Effects of Balloon Injury on Neointimal Hyperplasia in Streptozotocin-Induced Diabetes and in Hyperinsulinemic Nondiabetic Pancreatic Islet–Transplanted Rats

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Background—The mechanisms of increased neointimal hyperplasia after coronary interventions in diabetic patients are still unknown.

Methods and Results—Glucose and insulin effects on in vitro vascular smooth muscle cell (VSMC) proliferation and migration were assessed. The effect of balloon injury on neointimal hyperplasia was studied in streptozotocin-induced diabetic rats with or without adjunct insulin therapy. To study the effect of balloon injury in nondiabetic rats with hyperinsulinemia, pancreatic islets were transplanted under the kidney capsule in normal rats. Glucose did not increase VSMC proliferation and migration in vitro. In contrast, insulin induced a significant increase in VSMC proliferation and migration in cell cultures. Furthermore, in VSMC culture, insulin increased MAPK activation. A reduction in neointimal hyperplasia was consistently documented after vascular injury in hyperglycemic streptozotocin-induced diabetic rats. Insulin therapy significantly increased neointimal hyperplasia in these rats. This effect of hyperinsulinemia was totally abolished by transfection on the arterial wall of the N17H-*ras*–negative mutant gene. Finally, after experimental balloon angioplasty in hyperinsulinemic nondiabetic islet-transplanted rats, a significant increase in neointimal hyperplasia was observed.

Conclusions—In rats with streptozotocin-induced diabetes, balloon injury was not associated with an increase in neointimal formation. Exogenous insulin administration in diabetic rats and islet transplantation in nondiabetic rats increased both blood insulin levels and neointimal hyperplasia after balloon injury. Hyperinsulinemia through activation of the *ras/MAPK* pathway, rather than hyperglycemia per se, seems to be of crucial importance in determining the exaggerated neointimal hyperplasia after balloon angioplasty in diabetic animals. **(*Circulation*. 2001;103:2980-2986.)**

Key Words: diabetes mellitus ■ balloon ■ angioplasty ■ insulin

Diabetes mellitus is a poor prognostic factor for all patients with coronary artery disease and specifically for those who undergo CABG or balloon angioplasty.[1–3] It is also well recognized that restenosis rates may be very high, varying from 49% to 71% among diabetic patients.[1–5]

In addition, patients with diabetes mellitus and multivessel coronary disease were found to have a significantly higher mortality rate with PTCA than with CABG.[1–6]

Extensive data are not yet available on coronary stents in patients with diabetes, but the use of internal mammary arteries with bypass grafting seems to be preferred, especially in patients with multivessel disease.[7–9]

Serial intravascular ultrasound analysis showed that the main mechanism for increased restenosis in diabetic patients was an exaggerated intimal hyperplasia both in the presence and in the absence of stent deployment.[10] In addition, the molecular mechanisms underlying this exaggerated vascular smooth muscle cell (VSMC) proliferation and the relative role of hyperglycemia versus hyperinsulinemia in promoting a greater restenosis after angioplasty or stenting have not yet been investigated.

Therefore, the aims of the present study were to assess (1) the effects of glucose and insulin on VSMC proliferation and migration in vitro, (2) response to balloon injury in rats with streptozotocin (STZ)-induced diabetes with or without adjunct insulin therapy, and (3) neointimal hyperplasia after vascular injury in nondiabetic rats with hyperinsulinemia after pancreatic islet transplantation.

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Methods

VSMC Proliferation In Vitro

To assess the effects of glucose and insulin, 29.5×10^4 VSMCs (A10, thoracic rat aorta) were plated and grown in DMEM/10% FCS in the presence of low (5.6 mmol/L), medium (14 mmol/L), and high (22.2 mmol/L) glucose or in the presence of different insulin concentrations (2, 20, and 200 nmol/L) or in the absence of the same (control). To evaluate glucose osmotic effects on VSMC proliferation, we included an additional control with an osmotically active nonmetabolized agent, sorbitol, at the same glucose concentrations. Cell numbers in all conditions were assessed every 48 hours for 6 days.

Mitogen-Activated Protein Kinase Assay

To assess the effect of insulin on the activation of the *ras/MAPKK* pathway, VSMCs were starved in DMEM/0.5% FCS for 48 hours. Then they were stimulated by different concentrations of insulin (2, 20, and 200 nmol/L) for 10 minutes. After this time, VSMCs were processed for mitogen-activated protein kinase (MAPK) assay, as previously described.[11]

VSMC Migration Assay

VSMC migration was assayed by a modification of the Boyden's chamber method as previously described.[12] VSMCs (20×10^3) were placed in the upper chamber, and 25 μL of serum-free medium containing a migration factor such as *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or insulin or glucose was placed in the lower chamber. At the end of the assay period, VSMCs that had migrated to the lower side of the filter were counted under a microscope ($\times$100) to quantify VSMC migration. Migration activity was expressed as the mean number of cells that had migrated per high-power field.

Animal Model of Diabetes

Animals in this study were handled according to the animal welfare regulation of Federico II University of Naples, and the protocol was approved by the animal use committee of this institution in accordance with the animal use principles of the American Society of Physiology.

Fifty Wistar rats (Charles River, Calco, Italy) were treated at 14 weeks of age with a single intraperitoneal injection of 100 mg/kg STZ (Sigma) in 0.05 mol/L citrate buffer. This model resembles insulin-dependent diabetes mellitus (IDDM) as previously described.[13]

The diabetic state was assessed starting the day after STZ injection by measurements of nonfasting plasma glucose concentration with a portable glucose meter (One Touch II; Johnson & Johnson). Only animals with plasma glucose concentrations >300 mg/dL were considered diabetics and were included in the present study.

Rat Islet Isolation and Transplantation

To obtain a rat model of hyperinsulinemia without diabetes, in 14 Fisher rats (Charles River, Calco, Italy) at 10 weeks after birth, pancreatic islets were transplanted under the kidney capsule by 1 investigator (A.D.) as previously described.[14]

Experimental Angioplasty Technique

Angioplasty of the carotid artery was performed as previously described and validated in our laboratory.[15–18]

Study Design

Protocol I

The animals with STZ-induced diabetes were randomized before balloon injury into 2 groups: STZ group (n=11) and STZ+INS group (n=11, with insulin therapy).

Protocol II

Balloon injury was performed at 14 weeks after birth in hyperinsulinemic nondiabetic rats after islet transplantation (Transp Hyperins group).

Protocol III

In 12 rats with STZ-induced diabetes treated with insulin, we evaluated the effects of the in vivo inhibition of cellular *ras* using a negative mutant gene (N17H-*ras*) at the moment of the balloon injury (STZ/INS/*ras* group) as previously described.[16]

Insulin Administration

In the STZ+INS and in STZ/INS/*ras* groups, the day after STZ injection and for the following 14 days, insulin (Monotard, Novo Nordisk) was administered twice a day, from 4 to 8 IU according to plasma glucose level determined every day by use of the One Touch system. Balloon injury was performed 48 hours after STZ in the STZ+INS and STZ/INS/*ras* groups; thus, insulin was administered for a total of 16 days in both groups.

Blood Glucose and Insulin Levels

Blood samples from the tail vein were taken at the time of balloon injury and when carotid arteries were removed. These samples were analyzed for levels of glycemia and insulinemia. Plasma glucose was determined with the One Touch system. Insulin concentration was determined with a double-antibody radioimmunoassay using guinea pig anti-rat insulin serum, a rat insulin standard, and ^{125}I-labeled insulin (Linco Research).

Statistical Analysis

All data are shown as mean±SEM. Statistical analysis between groups was performed by 1-way ANOVA with an SPSS 10.0 program. When a significant overall effect was detected, Tukey's test was applied to compare single mean values. A value of $P<0.05$ was considered significant.

Results

Effects of Glucose and Insulin on VSMC Proliferation In Vitro

Figure 1a shows that high glucose concentration markedly inhibited VSMC proliferation. We observed similar results in an additional control with an osmotically active nonmetabolized agent, sorbitol, at the same glucose concentrations (Figure 1b). In contrast, insulin increased VSMC proliferation in a dose-dependent manner (Figure 2a).

Effects of Insulin on the Activation of the *ras/MAPK* Pathway

Insulin increased *ras/MAPK* activation, as demonstrated by the increase in extracellular signal–regulated kinase (ERK) activation at all insulin concentrations (Figure 2b). Only at nonphysiological concentration did insulin weakly activate c-Jun NH2-terminal kinase (JNK) and produce an evident increase of P38 activation (Figure 2b). These data suggest that ERK downstream *ras* is the principal intracellular target of the action of insulin on VSMCs.

Effects of Glucose and Insulin on VSMC Migration In Vitro

Figure 3 shows that insulin significantly increased VSMC migration in a dose-dependent manner (Figure 3a). In contrast, cultured VSMCs were unaffected by glucose (Figure 3b).

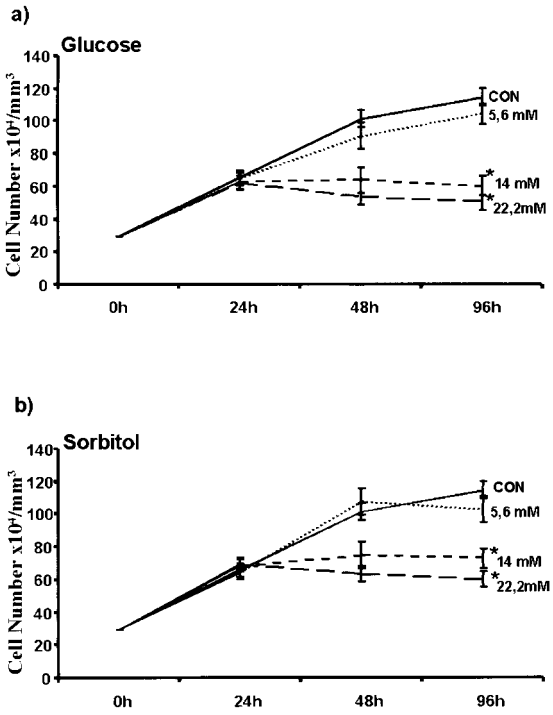

Figure 1. a, Effects of low (5.6 mmol/L), medium (14 mmol/L), and high (22.2 mmol/L) glucose concentrations on VSMC growth. Glucose at different concentrations (except for low) induced evident inhibition of VSMC proliferation vs control. Proliferation activities are mean±SD of 10 measurements. **P*<0.001 vs CON. b, Effects of sorbitol at concentrations of 5.6, 14, and 22.2 mmol/L on VSMC growth. Sorbitol induced evident inhibition of VSMC proliferation vs control. Proliferation activities are mean±SD of 10 measurements. **P*<0.001 vs CON.

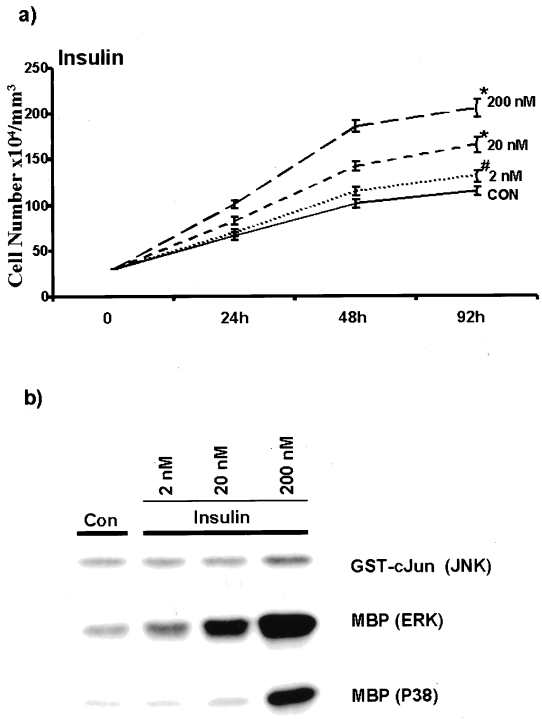

Figure 2. a, Effects of increased concentrations of insulin (2, 20, 200 nmol/L) on VSMC growth. Insulin induced evident dose-dependent increase of VSMC proliferation vs control. Effect is significant even at low concentration of 2 nmol/L. Proliferation activities are mean±SD of 10 measurements. #*P*<0.05 vs CON; **P*<0.001 vs CON. b, MAPK assays showing effect of insulin on MAPK activation. Insulin increased MAPK activation, as demonstrated by increase in ERK activation, at all concentrations. Only at highest concentrations did insulin weakly activate JNK and produce evident increase on P38 activation.

Effects of Vascular Injury on Neointimal Hyperplasia in Rats With STZ-Induced Diabetes (Protocol I)

After STZ injection, a 30% mortality was observed. A reproducible neointimal formation was found 14 days after balloon injury in the control group (CON group, n=12; neointima 0.113±0.026 mm^2; neointima/media ratio 1.123±0.229) (Figures 4 and 5). Surprisingly, in animals treated with STZ (STZ group, n=9), we observed a significant reduction of both neointimal area and neointima/media ratio (neointima 0.070±0.029 mm^2, *P*<0.001 versus CON; neointima/media ratio 0.508±0.201, *P*<0.001 versus CON) (Figures 4 and 5). After balloon injury, we had a 20% mortality in rats of the control group and in rats with STZ-induced diabetes.

Effect of Vascular Injury on Neointimal Hyperplasia in STZ-Induced Diabetic Rats With Insulin Therapy (Protocol I)

After balloon injury in animals with insulin-treated STZ-induced diabetes (STZ+INS group, n=9), we observed a significant increase of both neointimal area and neointima/media ratio (neointima 0.217±0.036 mm^2, *P*<0.001 versus CON; neointima/media ratio 1.722±0.364, *P*<0.001 versus CON) (Figures 4 and 5).

Effect of Islet Transplantation on Neointimal Hyperplasia After Vascular Injury (Protocol II)

In nondiabetic animals with hyperinsulinemia after islet transplantation (Transp Hyperins, n=5), we detected a significant increase of both neointimal area and neointima/media ratio after balloon injury (neointima 0.147±0.009 mm^2, *P*<0.05 versus CON; neointima/media ratio 1.545±0.082, *P*<0.001 versus CON) (Figures 4 and 5). In these animals, a 30% mortality after balloon injury was observed.

Effect of *ras* Inhibition on Neointimal Hyperplasia After Vascular Injury in Rats With STZ-Induced Diabetes (Protocol III)

In an additional 12 rats with STZ-induced diabetes under insulin therapy, the transfection on the vascular wall of a *ras*-negative mutant gene (N17H-*ras*) at the moment of the balloon injury (STZ/INS/*ras* group, n=9) caused a significant reduction of both neointimal area and neointima/media ratio compared with both the CON and the STZ+INS groups (neointima 0.069±0.018 mm^2, *P*<0.001 versus CON and STZ+INS; neointima/media ratio 0.539±0.114, *P*<0.001 versus CON and STZ+INS) (Figures 4 and 5).

Blood Glucose and Insulin Levels

Control rats (CON group) had a glycemia of 144±19.3 mg/dL and an insulinemia of 67.2±8.6 μU/mL (Figure 6).

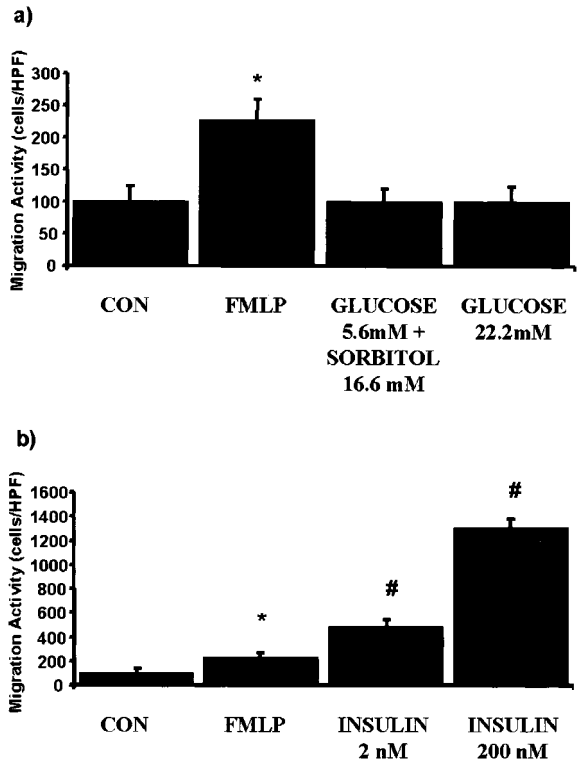

Figure 3. a, Effects of glucose on VSMC migration vs FMLP. Neither low nor high glucose concentrations affected VSMC migration vs control and FMLP. Migration activities are expressed as number of cells per high-power field (HPF) and are mean±SD of 10 measurements. *$P<0.01$ vs all. b, Effects of insulin (2, 200 nmol/L) on VSMC migration vs FMLP. Insulin increased VSMC migration vs control and FMLP. Effect is significant even at low concentration of 2 nmol/L. Migration activities are expressed as number of cells per HPF and are mean±SD of 10 measurements. *$P<0.01$ vs CON; #$P<0.001$ vs CON, FMLP.

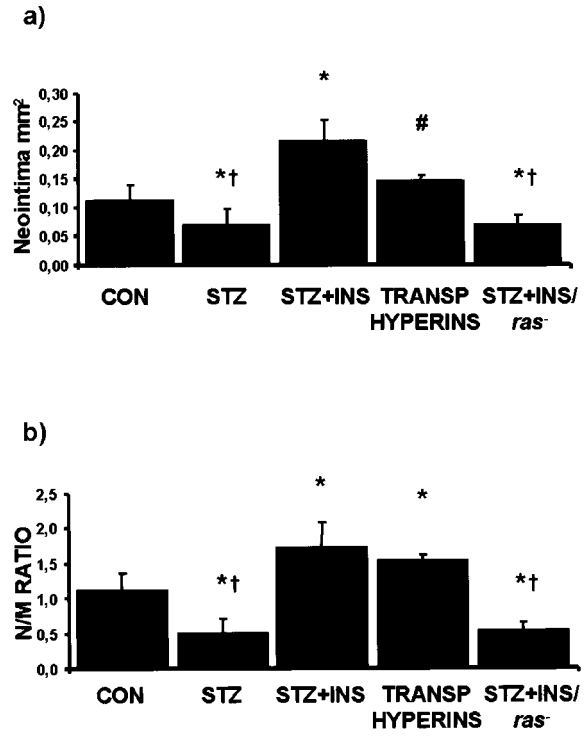

Figure 4. a, Bars represent neointimal cross-sectional area of common carotid arteries after balloon injury from rats subjected to only balloon injury (CON), rats treated with STZ), insulin-treated STZ-induced diabetic rats (STZ+INS), hyperinsulinemic nondiabetic islet-transplanted rats (Transp Hyperins), and insulin-treated rats with STZ-induced diabetes with transfection of *ras*-negative mutant gene (STZ+INS/*ras*⁻). *$P<0.001$ vs CON; #$P<0.05$ vs CON; †$P<0.001$ vs STZ+INS and Transp Hyperins. b, Bars represent neointima/media ratio of common carotid arteries after balloon injury from each group of animals studied. *$P<0.001$ vs CON; †$P<0.001$ vs STZ+INS and Transp Hyperins.

STZ-induced diabetic rats without insulin therapy showed a significant increase of plasma glucose levels (STZ group, glycemia 428.3±47.6 mg/dL, $P<0.001$ versus CON) and a significant reduction of insulin blood levels (STZ group, insulinemia 36.8±3.7 μU/mL, $P<0.001$ versus CON) (Figure 6). Insulin therapy reduced glycemia in the STZ+INS group (glycemia 266.7±18.2 mg/dL, $P<0.001$ versus STZ), with a significant increase in insulin levels (insulinemia 136.8±9.1 μU/mL, $P<0.01$ versus CON) (Figure 6). Furthermore, rats with transplants had a slight but significant reduction of glycemia (Transp Hyperins group, glycemia 98.2±5 mg/dL, $P<0.05$ versus CON) and a significant increase in blood insulin levels (Transp Hyperins group, insulinemia 133.1±3.6 μU/mL, $P<0.001$ versus CON) (Figure 6). Finally, also in the STZ/INS/*ras* group, insulin therapy reduced glycemia (glycemia 231.0±16.2 mg/dL, $P<0.001$ versus STZ), with a significant increase in insulin level (insulinemia 138.0±14.6 μU/mL, $P<0.01$ versus CON) (Figure 6).

Discussion

The major findings of the present study are that (1) hyperglycemia per se does not seem to be sufficient to induce an increased neointimal formation after balloon injury, which in hyperglycemic STZ-induced diabetic rats actually decreased; and (2) hyperinsulinemia plays a major role in determining the exaggerated neointimal hyperplasia after vascular injury in rats; ERK downstream *ras* is the principal intracellular target of the insulin actions on VSMCs.

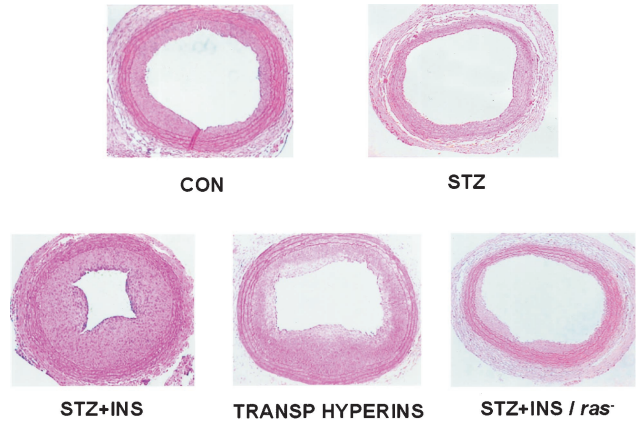

Figure 5. Representative hematoxylin-eosin–stained histological sections of common carotid arteries after balloon injury from each group of animals studied (abbreviations as in Figure 4).

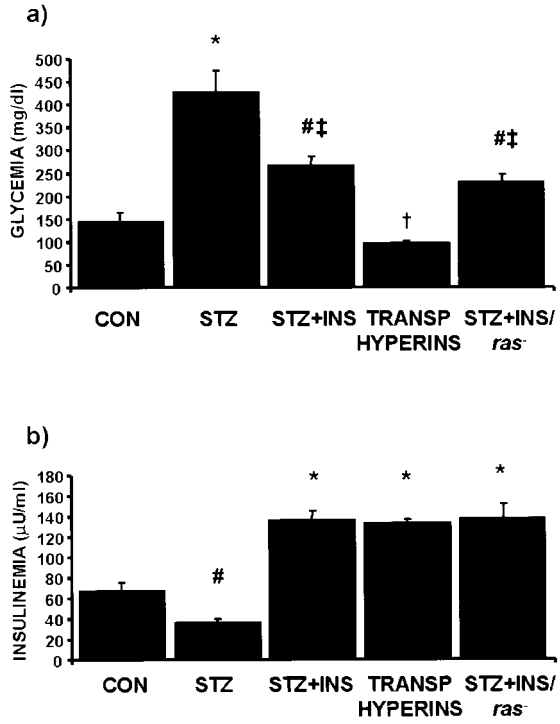

Figure 6. a, Bars represent plasma glucose levels from each group of animals studied. Abbreviations as in Figure 4. *$P<0.001$ vs CON; #$P<0.01$ vs STZ; †$P<0.05$ vs CON; ‡$P<0.01$ vs CON. b, Bars represent insulin levels from each group of animals studied. *$P<0.001$ vs CON; #$P<0.001$ vs all.

Role of Hyperglycemia on VSMCs In Vitro and In Vivo

Although hyperglycemia may increase the expression of several growth factors, such as platelet-derived growth factor, and their effects on VSMC growth,[12,19] our data demonstrated that high glucose concentrations alone did not affect VSMC migration and decreased VSMC proliferation in vitro (the 2 main mechanisms responsible for neointima formation after balloon injury). Glucose effects were entirely nonspecific to glucose metabolism as an inhibition of cell migration, and proliferation was also obtained with an osmotically active nonmetabolized agent, sorbitol. Therefore, we can simply speculate that glucose effects on VSMC growth in vitro seem to be related to an osmotic action.

Our in vivo data consistently demonstrated a significant reduction in neointimal thickness after experimental angioplasty in hyperglycemic rats with STZ-induced diabetes, although the glucose levels were ≈400 to 500 mg/dL. Recent data have also shown that in rabbits with alloxan-induced diabetes, high glucose levels were associated with reduced neointimal hyperplasia after balloon injury.[20] This effect was probably due to improved endothelial cell regrowth.[20]

Role of Hyperinsulinemia on VSMCs In Vitro and on Neointimal Hyperplasia After Balloon Injury In Vivo

High circulating insulin levels were associated with an increased risk for coronary artery diseases.[21,22] It has also been hypothesized, but never proved, that hyperinsulinemia in non–insulin-dependent diabetes mellitus (NIDDM) exerts a detrimental effect on injured vessels and underlies the high mortality in diabetic patients[23] after percutaneous transluminal coronary angioplasty. Likewise, hyperinsulinemia in nondiabetic patients seems to be a good predictor of restenosis after coronary stenting.[24] Furthermore, it has also been suggested that increased levels of plasminogen activator inhibitor type 1 secondary to high insulin levels are likely to mediate the adverse effects of insulin on vessel wall.[25] The BARI trial showed an increased mortality only in patients who were treated with oral hypoglycemic agents or insulin.[1] These observations imply that higher insulin levels in these patients underlie the adverse outcome after angioplasty.[1] Moreover, atherosclerosis-like lesions were documented when long-term insulin injections were performed in rats.[26] In addition, the intensive treatment of diabetes with insulin was not associated with a reduction in the occurrence of coronary artery disease in either the DCCT trial[27] or the UKPDS Study.[28]

In many NIDDM patients, insulin levels are higher than those in nondiabetic subjects.[23] In the presence of insulin resistance, when the metabolic action of insulin is ineffective, the proliferative response to hyperinsulinemia may still occur.[23]

In IDDM, insulin therapy is delivered in nonphysiological ways with respect to route and control of delivery. Thus, in many patients with IDDM, tissues may be exposed to higher levels of insulin than in those without diabetes.

VSMC migration and proliferation play a major role in the pathogenesis of restenosis in diabetic patients. Our data demonstrated that insulin increases VSMC migration and proliferation. The primary physiological substrate of the insulin receptor tyrosine kinase is a protein called insulin receptor substrate-1 (IRS-1). IRS-1 is associated with the adaptor protein GRB-2, which links IRS-1 to the *ras-raf-MAPK* cascade, which is the central pathway for VSMC growth. Insulin increases the amount of farnesylated p21*ras* in VSMCs.[29] Furthermore, recent data demonstrated increased amounts of farnesylated p21*ras* in tissues of hyperinsulinemic animals.[29] Finally, our laboratory previously demonstrated that the inhibition of *ras* or *MAPK* genes prevented neointimal formation after balloon angioplasty in rats.[16,18,30,31]

The role of hyperinsulinemia in promoting restenosis, however, is still a controversial issue.[32–34] The diabetic state affects different stages of the restenotic process.[32] In fact, it has been suggested that many of the potential mechanisms promoting restenosis in diabetic patients correlate with hyperglycemia rather than hyperinsulinemia.[32,33]

A recent study demonstrated an association between MAPK activity and VSMC proliferation after vascular injury in diabetic rabbits independent of insulin-like growth factor-1.[35] In agreement with these data, we have demonstrated that insulin increased MAPK activation; furthermore, we have also shown that ERK downstream *ras* is the principal intracellular target of the insulin actions on VSMCs. Nevertheless, it should be pointed out that the insulin concentrations used in our study were clearly much higher than those encountered in vivo, but this is not surprising, because such high concentra-

tions are required in view of the known differences between rodents and humans.

Our in vivo data demonstrated that insulin therapy in diabetic rats reduced plasma glucose levels at the expense of a significant increase in insulin blood levels. This increase in insulin blood levels was associated with the exaggerated neointimal hyperplasia observed after balloon injury.

To increase the insulin levels in nondiabetic rats and to obtain a model of hyperinsulinemia as the only risk factor for exaggerated neointimal hyperplasia, we performed an additional protocol in which pancreatic islets were transplanted in normal nondiabetic animals under the kidney capsule.[14] In this group, we observed an increase of insulin blood levels similar to that in STZ-induced diabetic rats with insulin therapy. In these animals, we also observed an exaggerated neointimal formation after experimental balloon angioplasty.

Finally, in hyperinsulinemic STZ-induced diabetic rats, the transfection of a *ras*-negative mutant gene reduced neointimal hyperplasia after balloon injury.

Taken together, these data further support the hypothesis that hyperinsulinemia, by increasing *ras/MAPK* pathway activation, is the main mechanism responsible for the greater rate of restenosis observed in diabetic patients.

Animal Models and Limitations of the Study

STZ administered to mature rats induces severe and permanent diabetes, with a decrease in insulin levels, to produce a cytotoxic model of diabetes very similar to IDDM.[13]

To further rule out a possible acute effect of STZ per se on VSMC proliferation in vivo, we also performed experiments in rats that had been rendered diabetic by STZ in the neonatal period (3 days after birth). In this same group of hyperglycemic and insulinopenic animals, the injury was performed at 14 weeks after birth, and we observed the same effects of balloon injury on neointimal hyperplasia as in the adult model of STZ-induced diabetes (data not shown).

Clinical adverse experience in subjects with diabetes undergoing coronary interventions is encountered predominantly in patients with type 2 diabetes because of the much higher prevalence of type 2 (accounting for >90% of diabetic patients) than type 1 diabetes. For this reason, caution should be used in extrapolating the present results in a clinical setting. Because of the greater duration of the disease, however, patients with IDDM risk higher cardiovascular morbidity and mortality than those with NIDDM. Furthermore, because of their insulin-requiring state, IDDM patients are exposed for a longer period to the potentially detrimental effects of insulin.

To obtain a rat model of hyperinsulinemia without diabetes, we used a standard technique to transplant pancreatic islets under the kidney capsule in nondiabetic rats.[14] This allowed us to have hyperinsulinemia as a single potential factor promoting neointimal hyperplasia after balloon angioplasty.

Finally, in the present study, the blood samples to assess glucose and insulin levels were obtained in anesthetized animals. It is likely that slightly different results could be obtained in conscious animals.

Clinical Relevance of the Study

Our experimental data support the hypothesis that rigorous glycemic control alone may not be sufficient to reduce the rate of restenosis in diabetic patients. Rather, hyperinsulinemia is responsible for the exaggerated neointimal proliferation observed in our animal models and could explain the increased restenosis rate observed after interventional procedures in diabetic patients. Therefore, it should be pointed out that in diabetic patients who require insulin therapy, good control of blood glucose should be obtained with the lowest possible levels of circulating insulin.

Further clinical trials are needed, however, to evaluate the impact of both hyperinsulinemia and tight glycemic control on the rate of restenosis after balloon angioplasty and coronary stenting in diabetic patients.

Acknowledgment

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