipose tissue of C57BL/6J and A/J mice. We did not detect statistical differences in GS activity ratios between the two strains (with or without insulin treatment); although, it should be noted that the activity ratios of GS were considerably low (compared with those of liver or muscle), which made the interpretation of the data unsatisfactory (data not shown). Our studies suggest that fat tissue may play a primary role in the development of insulin resistance and type 2 diabetes. It has long been known that free fatty acids are elevated in obese subjects and that elevation of plasma free fatty acids will inhibit glucose utilization in skeletal muscle and liver (30). Development of insulin resistance in adipose tissue, and its subsequent failure to suppress lipolysis, may be directly coupled to the changes in insulin sensitivity of the peripheral tissues (31).

The mechanism for increased GSK-3 activity in the adipose tissue of diabetic C57BL/6J mice is not clear at this point. The fact that protein expression levels did not change suggested that mechanisms regulating GSK-3 activity might be the cause. Because GSK-3 is inhibited by phosphorylation of serine 9 (24), and because insulin mediates this type of inhibition via activation of PKB (6,20), it is tempting to suspect that decreased PKB activity in fat tissues of diabetic animals is the cause. Our results, however, suggest that this is not the case. An alternative explanation for increased GSK-3 activity is dephosphorylation of serine 9 achieved by hyperactivity of a serine/threonine protein phosphatase. Indeed, protein phosphatase 2A (PP2A) catalytic subunit could reactivate GSK-3 β via dephosphorylation of serine 9 in vitro (24). In addition, PP2A activity was reported to be markedly enhanced in adipocytes isolated from type 2 diabetic Goto-Kakizaki (GK) rats (32). Future studies will examine the possibility that PP2A is coupled to development of insulin resistance in C57BL/6J diabetes mice.

In summary, our findings suggest that GSK-3 may contribute to the impairment of insulin action in vivo. Future studies will determine whether GSK-3 is directly coupled to the development of obesity and type 2 diabetes.

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