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Hexosamines Are Unlikely to Function as a Nutrient-Sensor in 3T3-L1 Adipocytes

A Comparison of UDP-Hexosamine Levels after Increased Glucose Flux and Glucosamine Treatment

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Whether the hexosamine biosynthesis pathway acts as a nutrient-sensing pathway is still unclear. Glucose is directed into this pathway by GFAT. Because the activity of GFAT is tightly regulated, we examined whether UDP-hexosamine levels can increase significantly and dose-dependently in response to elevated glucose concentrations. In glucosamine-treated 3T3-L1 adipocytes, inhibition of insulin-stimulated glucose uptake was highly correlated with UDP-hexosamine levels ($r = -0.992$; $p < 0.0001$ for UDP-GlcNAc and $r = -0.996$; $p < 0.0001$ for UDP-GalNAc). Incubation of 3T3-L1 adipocytes with 0.1 μ*M* insulin for 24 h in medium containing 1 and 5 m*M* glucose increased the rate of glucose uptake by 365% and 175% compared to untreated cells, respectively. This increase was not observed when the cells were incubated for 24 h with insulin in medium containing 10 or 25 m*M* glucose. However, treatment of cells with insulin and 1, 5, 10, or 25 m*M* glucose resulted in similar increases in levels of UDP-GlcNAc and UDP-GalNAc that always amounted to approx 30–40% above baseline values. This led us to conclude that despite exposure of adipocytes to conditions of extreme and prolonged glucose disposal, the increases in cellular UDP-hexosamines were minimal and not dependent on the extracellular glucose concentration. Taken together, our results are in line with the hypothesis that in glucosamine-treated adipocytes UDP-hexosamines influence insulin-stimulated glucose uptake. However, our observations in glucose-treated adipocytes argue against the possibility that UDP-hexosamines function as a nutrient-sensor, and question the role of the hexosamine biosynthesis pathway in the pathogenesis of insulin resistance.

Key Words: 3T3-L1 adipocytes; hexosamine biosynthesis pathway; UDP-*N*-acetyl glucosamine; UDP-*N*-acetyl galactosamine; glucose uptake.

Introduction

Glucosamine treatment, which increases the intracellular levels of metabolites of the hexosamine biosynthesis pathway, effectively attenuates insulin-stimulated glucose uptake in many different insulin-sensitive models *(1–6)*. This has led to the hypothesis that this pathway functions as a nutrient-sensing pathway and may be involved in the pathogenesis of type 2 diabetes mellitus. The idea is that an oversupply of cells with glucose would lead to accumulation of UDP-hexosamines, which in turn would down-regulate insulin-stimulated glucose uptake (reviewed in refs. *7* and *8*).

The hexosamine biosynthesis pathway starts with the conversion of fructose-6-phosphate into glucosamine-6-phosphate through the action of the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) *(9)*. The end-products of this pathway are uridinediphosphate-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylgalactosamine (UDP-GalNAc). In contrast to glucosamine, which bypasses GFAT, glucose enters the hexosamine biosynthesis pathway through the action of the rate-limiting enzyme GFAT and it is therefore questionable whether high glucose levels result in similarly increased UDP-hexosamine levels as observed with glucosamine treatment.

Nevertheless, a number of studies demonstrated that glucose can induce insulin resistance via the hexosamine biosynthesis pathway. In transgenic mice overexpressing GFAT, an increased flux of glucose is directed through the hexosamine biosynthesis pathway. These transgenic mice develop insulin resistance, reflected by a decreased glucose disposal rate during a hyperinsulinemic-euglycemic clamp *(10)* and reflected by a significantly lower uptake of 2-deoxyglucose in hindlimb muscle, measured during hyperinsulinemia *(11)*.

However, the transgenic mouse model does not provide evidence that, under circumstances in which GFAT activity

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is not artificially increased, glucose can induce insulin resistance via the hexosamine biosynthesis pathway as the activity of GFAT is allosterically inhibited through feedback by the downstream product UDP-GlcNAc (reviewed in ref. *12*). In line with the presence of such feedback inhibition, Nelson et al. *(13)* observed that an increased glucose flux into 3T3-L1 adipocytes clearly attenuated insulin-stimulated glucose uptake but increased the intracellular amounts of UDP-hexosamines by only 30%. These authors also showed that treatment of 3T3-L1 adipocytes with glucosamine lowered insulin-stimulated glucose uptake only when the amounts of UDP-hexosamines in the adipocytes increased by more than 400%. These results suggest that an increased glucose flux into 3T3-L1 adipocytes does not induce insulin resistance via the hexosamine biosynthesis pathway.

Because the activity of GFAT is tightly regulated *(12)*, we questioned whether major increases in the cellular amounts of UDP-hexosamines can occur in response to elevated glucose concentrations. To this end, we treated 3T3-L1 adipocytes for a prolonged time (24 h) with a very high insulin concentration (0.1 μ*M*) in medium containing various glucose concentrations. As such, we exposed the adipocytes to conditions of extreme and prolonged glucose disposal. If the UDP-hexosamines function as a nutrient sensor, the increase in cellular UDP-hexosamines should be dependent on the glucose concentration.

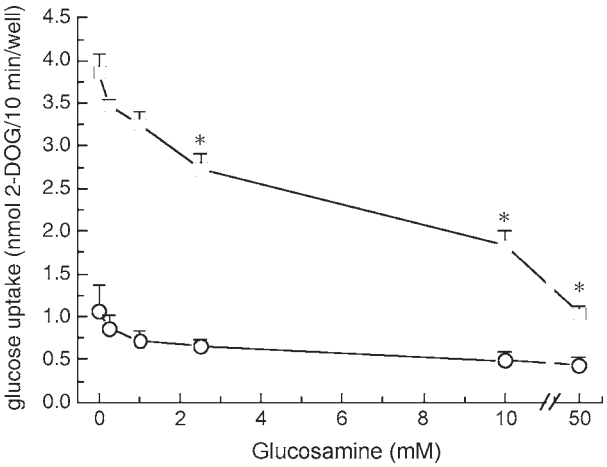

Fig. 1. Effect of glucosamine treatment on insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated for 1 h in reaction buffer containing 0, 0.25, 1, 2.5, 10, or 50 m*M* glucosamine in the absence of insulin and glucose. Subsequently, cells were washed twice and incubated without (circles) or with (squares) 0.1 μ*M* insulin. After 10 min the rate of glucose uptake was determined. The values presented are the mean + SEM of four experiments. *Significantly different from the increase in the rate of glucose uptake by insulin in cells incubated in the absence of glucosamine.

Results

The Rate of Glucose Uptake and the Amounts of UDP-Sugars in Glucosamine-Treated 3T3-L1 Adipocytes

First, we measured the insulin-stimulated rate of glucose uptake in glucosamine-treated cells. Figure 1 shows that in 3T3-L1 adipocytes not treated with glucosamine, the unstimulated rate of glucose uptake was 1.07 ± 0.29 nmol 2-DOG/10 min/well. Addition of 0.1 μ*M* insulin for 10 min increased the rate of glucose uptake to 3.86 ± 0.21 nmol 2-DOG/10 min/well. Hence, insulin stimulated the rate of glucose uptake by 2.79 ± 0.08 nmol 2-DOG/10 min/well. Glucosamine inhibited the insulin-stimulated glucose uptake in a dose-dependent manner. In comparison with untreated cells, treatment with 2.5, 10, and 50 m*M* glucosamine significantly lowered insulin-stimulated glucose uptake to 2.07 ± 0.26 (26% reduction, $p = 0.033$), 1.34 ± 0.21 (52% reduction, $p = 0.02$), and 0.81 ± 0.08 (71% reduction, $p < 0.0005$) nmol 2-DOG/10 min/well, respectively.

Next, we investigated the amounts of nucleotide-linked sugars in 3T3-L1 adipocytes under these conditions. Figure 2 shows that 3T3-L1 adipocytes not treated with glucosamine contained 0.76 ± 0.01 nmol UDP-GlcNAc/well, 0.31 ± 0.01 nmol UDP-GalNAc/well, and 0.41 ± 0.02 nmol UDP-glucose/well. Treatment with glucosamine dose-dependently increased the amounts of the two UDP-hexosamines and decreased the amount of UDP-glucose. In comparison with untreated cells, the amount of UDP-GlcNAc ($p = 0.006$) and the amount of UDP-GalNAc ($p = 0.044$) were already significantly increased following 1 h of 0.25 m*M* glucosamine treatment. When cells were treated with 2.5 m*M* glucosamine for 1 h, which significantly lowered insulin-stimulated glucose uptake, the amounts of UDP-GlcNAc and UDP-GalNAc were increased by 56 ± 3% and 54 ± 5%, respectively. The amount of UDP-glucose was 0.14 ± 0.01 nmol/well after 1 h of 50 m*M* glucosamine, which was significantly decreased ($p = 0.014$) compared to the amount in untreated cells. Figure 3 shows that there is a strong negative correlation ($r = -0.992$; $p < 0.0001$ for UDP-GlcNAc and $r = -0.996$; $p < 0.0001$ for UDP-GalNAc) between the rate of insulin-stimulated glucose uptake and the amounts of UDP-GlcNAc and UDP-GalNAc at the different glucosamine concentrations.

The Rate of Glucose Uptake and the Amounts of UDP-Sugars in 24 h Insulin-Treated 3T3-L1 Adipocytes

The rate of glucose transport of insulin-treated 3T3-L1 adipocytes incubated at different glucose concentrations was determined. Figure 4 shows that incubations of 3T3-L1 adipocytes in medium containing 1, 5, 10, and 25 m*M* glucose for 24 h did not affect basal or insulin-stimulated glucose uptake. The average increase in the rate of glucose uptake by 10 min of insulin at these four glucose concentrations was 3.60 ± 0.10 nmol 2-DOG/10 min/well (increase of 543%). In comparison with cells not exposed to insulin, treatment with 0.1 μ*M* insulin for 24 h significantly increased

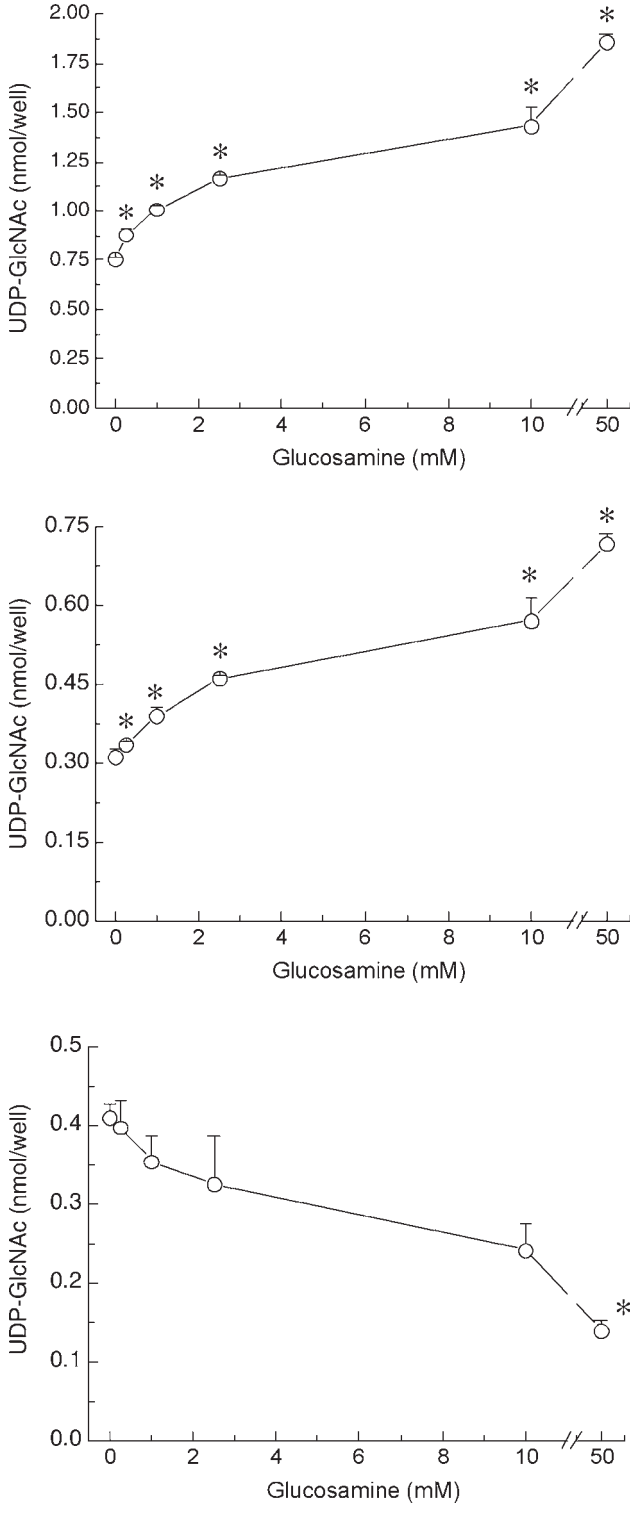

Fig. 2. Effect of incubation with glucosamine on the amounts of UDP-hexosamines and UDP-glucose in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated for 1 h in medium containing 0, 0.25, 1, 2.5, 10, or 50 m*M* glucosamine in the absence of insulin and glucose. Subsequently, cells were washed twice and harvested, and the amounts of UDP-GlcNAc (upper graph), UDP-GalNAc (middle graph) and UDP-glucose (lower graph) in the cells were determined. The values presented are the mean + SEM of four experiments. *Significantly different from the amounts of UDP-sugars in the cells incubated in the absence of glucosamine.

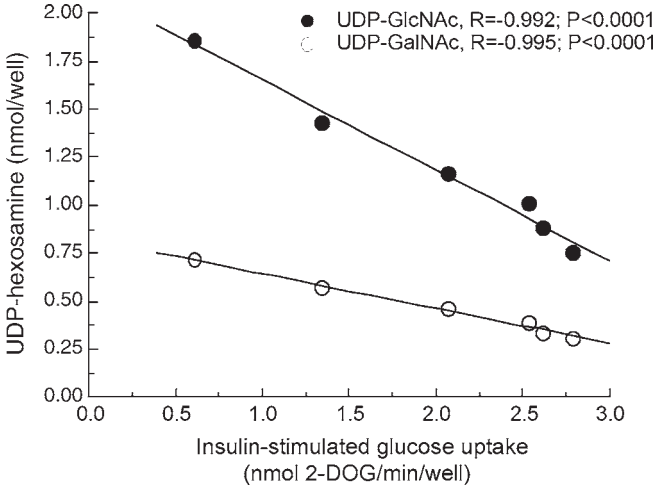

Fig. 3. Relation between the amounts of UDP-GlcNAc and UDP-GalNAc and the rate of insulin-stimulated glucose uptake. The amounts of UDP-GlcNAc (closed circles) and UDP-GalNAc (open circles) in glucosamine-treated 3T3-L1 adipocytes were plotted against the rate of insulin-stimulated glucose uptake, which was corrected for the rate of basal glucose uptake. With increasing cellular amounts of UDP-GlcNAc and UDP-GalNAc, the rate of insulin-stimulated glucose uptake declines.

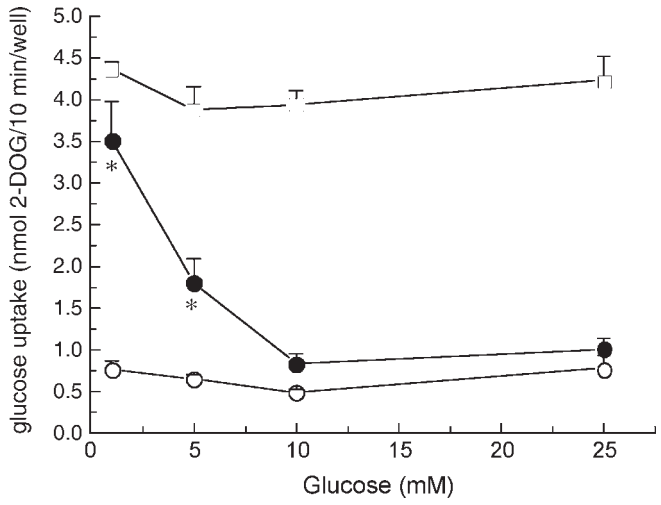

Fig. 4. Effect of 24 h insulin treatment on glucose uptake in 3T3-L1 adipocytes exposed to different glucose concentrations. The 3T3-L1 adipocytes were incubated in medium containing 1, 5, 10, and 25 m*M* glucose in the absence of 0.1 μ*M* insulin for 24 h (open symbols). Subsequently, cells were washed and incubated in reaction buffer in the absence of glucose and insulin. After 1 h the reaction buffer was added without (circles) or with (squares) 0.1 μ*M* insulin. After 10 min the rate of glucose uptake was determined. In addition, 3T3-L1 adipocytes were incubated in medium containing 1, 5, 10, and 25 m*M* glucose in the presence of 0.1 μ*M* insulin (closed circles) for 24 h. Subsequently, cells were washed and incubated in reaction buffer in the absence of glucose and insulin. After 1 h the reaction buffer was refreshed and after 10 min the rate of glucose uptake was determined. The values presented are the mean + SEM of four or five experiments. *Significantly different from corresponding cells treated for 24 h without insulin.

the rate of glucose uptake by 365 ± 49% ($p = 0.008$) and 175 ± 16% ($p = 0.012$) when the cells were incubated in medium containing 1 and 5 m*M* glucose, respectively. The rate of glucose uptake was not significantly increased when the 24 h insulin treatment occurred with medium containing 10 or 25 m*M* glucose.

Next, we incubated cells for 24 h in medium with varying glucose concentrations in the absence or presence of 0.1 µ*M* insulin after which the amounts of nucleotide-linked sugars were assessed. The amounts of UDP-GlcNAc and UDP-GalNAc in untreated as well as in insulin-treated 3T3-L1 adipocytes tended to increase upon incubation in medium with increasing glucose concentrations for 24 h (Fig. 5). However, it did not reach statistical significance. In comparison with cells treated with 0.5 m*M* glucose, the amount of UDP-glucose was significantly higher in 3T3-L1 adipocytes following 24 h incubation in medium with 5 and 10 m*M* glucose. The latter was the case for both untreated as well as insulin-treated adipocytes.

Compared to untreated cells, the increase in the amounts of UDP-GlcNAc, UDP-GalNAc, and UDP-glucose following 24 h treatment with insulin at all glucose concentrations was 36.8 ± 7.3% (ranging from 12 to 49%; $p = 0.019$), 38.0 ± 8.0% (ranging from 0 to 54%; $p = 0.025$), and 46.0 ± 8.0% (ranging from 20 to 71%; $p = 0.013$), respectively. Of note, the increases of the UDP-sugars were not affected by the 1 h incubation in insulin- and glucose-free medium (data not shown).

Prolonged Insulin Treatment Stimulates the Production of UDP-GlcNAc in Glucosamine-Treated 3T3-L1 Adipocytes

Because we found only a marginal increase in the amount of UDP-GlcNAc during a prolonged period of excessive glucose uptake, we examined whether prolonged insulin treatment affects the activity of the hexosamine biosynthesis pathway distal from GFAT. Therefore, 3T3-L1 adipocytes were treated for 24 h with 25 m*M* glucose in the absence or presence of 0.1 µ*M* insulin and subsequently treated for 1 h without or with 50 m*M* glucosamine after which the amount of UDP-GlcNAc was determined. Figure 6 shows that 24 h treatment of 3T3-L1 adipocytes with insulin significantly increased the amount of UDP-GlcNAc by 70 ± 19% ($p = 0.02$) compared to untreated cells. Treatment of cells with 50 m*M* glucosamine for 1 h significantly increased the amount of UDP-GlcNAc by 218 ± 24% ($p = 0.002$) compared to untreated cells. However, when glucosamine was added to cells which were first treated with insulin for 24 h, the amount of UDP-GlcNAc significantly increased by 530 ± 43% ($p = 0.008$).

Discussion

We investigated the possibility that the hexosamine biosynthesis pathway operates as a nutrient-sensing pathway in 3T3-L1 adipocytes by investigating whether UDP-hexos-

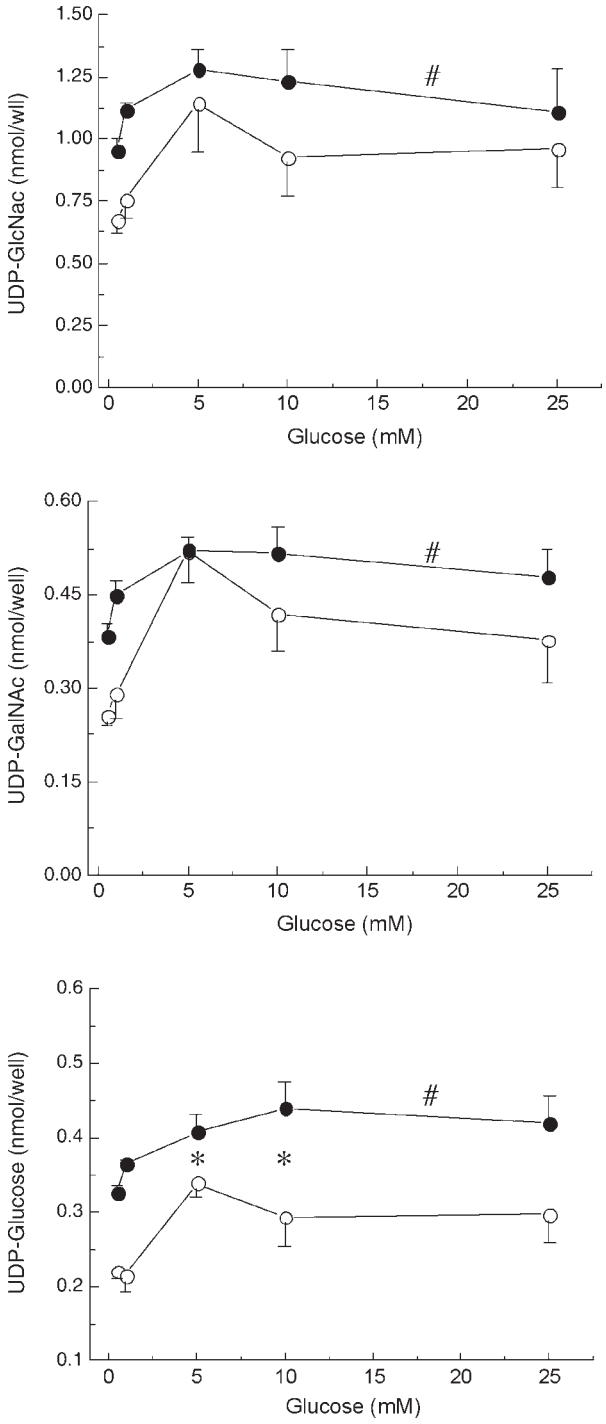

Fig. 5. Effect of 24 h insulin treatment on the amounts of UDP-hexosamines and UDP-glucose in 3T3-L1 adipocytes exposed to different glucose concentrations. Fully differentiated adipocytes were incubated in medium containing 0.5, 1, 5, 10, and 25 m*M* glucose in the absence (open symbols) or presence (closed symbols) of 0.1 µ*M* insulin for 24 h. Subsequently, cells were washed twice and incubated in reaction buffer in the absence of glucose and insulin. After 1 h cells were harvested and the amounts of UDP-GlcNAc (upper graph), UDP-GalNAc (middle graph), and UDP-glucose (lower graph) were determined. The values presented are the mean ± SEM of four or five experiments. *Significantly different from the amount of UDP-glucose in cells incubated in 0.5 m*M* glucose. #Significantly different from the amount of UDP-sugars in cells treated without insulin for 24 h.

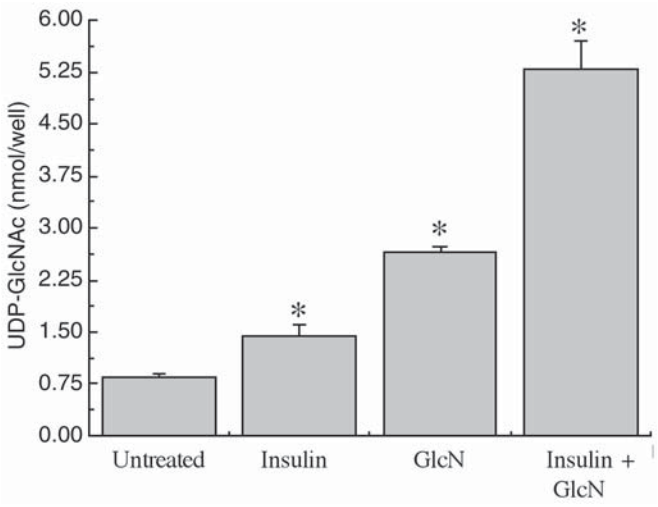

Fig. 6. Effect of 24 h insulin followed by 1 h glucosamine treatment on the amount of UDP-GlcNAc. Fully differentiated 3T3-L1 adipocytes, incubated in medium containing 25 m*M* glucose, were washed twice and incubated without (first bar) or with (third bar) 50 m*M* glucosamine for 1 h in glucose- and insulin-free medium. In addition, cells were treated with 0.1 μ*M* insulin for 24 h, washed with PBS, and treated without (second bar) or with (fourth bar) glucosamine (1 h, 50 m*M*). Next, cells were harvested and the amount of UDP-GlcNAc was determined. The values presented are the mean + SEM of four experiments. *Significantly different from untreated cells.

amine levels reflect the amounts of glucose to which the adipocytes are exposed. From our results it appears that during conditions of extreme glucose disposal the cellular amounts of UDP-hexosamines remain relatively constant. In complete contrast, in glucosamine-treated 3T3-L1 adipocytes the amounts of UDP-hexosamines increase in a dose-dependent manner and correlate almost perfectly with the decrease in insulin-stimulated glucose uptake. Taken together, our results are in line with the hypothesis that in glucosamine-treated adipocytes cellular UDP-hexosamine levels influence insulin-stimulated glucose uptake. However, our observations in glucose-treated adipocytes argue against the possibility that UDP-hexosamine levels function as a nutrient sensor, and question the role of the hexosamine biosynthesis pathway in the pathogenesis of insulin resistance.

Treatment of 3T3-L1 adipocytes with glucosamine for 1 h resulted in a rapid decrease in insulin-stimulated glucose uptake. Incubation with 50 m*M* glucosamine almost completely prevented an insulin-stimulated increase in the rate of glucose transport. These observations are in agreement with those of Heart et al. *(15)*, who reported that glucosamine dose-dependently decreases insulin-stimulated glucose uptake in 3T3-L1 adipocytes and that 50 m*M* glucosamine was required to maximally inhibit insulin-stimulated glucose transport. Of note, we *(16)* and others *(17)* did not identify a decrease in insulin sensitivity in humans during short-term glucosamine infusion, which questions whether in humans UDP-hexosamines can down-regulate insulin signaling. We quantified the amounts of UDP-GlcNAc and UDP-GalNAc in the murine adipocytes treated with glucosamine and observed a strong negative correlation between the rate of insulin-stimulated glucose uptake, on one hand, and the amounts of UDP-GlcNAc and UDP-GalNAc, on the other hand. This is in line with the hypothesis that in glucosamine-treated 3T3-L1 adipocytes increases in the amounts of UDP-hexosamines cause a decrease in insulin-stimulated glucose uptake. However, it has been demonstrated that treatment of 3T3-L1 adipocytes with glucosamine decreases intracellular ATP levels *(18)*. Moreover, the decreases in ATP levels and inhibition of insulin-stimulated glucose transport were highly correlated. Interestingly, glucosamine-induced insulin resistance was prevented upon addition of inosine, a compound that stimulates ATP production. Based on these observations, it is suggested that glucosamine inhibits insulin-stimulated glucose transport by decreasing cellular ATP levels and not through elevated UDP-hexosamine levels. Of note, the production of UDP-hexosamines in the presence or absence of inosine was not determined. Therefore, it cannot be excluded that, in the presence of inosine, UDP-hexosamines are less effectively produced in glucosamine-treated 3T3-L1 adipocytes. In contrast to the observations of Hresko et al. *(18)*, others reported that severe cellular ATP depletion in 3T3-L1 adipocytes did not affect insulin-stimulated glucose uptake *(19)*. The latter study recognize their opposite findings compared to Hresko et al. *(18)*, and suggests that this might be due to small but significant differences in the experimental conditions. Nevertheless, it remains questionable whether glucosamine inhibits insulin-stimulated glucose uptake in 3T3-L1 adipocytes by decreasing the ATP levels.

The amount of UDP-glucose in 3T3-L1 adipocytes decreased in a dose-dependent manner when the cells were treated with glucosamine. This might indicate that activation of the hexosamine biosynthesis pathway by incubation of the cells with glucosamine either stimulates processing of UDP-glucose to generate glycogen *(20)* or that the UDP from UDP-glucose is released for the synthesis of UDP-hexosamines *(21)*.

Nelson et al. *(13)* reported that overnight treatment of 3T3-L1 adipocytes with 2.5 m*M* glucosamine increased the amounts of UDP-hexosamines by 400% but did not affect insulin-stimulated glucose uptake. However, when insulin was present during treatment with 2.5 m*M* glucosamine, the increase in UDP-hexosamines amounted to 900% and insulin-stimulated glucose uptake was reduced in comparison with cells not exposed to insulin. Therefore, these authors concluded that to inhibit insulin-stimulated glucose uptake in 3T3-L1 adipocytes the increase in cellular UDP-hexosamine content should be more than 400%. In contrast, we observed that incubation with only glucosamine (2.5 m*M* and higher doses) for 1 h already inhibited insulin-stimulated glucose uptake. Moreover, insulin-stim-

ulated glucose uptake was already significantly reduced when the amounts of UDP-GlcNAc and UDP-GalNAc were increased by 50%. When we treated 3T3-L1 adipocytes for 1 h with 50 m*M* glucosamine, which almost completely inhibited insulin-stimulated glucose uptake, the amount of UDP-GlcNAc increased by 218%. However, the amount of UDP-GlcNAc increased further to 530% in cells treated with 50 m*M* glucosamine for 1 h when these cells were first exposed for 24 h to 0.1 μ*M* insulin and 25 m*M* glucose. This might explain why Nelson et al. *(13)*, who treated the 3T3-L1 adipocytes simultaneously with glucose, glucosamine, and insulin, observed such large increases in UPD-hexosamine levels.

We found that the rate of glucose uptake was significantly increased in 3T3-L1 adipocytes treated with 0.1 μ*M* insulin for 24 h when the cells were incubated in medium with 1 and 5 m*M* glucose compared to cells not treated with insulin. When the glucose concentration was increased to 10 and 25 m*M*, prolonged insulin treatment did not increase the rate of glucose uptake. Others (*14,22*) also demonstrated that prolonged exposure of 3T3-L1 adipocytes to insulin (0.01–0.1 μ*M*) increases the rate of glucose transport at low concentrations of glucose. Moreover, it was demonstrated that this increase in the rate of glucose transport was accompanied with elevated cellular levels of GLUT-1 and not GLUT-4. In addition, it was shown that the amount of GLUT-1, and not GLUT-4, at the plasma membrane was increased of these long-term (hours) insulin-treated cells, which is in contrast to short-term (minutes) insulin-treated cells where predominantly the amount of GLUT-4 increases at the plasma membrane. Hence, the stimulatory effect of long-term insulin treatment on the amount of GLUT-1 at the plasma membrane most probably explains the increase in the rate of glucose uptake of our 24 h insulin-treated 3T3-L1 adipocytes in the presence of 1 and 5 m*M* glucose. The observation that the rate of glucose uptake following treatment with insulin for 24 h is dependent on the glucose concentration, demonstrates that the amount of glucose that has been taken up by the adipocytes is different between cells incubated in low (1 and 5 m*M*) and cells incubated in high (10 and 25 m*M*) glucose concentrations.

In earlier studies investigating glucose-induced insulin resistance in 3T3-L1 adipocytes (*13,14,23,24*), the amount of insulin that was added for a prolonged period of time was relatively low (0.1–5 n*M*) compared to the concentration in our experimental setting (0.1 μ*M*). We recognize that the high concentration of insulin for 24 h is unsuitable to study the mechanism of glucose-induced insulin resistance, but this concentration ensures a prolonged and profound increased uptake of glucose during the 24 h treatment. In contrast to glucosamine treatment, 24 h treatment of 3T3-L1 adipocytes with insulin (0.1 μ*M*) and glucose only marginally increased the amounts of UDP-GlcNAc and UDP-GalNAc by approx 30–40%. The fact that addition of 50 m*M* glucosamine for 1 h to the 24 h insulin-exposed cells profoundly increased the amounts of UDP-hexosamines rules out the possibility that the 24 h insulin-treatment downregulates the activity of the hexosamine biosynthesis pathway. Although we do not provide direct evidence for this, the difference in the increases of the amounts of UDP-hexosamines between glucosamine and glucose treatment is most likely due to the fact that GFAT activity is inhibited by UDP-GlcNAc. Moreover, it appears that the GFAT activity is controlled to such an extent that incubation of cells with a high concentration of insulin and a high concentration of glucose did not yield more UDP-hexosamines compared to cells incubated with the same high concentration of insulin but with a low concentration of glucose.

In glucose-induced insulin-resistant 3T3-L1 adipocytes, the cellular amounts of UDP-hexosamines increased by 30% *(13)*. In our glucosamine-treated adipocytes, an increment of 50% in the amounts of UDP-GlcNAc and UDP-GalNAc coincided with a significantly lowered insulin-stimulated glucose uptake, suggesting that small increases in UDP-hexosamine levels already affect insulin signaling. Therefore, it cannot be totally excluded that the hexosamine biosynthesis pathway contributes to some degree in glucose-induced insulin resistance.

To conclude, our data show that glucosamine-induced insulin resistance in 3T3-L1 adipocytes is highly related with intracellular UDP-hexosamine levels. Prolonged insulin-mediated glucose loading in the adipocytes however, increases the amounts of UDP-GlcNAc and UDP-GalNAc only marginally and to a similar extent at all glucose concentrations. Taken together, our results are in line with the hypothesis that in glucosamine-treated adipocytes cellular UDP-hexosamine levels influence insulin-stimulated glucose uptake. However, our observations in glucose-treated adipocytes argue against the possibility that UDP-hexosamines act as a nutrient sensor, and question the role of the hexosamine biosynthesis pathway in the pathogenesis of insulin resistance.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was from ICN Biomedicals (Irvine, CA, USA) and glucose-free DMEM was from Life Technologies (Breda, The Netherlands). Fetal calf serum (FCS) was from PAA laboratories (Linz, Austria). Glucosamine, 2-deoxyglucose (2-DOG), 3-isobutyl-1-methylxanthine (IBMX), and dexamethasone were obtained from Sigma (St. Louis, MO, USA). Human recombinant insulin was obtained from Eli Lilly (Nieuwegein, The Netherlands) and [1-^{3}H]-2-DOG (spec. act. 481 GBq/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were of analytical grade.

Adipogenesis of 3T3-L1 Cells

The 3T3-L1 cells (ATCC, Manassas, VA, USA) were seeded in 12-well plates and grown to confluence in DMEM with 25 m*M* glucose, 2 m*M* glutamine, and 10% (v/v) FCS for 10 d. Subsequently, insulin (1.7 µ*M*), IBMX (1 µ*M*), and dexamethasone (25 pM) were added. After 48 h, IBMX and dexamethasone were omitted from the culture medium and, after an additional 6 d of culture, insulin was also omitted. After another 7 d of culture, the 3T3-L1 cells were fully differentiated into adipocytes (more than 90% of the cells contained lipid droplets) and used for analysis. The amount of protein in the 12-wells was 328 ± 22 µg/well (mean ± SD).

Treatment of 3T3-L1 Adipocytes with Glucosamine or Glucose

In case of glucosamine treatment, 3T3-L1 adipocytes were incubated in DMEM with 25 m*M* glucose, 2 m*M* glutamine but without FCS for 3 h. Subsequently, cells were washed twice with phosphate buffered saline (PBS) and incubated at 37°C for 1 h in reaction buffer containing 138 m*M* NaCl, 1.85 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4$, 4.8 m*M* KCl, 0.2% (w/v) BSA, and 50 m*M* *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) adjusted to pH 7.4. In addition, cells were incubated for 1 h in reaction buffer containing 0.25, 1, 2.5, 10, or 50 m*M* glucosamine.

In case of glucose and insulin treatment, cells were kept in DMEM with 25 m*M* glucose, 2 m*M* glutamine, and 10% (v/v) FCS and 0.5, 1, 5, 10, or 25 m*M* glucose in the absence or presence of 0.1 µ*M* insulin for 24 h. The FCS was omitted during the last 3 h of incubation. Next, cells were washed twice with PBS and incubated at 37°C for 1 h in reaction buffer in the absence of glucose and insulin, to re-create unstimulated cells as described by Thomson et al. *(14)*.

After glucosamine or glucose and insulin treatment, cells were washed with reaction buffer and, subsequently, the rate of glucose uptake or the levels of the different UDP-sugars were measured in these cells.

Glucose Uptake

To assess the rate of glucose uptake, cells were refreshed with 500 µL reaction buffer without or with insulin (0.1 µ*M*) and after 10 min, reaction buffer (250 µL) containing 27.8 kBq [1-^{3}H]-2-DOG, and 1.5 m*M* 2-DOG without or with 0.1 µ*M* insulin was added. After 10 min of incubation at 37°C, glucose uptake was terminated upon washing three times with ice-cold PBS containing 10 m*M* glucose. Subsequently, cells were lysed in 1% (w/v) sodium dodecyl sulfate and 0.2 *M* NaOH. Internalized radioactivity was measured by liquid scintillation spectrometry.

Analysis of UDP-Hexosamines and UDP-Glucose

Analyses were performed as described previously *(25)*. In brief, adipocytes were washed twice with PBS, scraped off the plate, and subsequently collected in 500 µL extraction buffer containing 100 m*M* KCl, 1 m*M* EDTA, and 50 m*M* KH_2PO_4, adjusted with KOH to pH 7.5. The homogenate was sonicated (Branson Ultrasonics Corporation, Model 250 sonifier, Danbury CT, USA) at 4°C by 10 bursts of 5 s (output 1) using a microtip. The mixture was centrifuged at 60,000*g* for 15 min at 4°C. The supernatant (400 µL) was deproteinized by adding 1 volume of 1.2 *M* perchloric acid. After 5 min on ice, the mixture was centrifuged for 10 min at 15,000*g* and 4°C. The supernatant was diluted with 10 volumes of 10 m*M* KH_2PO_4, adjusted with H_3PO_4 to pH 2.5 and applied to LC-SAX ion-exchange columns (Amersham, Buckinghamshire, UK). After washing, the samples were eluted with 1.5 mL KH_2PO_4 (150 m*M*), adjusted with KOH to pH 7.5. Finally, samples (150 µL) were injected on two LC-18T columns in series (25 cm × 4.6 mm, 5 µ*M*; Supelco, Zwijndrecht, The Netherlands) and separated by high-performance liquid chromatography. Detection of UDP-glucose, UDP-*N*-acetylglucosamine, and UDP-*N*-acetylgalactosamine was performed by UV at 262 nm.

Analysis of the Data

The conditions belonging to an experiment were applied on one 12-well plate. An experiment was replicated four or five times. Effects of the various experimental conditions were tested by means of either one-way or two-way repeated measures ANOVA and within subject factor contrasts were tested for statistical significance ($p < 0.05$). Where appropriate, Spearman correlation coefficients (r) were determined or statistical analyses were performed with parallel pair-wise *t*-test with correction according to Bonferroni.

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