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Insulin Glargine reduces carotid intimal hyperplasia after balloon catheter injury in Zucker fatty rats possibly by reduction in oxidative stress

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Abstract

Diabetes and impaired glucose tolerance are associated with increased cardiovascular disease morbidity and mortality particularly after vascular injury. Since insulin is frequently used in such patients, the effect of glulisine (short acting) and glargine (long acting) were tested in Zucker fatty rat carotid artery subjected to balloon catheter injury. Insulin resistant Zucker fatty rats were sc injected 0.45 mg/kg of glargine (once) or glulisine (twice) for 1 week before, and 3 weeks after balloon injury. Fasting and postprandial glucose was measured twice weekly. Injured and uninjured carotid arteries, liver and aorta were harvested after 3 weeks of injury. Carotid sections were H & E stained for measuring intima/media ratio or immunostained for nitrotyrosine. Serum and aortic protein were analyzed for IGF-1 and 8-isoprostane respectively. Carotid intima/media ratio was significantly reduced in the glargine group [0.9 ± 0.1 -control; 0.6 ± 0.1 -glulisine; 0.4 ± 0.1 -glargine, $p < 0.05$]. Serum IGF-1 levels were higher in both insulins, but significant only in glargine group [567 ± 121 (ng/ml)-control; 1059 ± 150 (ng/ml)-glargine; $p < 0.05$]. The aortic 8-isoprostane levels decreased significantly in the glargine group [(921 vs. 2566 pg/mg protein; $p < 0.05$]. Compared to control nitrotyrosine staining intensity was significantly lower in both groups of insulin treated rats; the lowest level was in the glargine group. Insulin glargine attenuates carotid intimal hyperplasia in non diabetic Zucker fatty rat independent of glucose levels and support a valuable function for insulin in vascular disease that merits additional investigations.

Keywords

Type 2 diabetes; Cardiovascular disease; Insulin resistance; Glargine; IGF-1; Balloon catheter injury; Intimal hyperplasia; I/M ratio; Oxidative stress

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Introduction

Cardiovascular disease (CVD) is a major problem in patients with type 2 diabetes, obesity and insulin resistance [1–3]. There has been considerable controversy on the role of various diabetes treatments and their impact on vascular abnormalities. In particular, the role of insulin has been controversial [4].

Epidemiologic studies suggest that high systemic plasma insulin levels may be associated with increased risk of cardiovascular events. This led to the misconception that exogenous insulin use may lead to cardiovascular disease [5–7]. Indeed, insulin is a growth factor and increases proliferation of vascular smooth muscle cells (SMCs) in vitro. However, SMCs growth stimulation takes place at very high insulin concentrations. In contrast, low dose insulin stimulates endothelial production of nitric oxide, which leads to vasodilatation. Recent studies have demonstrated an anti-inflammatory effect of administration of insulin in patients with myocardial infarction, provided the blood glucose is normal [8]. The new guidelines recommend insulin use early in the natural course of the disease, and studies are needed to determine the effect of insulin treatment on the vasculature early in the process. A clinical trial [9] is in progress for determining the effect of insulin on cardiovascular events in patients with prediabetes and newly diagnosed diabetes. Also, insulin is known to increase the production of potent vasodilators including nitric oxide, and in vitro insulin has been shown to stimulate the upregulation of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) in a receptor kinase mediated manner [10]. Further, clinical studies have shown the acute activation of endothelium-dependent vasodilatation in subjects (with normal/healthy vasculature) by both regular human insulin and insulin glargine [11].

In patients with diabetes and other subjects with vascular disease, a consistent blunting of the endothelial dependent increase in blood flow in response to acetylcholine (ACh) has been demonstrated [12]. However, ACh-induced vasodilatation in patients with type 2 diabetes has been shown to be enhanced during chronic insulin therapy [13–15]. It is proposed that the normal physiologic actions of insulin could be anti-atherogenic, however insulin resistance with higher levels of insulin with its associated loss of this potential vasculoprotective effect increases the risk of CVD. Increasing evidence suggests that insulin resistance is associated with incidence of CVD [16–17]. Several new insulin analogs have been developed in the last decade. These are designed to be either very short acting or long acting, both of which have advantages in clinical practice [18–19]. Thus, short acting analogs are used predominantly with meals, whereas long acting analogs are often the treatment of choice for basal insulin replacement. Whether targeting postprandial or fasting glucose is more likely to prevent cardiovascular events also remains an important and controversial question [20–22].

In order to improve our understanding of the role of insulin in vascular healing following balloon catheter injury, as well as to determine differences between several insulin preparations following vascular injury, we carried out a study using an established animal model of vascular injury. This animal model, the Zucker fatty rat was chosen because it has been previously demonstrated that this model has increased propensity for the development of intimal hyperplasia [23]. In addition, Zucker fatty rats are obese, insulin-resistant, hyperinsulinemic, and have elevated triglyceride levels. Further, these animals have the advantage of having a normal blood sugar so that the administration of insulin does not lead to alterations caused by changes in blood glucose. Intimal hyperplasia is easily demonstrated in a reproducible manner in insulin resistant animals, as compared to those who are insulin deficient [24]. Finally, insulin resistance is a (possible) major contributor to cardiovascular disease in diabetes, rather than insulin deficiency and/or hyperglycemia. In the present study, we tested the effect of a short and a long acting insulin analogs, glulisine and glargine

on intracellular events potentially mediating vascular recovery following balloon catheter injury in Zucker fatty rats using morphometric and biochemical techniques. Our hypothesis was that insulin would attenuate the intimal hyperplasia that occurs in insulin resistant animals following vascular injury. Furthermore, we proposed that there may be differences in intimal hyperplasia if only basal insulin was provided as opposed to provision of insulin only in relation to meals.

Methods

The protocol used for animal experiments in the present study was approved by the Institutional Animal Care and Use Committee of the Tulane University Health Sciences Center, New Orleans, LA. Nine to ten week old female Zucker fatty rats were obtained from Harlan Sprague Dawley Inc., Indianapolis, IN; each group contained 9–12 rats to begin with. The Zucker fatty rat is a well-established model for insulin resistance/type-2 diabetes, and exhibits excessive body weight, hyperinsulinemia, and hypertriglyceridemia [25]. The obese Zucker rat carotid artery injury model has been considered to be an appropriate animal model for the study of the mechanism and treatment of increased restenosis associated with type II diabetes. Further, it has been suggested that an insulin-resistant model is associated with a propensity for neointimal proliferation as this was not seen in an insulin-deficient model with or without exogenous insulin administration [24].

The rats were housed individually with free access to food and water, and were maintained in a temperature and humidity regulated room with 12-hour light dark cycle. After an acclimatization period, the rats were divided into control glulisine and glargine groups and administered n-saline or 0.45 mg/kg/day of the insulin preparation by sc injection (baseline insulin was 12.5 ± 2.7 ng/ml).

Insulin glargine was administered 0.45 mg/kg/day once a day, a similar dose of glulisine in two equal doses was administered before feeding; the insulin dose was determined on the basis of literature reports [26]. Typically, the rats were fasted overnight and between 8.00 and 9.00 am were given the insulin injection and the feed. The feed was taken away after 45 min and after ensuring the rats had eaten and were away from the feed. Between 7.00 and 8.00 pm, the second dose of insulin was injected and the feed was provided for 45 min. The rats were thus conditioned to eat when the feed was made available. Although glargine could be administered anytime during the day, for consistency this was also given in the morning. These rats were fasted overnight when the fasting glucose was measured. Body weights of rats were measured at weekly intervals in addition to measuring the fasting and postprandial glucose levels by tail vein puncture. Typically, the rats were fasted overnight for measuring the fasting glucose and sc injected with insulin and given the feed. Between 90 to 120 min after giving the feed, the postprandial glucose was measured. In addition to blood glucose and serum IGF-1 measurements, the carotid artery sections were either H&E stained for histological evaluation or used for immunodetection of nitrotyrosine; the aortic protein was used for assaying 8-isoprostane by ELISA (Cayman Chemical, Ann Arbor, MI) and quantitation of eNOS by Western blot methods as previously described [27].

Balloon catheter injury

After one week of stabilization, balloon injury was induced as previously described [23]. Briefly, the rats were anesthetized with 5% isoflurane in oxygen inhalation and the femoral artery was surgically approached. Using standard methods, a Maverick over the wire catheter (20 X 2.0 mm; Boston Scientific, Natick, MA) was fluoroscopically guided up the aorta to the carotid artery. The balloon was inflated and maintained at 4.0 atmospheres for exactly 20 seconds. The pressure was then reduced to 2 atmospheres for denuding the endothelium and the catheter was dragged to the descending aorta. At this point, the catheter

was deflated totally and the pressure made negative. The catheter was then gently withdrawn before tying the artery. The incision was closed with Vicryl suture and the animal was allowed to recover before being returned to the vivarium. The administration of insulin or saline was continued for 3 weeks after the procedure. On day 22 after the procedure, the rats were sacrificed for harvesting both injured and the contralateral carotid arteries, aortic arch and other tissues. The carotid arteries were processed by standard methods for histological evaluation and the aortic arch and other tissue samples were frozen and subsequently used for biochemical analysis.

Morphometric measurements

Following the processing for dehydration, the carotid artery segment between the carotid bifurcation and the aortic arch was carefully separated and divided into four equal segments for embedding in the paraffin. From these paraffin blocks 6 μ sections were obtained by microtome and used for staining. All four sections of the artery (individual) were selected for measurements and the analysis on the stained sections was done with a magnification of X10. Computerized digital microscopic software (Image-Pro plus 4) was used to obtain measurements of the intimal and medial areas by a previously described method [19]. The intima/media (I/M) ratio was calculated for all specimens for comparison between various treatment groups. The unstained sections of the carotid arteries were used for immunohistochemical analysis of nitrotyrosine.

Statistical Analysis

Statistical analysis was performed with Sigma Stat software (SSPS, Chicago, IL). One way ANOVA, all pair wise multiple comparison procedures (Tukey test) was used. Data are reported as mean $\pm$ SEM. Statistical significance was set at an α of <0.05 .

Results

The effects of insulin treatment on glucose, intimal hyperplasia and IGF-1 are summarized in table 1. Neither fasting nor postprandial blood glucose levels varied significantly between control and insulin treated rats. Representative micrographs of sections from the carotid arteries of rats sacrificed 21 days after balloon catheter injury are shown in figure 1. When compared to control the intima/media (I/M) ratio was reduced in the glargine and glulisine treated groups but the reduction in I/M ratio was statistically significant only in the glargine treated rats [0.9 ± 0.1 -control; 0.6 ± 0.1 -glulisine; 0.4 ± 0.1 -glargine, $p<0.05$] (figure 2).

The serum IGF-1 levels were higher in both groups of rats treated with insulin however this increase was only significant in the glargine treated rats [567 ± 121 (ng/ml)-control; 763 ± 161 (ng/ml)-glulisine; 1059 ± 150 (ng/ml)-glargine, $p<0.05$] (figure 3).

In order to determine potential contribution of intracellular events aortic 8-isoprostane levels were determined and found to be decreased significantly in the glargine and glulisine treated rats [921 and 1116 pg/mg protein respectively vs. 2566 pg/mg protein in control; $p<0.05$] (figure 4).

To further characterize potential contribution of intracellular events associated with the effect of insulin analogs immunohistochemical staining for nitrotyrosine was determined and found to be statistically significant and lower in both the insulin treated animals compared to control and the lowest level of nitrotyrosine staining was seen in the glargine treated animals (figure 5).

Discussion

The results of the present study show that treatment with the insulin analog glargine decreases intimal hyperplasia following carotid artery balloon catheter injury in the Zucker fatty rat. The attenuation of intimal hyperplasia was associated with an increase in serum IGF-1 levels and a decrease in tissue 8-isoprostane levels and vascular nitrotyrosine staining. The beneficial effect of glargine treatment occurred in the absence of an effect on blood glucose levels. The study also showed a modest decrease in intimal hyperplasia with short acting glulisine which was not statistically significant. Whether this difference between insulin preparations is due to the shorter duration of action of glulisine (and therefore less than 24-hour coverage) needs further investigation. The data however, failed to demonstrate any advantage of prandial insulin administration in this model.

The beneficial effect of glargine was associated with a significant increase in IGF-1 levels, which is compatible with other studies [18]. The effect of glulisine on IGF-1 was not statistically significant. Several insulin analogs are known to either stimulate IGF-1 production or stimulate the IGF-1 receptor to a greater degree than human insulin [28]. Insulin and IGF-1 receptors share structural and functional homology [29–32]. Unlike insulin, IGF-1 is synthesized by vascular smooth muscle cells and cardiomyocytes and is regulated by insulin, angiotensin II, platelet derived growth factor and mechanical forces such as shear stress [30, 33]. In experimental animals and humans, IGF-1 has been shown to decrease vascular resistance thus bringing about specific physiologic effects on vascular function [34–36].

Although growth factors such as IGF-1 have traditionally been considered to contribute to vascular injury by stimulating smooth muscle cell proliferation and migration a recent study has shown that IGF-1 treatment reduces the vascular inflammatory response, oxidative stress and atherosclerosis progression in ApoE-deficient mice [27]. In our experiments when compared to control rats, the serum IGF-1 levels in both glulisine as well as glargine treated rats were increased but the increase was significant only in the glargine animals. The serum IGF-1 levels however were higher than those in the IGF-1 infused ApoE-deficient mice. Thus, it is possible that the beneficial effect of glargine treatment may in part be mediated by the increased levels of IGF-1 and the resulting IGF-1 mediated reductions in intracellular oxidative stress. The animal model used in the present study is euglycemic but exhibits hypertriglyceridemia, obesity, hyperinsulinemia and insulin resistance; we could thus eliminate the glucose mediated changes as glucose peaks and spikes have been shown to have pronounced effects on IGF-1 release [37].

In order to more directly address the effect of glargine on intimal hyperplasia is due to its effect on IGF-1, the effect of glargine in animals lacking the IGF-1 receptor would prove useful. Unfortunately, attempts at creating an IGF-1 knockout animal have failed as this is essential for growth and lack of the receptor leads to failure to thrive. One possible approach would be to selectively knock out the IGF-1 receptor in the vasculature alone. A less definitive approach would be to infuse IGF-1, although this would only prove an IGF-1 effect in the vasculature – which we have previously demonstrated in another model of atherosclerosis [27]. It is important to recognize that plasma levels of IGF-1 and the effect of IGF-1 administration is the subject of controversy [38–42]. There is, however, consensus that a low plasma IFG-1 is associated with an increase in risk of vascular disease. A recent study has demonstrated that IGF-1 infusion decreased atherosclerotic plaque progression and macrophage infiltration into lesions in a mouse model of atherosclerosis [27]. Furthermore, IGF-1 decreased vascular expression of the proinflammatory cytokines interleukin-6 and tumor necrosis factor-alpha, reduced aortic superoxide formation and urinary 8-isoprostane levels, and increased aortic phosphor-Akt and eNOS expression and

circulating endothelial progenitor cells, consistent with an anti-inflammatory, antioxidant, beneficial effect on the vasculature. The present data support these findings and suggest that the glargine induced increase in circulating IGF-1 reduced vascular inflammatory responses, vascular oxidant stress and possibly thereby decreased intimal hyperplasia. Aortic 8-isoprostane levels were decreased significantly in the glargine and glulisine treated rats. Immunohistochemical staining for nitrotyrosine was also significantly lower in the glargine treated animals compared to control while that in glulisine treated rats was not significantly lowered. Additionally, we found a non-significant increase in the expression of eNOS in the aortic protein of rats treated with glargine (data not shown).

A potent stimulatory effect of IGF-1 on blood flow has been reported by earlier workers [43]. It is therefore possible that the effects of glargine on vascular function are similar to those of IGF-1. In a recent study, the effects of 3.5 years of addition of insulin glargine to metformin (former) therapy was determined on endothelial function in patients with type 2 diabetes. The data were compared with data from a group of normal subjects and show that insulin glargine had a beneficial effect [44].

After several decades of perpetuation of the myth that insulin may cause vascular disease (5–7), this controversy is being put to rest with important data from a variety of studies [45–46]. In a large number of managed care patients, insulin treatment was associated with a lower incidence of cardiovascular events. In the Diabetes Control and Complications Trial (DCCT), follow-up Epidemiology of the DIabetes Complications (EDIC) study after several years of glycemic control with multiple insulin injections and possibly higher insulin doses and levels, the intensive treatment group had less carotid intimal-thickness and less CV events on follow up. It is difficult to determine whether the benefits seen in the EDIC study relate to higher insulin use or better glycemic control. In this context, our study is important since in the animal model used, blood glucose was not elevated and there were no differences in blood glucose levels between the various groups of rats. An analysis of a large claims database determined that patients treated with insulin had a lower incidence of cardiovascular events [46].

The present data support other findings of a beneficial effect on the vasculature that may have implications for therapy. Recent guidelines recommend insulin use early in the natural history of the disease, and studies are needed to determine the effect of insulin treatment on the vasculature early in the process. Indeed, a clinical trial [9] is in progress studying the effect of insulin on cardiovascular events in patients with prediabetes and newly diagnosed diabetes. Also, insulin is known to increase the production of potent vasodilators including nitric oxide, and *in vitro* insulin has been shown to stimulate the upregulation of eNOS and NO in a receptor kinase mediated manner [10]. Further, clinical studies have shown the acute activation of endothelium-dependent vasodilation in subjects (with normal/healthy vasculature) by both regular human insulin and insulin glargine [11]. In contrast, in diabetic subjects and other subjects with vascular disease a consistent blunting of the endothelial dependent increase in blood flow in response to ACh has been demonstrated [12]. In addition, ACh-induced vasodilation in patients with type 2 diabetes has been shown to be enhanced during chronic insulin therapy [13–15]. These findings provide support for the use of insulin therapy for improving endothelial function in patients with diabetes. Importantly, an antiinflammatory effect of insulin was observed in obese insulin resistant patients with a low dose insulin infusion [47]. An anti-atherosclerotic and reactive oxygen species suppressing effect of insulin in Apo-E knock out mice is reported [48]. Further it has been proposed that insulin therapy may, however, reverse or ameliorate several consequences of insulin resistance such as diabetic dyslipidemia and endothelial dysfunction [49] without significant changes in the blood pressure. It has been reported increases in plasma insulin levels to high physiological concentrations do not increase blood pressure because increases

in cardiac output are offset by decreases in peripheral vascular resistance that can be attributed at least in part to vasodilation in skeletal muscle [50].

The time course of neointimal growth following balloon catheter injury in the rat carotid artery has been established and the effect of insulin treatment at the peak of intimal growth following injury has been investigated [51, 43]. The present results are in contrast with other studies that demonstrate increased intimal growth 14 days after balloon catheter injury in rats in whom diabetes was first induced by streptozocin and then corrected with transplantation of the pancreas. We believe that the time course of measurement after the injury may not have been optimal and furthermore, the animal model used was somewhat artificial [52]. Another study found increased neo-intimal thickness in insulin-treated rats. However, these rats were fed oral sucrose and fructose, which may have induced the intimal hyperplasia [53]. Indeed, it has been suggested that much of the intimal growth associated with vascular injury relates to insulin resistance [54]. Moreover it has been suggested the development of desperate forms of intimal hyperplasia mediated by a variety of mechanisms occurs resulting in the formation of different forms of intimal hyperplasia [25]. Our model is one of insulin resistance, which is associated with increased intimal hyperplasia, and therefore the benefit of insulin therapy is more likely to be seen. The rationale for using the female rats was to study the IR state and not have the confounding effect of high glucose. Male Zucker fatty rats are more prone to become diabetic much earlier (around 10 weeks) whereas the female rats are insulin resistant and become diabetic much later. The females develop the same obese phenotype as males [55].

The results of the present study lend support to Outcome Reduction with Initial Glargine Intervention (ORIGIN) a large clinical trial in which insulin glargine is administered in contrast to usual care in patients with newly diagnosed diabetes or impaired fasting glucose or impaired glucose tolerance [9]. Most of these patients are likely to be insulin resistant and have very mild hyperglycemia. We conclude that insulin has beneficial effects on the vasculature and that this benefit is greatest with insulin glargine, possibly mediated by its effects on increasing serum IGF-1, although definitive proof of this mechanism is currently lacking and difficult to obtain.

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Balloon catheter injured and untreated

Figure 1. Representative photomicrograph sections of balloon catheter injured H & E stained carotid artery from Zucker fatty rats. The intimal hyperplasia seen in the saline treated control rats (a) was reduced in the glulisine treated rats, but this was not significant (b). The hyperplasia was significantly reduced in the glargine treated rats (c).

Figure 2.

Bar graphs showing intimal hyperplasia, a normal response seen after balloon catheter injury in carotid artery in control rats treated with normal saline (0.9 ± 0.1). sc injections of glutamine 0.45 mg/kg/d in two divided doses reduced the hyperplasia but this was not significant compared to controls (0.6 ± 0.1 vs 0.9 ± 0.1). Treatment with glargine 0.45 mg/kg/d reduced the hyperplastic response and this was significantly different from control (0.4 ± 0.1 vs 0.9 ± 0.1 ; $p<0.05$).

Figure 3.

Bar graphs showing serum IGF-1 levels measured by ELISA using a kit from Cayman Chemicals, Ann Arbor, MI. The serum IGF-1 levels were higher in both groups of rats treated with insulin however the increase was only significant in the glargine treated rats [567 ± 121 (ng/ml)-control; 763 ± 161 (ng/ml)-glulisine; 1059 ± 150 (ng/ml)-glargine, $p < 0.05$].

Aortic 8- isoprostane in balloon injured rats

Figure 4.

The aortic arch from balloon catheter injured rats was dissected from the common carotid artery and protein from this source was used for measuring 8-isoprostane levels (marker of oxidative stress). When compared to controls, the levels were significantly reduced in both glargine as well as glutamine treated animals [(921 and 1116 pg/mg protein respectively vs. 2566 pg/mg protein in control; $p<0.05$).

N-tyrosine levels in carotid artery of insulin analog-treated rats

Figure 5.

The unstained section of carotid arteries from normal saline treated (control) and glulisine and glargine treated animals were evaluated for nitrotyrosine staining by immunohistochemical methods. The top panel shows representative sections and the quantitation is depicted in the bottom panel. When compared to controls, the staining intensity was lower in glulisine and glargine treated animals but the difference was significantly different in the glargine treated rats ($p<0.05$).

Table 1

Biochemical and histological parameters

	Pre Injury glucose mg/dL		Post Injury glucose mg/dL		Body Weights		I/M	IGF-1 (ng/ml)
	Fasting	Postprandial	Fasting	Postprandial	Initial	Final		
Control	89 ± 6	118 ± 7	94 ± 6	122 ± 11	349 ± 20	442 ± 27	0.9 ± 0.1	567 ± 121
Glulisine	91 ± 7	93 ± 8	84 ± 4	78 ± 5	373 ± 16	435 ± 8	0.6 ± 0.1	763 ± 161
Glargine	85 ± 6	77 ± 14	92 ± 8	88 ± 6	352 ± 8	444 ± 7.6	0.4 ± 0.1 ^a	1059 ± 150 ^a

^a p<0.05