Differential Effects of Troglitazone and D-Chiroinositol on Glucosamine-Induced Insulin Resistance In Vivo in Rats

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Troglitazone and D-chiroinositol have been shown to exert antidiabetic effects by either potentiating or mimicking insulin action. We studied whether pretreatment with these compounds can prevent the deleterious effects of glucosamine on insulin action that may play an important role in hyperglycemia-induced insulin resistance. Normal Wistar rats were pretreated with troglitazone (100 mg/kg/d), D-chiroinositol (100 mg/kg/d), or placebo (saline) for 7 days. Glucosamine (50 μmol/kg/min) was then infused for 210 minutes, and a euglycemic glucose clamp was performed during the last 120 minutes. Pretreatment with troglitazone or D-chiroinositol had no effect on fasting plasma glucose or insulin or basal hepatic glucose output (HGO). Under the euglycemic-hyperinsulinemic (956 ± 93 pmol/L) clamp condition, HGO in glucosamine-infused placebo-treated rats was not suppressed, but instead was increased over the basal level, indicative of hepatic insulin resistance. In contrast, HGO failed to increase during glucosamine infusion in rats pretreated with troglitazone but was not normally suppressed. This may indicate a partial improvement in the hepatic insulin resistance. D-Chiroinositol pretreatment had no effect on the glucosamine-induced increase in HGO. The glucose disposal rate (GDR) was 25% lower in rats infused with glucosamine versus saline-infused rats (25.5 ± 2.5 *v* 34.1 ± 2.0 mg/kg/min), indicative of peripheral insulin resistance. Pretreatment with D-chiroinositol (34.5 ± 2.3 mg/kg/min) prevented the glucosamine-induced decrease in the GDR, indicating an improvement in peripheral insulin resistance. Troglitazone (25.2 ± 3.3 mg/kg/min) was without effect. In conclusion, (1) in normal control rats, glucosamine infusion induced hepatic and peripheral insulin resistance; (2) D-chiroinositol, but not troglitazone, pretreatment prevented glucosamine-induced peripheral insulin resistance; and (3) troglitazone, but not D-chiroinositol, partially blocked the glucosamine-induced hepatic insulin resistance. D-Chiroinositol may provide a novel pharmacological approach to hexosamine-induced peripheral insulin resistance.

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INSULIN RESISTANCE plays a crucial role in the pathophysiology of type 2 diabetes mellitus.[1] The etiology of insulin resistance is heterogeneous. It may be determined genetically or acquired through the influences of various nutritional, physical, and humoral factors. Understanding the multiple mechanisms of insulin resistance and the development of specific pharmacological treatments should provide better approaches for the therapy of type 2 diabetes.

It is well known that hyperglycemia is not merely a result of metabolic derangement, but rather, hyperglycemia itself can cause an impairment of insulin-mediated glucose disposal in peripheral tissues (skeletal muscle and adipose tissue). Glucose toxicity has important clinical implications in that hyperglycemia self-perpetuates the diabetic state.[2,3] Marshall et al[4] have proposed that increased glucose flux via the hexosamine synthesis pathway, initiated by the conversion of fructose-6-phosphate to glucosamine-6-phosphate by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT), is required for hyperglycemia-induced downregulation of glucose transport in adipocytes. Glucosamine, which enters the pathway distal to GFAT, has been reported to impair insulin-mediated glucose uptake in vivo in rats,[5-9] as well as in vitro in adipocytes[4] and skeletal muscles.[10,11]

The present study was designed to clarify whether two insulin-sensitizing or -mimicking agents can ameliorate the insulin resistance caused by the hexosamine synthesis pathway. For this purpose, we selected troglitazone and D-chiroinositol. Troglitazone, a thiazolidinedione, reduces hyperglycemia by either mimicking or potentiating insulin action in animals and humans with obesity and type 2 diabetes.[12] D-Chiroinositol is a component of an inositol glycan that has been proposed as a putative mediator of insulin action.[13] A decrease in the urinary excretion and skeletal muscle content of D-chiroinositol was noted in patients with type 2 diabetes,[14] and urinary excretion of D-chiroinositol correlated well with insulin sensitivity in rhesus monkeys[15] and in patients with type 2 diabetes.[16] Administration of D-chiroinositol and D-chiroinositol–containing inositol glycan mediators to insulin-resistant monkeys and streptozotocin-diabetic rats has been shown to decrease plasma glucose without increasing plasma insulin.[17-19] In recent clinical trials, treatment with D-chiroinositol improved glucose tolerance in women with the polycystic ovary syndrome[20] and in human subjects with impaired glucose tolerance.[21] Troglitazone and D-chiroinositol share a common characteristic, that is, they reduce hyperglycemia without increasing plasma insulin. However, a clear distinction exists in their potential for the treatment of diabetes. Troglitazone has been shown to be effective for hyperinsulinemic hyperglycemia but not for hypoinsulinemic hyperglycemia,[12] while D-chiroinositol has been reported to reduce glycemia in both hyperinsulinemic and hypoinsulinemic animals.[17]

In the present study, we pretreated rats with troglitazone or D-chiroinositol for 7 days and then measured the effects of acute glucosamine infusion on insulin-mediated glucose metabolism using the euglycemic clamp technique to separately measure the effects on hepatic glucose output (HGO) and the glucose disposal rate (GDR).

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MATERIALS AND METHODS

Animals

Seven-week-old male Wistar rats (CLEA, Tokyo, Japan) were housed individually in separate cages in an environmentally controlled room with a 12-hour light/dark cycle (lights on 6 AM to 6 PM). The rats had free access to laboratory chow (CE-2; CLEA) and water. After 1 week of acclimation, they were randomly allocated to one of the following four experimental groups: (1) glucosamine-infused rats pretreated with a placebo (GlcN), (2) glucosamine-infused rats pretreated with troglitazone (TROG/GlcN), (3) glucosamine-infused rats pretreated with D-chiroinositol (DCI/GlcN), and (4) saline-infused rats pretreated with a placebo (control). Rats assigned to the TROG/GlcN group or DCI/GlcN group received troglitazone (100 mg/kg/d) suspended in 0.5% carboxymethylcellulose saline or D-chiroinositol (100 mg/kg/d) dissolved in tap water, respectively, by gastric gavage for 7 days. Troglitazone and D-chiroinositol were kindly provided by Sankyo Pharmaceutical (Tokyo, Japan) and Kaken Pharmaceutical (Tokyo, Japan), respectively. Rats in the GlcN group and the control group received saline by gastric gavage for 7 days. The knowledge about the dose-response profiles of troglitazone and D-chiroinositol for insulin resistance in experimental animals is limited. The dose of both agents used in this study is well within the range of the lowest and highest doses of each agent (1 to 150 mg/kg for troglitazone[22,23] and 1 to 500 mg/kg for D-chiroinositol[24,25]) that have been proven to be effective in humans and animals with insulin resistance.

On the morning after completion of these treatments, a euglycemic-hyperinsulinemic clamp study was performed with chronically catheterized rats.[26] Four days before the clamp study, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Single- and triple-lumen catheters prepared by heat-connecting PE-10 and PE-50 polyethylene tubing (Becton Dickinson, Parsippany, NY) were placed into the right carotid artery and jugular vein, respectively. The catheters were prefilled with polyvinylpyrrolidone (PVP) solution containing 500 mg PVP (K-90; Nakalai Tesque, Kyoto, Japan), 10 mg cefmetazol sodium (Sankyo Pharmaceutical), 1,000 U heparin sodium (Shimizu Pharmaceutical, Shizuoka, Japan), and 0.7 mL distilled water. The solution was aspirated immediately before the experiments. The tip of the arterial catheter was extended to the aortic arch, and the venous catheter was advanced into the left atrium. The triple-lumen catheters were used to infuse insulin, D-[3-^{3}H]-glucose, unlabeled glucose, and glucosamine simultaneously. The arterial line was used for blood sampling. These catheters were tunneled under the skin and exteriorized in the nuchal area.

To determine the effect of glucosamine infusion on insulin's effect to suppress HGO and stimulate peripheral glucose disposal, we infused D-glucosamine (Sigma, St Louis, MO) diluted in saline for 210 minutes in a primed (50 μmol/kg)- continuous (50 μmol/kg/min) fashion in the placebo-treated rats, and the euglycemic clamp studies were performed during the last 120 minutes of glucosamine infusion. The troglitazone-treated or D-chiroinositol–treated rats were also infused with glucosamine and studied similarly. The control rats were infused with saline for 210 minutes instead of glucosamine. Glucosamine infusion in normal rats at this rate is expected to exert maximal inhibition of insulin-mediated glucose disposal.[7] The method for the glucose clamp is described elsewhere.[26] One hour before glucosamine infusion, the overnight-fasted rats were brought to the laboratory in a box in which they were unrestrained and freely moving. After obtaining basal blood samples for glucose and insulin, a primed-continuous infusion of glucosamine and D-[3-^{3}H]-glucose (6 mCi, 0.15 mCi/min; Du Pont-New England Nuclear, Boston, MA) was initiated 90 minutes and 60 minutes before the clamp studies and continued throughout the study.

The euglycemic-hyperinsulinemic clamp was started by infusing insulin (Humulin R; Eli Lilly & Co, Indianapolis, IN) in a primed (40 mU/kg)-continuous (8 mU/kg/min) fashion. This insulin infusion rate was selected to increase plasma insulin to the level at which a nearly half-maximal stimulatory effect of insulin on whole-body glucose disposal was demonstrated.[27,28] The infusion rate of 30% unlabeled glucose was variably adjusted based on plasma glucose values measured every 5 minutes according to a previously reported algorithm[29] to clamp plasma glucose at 110 mg/dL. Blood samples for tracer kinetic studies were obtained at 80, 85, and 90 minutes of the pre–glucose clamp period of glucosamine infusion and during the last 30 minutes of the glucose clamp. Basal HGO was calculated from the data obtained at 80, 85, and 90 minutes after initiation of glucosamine or saline infusion. The GDR and HGO under the glucose clamp condition were calculated from the values obtained at 90, 105, and 120 minutes.

Analytical Procedures

The plasma glucose concentration was measured by the glucose oxidase method using a glucose analyzer (Glucose Analyzer 2; Beckman Instruments, Palo Alto, CA), and the plasma insulin concentration was measured by a double-antibody radioimmunoassay (Insulin Eiken; Eiken, Tokyo, Japan) using rat insulin (kindly supplied by Eli Lilly & Co) as the standard. Plasma D-[3-^{3}H]-glucose radioactivity was measured in duplicate. The supernatant of $Ba(OH)_2$ and $ZnSO_4$ precipitation of plasma samples was counted after evaporation to dryness to eliminate tritiated water.

The data are presented as the mean ± SE and were subjected to one-way ANOVA. Fisher's test was used for comparison of mean values among different groups. Differences were considered significant at a P level less than .05.

RESULTS

The body weight and fasting plasma glucose and insulin concentrations of each group were all similar (Table 1). During the clamp, plasma insulin was increased to approximately 900 pmol/L. There were no significant differences among the four groups for steady-state plasma glucose and insulin concentrations calculated as the mean value of three determinations during the last 30 minutes of the clamp. The glucose infusion rates (GIRs) required to maintain plasma glucose at 6.3 mmol/L during the course of the clamp studies in each group are shown in Fig 1. The GIRs during glucosamine infusion in placebo-pretreated rats (GlcN group) were already clearly lower than the GIRs during the saline infusion (control group) at 10 minutes and remained so until the end of the study (120 minutes). Both TROG/GlcN and DCI/GlcN rats showed intermediate values between the GlcN and control values. Their GIRs were significantly lower than the GIRs in the control group during the last 30 minutes of the clamp study. The GIRs in the DCI/GlcN group were higher at 110 and 120 minutes versus the GlcN

Table 1. Characteristics of Rats in Each Experimental Group

Characteristic	Control	GlcN	TROG/GlcN	DCI/GlcN
No. of rats	8	8	8	8
Weight (g)	202 ± 2	206 ± 5	203 ± 5	208 ± 3
Glucose (mmol/L)				
Fasting	7.4 ± 0.3	7.7 ± 0.3	7.5 ± 0.3	6.9 ± 0.3
Clamp	6.2 ± 0.1	6.5 ± 0.2	6.4 ± 0.1	6.3 ± 0.1
Insulin (pmol/L)				
Fasting	71 ± 6	99 ± 12	98 ± 15	89 ± 14
Clamp	810 ± 47	956 ± 93	890 ± 44	840 ± 67

NOTE. Data are the mean ± SE.

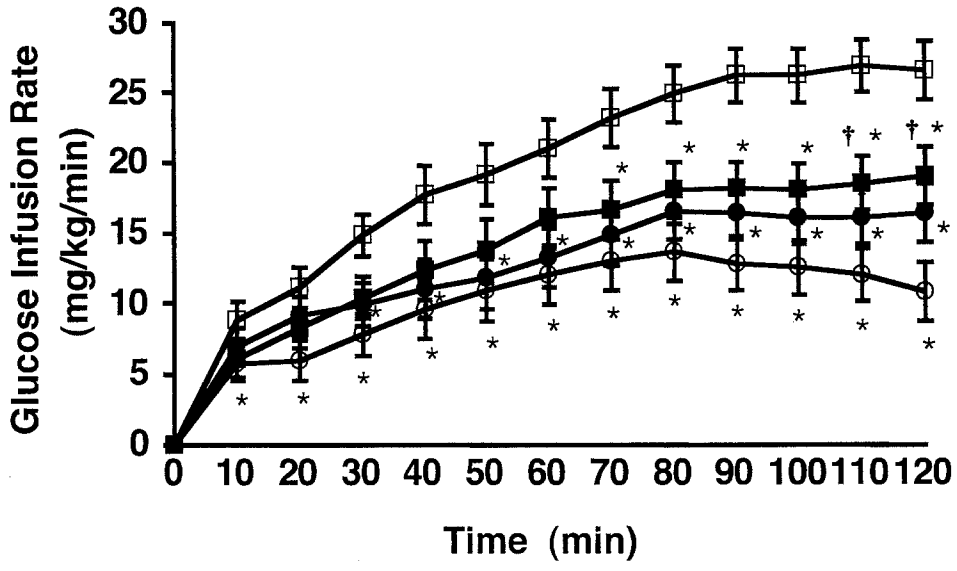

Fig 1. Effect of glucosamine infusion on the GIR during the glucose clamp in TROG/GlcN (●), DCI/GlcN (■), or placebo (GlcN, ○) groups. Control rats (□) received saline infusion. The GIR in the DCI/GlcN group was higher at 110 and 120 minutes *v* the GlcN group. Values are the mean ± SE; 8 rats in each group. *$P <$ .05 *v* control, †$P <$ .05 *v* GlcN.

group. GIRs in the TROG/GlcN group did not differ from the values in the GlcN group at any point during the clamp.

GDRs during the last 30 minutes of the clamp are shown in Fig 2. The GDR decreased by 25% in the GlcN group compared with the control rats (34.1 ± 2.0 *v* 25.5 ± 2.5 mg/kg/min, $P < .05$). Pretreatment with D-chiroinositol (34.5 ± 2.5 mg/kg/min) prevented the decrease in the GDR. In contrast, troglitazone (25.2 ± 3.3 mg/kg/min) had no effect.

Basal HGO was measured 80 to 90 minutes after initiation of the glucosamine or saline infusion. Glucosamine infusion for 90 minutes did not produce significant changes in HGO compared with saline infusion (10.3 ± 1.0 *v* 12.1 ± 1.1 mg/kg/min; Fig 3). Neither troglitazone nor D-chiroinositol affected basal HGO. In the control group, HGO decreased to 70% of the basal value during the last 30 minutes of the clamp when plasma insulin was increased to 810 ± 47 pmol/L. In the GlcN group, an increase in HGO instead of a decrease was noted during the clamp. Thus, glucosamine infusion for up to 90 minutes did not affect HGO, but prolonged infusion (180 to 210 minutes) increased HGO even in the hyperinsulinemic clamp condition. Despite the infusion with glucosamine, rats in the TROG/GlcN group did not show an increase in HGO during the last 30 minutes of the clamp. Nevertheless, they did not exhibit the normal suppression of HGO found in the control group. It appears that under the present conditions, glucosamine-induced hepatic insulin resistance was not completely blocked by troglitazone pretreatment. In contrast, pretreatment with D-chiroinositol was without effect on HGO.

DISCUSSION

Short-term glucosamine infusion in rats decreased the insulin-mediated GDR by 27% during the euglycemic clamp in the present study. The magnitude of impairment agrees well with previous studies.[5-9] Hepatic insulin action was also impaired, since an increase in HGO over the basal level was noted instead of the normal suppression. In this regard, other investigators

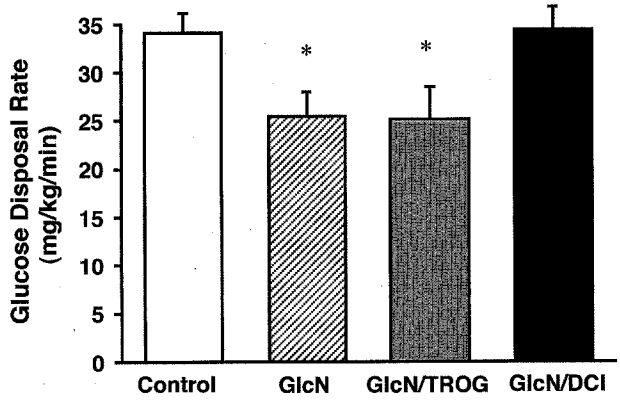

Fig 2. Steady-state GDR during the last 30 minutes of the hyperinsulinemic-euglycemic glucose clamp study in TROG/GlcN, DCI/GlcN, or placebo (GlcN) groups. Control rats received saline infusion. Values are the mean ± SE; 8 rats in each group. *$P <$ .05 *v* control and DCI groups.

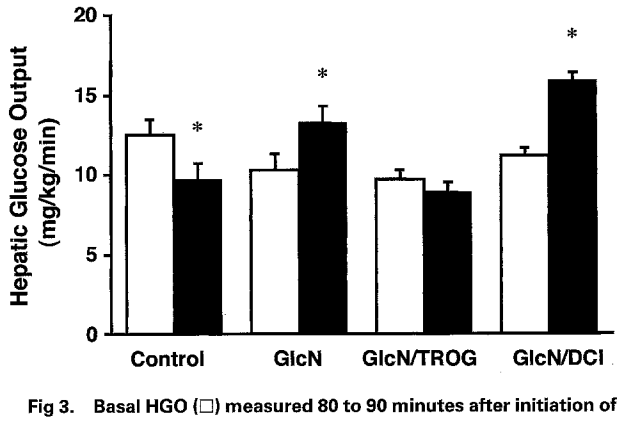

Fig 3. Basal HGO (□) measured 80 to 90 minutes after initiation of glucosamine or saline infusion and clamp HGO (■) measured during the last 30 minutes of the glucose clamp study in TROG/GlcN, DCI/GlcN, or placebo (GlcN) groups. Control rats received saline infusion. Values are the mean ± SE; 8 rats in each group. *$P <$ .05 *v* basal state for each treatment group.

reported normal[6,8] and reduced[9] hepatic insulin action during a euglycemic clamp in rats receiving glucosamine infusion. Glucosamine has been shown to inhibit hepatic glucokinase in vitro and to impair the ability of hyperglycemia to suppress HGO in vivo.[30] The liver has been shown to contain GFAT by immunohistochemistry,[31] and a higher GFAT activity in the liver versus the skeletal muscle and fat has been reported.[8,32] However, the cellular content of uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), a metabolite of the hexosamine synthesis pathway, is not increased in the liver of several animal models with hyperglycemia.[33,34] Therefore, it is still unclear as to whether glucose flux via the hexosamine synthesis pathway in the liver is increased in the diabetic state.

Pretreatment of rats with troglitazone did not alter glucosamine's inhibitory effect on insulin-mediated glucose disposal (Fig 2). This may indicate that troglitazone is less effective for preventing glucosamine-induced peripheral insulin resistance than for correcting HGO. Alternatively, it is possible that the dose and/or length of troglitazone administration was not sufficient to prevent glucosamine's effects. However, the dose used in this study has been shown to be effective in various animal models of diabetes and insulin resistance.[26,35,36] In fact, the dose is more than 10 times higher than that used clinically for human type 2 diabetes.[37] Most recently, Miles et al[38] reported the failure of troglitazone to prevent glucosamine-induced insulin resistance even with a 1.5-fold larger dose and a twofold longer treatment period than our study. In several in vitro studies, troglitazone and other thiazolidinediones were reported to restore high glucose–induced perturbations in insulin action at the level of glucose uptake[39] in cultured coronary smooth muscle cells, glycogen synthase activation[40] in isolated rat adipocytes, and insulin receptor kinase[41] in rat-1 fibroblasts overexpressing human insulin receptors. These results and our current results are not necessarily mutually exclusive. In fact, glucosamine does not share the upstream impairments in insulin signaling at the level of insulin receptors caused by hyperglycemia.[10] Thus, multiple mechanisms operate for the induction of insulin resistance by hyperglycemia, and some of them may be modified by thiazolidinediones.

We found that troglitazone treatment partially blocked glucosamine's effects in impairing the insulin-mediated suppression of HGO (Fig 3), but it was without effect on peripheral glucose disposal (Fig 2). Direct hepatic actions of troglitazone[42,43] have been reported. A similar pattern of response to thiazolidinediones, ie, amelioration of hepatic insulin action in the face of unaltered peripheral insulin resistance, has been reported in Goto-Kakizaki rats,[44] alloxan-diabetic dogs,[45] and streptozotocin-diabetic rats.[46]

The most interesting finding in the present study is that pretreatment of normal rats with D-chiroinositol clearly prevented the decrease in insulin-mediated peripheral glucose disposal (Fig 2) during glucosamine infusion but was without effect on HGO (Fig 3). Our results raise the possibility that D-chiroinositol could be selectively effective for peripheral insulin resistance induced by increased glucose flux through the hexosamine synthetic pathway.

The role of the hexosamine synthetic pathway initially gained attention in the context of hyperglycemia-induced insulin resistance.[5] Recent studies extended the view beyond glucose toxicity.[47] Overactivity of the pathway may play a role in various insulin-resistant conditions not necessarily associated with overt hyperglycemia, such as in ob/ob mice[34] and in rats infused with free fatty acids.[48]

As to the molecular mechanism of the hexosamine synthetic pathway for regulating cellular glucose metabolism, researchers are now focusing on protein glycosylation.[10,32,47,49] Glycosylphosphatidylinositol (GPI)-anchored proteins, which are among a major class of glycoproteins, have not been discussed thus far in this context. Their synthesis is initiated by the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to phosphatidylinositol (PI) to form GlcNAc-PI.[50] It has been postulated that inositol glycan insulin mediators are generated by the insulin-dependent hydrolysis of GPI-anchored proteins or protein-unbound free GPI.[51] Notably, Müller et al[52] have reported that prolonged exposure of rat adipocytes to 20 mmol/L glucose inhibited both Glut-4 translocation and the insulin-dependent cleavage of GPI anchors. This raises the possibility that hyperglycemia impairs the generation of inositol glycan insulin mediators, and there may be a link between hyperglycemia-induced downregulation of glucose transport and a defect in the generation of inositol glycan insulin mediators. Furthermore, it is of interest to note that an increase in the activity of the hexosamine synthetic pathway has been reported, in the skeletal muscle of ob/ob mice,[34] streptozotocin-diabetic rats,[33] and type 2 diabetic humans,[53] and in each of these animal models and human type 2 diabetes, abnormalities in the synthesis or metabolism of inositol glycan insulin mediators have been suggested.[54-57] Although these results may suggest a link between the hexosamine synthetic pathway and the biosynthetic mechanism of inositol glycan insulin mediators, the mechanism for the effect of D-chiroinositol to prevent glucosamine-induced insulin resistance remains unknown and open to further investigations.

It should be noted that there are limitations in the interpretation of our results. First, the observed tissue selectivity of the effects of both troglitazone and D-chiroinositol occurred at a single dose and therefore could be dose-dependent. Second, the glucosamine infusion rate we used was three times higher than the rate[7] that was shown to inhibit whole-body glucose disposal maximally under the euglycemic clamp in normal rats. Although it is unlikely, it cannot be excluded that pretreatment of animals with troglitazone or D-chiroinositol affects the pharmacokinetics of glucosamine and reduces its plasma concentration, which in turn results in an apparent amelioration of hepatic or peripheral insulin resistance.

In conclusion, the glucosamine-induced impairment in insulin-mediated glucose disposal in vivo in rats is prevented by pretreatment with D-chiroinositol, but not with troglitazone. In contrast, the deterioration in hepatic insulin action with glucosamine was partially blocked by pretreatment with troglitazone, but not with D-chiroinositol.

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