Glucosamine and glucose induce insulin resistance by different mechanisms in rat skeletal muscle

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Han, Dong-Ho, May M. Chen, and John O. Holloszy. Glucosamine and glucose induce insulin resistance by different mechanisms in rat skeletal muscle. Am J Physiol Endocrinol Metab 285: E1267–E1272, 2003. First published September 3, 2003; 10.1152/ajpendo.00255.2003.—It has been hypothesized that glucose-induced insulin resistance is mediated by accumulation of UDP-N-acetylglucosamines (UDP-HexNAcs). In a previous study on rat epitrochlearis muscles incubated with high concentrations of glucose and insulin (Kawanaka K, D-H Han, J Gao, LA Nolte, and JO Holloszy. J Biol Chem 276: 20101–20107, 2001), we found that insulin resistance developed even when the increase in UDP-HexNAcs was prevented. Furthermore, actinomycin D completely prevented glucose-induced insulin resistance despite a greater accumulation of UDP-HexNAcs. In the present study, we used the same epitrochlearis muscle preparation, as well as the rat hemidiaphragm, to determine whether, like glucose, glucosamine causes insulin resistance by an actinomycin D-inhibitable process. Incubation of diaphragm muscles with 10 mM glucosamine for 3 h resulted in an approximately fivefold increase in UDP-HexNAcs, an ~50% reduction in insulin responsiveness of glucose transport, and a 58% reduction in ATP concentration. These effects of glucosamine were not prevented by actinomycin D. Incubation of epitrochlearis muscles with 20 mM glucosamine for 3 h or with 10 mM glucosamine for 5 h also caused large decreases in insulin responsiveness of glucose transport but with no reduction in ATP concentration. Actinomycin D did not prevent the glucosamine-induced insulin resistance. The insulin-induced increases in tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and the binding of PI 3-kinase to IRS-1 were decreased ~60% in epitrochlearis muscles exposed to glucosamine. This is in contrast to glucose-induced insulin resistance, which was not associated with impaired insulin signaling. These results provide evidence that glucosamine and glucose induce insulin resistance by different mechanisms.

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When large amounts of glucose are rapidly transported into skeletal muscle or fat cells, the glucose transport process becomes resistant to stimulation by insulin (7, 9, 13, 14, 26, 32, 33, 39, 40, 44) and, in the case of muscle, also to contractile activity (27). This form of insulin resistance, which has been termed “glucose toxicity,” is important because it can reverse the beneficial effects of exercise and exercise training on muscle insulin sensitivity and responsiveness (4, 21, 26, 27). Glucose toxicity may also sometimes contribute to impaired insulin action in diabetes (47). A widely accepted hypothesis regarding the mechanism responsible for glucose-induced insulin resistance is that glucose toxicity is mediated by increased flux of glucose into the hexosamine biosynthetic pathway, resulting in accumulation of UDP-N-acetylglucosamines (UDP-HexNAcs) (1, 32–34, 41, 43, 45).

However, in a study of this phenomenon in rat epitrochlearis muscles exposed to high concentrations of glucose and insulin (25), we found that insulin resistance developed even when the increase in UDP-HexNAcs was prevented by inhibition of the rate-limiting enzyme glutamine fructose-6-phosphate amidotransferase (GFAT). Actinomycin D and cycloheximide completely prevented glucose-induced insulin resistance despite a greater accumulation of UDP-HexNAcs (25). In addition, Hresko et al. (22) found that exposure of fat cells to glucosamine resulted in insulin resistance by causing a depletion of ATP, and Nelson et al. (35) reported that defective protein kinase B activation is associated with glucose-induced, but not glucosamine-induced, insulin resistance in adipocytes.

These findings raised the question that we addressed in this study: are different mechanisms involved in the induction of insulin resistance by glucosamine and glucose, or does glucosamine, like glucose, cause insulin resistance by an actinomycin D-inhibitable process?

MATERIALS AND METHODS

Materials. 2-Deoxy-d-[1,2-3H]glucose was purchased from American Radiolabeled Chemicals, and [U-14C]mannitol was obtained from DuPont-NEN. Pork insulin was purchased from Eli Lilly. Antibodies against insulin receptor substrate (IRS)-1, phosphatidylinositol (PI) 3-kinase, phospho-Akt1/PKBα (Ser473) and Thr308, and phospho-Oscrete were from Upstate Biotechnology. Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories. Reagents for SDS-PAGE were obtained from Bio-Rad. All other reagents were purchased from Sigma-Aldrich.

Animals. Male Wistar rats weighing ~100 g were obtained from Charles River Laboratories and provided with Purina rat chow and water ad libitum. This research was approved by the Washington University Animal Care Committee.

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by the Animal Studies Committee of Washington University School of Medicine.

Muscle preparations and incubations. After an overnight fast, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and the epitrochlearis and diaphragm muscles were dissected out. The diaphragm preparation used in this study was the intact hemidiaphragm with rib cage attached (29).

Hemidiaphragms were placed in 6 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHB; containing 5 mM glucose, 10 mM glucosamine, 25 mM mannitol, and 0.1% radioimmunoassay-grade bovine serum albumin, with or without 2 mM glucose, 20 mM glucosamine, 15 mM mannitol, and 0.1% albumin, with or without 2 mM insulin) for 3 h. Epitrochlearis muscles were incubated with shaking at 35°C for 3 h. Epitrochlearis muscles were incubated with shaking at 35°C in 2 ml of oxygenated KHB (containing 5 mM glucose, 10 or 20 mM glucosamine, 25 or 15 mM mannitol, and 0.1% albumin, with or without 2 mM insulin) for 3 h, or in the same medium with 10 mM glucosamine for 5 h. For some of the muscles, the incubation medium also contained 2 μM actinomycin D. Control muscles were incubated in the same medium without glucosamine.

Measurement of glucose transport activity. After the initial incubation, the muscles were transferred to flasks containing KHB with 2 mM sodium pyruvate, 2.0 mM insulin (if it was present in the previous incubation), and 36 mM mannitol and incubated with shaking at 30°C for 20 min to remove glucosamine and glucose from the extracellular space. Glucose transport activity was then measured using 2-deoxy-D-glucose (2DG), as described previously (15, 48). Briefly, the muscles were incubated for 20 min at 30°C in oxygenated KHB containing 4 mM 2-deoxy-[1,2-3H]glucose, 1.5 μCi/ml, and 36 mM [U-14C]mannitol, 0.2 μCi/ml, and if insulin was present in the previous incubations, it was also present in the transport assay.

Insulin signaling. After incubation for 5 h with 20 mM glucosamine and 1 mM insulin, as described in Muscle preparations and incubations, epitrochlearis muscles were used for measurement of IRS-1 tyrosine phosphorylation (16), IRS-1-associated PI 3-kinase (36), and phosphorylation of protein kinase B (27), as described previously.

Analytical procedures. UDP-linked hexosamines were measured in diaphragm muscles with high-performance liquid chromatography (20, 26). Diaphragm muscle ATP concentration was measured fluorometrically.

Statistical analysis. Results are expressed as means ± SE. The significance of differences between two groups was assessed using Student’s unpaired t-test. For multiple comparisons, significance was determined by analysis of variance. A Newman-Keuls post hoc test was used to locate significant differences between means.

RESULTS

Incubation of hemidiaphragm muscle with glucosamine. As shown in Fig. 1, incubation of rat hemidiaphragm muscle preparations with 10 mM glucosamine for 3 h resulted in a reduction of insulin responsiveness of glucose transport of ~50%. In contrast to our finding that 2 μM actinomycin D completely blocks the development of glucose-induced insulin resistance (25), glucosamine-induced insulin resistance was not prevented by actinomycin D.

Hexosamine pathway end products. Treatment of diaphragms with 10 mM glucosamine for 3 h resulted in an approximately fivefold increase in UDP-HexNAs [24.5 ± 1.2 vs. 124.7 ± 3.8 (SE) nmol/g for 7 muscles/group].

ATP concentration in muscles treated with glucosamine. Hresko et al. (22) found that exposure of adipocytes to glucosamine caused ATP depletion. They provided evidence that the severe insulin resistance that developed in the adipocytes exposed to glucosamine was mediated by the ATP depletion. We therefore determined the effect of 3 h of incubation with 10 mM glucosamine on ATP concentration in rat hemidiaphragms. As shown in Fig. 2, ATP concentration was decreased ~58% in the glucosamine-treated diaphragms.
by hexokinase requires ATP. The diaphragm is covered by connective tissue, and respiration of the muscle fibers could be limited by inadequate oxygen diffusion, making anaerobic glycolysis a major source of ATP production. Thus it seemed possible that inadequate glucose availability may have mediated the decrease in ATP.

We therefore also examined the effect of glucosamine in the epitrochlearis muscle. In the resting state, the epitrochlearis muscle is well oxygenated under our experimental conditions, as evidenced by maintenance of normal ATP and creatine phosphate concentrations in the presence of low substrate levels (2 mM glucose) for 5 h (unpublished data) or for 9 h with 8 mM glucose even in the absence of insulin (12).

As shown in Fig. 3, incubation of epitrochlearis muscles with 10 mM glucosamine for 3 h had no effect on the magnitude of the increase in 2DG transport induced by 2 mU/ml insulin. However, increasing the duration of exposure to 10 mM glucosamine for 5 h, or increasing the concentration of glucosamine to 20 mM, did result in significant decreases in insulin responsiveness of glucose transport. This glucosamine-induced decrease in insulin-stimulated glucose transport in the epitrochlearis was not mediated by a decrease in high-energy phosphates, because ATP \( 4.77 \pm 0.14 \) (SE) \( \mu \text{mol/g muscle} \) for controls and \( 4.96 \pm 0.21 \) (SE) \( \mu \text{mol/g} \) for muscles exposed to 10 mM glucosamine for 5 h; 8 muscles/group and creatine phosphate \( 15.83 \pm 0.36 \) (SE) \( \mu \text{mol/g} \) for controls and \( 15.92 \pm 0.35 \) (SE) \( \mu \text{mol/g} \) for muscles exposed to 10 mM glucosamine for 5 h; 8 muscles/group) concentrations were unchanged. In contrast to our finding (25) that the insulin resistance induced in epitrochlearis muscles by exposure to high glucose and insulin concentrations was completely prevented by actinomycin D, actinomycin D had no effect on the decrease in insulin responsiveness induced by glucosamine (Fig. 3).

**Insulin signaling.** As shown in Fig. 4A, insulin-induced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) was determined as described in MATERIALS AND METHODS in epitrochlearis muscles incubated for 5 h in the presence or absence of 10 mM GluN. Values are means ± SE of 10–12 muscles/group. \* \( P < 0.01 \) vs. insulin alone. B: insulin-induced binding of phosphatidylinositol 3-kinase (PI3K) to IRS-1 was determined in epitrochlearis muscles incubated in the presence or absence of 10 mM GluN for 5 h. Values are means ± SE of 11 muscles/group. \* \( P < 0.01 \) vs. insulin alone. C: insulin-induced phosphorylation (p) of PKB on Thr\(^{308} \) and Ser\(^{473} \). Values are means ± SE of 12 muscles/group.
ulated phosphorylation of protein kinase B was not reduced in the glucosamine-treated muscles (Fig. 4C).

**DISCUSSION**

Glycogen-depleting exercise followed by carbohydrate feeding results in a large increase in muscle glycogen that greatly exceeds the glycogen concentration found in muscle in the fed, sedentary state (2, 6). This phenomenon, which has been called glycogen supercompensation, is mediated by a massive influx of glucose that results from a large increase in glucose transport activity. The increase in glucose transport is initially a consequence of persistence of an exercise-induced stimulation of glucose transport and, as this effect wears off, a result of an exercise-induced enhancement of insulin action (4, 10, 19, 23, 38, 46).

Concomitant with the glycogen supercompensation, the exercised muscles develop resistance to insulin that results in reversal of the beneficial effects of exercise on insulin sensitivity and responsiveness (4, 9, 18, 21, 26, 27, 39). This glucose toxicity form of insulin resistance can also be induced by prolonged glucose infusions that result in high levels of blood glucose and insulin (7, 25, 31) and by incubation of isolated muscles or fat cells with high concentrations of glucose and insulin (11, 24, 25, 30). Glucose toxicity insulin resistance may also develop in type 1 diabetics with high blood glucose levels whose hyperglycemia is reversed by insulin therapy (47).

A number of hypotheses have been proposed regarding the mechanism responsible for glucose-induced insulin resistance. One is that accumulation of large amounts of glycogen mediates the insulin resistance, possibly as a result of binding of GLUT4-containing vesicles to glycogen particles (5). Another is that glucose-induced insulin resistance is caused by increased entry of glucose into the hexosamine synthetic pathway, resulting in accumulation of UDP-HexNAc (1, 33, 34, 41, 43, 45).

The hypothesis that hexosamine pathway end products cause insulin resistance was based on studies by Marshall and coworkers (32, 33, 44), who found that adipocytes exposed to high concentrations of glucose and insulin became insulin resistant, but only in the presence of glutamine. The finding that glutamine was necessary raised the possibility that the hexosamine pathway is involved. Evidence in support of this hypothesis came from experiments in which inhibition of GFAT, the rate-limiting step in the hexosamine pathway, protected against glucose-induced insulin resistance (33). They also showed that glucosamine, which enters the pathway beyond GFAT, is more potent than glucose in producing insulin resistance (33). Further support for the hypothesis that hexosamine pathway end products mediate glucose toxicity was provided by studies showing that infusion of rats with glucosamine (1, 43) or incubating muscles with glucosamine (28, 41, 42) causes insulin resistance of glucose transport. Overexpression of GFAT in muscle and adipocytes also resulted in a decrease in glucose disposal (17).

In a previous study (25), we evaluated the hypothesis regarding the mechanisms responsible for glucose toxicity in an in vitro isolated muscle preparation. Our experimental approach was to incubate rat epidermal muscles with very high concentrations of glucose and insulin for 5 h. This relatively brief treatment resulted in a decrease in insulin responsiveness of glucose transport similar in magnitude, ~60%, to that induced by a 24-h-long glucose infusion that raised plasma glucose to 13 mM and insulin to ~460 μU/ml. Using this model we found that, in contrast to previous studies in fat cells (33), inhibition of GFAT did not protect against the development of insulin resistance despite complete prevention of the increase in UDP-HexNAc (25). Also, in contrast to the finding in fat cells that glutamine is necessary for development of glucose toxicity, glucose-induced insulin resistance developed despite the absence of glutamine in the incubation medium.

Marshall et al. (32) showed that inhibition of mRNA synthesis protects against glucose toxicity in fat cells. We confirmed this finding in our muscle preparation, in which both the inhibitor of mRNA synthesis, actinomycin D, and the inhibitor of protein synthesis, cycloheximide, completely prevented the glucose-induced insulin resistance. As a consequence of prevention of insulin resistance, more glucose flooded into the muscles treated with actinomycin D or cycloheximide, resulting in two- to threefold larger increases in UDP-HexNAc and glycogen than in the muscles not treated with actinomycin D or cycloheximide that became severely insulin resistant (25). On the basis of these findings and similar results obtained by Davidson et al. (8) in L6 myotubes, we concluded that glucose-induced insulin resistance in skeletal muscle is mediated by synthesis of a protein with a short half-life, not by accumulation of UDP-HexNAc or glycogen.

Other studies have shown that 1) glucosamine causes impairment of insulin-stimulated IRS-1 tyrosine phosphorylation and association of the p85 regulatory subunit of PI3K with IRS-1 (28, 37), whereas glucose toxicity does not (25); 2) defective protein kinase B activation by insulin is associated with glucose-but not glucosamine-mediated insulin resistance in adipocytes (35); and 3) glucosamine, but not glucose, can cause high-energy phosphate depletion (22). These findings, together with the evidence from our experiments on muscle indicating that glucose-induced insulin resistance is not mediated by accumulation of UDP-HexNAc (25), raised the question: are different mechanisms involved in the induction of skeletal muscle insulin resistance by glucose and glucosamine?

Our experiments on rat hemidiaphragms show that, as in adipocytes (22), high concentrations of glucosamine can cause insulin resistance by means of ATP depletion. This phenomenon, which appears to be mediated by increased high-energy phosphate (−P) utilization and decreased glucose availability, is not the mechanism by which glucosamine causes insulin resistance in well-oxygenated tissues provided with adequate substrate. This is evidenced by the findings that
glucosamine infusion into rats causes muscle insulin resistance without lowering $\sim$P (37), and, in the present study, that exposure of epitrochlearis muscles to glucosamine causes insulin resistance despite no decrease in ATP.

We previously found (25) that prevention of an increase in UDP-HexNAc by inhibition of GFAT does not prevent glucose-induced insulin resistance and that inhibition of protein synthesis prevented glucose-induced insulin resistance despite enhanced UDP-HexNAc accumulation in skeletal muscle. This latter finding suggested the possibility that, like glucose, glucosamine might cause insulin resistance by inducing synthesis of a protein that mediates the insulin resistance. However, in contrast to the glucose-induced decrease in insulin responsiveness, the insulin resistance produced by exposing epitrochlearis muscles to glucosamine was not prevented by actinomycin D. As in previous studies on rats infused with glucosamine (28, 37), exposure of epitrochlearis muscles to glucosamine resulted in a marked reduction of stimulation by insulin of IRS-1 tyrosine phosphorylation and IRS-1 association with PI3K. Under similar experimental conditions, glucose-induced insulin resistance was not associated with development of insulin resistance of these insulin-signaling events in epitrochlearis muscles (25).

It appears evident from these major differences that glucose and glucosamine induce skeletal muscle insulin resistance by different mechanisms. It has been proposed that glucosamine causes insulin resistance by impairing the functions of insulin-signaling proteins and GLUT4 by means of increased protein $O$-GlcNAcylation (3, 41). Although it seems likely that this is the mechanism by which glucosamine causes insulin resistance, it is not clear whether this phenomenon has physiological relevance. The high concentrations of glucosamine required to produce muscle insulin resistance in animals infused with glucosamine and in muscles incubated with glucosamine in vitro are an experimental artifact, as glucosamine is normally too low to detect in plasma. In agreement with our finding that flooding of glucose into muscle can produce insulin resistance by a mechanism that does not involve hexosamine pathway end products (25, 26), Robinson et al. (42) reported that an extreme degree of hyperglycemia resulted in insulin resistance despite only an $\sim$15% increase in UDP-HexNAcs.

In conclusion, the results of this investigation and of previous studies (8, 28, 30, 35) provide evidence that glucosamine and glucose induce insulin resistance by different mechanisms. In light of this evidence, and because exposure of tissues to glucosamine is unphysiological, it would appear that the appropriate approach to investigating the mechanisms underlying glucose toxicity is to flood muscle or adipose tissue with glucose, not glucosamine.

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DISCLOSURES

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REFERENCES


