Allosteric Regulation of Glycogen Synthase and Hexokinase by Glucosamine-6-Phosphate During Glucosamine-Induced Insulin Resistance in Skeletal Muscle and Heart

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Glucosamine infusion induces insulin resistance in vivo, but the effect of glucosamine on intracellular metabolites of the hexosamine pathway, especially glucosamine-6-phosphate (GlcN6P) is unknown. Because of the structural similarity of glucose-6-phosphate (G-6-P) and GlcN6P, we hypothesized that accumulation of this metabolite might alter the activities of enzymes such as glycogen synthase and hexokinase. We infused glucosamine (30 µmol · kg^{-1} · min^{-1}) to induce insulin resistance in rats during a euglycemic-hyperinsulinemic clamp. Glucosamine induced whole-body insulin resistance, which was apparent after 90 min and continued progressively for 360 min. Despite inducing severe whole-body insulin resistance and decrease in glycogen synthase fractional activity in rectus abdominis muscle (69 ± 3 vs. 83 ± 1%, *P* < 0.01) and heart (7 ± 1 vs. 32 ± 4%, *P* < 0.001), glucosamine did not change the glycogen content in rectus and even increased it in the heart (209 ± 13 vs. 117 ± 9 mmol/kg dry wt, *P* < 0.001). Glucosamine increased tissue concentrations of UDP-GlcNAc 4.4- and 4.6-fold in rectus abdominis and heart, respectively. However, GlcN6P concentrations increased 500- and 700-fold in glucosamine-infused animals in rectus abdominis (590 ± 80 vs. 1.2 ± 0.1 µmol/kg wet wt, *P* < 0.001) and heart (7,703 ± 993 vs. 11.2 ± 2.3 µmol/kg wet wt, *P* < 0.001). To assess the possible significance of GlcN6P accumulation, we measured the effect of GlcN6P on glycogen synthase and hexokinase activity in vitro. At the GlcN6P concentrations measured in rectus abdominis and heart in vivo, glycogen synthase was activated by 21 and 542%, while similar concentrations inhibited hexokinase activity by 5 and 46%, respectively. This study demonstrates that infusion of glucosamine during a euglycemic-hyperinsulinemic clamp results in marked accumulation of intracellular GlcN6P. The GlcN6P concentrations in the heart and rectus abdominis muscle reach levels sufficient to cause allosteric activation of glycogen synthase and inhibition of hexokinase. *Diabetes* 48:1101–1107, 1999

Hyperglycemia is a major cause of insulin resistance in diabetic patients, but the mechanisms underlying this phenomenon of "glucose toxicity" are currently poorly understood (1). Studies performed in adipocytes have shown that glucose-induced desensitization of insulin-stimulated glucose transport is glutamine-dependent (2) and can be blocked by glutamine analogs such as *O*-diazoacetyl-L-serine or 6-diazo-5-oxonorleucine (2). It is also known that glucosamine, which enters the hexosamine pathway by bypassing the rate-limiting enzyme of the hexosamine pathway, glutamine:fructose-6-phosphate amidotransferase (GFA) is, on a molar basis, more effective than glucose in inducing insulin resistance of glucose uptake. Glucosamine induces insulin resistance in multiple tissues in normal rats (3) but fails to do so in diabetic rats, possibly because chronic hyperglycemia has already activated the hexosamine pathway (4).

Although the above data suggest that the hexosamine pathway may mediate glucose toxicity, several aspects of glucosamine-induced insulin resistance differ from that in diabetic animals. In fibroblasts overexpressing GFA, basal glycogen synthase activity is higher than in control cells, although the transfected cells have a severe impairment in glycogen synthesis (5). In fibroblasts overexpressing the insulin receptor, preexposure to glucosamine increases glycogen synthase fractional activity despite inducing a defect in glycogen synthesis (6). These data contrast with the uniform finding of a low glycogen synthase fractional activity and defective glycogen synthesis in both type 1 (7) and type 2 (8,9) diabetic patients. Effects of glucosamine on muscle glycogen content also appear to be heterogeneous. In the heart, glucosamine decreases insulin stimulated glucose uptake despite increasing the glycogen content (3). In skeletal muscles, glucosamine seems to decrease the glycogen content in soleus muscle (3) and either to decrease (4,10) or not change (3) glycogen content in rectus abdominis muscle. The reasons for these variable effects of glucosamine on glycogen synthase activity and glycogen synthesis are unclear.

Glucosamine is transported and phosphorylated to glucosamine-6-phosphate (GlcN6P) by the same mechanisms as

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G-6-P, glucose-6-phosphate; Gal, galactose; GalNAc, *N*-acetyl-galactosamine; GFA, glutamine:fructose-6-phosphate amidotransferase; Glc, glucose; GlcN6P, glucosamine-6-phosphate; GlcNA, glucosamine-specific activity; GlcNAc, *N*-acetyl-glucosamine; GLYSA, glycogen-specific activity; GSA, glucose-specific activity; HPLC, high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; WSA, water-specific activity.

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glucose (4,11). GlcN6P is then thought to be rapidly acetylated and converted via a series of non–rate-limiting reactions to UDP-*N*-acetylglucosamine (UDP-GlcNAc) (6,12). The validity of the assumption of rapid acetylation of GlcN6P, however, has not been tested in studies where glucosamine has been used to induce insulin resistance (4,6,13). Several hexose phosphates, including GlcN6P, have been shown to activate glycogen synthase in polymorphonuclear leukocytes in vitro (14). If accumulation of GlcN6P would be considerable, it might stimulate glycogen synthase allosterically. GlcN6P accumulation might also inhibit hexokinase activity, although a concentration of 1 mmol/l has been reported not to change hexokinase activity in the brain (15).

The present study was undertaken to determine whether GlcN6P accumulates in skeletal muscle or the heart during glucosamine-induced insulin resistance and if so, whether its magnitude is sufficient to allosterically regulate glycogen synthase and hexokinase activities. We induced insulin resistance in vivo in chronically catheterized, freely moving awake rats using glucosamine. Glycogen synthesis was determined using both labeled glucose and glucosamine, and GlcN6P and key intermediates of the hexosamine and glucose pathway were measured in rectus abdominis muscle and heart. We then examined the effect of various GlcN6P concentrations on glycogen synthase and hexokinase activities in vitro. These data demonstrate that a massive increase in GlcN6P concentrations occurs during glucosamine infusions in vivo, and that its magnitude when measured in vitro is sufficient to allosterically activate glycogen synthase in both the heart and skeletal muscle and inhibit hexokinase activity in the heart.

RESEARCH DESIGN AND METHODS

Preparation of animals. Male Wistar rats (250–300 g) were housed in a 12-h light-dark cycle and fed standard rodent food (Altromin 1323; Feedcon, Helsinki, Finland). At 6 days before the study, the animals were anesthetized and catheters were inserted into the aortic arch for blood sampling and the right atrium for infusions as previously described in detail (16).

Study design. Two groups of animals were studied under normoglycemic-hyperinsulinemic conditions (insulin infusion rate 18 mU · kg^{-1} · min^{-1}). The animals received, in addition to glucose and insulin, an infusion of either glucosamine or saline. The rats, which were studied after a 16-h fast, were placed into metabolic cages, and the catheters were connected to infusion pumps. The rats were allowed to move freely for 1 h before the infusions were started. A primed-continuous infusion of insulin (10 µl/min, 18 mU · kg^{-1} · min^{-1}) was started at 0 min and continued for 360 min. Plasma glucose was measured every 5 min (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA), and the rate of a 20% glucose infusion was adjusted to maintain the plasma glucose concentration at ~5–6 mmol/l. A primed (345 µmol/kg) continuous (30 µmol · kg^{-1} · min^{-1}) infusion of glucosamine was started at 0 min and maintained throughout the study. [3-^{3}H]Glucose was infused to measure rates of whole-body glucose kinetics, glycolysis and glycogen synthesis, and in vivo glycogen synthesis in the heart and skeletal muscle. In addition, [U-^{14}C]glucosamine was infused to determine plasma levels of glucosamine and to explore the possibility that glucosamine is incorporated into glycogen via either formation of UDP-glucosamine (17,18) or reversal of the GFA reaction (19). [3-^{3}H]Glucose (15 µCi, 0.2 µCi/min) was mixed with the insulin infusion and [U-^{14}C]glucosamine (30 µCi, 0.5 µCi · kg^{-1} · min^{-1}) with the glucosamine infusion. Samples for determination of glucose-specific activity (GSA) and glucosamine activity (GlcNA) were drawn at 0, 30, 60, 120, 180, 240, 300, and 360 min. At the end of the study, the rats were anesthetized and rectus abdominis and heart muscles were freeze-clamped in situ with aluminum tongues precooled in liquid nitrogen for measurement of the glycogen concentration, hexokinase and glycogen synthase activities, rates of glycogen synthesis from glucose and glucosamine, and metabolite concentrations. Tissue samples were stored at –80°C until analysis.

Measurements of whole-body glucose kinetics, glycolysis and glycogen synthesis, and plasma glucosamine concentrations. Aliquots of plasma (50 µl) for the determination of [^{3}H]GSA, [^{3}H]WSA (tritiated water-specific activity), and [^{14}C]GlcNA were deproteinized with 100 µl $Ba(OH)_2$ and 100 µl $Zn(SO)_4$ (20). The protein-free supernatant was divided into two 40-µl aliquots, one of which was counted directly to measure the total [^{3}H] and [^{14}C] radioactivity concentrations, after adding liquid scintillation fluid (OptiPhase, Wallac, U.K., and Hisafe 3, Milton Keynes, U.K.). The other aliquot was counted after evaporation to dryness and reconstitution in water to determine the concentration of [^{3}H]glucose radioactivity (21). [^{3}H]GSA was calculated by dividing [^{3}H] radioactivity in the dried reconstituted aliquots by the plasma glucose concentration. WSA was calculated by subtracting [^{3}H] radioactivity in the dried aliquot from total [^{3}H] radioactivity (21). Plasma water was assumed to be 93% of total plasma volume and total water mass 65% of the body mass (21). Plasma glucosamine concentrations were measured by dividing plasma [^{14}C] radioactivity (dpm/ml) by the specific activity of glucosamine in the infusate (dpm/mmol). This method was based on an assumption that glucosamine metabolism does not include significant amounts of metabolites, which would be released into the circulation (22). Although the distribution of radioactivity was not analyzed in plasma samples, radioactivity reached a rapid plateau within 30 min, and the concentrations were similar to those previously measured with high-performance liquid chromatography (HPLC) during infusion of a similar glucosamine dose plateau rapidly after priming dose (<30 min) and remained constant thereafter, suggesting that the assumption was correct.

Glycogen concentration and in vivo rates of glycogen synthesis in heart and skeletal muscle. The glycogen concentration was measured after amyloglucosidase treatment of neutralized alkali extracts of the freeze-dried samples, as previously described (16). For determination of glycogen-specific activity (GLYSA), glycogen was precipitated with 66% ice-cold ethanol after alkaline extraction as previously described in detail (16). The precipitate was washed thoroughly with ethanol, redissolved in alkali, neutralized, and treated with amyloglucosidase. An aliquot was counted for [^{3}H] and [^{14}C] radioactivity, and glucose was measured in another aliquot. GLYSA (dpm/mmol) was calculated by dividing the [^{3}H] or [^{14}C] radioactivity by the glycogen concentration (mmol/ml) in the ethanol precipitate. The amount of radioactivity incorporated into glycogen per dry muscle (dpm_{gly}) was calculated by multiplying GLYSA (dpm/mmol) by the tissue glycogen concentration (mmol/kg dry wt).

Metabolite assays. For determination of GlcN6P, glucose-6-phosphate (G-6-P), ATP, UDP-Glc, UDP-Gal, UDP-GlcNAc, and UDP-GalNAc, the freeze-dried tissue samples were ground in a mortar and extracted with ice-cold 7% perchloric acid for 30 min, neutralized with 2.2 mol/l $KHCO_3$, and kept at –80°C until analysis. GlcN6P concentrations were measured using HPLC after *o*-phthaldialdehyde derivatization and fluorescence detection as previously described in detail (3,23). G-6-P and ATP concentrations were determined using enzymatic microfluorometric methods as described by Passonneau and Lowry (24).

The concentrations of UDP-Glc, UDP-Gal, UDP-GlcNAc, and UDP-GalNAc were measured after delipidation of the neutral muscle extract as described by Robinson et al. (6) and Hebert et al. (25) with slight modifications. HPLC was performed using two Spherisorb amino columns (25 cm × 3 mm; Phase Separation, Norwalk, CT) connected in series. The columns were eluted with concave gradient from 80 mmol/l potassium phosphate (pH 2.8), 35% acetonitrile, to 800 mmol/l potassium phosphate (pH 3.6) over 70 min at a flow rate of 0.3 ml/min at room temperature. Under these conditions, UDP-GlcNAc, UDP-GalNAc, UDP-Glc, and UDP-Gal eluted as separate peaks between 40 and 45 min. Their concentrations were quantified by ultraviolet absorption (254 nm) and by comparison to external standards. The standard curves were linear over a concentration range of 1 to 20 nmol per peak. The recovery of standards added to muscle homogenates was >95%. The purity of GlcN6P, UDP-hexoses, and UDP-hexosamines was verified by HPLC.

Glycogen synthase and hexokinase activities. For measurement of glycogen synthase activity, frozen tissue (10–20 mg) was homogenized in a 10-fold excess (vol/wt) 50 mmol/l HEPES buffer (pH 7.4) containing 1% Triton X-100, 50 mmol/l sodium pyrophosphate, 10 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium orthovanadate, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mmol/l benzamidine, and 2 mmol/l phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 50,000 rpm for 1 h. The supernatant was stored for analysis at –80°C. Glycogen synthase activity was then determined using a radiometric method as described by Bogardus et al. (26). Glycogen synthase fractional activity was determined from the ratio of glycogen synthase activities measured at 0.1 and 10 mmol/l G-6-P. An increase in this ratio reflects a decrease in the phosphorylation state of glycogen synthase (26). All assays were normalized to protein. Counts originating from infused [3-^{3}H]glucose did not contribute significantly to background radioactivity in the assay. In vitro dose-response curves for activation of glycogen synthase by GlcN6P were constructed by measuring glycogen synthase activity in heart and rectus abdominis muscle from tissues of the glucosamine-infused animals in the presence of 0, 0.05, 0.1, 0.5, 1, 2, 5, and 10 mmol/l GlcN6P at a G-6-P concentration of 0.1 mmol/l in the final incubation mixture. Glycogen synthase activity was also measured in the same samples in the presence of 0.5 and 10 mmol/l G-6-P without GlcN6P addition. For measurement of the effect of GlcN6P on hexokinase activity, frozen samples of heart and rectus abdominis muscle were homogenized in 50 mmol/l potassium phosphate buffer (pH 7.4) containing 1 mmol/l dithiothreitol, 28 mmol/l EDTA, 33 mmol/l sodium fluoride, 0.12 mmol/l leupeptin,

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500 U/ml trypsin inhibitor, 0.6 mmol/l benzamidine, 0.5 mmol/l *N-P*-tosyl-L-lysine chloromethyl ketone, 0.5 mmol/l *N*-tosyl-L-phenylalanine chloromethyl ketone, and 2.4 mmol/l PMSF. Fifty microliters of this homogenate was added to 1 ml of reaction mixture containing 0.125 mmol/l glucose, 10 mmol/l ATP, 385 mg/l actractylate, and 33 mmol/l sodium fluoride in the presence of 1 µCi [3-^{3}H]glucose. The reaction was stopped by adding 550 µl of 1.45 N perchloric acid to each tube. The extracts were then neutralized with $KHCO_3$ and centrifuged, and the supernatant was applied to ion-exchange chromatography to separate neutral glucose from G-6-P. The columns (Agl-X8; BioRad, Hercules, CA) were washed three times with distilled water and thereafter eluted with 1 N HCl. The eluted radioactivity was counted for [^{3}H] radioactivity. The rate of G-6-P formation was calculated by dividing the recovered [^{3}H] radioactivity by the specific activity of glucose and time. All assays were done in triplicate. All chemicals used in metabolite and enzyme assays were from Sigma (St. Louis, MO).

Calculations. The rate of glucose turnover (R_t, micromoles per kilogram per minute) was calculated from the formula $R_t = (F/\text{GSA})/W$, where F denotes the isotope infusion rate (dpm per minute), GSA the mean specific activity of glucose (dpm per micromole) in plasma, and W body weight (kilograms). The rate of glycogen synthesis (incorporation of glucosyl units into glycogen) (R_s; micromoles per kilogram per minute) was calculated from the formula $R_s = [(\text{dpm}_{gly})/dt)]/\text{GSA}$, where dt is the time period (360 min). The rate of whole-body glycolysis was calculated from the increment in WSA (dpm per milliliter per minute) multiplied by total body water and divided by the mean GSA (21).

Statistics. Data between the two groups were analyzed using the unpaired Student's t test. Simple correlations were calculated using Pearson's correlation coefficient. All calculations were made using the Sigma Plot statistical software (version 4.00; SPSS, Chicago, IL). All data are expressed as mean ± SE.

RESULTS

Effect of glucosamine on whole-body insulin-stimulated glucose uptake, glycolysis, and glycogen synthesis. Plasma glucose concentrations averaged 5.5 ± 0.1 and 5.5 ± 0.1 mmol/l (NS) during insulin and glucosamine + insulin infusions. Serum insulin concentrations averaged 1.6 ± 0.3 and 1.5 ± 0.2 nmol/l, respectively. Infusion of glucosamine increased circulating glucosamine concentrations to 1.6 ± 0.3 mmol/l; they remained constant during the 6 h study period (Fig. 1). Plasma glucosamine was undetectable in animals not receiving glucosamine. Glucosamine induced severe insulin resistance, as evidenced by the lower glucose infusion rate required to maintain normoglycemia (Fig. 1). A statistically significant difference in the glucose infusion rate was observed after 90 min. The glucose infusion rate averaged 130 ± 11 and 168 ± 8 µmol · kg^{-1} · min^{-1} ($P < 0.01$) during the entire 6 h study period ($P < 0.001$), and 122 ± 15 and 187 ± 8 µmol · kg^{-1} · min^{-1} ($P < 0.001$) during the last hour of the study in the rats infused with glucosamine + insulin and insulin, respectively (Fig. 1). The rate of glucose turnover, measured with [3-^{3}H]glucose, was lower in the animals infused with glucosamine + insulin (109 ± 10 µmol · kg^{-1} · min^{-1}) than with insulin (145 ± 8 µmol · kg^{-1} · min^{-1}, $P < 0.001$). Whole-body glycolysis remained unchanged (52 ± 4 vs. 57 ± 6 µmol · kg^{-1} · min^{-1}, NS), whereas whole body-glycogen synthesis was significantly decreased (57 ± 9 vs. 88 ± 11 µmol · kg^{-1} · min^{-1}, $P < 0.05$).

Glycogen metabolism in rectus abdominis and heart muscles. Glycogen content in rectus abdominis muscle was comparable in animals infused with glucosamine + insulin and insulin (330 ± 19 and 283 ± 10 mmol/kg dry wt). In the heart, glycogen content was significantly increased by glucosamine (209 ± 13 vs. 117 ± 9 mmol/kg dry wt, $P < 0.001$). Glycogen synthesis during the whole study period, as determined from the rate of incorporation of [3-^{3}H]glucose into glycogen in rectus abdominis muscle, was not different between the animals infused with glucosamine + insulin (253 ± 24 µmol · kg^{-1} dry wt · min^{-1}) and with insulin alone (298 ± 71 µmol · kg^{-1} dry wt · min^{-1}, NS). In the heart, the animals infused with glucosamine + insulin had a significantly higher rate of glucose incorporation into glycogen than animals infused with insulin alone (181 ± 43 vs. 120 ± 12 µmol · kg^{-1} dry wt · min^{-1}, $P < 0.05$). The incorporation of [^{14}C] radioactivity into glycogen from infused [U-^{14}C]glucosamine was negligible in both rectus abdominis and heart muscles and did not contribute to changes in glycogen content (data not shown).

Infusion of glucosamine + insulin significantly decreased glycogen synthase fractional activity in both rectus abdominis muscle and heart compared with the insulin-infused animals. Glycogen synthase fractional activity averaged 69 ± 3% in the animals infused with glucosamine + insulin and 83 ± 1% ($P < 0.01$) in the insulin-infused animals in rectus abdominis muscle. In the heart, glycogen synthase fractional activity was 77% lower in the animals infused with glucosamine + insulin (7 ± 1%) than with insulin (32 ± 4%, $P < 0.001$).

Metabolite concentrations. GlcN6P concentrations were low in the insulin-infused animals both in rectus abdominis muscle and heart, although the GlcN6P concentrations were ~10-fold higher in heart than in rectus abdominis (Table 1). Infusion of glucosamine increased these concentrations 700- and 500-fold, respectively (Table 1). These increases were more than two magnitudes larger than the 4.4- and 4.6-fold increases in UDP-GlcNAc concentrations in rectus abdo-

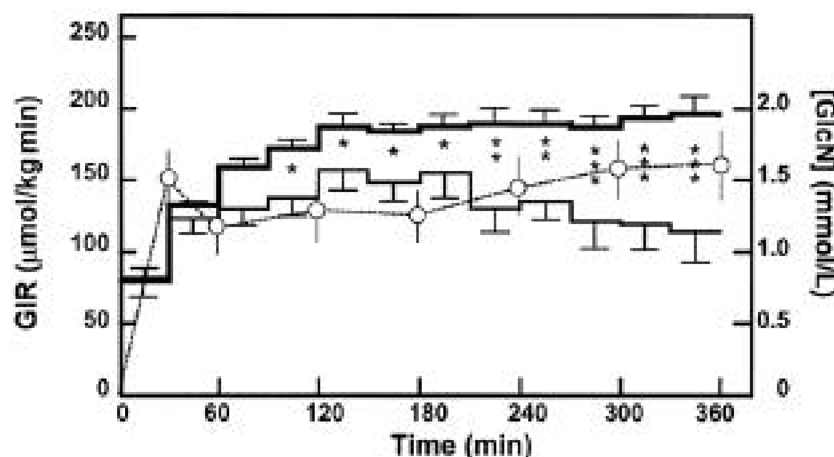

FIG. 1. Glucose infusion rate (GIR) in rats infused with insulin (▬▬) and insulin + glucosamine (——) and serum glucosamine concentrations in glucosamine-infused rats (– – –) during the euglycemic-hyperinsulinemic clamp. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, difference in glucose infusion rate between glucosamine + insulin vs. insulin-infused animals.

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minis and heart (Table 1). The UDP-Glc and UDP-Gal concentrations were significantly lower in the animals infused with glucosamine + insulin than with insulin (Table 1).

Effects of GlcN6P on glycogen synthase activity in vitro. The data presented above demonstrated tissue-specific and paradoxical effects of glucosamine on glycogen synthesis. Thus, while glycogen synthesis was decreased at the level of the whole body, it was not altered in rectus abdominis muscle and was markedly increased in the heart. The latter two changes were observed despite a decrease in glycogen synthase fractional activity, suggesting that mechanisms other than those mediated by a change in glycogen synthase phosphorylation regulate glycogen synthesis during glucosamine infusions. To determine to what extent the observed increases in GlcN6P concentrations were able to activate glycogen synthase, we measured glycogen synthase activity in vitro at different GlcN6P concentrations. These measurements were performed in pooled samples (similar amount of protein from each animal, $n = 5$) of glucosamine-infused rats (Fig. 2). In rectus abdominis muscle (Fig. 2*A*), the origin of the curve (at a G-6-P concentration of 0.1 mmol/l, without GlcN6P added to the reaction mixture) was at 71%, which was comparable to the fractional activity measured in individual samples (69 ± 3%). When the GlcN6P concentration was increased to 0.5 mmol/l, which corresponded to the measured GlcN6P concentration in rectus abdominis muscle, glycogen synthase fractional activity increased to 86%. By further increasing the GlcN6P concentration, maximal velocity of the enzyme (defined as glycogen synthase activity at a maximally effective G-6-P concentration of 10 mmol/l), could be reached but not overcome as the velocity plateaued at 5 mmol/l of GlcN6P (106%, 10 mmol/l GlcN6P 104%).

In the heart, GlcN6P had a greater effect on glycogen synthase fractional activity than in rectus abdominis muscle. Basal glycogen synthase fractional activity averaged 12% and was comparable to that measured in individual samples in the glucosamine-infused rats (7 ± 1%). By increasing the GlcN6P concentration to 0.5 mmol/l, enzyme activity was doubled (24%) and at concentrations measured in the heart in the glucosamine-infused animals (7.7 mmol/l), GlcN6P increased glycogen synthase fractional activity to 65%, which was two times higher than the activity measured in hearts of insulin-infused animals (32%). GlcN6P was a weaker stimulator of glycogen synthase than G-6-P (Fig. 2*B*). Even very high concentrations of GlcN6P were unable to activate glycogen synthase to the same extent as G-6-P, since a plateau in activation (fractional activity 67%) was obtained at a GlcN6P concentration of 10 mmol/l.

Effect of GlcN6P on hexokinase activity in vitro. To determine whether accumulation of GlcN6P during glucosamine infusions was sufficient to inhibit hexokinase activity, we measured hexokinase activity in vitro at different GlcN6P concentrations. Hexokinase activity was measured at a low glucose concentration (0.125 mmol/l), which resulted in negligible accumulation of G-6-P itself. Hexokinase activity in the absence of GlcN6P was set at 100%. By increasing GlcN6P concentrations in the reaction mixture, linear inhibition of hexokinase activity was observed over a wide range of GlcN6P concentrations (0 to at least 10 mmol/l) (Fig. 3). From the regression line, it was calculated that hexokinase activity decreased by 6% for every millimole increase per liter GlcN6P.

DISCUSSION

Glucosamine induces severe insulin resistance of glucose uptake over several hours. The insulin resistance is thought to result from activation of the hexosamine pathway, because glucosamine is metabolized via this pathway and because activation of the hexosamine pathway, by overexpressing its rate-limiting enzyme GFA, results in severe insulin resistance (25). There are also, however, other possible mechanisms by which glucosamine might alter glucose metabolism. In the present study, we found infusion of glucosamine to increase GlcN6P, the first product of glucosamine metabolism, 500- and 700-fold in rectus abdominis and heart muscles. The increases in UDP-GlcNAc of the hexosamine pathway were trivial (4.4- and 4.6-fold) compared with those of GlcN6P. In vitro, the increases in GlcN6P were sufficient to allosterically stimulate glycogen synthase activity in both tissues. Provided effects of GlcN6P in vitro mimic

TABLE 1
Glycogen, G-6-P, GlcN6P, and ATP concentrations in rectus abdominis and heart

	GlcN	Rectus abdominis	Heart
Glycogen (mmol/kg dry wt)	–	283 ± 10	117 ± 9
	+	330 ± 19	209 ± 13*
G-6-P (mmol/kg wet wt)	–	0.38 ± 0.08	0.79 ± 0.21
	+	0.42 ± 0.09	0.40 ± 0.07
GlcN6P (mmol/kg wet wt)	–	0.0012 ± 0.0001	0.0112 ± 0.0023
	+	0.59 ± 0.08†	7.7 ± 1.0†
ATP (mmol/kg wet wt)	–	5.3 ± 0.2	3.7 ± 0.2
	+	5.6 ± 0.2	3.9 ± 0.2
UDP-Glc (µmol/kg wet wt)	–	12.8 ± 1.9	46 ± 5
	+	8.8 ± 0.6*	23 ± 3‡
UDP-Gal (µmol/kg wet wt)	–	6.8 ± 1.5	17 ± 2
	+	3.0 ± 0.2*	9 ± 1‡
UDP-GlcNAc (µmol/kg wet wt)	–	23.0 ± 1.0	51 ± 5
	+	101 ± 7†	236 ± 10†
UDP-GalNAc (µmol/kg wet wt)	–	9.9 ± 1.2	24 ± 3
	+	19.4 ± 1.2†	77 ± 5†

Data are means ± SE. n = 6–7 rats per group. *$P < 0.05$, †$P < 0.001$, ‡$P < 0.01$ without vs. with GlcN.

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its effects in vivo, this finding may provide an explanation for the paradoxical increase in glycogen concentrations in the heart during glucosamine infusions.

Glucosamine and glucose transport and phosphorylation. GlcN6P accumulated in rectus abdominis muscle and heart in amounts sufficient to inhibit hexokinase activity slightly in skeletal muscle and more clearly (by 46%) in the heart. In a previous study performed in polymorphonuclear leukocytes, GlcN6P was ineffective in inhibiting brain hexokinase activity (15). That finding is not necessarily inconsistent with the present data, since the highest concentration in the latter study was 1 mmol/l. That concentration, which already exceeds physiologic concentrations in muscle by ~1,000-fold and in the heart by 100-fold (Table 1), inhibited hexokinase activity by only 5–10% in the present study. The findings of accumulation of GlcN6P in the heart and in skeletal muscles and the allosteric effects of this metabolite in vitro on hexokinase activity prompt us to reconsider the mechanism by which glucosamine induces insulin resistance in vivo. Glucosamine is transported and phosphorylated by the same carriers and enzymes as glucose in adipocytes (27), vascular smooth muscle cells (28), and glomerular mesangial cells (27). The affinity of the glucose transporter has been estimated to be two- to fourfold higher for glucose than for glucosamine in rat diaphragm muscle (29). This information, combined with the plasma concentration of glucose, which was ~3.5-fold higher for glucose than for glucosamine, makes simple competition between glucose and glucosamine for glucose transporters an unlikely possibility to explain glucosamine-induced insulin resistance. The slow time course of glucosamine-induced insulin resistance in the face of a constant plasma glucosamine concentration also argues against that possibility (Fig. 1). Regarding phosphorylation, the large increase in GlcN6P and its inhibitory effects on hexokinase raise the possibility that glucosamine accumulated intracellularly during the glucosamine infusion. It is unknown whether this occurred in the present study, since the in vivo experimental design did not allow determination of extra- and intracellular glucosamine concentrations. Marked accumulation seems unlikely based on previous in vitro studies in the isolated rat diaphragm, in which insulin increased GlcN6P threefold to 2.7 mmol/l within 10 min but did not increase intracellular glucosamine concentrations (29). Regarding the heart, we have previously shown, using a protocol identical to that in the present study, that glucosamine decreases insulin-stimulated glucose uptake by ~40%, when determined using [^{14}C]2-deoxy-glucose during the last hour of a 6-h glucosamine infusion (3). In the present study, we observed massive accumulation of GlcN6P and 50% inhibition of hexokinase activity in vitro in the heart of glucosamine-infused animals. Although it would be tempting to speculate that accumulation of GlcN6P was responsible for insulin resistance in the heart, this cannot be concluded from the present data. Additional studies aimed at quantitation of rates of glucose transport and phosphorylation in vivo and determination of the relative affinities of glucose and

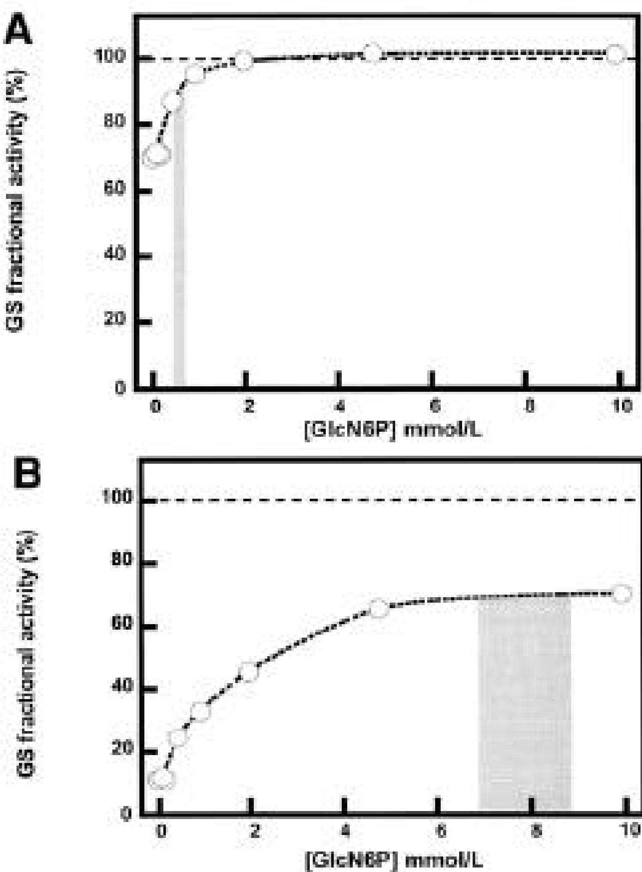

FIG. 2. In vitro stimulation of glycogen synthase (GS) by GlcN6P in rectus abdominis (*A*) and heart (*B*). Pooled samples of the tissue of interest from glucosamine + insulin–infused animals were analyzed for glycogen synthase fractional activity. The maximal glycogen synthase activity observed at high (10 mmol/l) G-6-P concentrations in the absence of GlcN6P was set at 100% (----). All enzymatic assays were done at low (0.1 mmol/l) G-6-P concentrations, and glycogen synthase fractional activity was assayed at increasing in vitro GlcN6P concentrations (0, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0, and 10.0 mmol/l). The shaded region depicts the measured tissue concentrations (± SE) in both tissues. All measurements were done in triplicate.

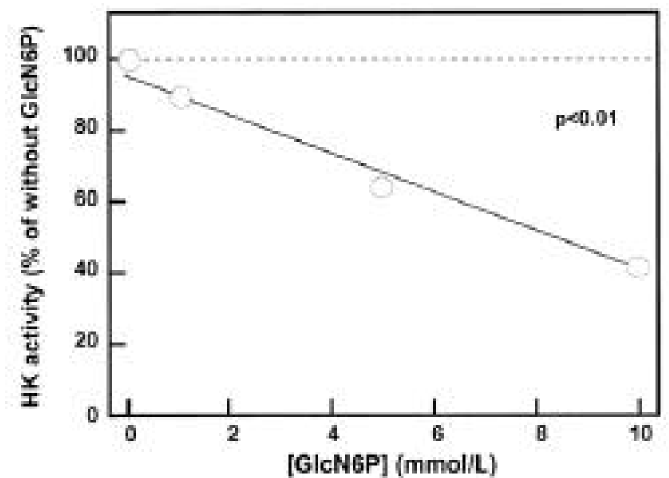

FIG. 3. In vitro inhibition of hexokinase (HK) by GlcN6P. Pooled samples from rectus abdominis muscle from insulin-infused rats were assayed for hexokinase activity at increasing concentrations of GlcN6P (0, 1, 5, and 10 mmol/l). The hexokinase assays were performed at low glucose concentrations (0.125 mmol/l) to prevent inhibition of hexokinase activity by G-6-P formation. All measurements were done in triplicate. *P* < 0.01, significance of correlation between hexokinase activity and GlcN6P concentrations.

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glucosamine for hexokinase would be needed to resolve this issue.

Effects of glucosamine on glycogen synthesis. Previous data regarding effects of glucosamine on glycogen synthase activity and glycogen synthesis have been inconsistent and have not mimicked the defects characterizing animals or humans with glucose-induced insulin resistance (3,4,7,8). In fibroblasts overexpressing GFA, Crook et al. (13) found basal glycogen synthase activity to be higher than in control cells, although the GFA-transfected cells exhibited resistance to insulin stimulation of glycogen synthesis. In fibroblasts overexpressing the insulin receptor, glucosamine increases glycogen synthase activity in both the presence and absence of insulin but inhibits insulin-stimulated glycogen synthesis (6). The present study, by demonstrating that GlcN6P accumulates in amounts that are sufficient to stimulate glycogen synthase fractional activity in both the heart and skeletal muscle, adds allosteric stimulation of glycogen synthase activity by GlcN6P to the list of potential explanations for these paradoxic findings. This effect was more pronounced in the heart, in which the GlcN6P concentration was higher than in skeletal muscle probably because of a several-fold higher rate of glucose uptake in heart than in skeletal muscle during glucosamine infusions (3). We did not explore effects of GlcN6P compared with G-6-P on rate-limiting enzymes of glycolytic or pentose phosphate pathways or on glycogen phosphorylase, and we cannot exclude modulation of glucose metabolism by GlcN6P accumulation in these pathways.

In the present study, as in a previous study by Rossetti et al. (4), the fractional activity of glycogen synthase, when measured in freeze-clamped tissues from glucosamine + insulin–infused rats, was significantly lower than the activity in animals that received only insulin. The reduction in glycogen fractional activity is consistent with a defect in insulin-induced dephosphorylation of the enzyme and with our recent data demonstrating that glucosamine induces defects in the insulin signaling pathway (30); in the latter study, the protocol was identical to the present study. Glucosamine impaired insulin signaling events downstream of IRS-1 phosphorylation until glycogen synthase kinase 3, which directly regulates glycogen synthase phosphorylation. The signaling defects appear not to involve insulin receptor tyrosine kinase activity (6,30) or S6-kinase activity (30). Although these data are all consistent with insulin resistance, the present data imply that they should not necessarily be translated into defects in glucose handling, since the latter is also dependent on tissue concentrations of allosteric regulators, which glucosamine may change considerably.

At least three additional factors need to be considered when interpreting glucosamine-induced changes in insulin-stimulated rates of glycogen synthesis. First, and as also demonstrated in the present study (Table 1), glucosamine infusion depleted the UDP-glucose pool (4,6). This depletion decreased glycogen synthesis, since UDP-glucose is a substrate for glycogen synthase. The decrease in UDP-glucose is thought to reflect, at least in part, trapping of UTP as UDP-GlcNAc and UDP-GalNAc (31). Another possibility is that GlcN6P can be incorporated into glycogen (32). This could occur via a reaction of UTP with GlcN6P, which results in the formation of UDP-glucosamine, a substrate for glycogen synthesis (17,18), or via reversal of the GFA reaction, which is catalyzed by GlcN6P deaminase (19). The possibility that glucosamine-derived carbons are incorporated into glycogen in skeletal muscle or the heart was tested in the present study by using labeled glucosamine. Consistent with an earlier study performed in the isolated rat diaphragm, negligible amounts of glucosamine label were found in glycogen (29). In the present study, whole-body glucose uptake and glycogen synthesis decreased significantly, while glycogen synthesis in rectus abdominis muscle remained unchanged and heart glycogen content increased. These data do not necessarily contradict each other, since the effect of glucosamine on glycogen content exhibits variation between tissues. For example, in a previous study, we found glucosamine to induce the greatest defect in glycogen synthesis in the liver (3). Whether the liver defect was sufficient to explain that in whole-body glycogen synthesis could not be determined because liver glucose uptake could not be determined using labeled 2-deoxy-glucose.

The dose of glucosamine used in the present study (30 μmol · kg^{-1} · min^{-1}) was chosen based on the calculation made by Rossetti et al. (4), who assumed some 2% of glucose to enter the hexosamine pathway and that a flux of ~8 μmol · kg^{-1} · min^{-1} of glucose would be needed to enter the pathway to induce insulin resistance. Rossetti et al. estimated the affinity of the glucose transporter to be fourfold lower for glucosamine than glucose and therefore infused glucosamine at a rate approximately fourfold higher than the glucose flux assumed to be metabolized via the hexosamine pathway (4). Data are controversial regarding the ability of lower doses of glucosamine, which would be expected to result in a smaller increase in GlcN6P, to cause insulin resistance. Baron et al. (33) found the ED_{50} for glucosamine's effect to reduce whole-body glucose uptake to be at or below 0.5 μmol · kg^{-1} · min^{-1}, while Miles et al. (34) found no effect of a glucosamine dose of 3 μmol · kg^{-1} · min^{-1}, and only a 13% lowering in whole body glucose uptake using a glucosamine dose of 15 μmol · kg^{-1} · min^{-1}. In humans, a glucosamine dose of 1.6 μmol · kg^{-1} · min^{-1} did not alter whole-body glucose uptake (35).

In a recent study by Hresko et al. (36), glucosamine treatment of 3T3-L1 adipocytes induced a clear and dose-dependent decrease in intracellular ATP concentrations, which correlated well with insulin resistance of glucose uptake. Glucosamine-induced insulin resistance could also be prevented by experimentally restoring ATP concentrations. In the present study, glucosamine did not change ATP concentrations in either skeletal muscle or heart. Therefore, ATP depletion did not contribute to the observed in vivo insulin resistance in the present study. This does not exclude the possibility that high concentrations of glucosamine or prolonged exposure of cells to glucosamine deplete ATP and induce insulin resistance via this mechanism.

The present study should not be misinterpreted to suggest that the hexosamine pathway is not important in mediating glucose-induced insulin resistance, at least in skeletal muscle. It demonstrates that glucosamine does not perfectly mimic glucose-induced alterations in insulin action in skeletal muscle or the heart. The massive accumulation of GlcN6P in these tissues should be considered when interpreting glucosamine-induced changes in glucose uptake, glycogen synthase activity, and glycogen synthesis.

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