

## *In Vivo* Administration of Glucosamine Inhibited Phosphatidylinositol 3-Kinase Activity without Affecting Tyrosine Phosphorylation of the Insulin Receptor or Insulin Receptor Substrate in Rat Adipocytes

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We examined insulin signaling in rat epididymal adipocytes which developed insulin resistance by the *in vivo* infusion of glucosamine. Insulin-stimulated 2-deoxyglucose uptake into the adipocytes isolated from rats which were infused glucosamine for 4 hours was diminished by 26%. To analyze insulin signaling in adipocytes, the epididymal fat tissues were harvested 5 minutes after insulin administration (10U/kg), which was administered immediately after glucosamine infusion. Glucosamine had no effect on the insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate (IRS)-1. Glucosamine infusion decreased insulin-stimulated phosphatidylinositol (PI) 3-kinase activity by 66%. Glucosamine infusion also inhibited insulin-stimulated PI 3-kinase activity associated with IRS-1, 2, 3 by 30%, 43%, and 44%, respectively. There was no difference in the association of the 85kDa subunit of PI 3-kinase with the IRS-1 and IRS-2 protein. PI 3-kinase activity in adipocytes from rats treated with glucosamine that were administered platelet derived growth factor (3 $\mu$ g/kg) for 5 minutes was also reduced by 39%. When we measured the kinase activity of protein kinase C (PKC)  $\lambda$ , which is the downstream effector of PI 3-kinase in isolated adipocytes, we found that glucosamine inhibited insulin stimulated PKC $\lambda$  kinase activity by 33%. These results suggest that glucosamine infusion contributes to the development of insulin resistance by mainly modulating the PI 3-kinase molecules.

Insulin resistance is a major contributing factor for pathogenesis of type 2 diabetes (9). It is well known that sustained hyperglycemia contributes to insulin resistance. One of the mechanisms by which hyperglycemia induces insulin resistance is the activation of the hexosamine pathway. The end product of this pathway, uridine diphosphate-*N*-acetylglucosamine, was shown to be a potent inducer of insulin resistance in peripheral tissues (6). Overexpression of glutamine:fructose-6-phosphate amidotransferase, which is a rate-limiting enzyme in this pathway in skeletal muscle and fat, in transgenic mice has been shown to lead to insulin resistance (8). When glucosamine is administered to cells, it is taken up by glucose transporters and is readily phosphorylated to glucosamine 6-phosphate by hexokinase. *In vivo* glucosamine infusion induced insulin resistance in normoglycemic rats, but not hyperglycemic, ones suggesting that both glucose and glucosamine induce insulin resistance by a common pathway (18). Glucosamine infusion also impaired insulin-induced glucose transporter (GLUT4) translocation to the plasma membrane in rat muscle (2). Though it is clear that

activation of the hexosamine pathway is an important mechanism in the induction of insulin resistance, it is unclear which step in the insulin signaling pathway is impaired by glucosamine infusion.

Insulin signaling involves a cascade of events initiated by insulin binding to its cell surface receptor. Binding of insulin receptor substrates (IRSs) to the regulatory subunit of phosphatidylinositol (PI) 3-kinase results in activation of PI 3-kinase, which is required for insulin-mediated glucose transport as well as insulin-induced up-regulation of glycogen synthesis and protein synthesis (13). Previous studies have suggested the possibility that activation of the hexosamine pathway may modulate IRS proteins or PI 3-kinase (11,15).

In this study, we analyzed the effects of glucosamine infusion in rats on insulin signaling in epididymal adipocytes.

## MATERIALS AND METHODS

**Materials** Anti-phosphotyrosine antibody (PY-20) was purchased from Transduction Labs (USA). Anti-85kDa subunit of PI 3-kinase (p85) antibody, and anti-IRS-1, 2, and 3 antibodies were purchased from Upstate Biotech Industry (USA). The anti-insulin receptor (IR) antibody and anti-protein kinase C (PKC)  $\lambda$  antibody were obtained from Santa Cruz Biotech (USA). [ $\gamma$ - $^{32}$ P] ATP was obtained from Amersham (USA) and the 2-[1- $^{14}$ C]-deoxyglucose was obtained from ICN (USA). Insulin (Novolin R) was purchased from Novo Nordisk (Denmark), the human platelet derived growth factor (PDGF)-BB was obtained from Collaborative Research (USA) and the collagenase from Worthington Biochemical Corporation (USA). Phosphatidylinositol and myelin basic protein (MBP) were obtained from Sigma (USA). Histone 2B was purchased from Boehringer Mannheim (Germany), while thin layer chromatography aluminum sheets were obtained from Merck (Germany). The BCA protein assay kit was purchased from Pierce (France).

**Animals** Our experimental protocol was approved by the Ethics Review Committee for Animal Experimentation at our university. Male Wistar rats were purchased from CLEA Japan (Osaka, Japan). The rats (7 weeks old) were fed standard rodent chow and water *ad libitum* and were housed in a room programmed for a 12-hours light-dark cycle. Sixteen hours before the beginning of the study, food was withdrawn from the rat cages. Rats were anesthetized with pentobarbital (50mg/kg) and a catheter was inserted into their right jugular veins. Rats were infused with either saline or glucosamine (6mg/kg/min) for 4 hours. When rat tissues were used in the kinase assay and for immunoblotting, the animals were sacrificed 5 minutes after insulin (5U/kg) or PDGF-BB (3 $\mu$ g/kg) administration, which was given immediately after the end of the infusion period. Epididymal fat tissue was rapidly removed, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analysis. The duration and dose of the glucosamine infusion were based on the data reported previously (2,7,18).

**2-deoxyglucose uptake** To evaluate glucose uptake into fat tissue, 2-deoxyglucose uptake was measured by the method of Olefsky (14). Isolated adipocytes were prepared from rats infused with either saline or glucosamine. Epididymal fat tissue was digested by collagenase for 30 minutes. Adipocytes were incubated with 100nM insulin for 10 minutes. 9.3kBq of 2-[1- $^{14}$ C]-deoxyglucose were then added and the additional incubation was continued for 10 minutes at  $37^{\circ}\text{C}$ . The reaction was then terminated by spinning the cell suspension through dinonylphthalate oil. Radioactivity was measured in a liquid scintillation counter.

**PI 3-kinase activity** PI 3-kinase activity was measured as described by Endemann *et al.* (4). Epididymal fat tissue (300mg) was homogenized in ice-cold lysis buffer (20mM Tris-HCl at pH7.6, 137mM NaCl, 2mM EDTA, 1% Nonidet P-40, 1mM sodium orthovanadate, 1mM

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phenylmethylsulfonyl fluoride, and 1mM dithiothreitol) using a Polytron homogenizer (Brinkmann Instruments, Switzerland). The homogenates were solubilized at 4°C for 30 minutes, and centrifuged at 18,000×g for 15 minutes at 4°C. The supernatant was then collected and assayed for protein concentration using a BCA protein assay kit. The supernatants containing 1mg amounts of protein were immunoprecipitated with anti-PY-20 (2μg), anti-IRS-1 (4μg), anti-IRS-2 (4μg), or anti-IRS-3 (4μg) antibodies for 16 hours at 4°C. Immunoprecipitates were then assayed for 10 minutes at room temperature in a reaction mixture containing PI (0.2mg/ml) and [ $\gamma$ -<sup>32</sup>P] ATP. The reaction was stopped by the addition of 15μl of 4N HCl and 130μl of chloroform-methanol (1:1). 30μl of phospholipid-containing chloroform phase was spotted onto a thin layer chromatography plate. The radioactivity in the spots corresponding to PI 3-monophosphate was analyzed using a BAS2000 image analyzer (Fuji, Japan).

**PKC $\lambda$  kinase assay** PKC $\lambda$  kinase activity was measured as described by Kotani et al. (12). 300mg of fat tissue were homogenized and the supernatant containing a 1mg amount of protein was immunoprecipitated with anti-PKC $\lambda$  antibody (2μg) for 3hours at 4°C. The kinase reaction was initiated by resuspending the protein G-Sepaharose coupled with anti-PKC $\lambda$  antibody in 30μl of the kinase mixture containing 12.5μg of MBP substrate and 14kBq of [ $\gamma$ -<sup>32</sup>P] ATP. As above, the amount of radioactivity was determined using a BAS2000 image analyzer.

**Immunoblot analysis** 500mg of fat tissue were homogenized in ice-cold lysis buffer (the same buffer used in the PI 3-kinase assay) using a Polytron homogenizer. After centrifugation, the supernatant (2mg of protein) was incubated with the immune complex of proteinG-Sepharose containing anti-IR (5μg), anti-IRS-1 (4μg), or anti-IRS-2 (4μg) antibody for 16 hours at 4°C. The immunocomplex was loaded onto SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed with an enhanced chemiluminescence detection kit (Amersham) and band intensities were quantified using NIH image 1.62 soft ware (NIH, USA).

**Statistical analysis** Data were presented as means  $\pm$  standard error. Statistical analysis was performed using Statview software (SAS Institute Inc., USA). The significance of the differences between means was evaluated using unpaired Student's *t*-tests.  $P < 0.05$  was considered significant.

## RESULTS

**Effects of glucosamine infusion on 2-deoxyglucose uptake** To confirm that glucosamine infusion into rats for 4 hours caused insulin resistance in adipocytes, we prepared isolated adipocytes from the epididymal fat and measured their uptake ability of 2-deoxyglucose. When rats were infused with saline, insulin induced an  $8.1 \pm 0.6$ -fold increase in 2-deoxyglucose uptake in these cells. However, in rats infused with glucosamine, 2-deoxyglucose uptake by adipocytes increased to only  $6.0 \pm 0.3$ -fold in response to insulin stimulation (Fig. 1). Thus, glucosamine infusion decreased insulin-stimulated 2-deoxyglucose uptake by 26% ( $P = 0.01$ ).

**Effects of glucosamine on tyrosine phosphorylation of the IR and IRS-1** We measured tyrosine phosphorylation of the IR and IRS-1 in adipocytes. Insulin-stimulated tyrosine phosphorylation of the IR and IRS-1 in saline-infused rats were increased  $4.5 \pm 0.7$ -fold and  $4.3 \pm 1.1$ -fold, respectively. On the other hand, insulin-stimulated tyrosine phosphorylation of the IR and IRS-1 in glucosamine-infused rats were increased  $3.1 \pm 1.1$ -fold and  $4.8 \pm 1.4$ -fold,

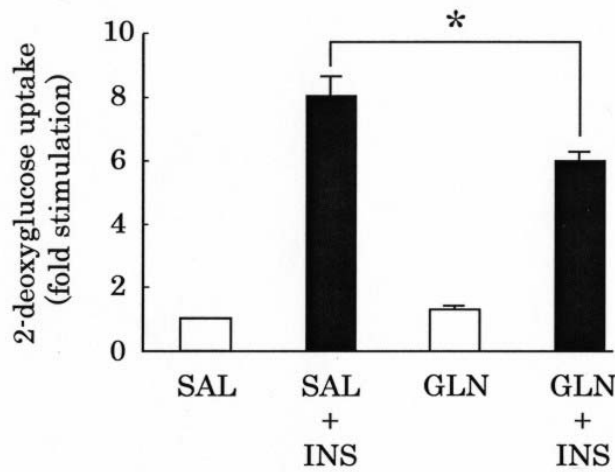


FIG. 1. Effects of glucosamine on 2-deoxyglucose uptake in the isolated adipocytes. Adipocytes were isolated from rat epididymal fat tissue obtained from animals infused with either saline (SAL) or glucosamine (GLN). Adipocytes were incubated with 100nM of insulin for 5 min, followed by an additional 10 min incubation with 2-[1-<sup>14</sup>C]-deoxyglucose (9.3kBq). Radioactivity was measured using a liquid scintillation counter. Data are means±SE from 4 separate experiments. \*, P<0.05; INS, insulin.

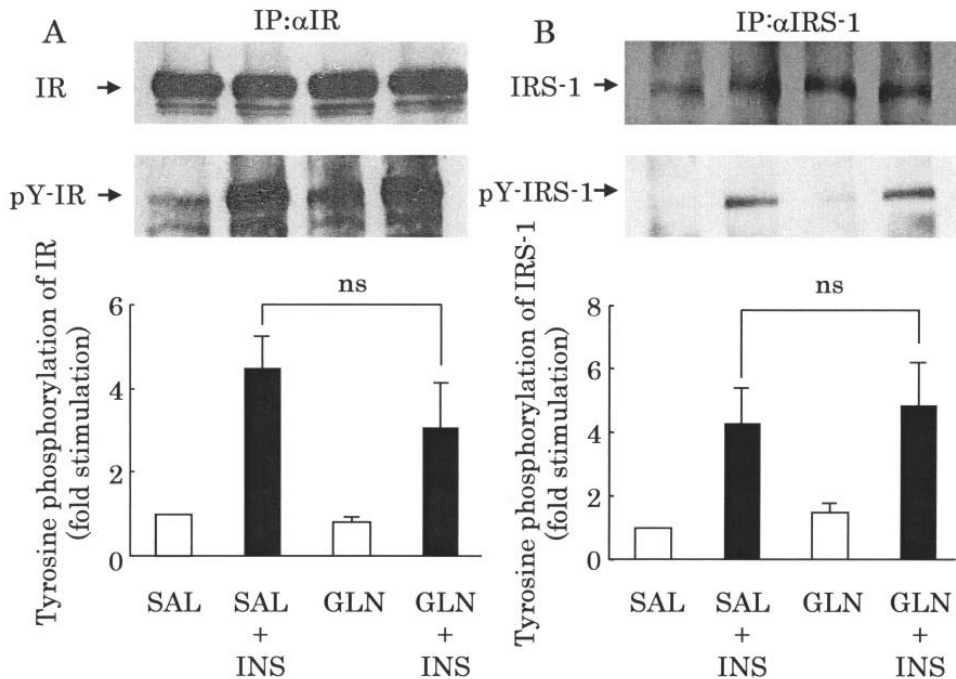


FIG. 2. Effects of glucosamine on tyrosine phosphorylation of the IR and IRS-1. Tissue homogenates were subjected to immunoprecipitation (IP) with anti-IR (A) and anti-IRS-1 (B) antibodies. The precipitated proteins were separated by SDS-PAGE and immunoblotted with PY-20 antibody. Data are means±SE from 3 separate experiments. ns, not significant; pY, tyrosine phosphorylated; SAL, saline; GLN, glucosamine; INS, insulin.

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respectively (Fig. 2A, B). Analysis showed that glucosamine infusion had no effect on tyrosine phosphorylation of the IR ( $P=0.33$ ) and IRS-1 ( $P=0.77$ ). The expression of IRS-2 protein in adipocytes was too low to allow for the detection of a change in its degree of tyrosine phosphorylation.

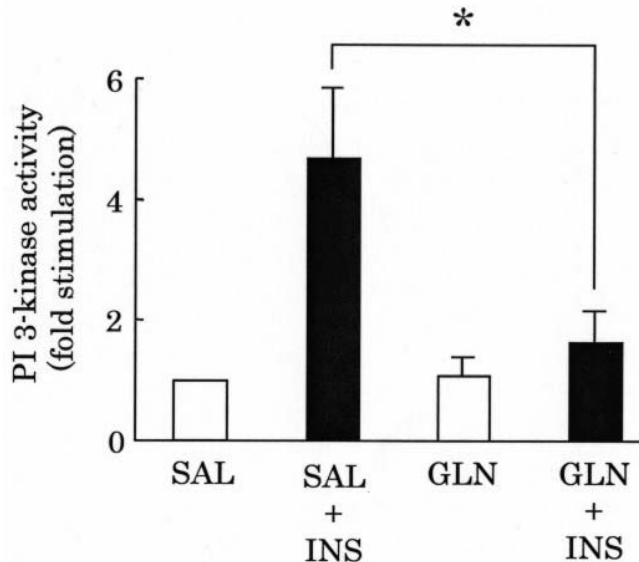


FIG. 3. Effects of glucosamine on PI 3-kinase activity. PI 3-kinase activity was determined by immunoprecipitation with anti-phosphotyrosine antibody (PY-20). After immunoprecipitation, PI 3-kinase activity associated with PY-20 was measured. Kinase assays were performed using PI as a substrate as described in the METHODS. Data are means  $\pm$  SE from 6 separate experiments. \*,  $P<0.05$ ; SAL, saline; GLN, glucosamine; INS, insulin.

**Effects of glucosamine infusion on PI 3-kinase activity** To assess the effect of glucosamine infusion on PI 3-kinase activity, we measured PI 3-kinase activity associated with PY-20 in adipocytes. Insulin increased PI 3-kinase activity associated with PY-20  $4.7\pm 1.2$ -fold in saline-infused rats, and  $1.6\pm 0.5$ -fold in glucosamine-infused rats. Infusion of glucosamine reduced insulin-stimulated PI 3-kinase activity associated with PY-20 by 66% ( $P=0.04$ ) (Fig. 3). We next examined PI 3-kinase activity associated with IRS-1, 2, and 3 in adipocytes. Insulin increased PI 3-kinase activity associated with IRS-1  $19.3\pm 0.8$ -fold in saline-infused rats, and  $13.5\pm 1.3$ -fold in glucosamine-infused rats (Fig. 4A). Infusion of glucosamine reduced insulin-stimulated PI 3-kinase activity associated with IRS-1 by 30% ( $P=0.01$ ). Insulin increased PI 3-kinase activity associated with IRS-2  $6.0\pm 0.9$ -fold in saline-infused rats, and  $3.4\pm 0.5$ -fold in glucosamine-infused rats (Fig. 4B). Insulin-stimulated PI 3-kinase activity associated with IRS-2 was decreased by 43% in glucosamine-infused rats ( $P=0.03$ ). Insulin increased PI 3-kinase activity associated with IRS-3  $4.5\pm 0.7$ -fold in saline-infused rats. In glucosamine-infused rats, insulin increased PI 3-kinase activity associated with IRS-3  $2.5\pm 0.4$ -fold (Fig. 4C). Infusion of glucosamine reduced insulin-stimulated PI 3-kinase activity associated with IRS-3 by 44% ( $P=0.04$ ). The amount of IRS-1 and IRS-2 that was immunoprecipitated with each specific antibody was not influenced by glucosamine infusion, respectively (data not shown). Since anti-IRS-3 antibody was not available for immunoblotting, the amount of IRS-3 which this antibody can immunoprecipitate was not evaluated.

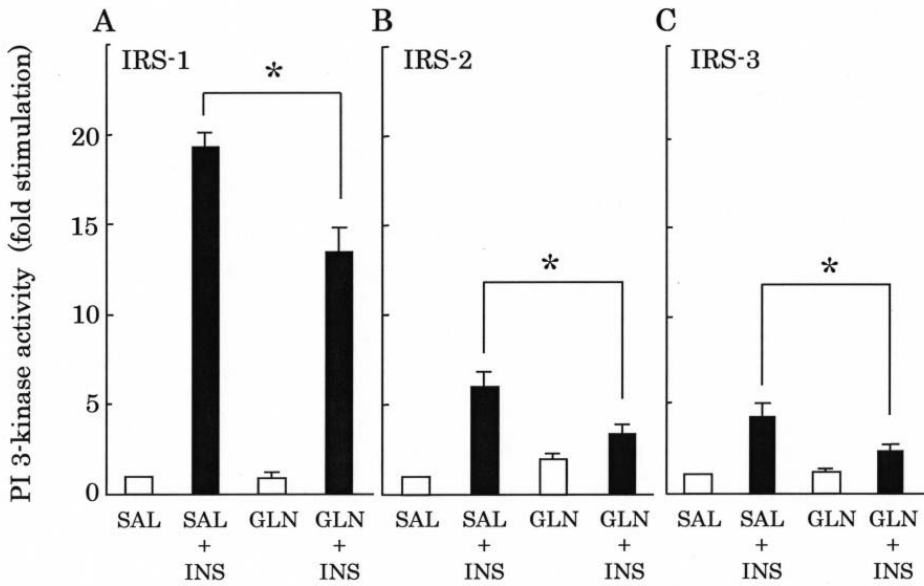


FIG. 4. Effects of glucosamine on PI 3-kinase activity associated with IRSs. PI 3-kinase activity was measured by precipitation with anti-IRS-1 (A), anti-IRS-2 (B), and anti-IRS-3 (C) antibodies. Kinase assays were performed using PI as the substrate as described in the METHODS. Data are means  $\pm$  SE from 6 separate experiments. \*,  $P < 0.05$ ; SAL, saline; GLN, glucosamine; INS, insulin.

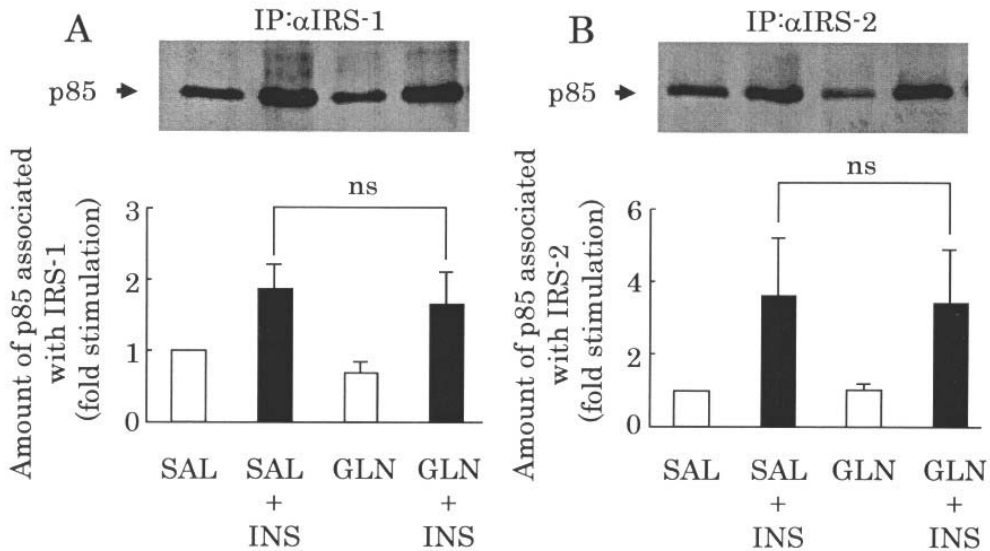


FIG. 5. Effects of glucosamine on the association of the 85kDa regulatory subunit of PI 3-kinase (p85) with IRS-1 and IRS-2. Tissue homogenates were subjected to immunoprecipitation (IP) with anti-IRS-1 (A) and IRS-2 (B) antibodies. The precipitated proteins were separated by SDS-PAGE and immunoblotted with anti-p85 antibody. Data are means  $\pm$  SE from 3 separate experiments. ns, not significant; SAL, saline; GLN, glucosamine; INS, insulin.

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**Effects of glucosamine infusion on the association of the 85kDa regulatory subunit of PI 3-kinase (p85) with IRS-1 and IRS-2** We analyzed whether glucosamine infusion affected the association of p85 with IRS-1 and IRS-2 in adipocytes. Insulin increased the amount of p85 associated with IRS-1 1.9±0.3-fold in control rats and 1.7±0.4-fold in glucosamine-infused rats above basal levels (Fig. 5A). Insulin also increased the amount of p85 associated with IRS-2 3.6±1.6-fold in control rats and 3.4±1.5-fold in glucosamine-infused rats (Fig. 5B). There were no differences in the amount of p85 associated with IRS-1 (p=0.73) and IRS-2 (p=0.92) in adipocytes obtained from rats treated with saline and glucosamine. The association of p85 with IRS-3 was not analyzed in the present study.

**Effects of glucosamine infusion on PI 3-kinase activity in response to PDGF stimulation** We investigated whether glucosamine infusion affected PI 3-kinase activity in response to PDGF stimulation in adipocytes. PDGF increased PI 3-kinase activity associated with PY-20 3.3±0.4-fold in saline-infused rats and 2.0±0.4-fold in glucosamine-infused rats (Fig. 6). Thus, PDGF-stimulated PI 3-kinase activity in glucosamine-infused rats was reduced to 61% of that of the control rats (P=0.04).

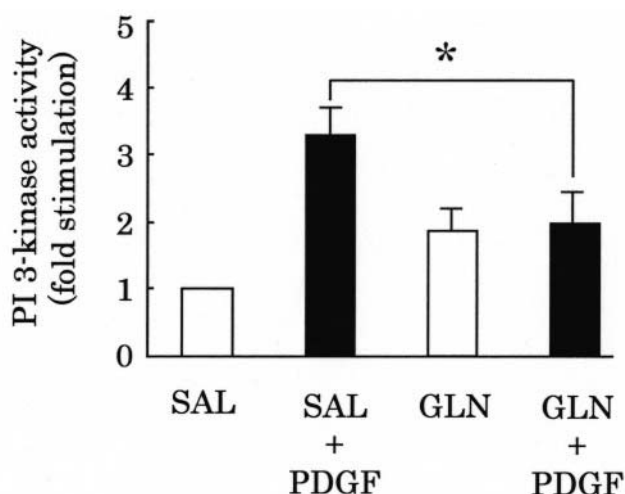


FIG. 6. Effects of glucosamine on PDGF-stimulated PI 3-kinase activity. PI 3-kinase activity was measured using PY-20 antibody immunoprecipitates. Kinase assays were performed using PI as the substrate as described in the METHODS. Data are means ± SE from 4 separate experiments. \*, P<0.05; SAL, saline; GLN, glucosamine.

**Effects of glucosamine infusion on PKCλ kinase activity** We analyzed whether glucosamine infusion affected PKCλ kinase activity. Insulin increased PKCλ kinase activity 2.0±0.2-fold in the saline-infused rats but only 1.3±0.2-fold in the glucosamine-infused rats (Fig. 7). Thus, insulin-stimulated PKCλ kinase activity in adipocytes was reduced by 33% in the rats infused with glucosamine, compared to control rats (P=0.04).

## DISCUSSION

“Glucose toxicity” induced by hyperglycemia is thought to be one of the pathogenic mechanisms responsible for the development of insulin resistance. Since it is not clear which step in insulin signaling is impaired in the insulin resistant state, we investigated insulin signaling in rat isolated adipocytes under conditions of insulin resistance induced by glucosamine administration.

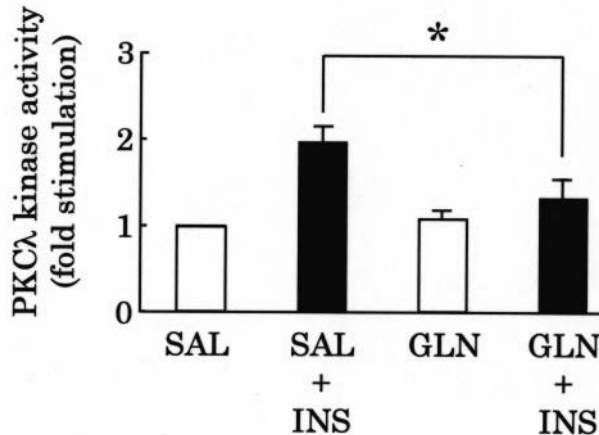


FIG. 7. Effects of glucosamine on PKCλ kinase activity. Tissue homogenates were subjected to immunoprecipitation with anti-PKCλ antibody. [<sup>32</sup>P] incorporation by MBP was quantified. Data are means ± SE from 4 separate experiments. \*, P<0.05; SAL, saline; GLN, glucosamine; INS, insulin.

We analyzed the effects of glucosamine on PI 3-kinase activity and showed that it reduced PI 3-kinase activity in adipocytes. This impairment in PI 3-kinase activity was observed in various insulin-resistant models (1,5,16). One of the possible explanations for this reduction in PI 3-kinase activity was suggested to be a decrease in tyrosine-phosphorylation of the IR and IRS-1 (5,16). It was reported that exposure of Rat1 fibroblasts to a high glucose concentration resulted in serine phosphorylation of the IR (16), which resulted in a decreased tyrosine-phosphorylation of the IR. Since we did not observe any changes in the tyrosine-phosphorylation level of the IR or IRS-1 in glucosamine treated adipocytes (Fig. 2), it was concluded that this decrease in PI 3-kinase activation did not result from changes in the tyrosine-phosphorylation of the IR and IRS-1.

We analyzed the molecular mechanism responsible for the inhibition of PI 3-kinase activity in adipocytes derived from rats infused with glucosamine. Although it was reported that the amount of PI 3-kinase associated with IRS was decreased in the skeletal muscle of high fructose-fed rats and in the skeletal muscle and fat tissue of high fat-fed rats (1,3), we did not observe a decrease in the insulin-stimulated association of IRS-1 and IRS-2 with p85 of PI 3-kinase in adipocytes of glucosamine-infused rats (Fig. 5). A decrease in the expression of PI 3-kinase and IRS proteins is thought to result in a reduction in PI 3-kinase activity. It was likely that a 4 hour glucosamine infusion was not long enough to affect the expression of these proteins. Since we did not evaluate the associations of the p50 or p55 subunit of PI 3-kinase with IRS proteins, we could not omit the possibility that the association of the p50 or p55 subunit with IRS proteins affected PI 3-kinase activity.

We examined whether the infusion of glucosamine modulated the PI 3-kinase molecule itself. Specifically, we analyzed the effects of glucosamine on PI 3-kinase activity by stimulating the IRS-independent pathway with PDGF. Following autophosphorylation of the PDGF-β receptor by PDGF, the p85 subunit of PI 3-kinase binds to tyrosine residues 740 and 751 of the PDGF-β receptor (10). We found that glucosamine infusion inhibited PDGF-stimulated PI 3-kinase activation in rat adipocytes (Fig. 6). Since the tyrosine phosphorylation of the PDGF receptor in glucosamine-treated 3T3-L1 adipocytes was reported to be unchanged, it was speculated that the PI 3-kinase molecule was modulated by infusion of glucosamine (17).



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In summary, we analyzed insulin signaling in adipocytes under insulin resistant conditions caused by glucosamine infusion in rats. We demonstrated that glucosamine inhibited insulin-stimulated PI 3-kinase activity that appeared to be due to a modulation in PI 3-kinase.

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