In Vivo Effect of Insulin on the Acute Proliferative Response of the Rat Aorta to Injury

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The objective of our study was to investigate the effect of hyperinsulinemia associated with either euglycemia, hypoglycemia, or hyperglycemia on the short-term mitotic activity of arterial smooth muscle cells (SMCs) after aortic injury. The proliferative reaction of arterial SMCs was provoked by the passage of an embolectomy catheter with a tightly inflated balloon. DNA synthesis was measured as DNA specific activity after incubation of the aorta in a medium containing $[^3]$H]thymidine. Unrestrained rats were rendered hyperinsulinemic (4-7 nM versus 0.3 nM in controls) immediately after aortic injury by insulin infusion (10 units/day) and either euglycemic (about 5.6 mM), hyperglycemic (14-17 mM), or hypoglycemic (about 2.8 mM) by adjusting the flow rate of hypertonic glucose (30% wt/vol) that was infused simultaneously. The infusion was performed via a catheter inserted into the jugular vein and lasted 2 or 4 days after the aortic injury. After the injury, SMC mitotic activity was dramatically increased on day 2 in control rats with deendothelialized aortas and declined between days 2 and 4. In euglycemic-hyperinsulinemic and hyperglycemic-hyperinsulinemic rats, SMC proliferation showed the same pattern as in controls. At no time was a significant difference observed among the three groups of rats. In hypoglycemic-hyperinsulinemic rats, SMC mitotic activity increased to a lesser extent than in controls on day 2 after deendothelialization. These data indicate that hyperinsulinemia with or without hyperglycemia does not stimulate the early stages of arterial SMC proliferation in the rat although a long-lasting effect of hyperinsulinemia cannot be excluded. Hypoglycemia, even in the presence of hyperinsulinemia, partially inhibits the proliferation of arterial SMCs induced by arterial injury. (Arteriosclerosis and Thrombosis 1992;12:633-638)

KEY WORDS • hyperinsulinemia • hyperglycemia • euglycemia • hypoglycemia • smooth muscle cells • proliferation

It is well established that in diabetes mellitus, the prevalence and the incidence of macroangiopathy are increased (for review, see Reference 1). Factors that predispose diabetic patients to macroangiopathy are far from being fully understood. However, several epidemiological studies suggest that hyperinsulinemia may be involved in atherogenesis (for review, see Reference 2). On the other hand, on the basis of in vitro observations, it has been found that insulin can stimulate smooth muscle cell (SMC) proliferation, one of the most important initial steps of atherogenesis. However, direct evidence of the growth-promoting effect of insulin on SMCs in vivo is still lacking.

Using an animal model of arterial injury with a balloon catheter (deendothelialization), it has previously been shown that injection of insulin into diabetic rats increased the proliferative response of SMCs to injury compared with nondiabetic rats. However, insulin treatment of control rats resulted in severe hypoglycemia, and in this case the mitotic response of SMCs to injury was depressed, thus suggesting that blood glucose concentrations might modulate the possible effects of insulin on SMC proliferation.

In an attempt to study in vivo the role of insulin under precise conditions, we investigated the effect of hyperinsulinemia associated with euglycemia on the acute SMC proliferative response to arterial injury. In addition, to clarify the relations between insulinemia and the variations of glycemia, we studied the effects of hyperinsulinemia associated with either hyperglycemia or hypoglycemia on the SMC proliferative response to deendothelialization.

After being subjected to aortic injury, rats were rendered hyperinsulinemic (Hi) and either euglycemic (Eu), hyperglycemic, (Her), or hypoglycemic (Ho) by simultaneous infusion of insulin and glucose. SMC proliferation was evaluated on days 2 and 4 of the infusion period.

Methods

Animals

Male Wistar rats weighing approximately 300 g were bred in the laboratory and fed ad libitum with a
standard diet (Usine d’Alimentation Rationnelle 113, Villemoisson sur Orge, France). The flexible technique for long-term infusion in unrestrained rats was used for simultaneous infusion of glucose and insulin. Deendothelialization of the thoracic aorta followed by catheterization of the jugular vein was performed under ketamine hydrochloride anesthesia (Imalgene, Merieux, France; 125 mg/kg i.p.).

Two hours after surgery, the rats were housed individually in cylindrical Plexiglas cages and were permanently connected to a peristaltic pump (Ismatec) delivering glucose and to a driving pump (Perfusor VI, Roucaire, Velizy, France) delivering insulin. A solution of insulin (Actrapid, Novo, Copenhagen, Denmark) at 37°C for 1 hour. The incubation medium was then replaced by a medium containing 20 \( \mu \)l/min (~7 g/day) to produce hyperinsulinemia. Hyperensive (30% wt/vol) sterile glucose (Chaix et Dumarais, France) was infused at an initial rate of 12 \( \mu \)l/min (~5 g/day), 17 \( \mu \)l/min (~7 g/day), or 35 \( \mu \)l/min (~14 g/day) to produce a hyperinsulinemic-hypoglycemic (Hi-Ho), a hyperinsulinemic-euglycemic (Hi-Eu), or a hyperinsulinemic-hyperglycemic (Hi-Her) state, respectively. Hypoglycemia (~2.8 mM), euglycemia (~5.6 mM), and hyperglycemia (14–17 mM) were maintained by adjusting the flow rate of glucose throughout the experiment. Control rats were deendothelialized and catheterized but not infused. Blood glucose levels of the rats were measured five times daily throughout the infusion period on blood samples collected from the tail vein. Plasma glucose concentration was immediately determined on a 10-\( \mu \)l aliquot, and the remaining plasma was kept at ~20°C until radioimmunoassay of insulin. The infusion period lasted 2 or 4 days for each group, except for the Ho-Hi rats, which were studied only until day 2.

**Aortic Injury**

Aortic injury was performed using a balloon embolectomy catheter according to the method of Baumgartner and Studer. As described previously, the passage of an embolectomy catheter with a tightly inflated balloon in the thoracic aorta of the rat completely removes the endothelium and provokes a stereotypical proliferative reaction of arterial SMCs that reaches its peak 2 days after formation of the lesion.

**Incubation and Preparation of the Thoracic Aorta**

The incubation, preparation, and chemical analysis of the thoracic aorta were performed as previously described. Briefly, the aortic segment from the left subclavian to the celiac arteries was incubated with Krebs-Henselet bicarbonate buffer (composition in mM: NaCl 120, KCl 4.8, CaCl$_2$ 1.8, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, and NaHCO$_3$ 25) with 5 mM glucose and 10 g/1 bovine serum albumin (BSA, Cohn fraction V; Sigma Chemical Co., St. Louis, Mo.) at 37°C for 1 hour. The medium was then replaced by a medium containing 20 \( \mu \)l \([^{3}H]\)thymidine (25 Ci/mmol; CEA, Gif-sur-Yvette, France) diluted in normal saline (0.2 mCi/ml); the final dilution of \([^{3}H]\)thymidine in the incubation medium was 2 \( \mu \)Ci/ml. After a 1-hour incubation, the incubation medium was replaced by 2 ml fresh buffer and the aorta was postincubated for an additional 1 hour.

After the adventitia was discarded, the DNA content and radioactivity of each intima-media sample were determined according to Goldberg et al, as previously described.

**Analytical Procedures**

Plasma glucose was determined by the glucose oxidase technique using a glucose analyzer (Beckman, Palo Alto, Calif.). Plasma immunoreactive insulin was estimated using a radioimmunoassay kit (CEA). The lower limit of the assay was 15 pM with an interassay and intra-assay coefficient of variation of 6%. The DNA content of the intima-media was measured according to the technique of Burton. DNA synthesis, as reflected by thymidine incorporation, was expressed in counts per minute per microgram of DNA.

**Statistics**

Results are expressed as mean±SEM. Statistical significance for plasma glucose and plasma insulin concentrations were analyzed by a repeated-measures analysis of variance. Statistical analysis for the DNA specific activity of the intima-media was performed with the Mann-Whitney U test.

For statistical analysis concerning specific activity, each group of Hi rats was compared with its corresponding control group. For blood parameters, Hi groups were compared with a “pooled control group,” i.e., all the values of the different control groups were pooled, and the mean was calculated and compared with that of the different groups of Hi rats.

**Results**

**Time Course of Plasma Glucose and Insulin Concentrations**

In control rats plasma glucose remained rather stable throughout the experiment (Figures 1 and 2). Plasma glucose concentrations in Ho-Hi rats (studied only until day 2) were decreased compared with controls and became significantly different from day 1 of the infusion. Except at the beginning of the infusion, plasma glucose concentrations were roughly similar in Eu-Hi rats and in control rats. In Her-Hi rats plasma glucose increased very sharply on day 0 and remained significantly much higher than in control, Eu-Hi rats, and Ho-Hi rats. As early as 2 hours after the beginning of the infusion, insulinemia was significantly increased in all insulin-infused rats compared with control rats, and it remained fairly stable throughout the infusion period (Figures 3 and 4).

**DNA Synthesis by the Intima-Media**

In control rats, DNA synthesis, as reflected by the incorporation of thymidine into the intima-media, followed its usual pattern: intima-media DNA synthesis was dramatically increased on day 2 after the injury compared with DNA synthesis in nondeendothelialized control rats and then began to decrease on day 4 (Figure 5).

On day 2 intima-media DNA synthesis was significantly lower in Ho-Hi rats (1,360±208 cpm/\( \mu \)g DNA) than in control rats (2,174±253 cpm/\( \mu \)g DNA).

In Eu-Hi rats as well as in Her-Hi rats, intima-media DNA synthesis showed the same pattern as in control rats: it increased on day 2 and declined from day 2 to day 4. At no time was a significant difference...
observed among Eu-Hi, Her-Hi, and control rats. The intima-media DNA content was similar in all deendothelialized groups on days 2 and 4 of the infusion period (data not shown).

Discussion

Our experimental approach was designed to study under precise conditions the in vivo effect of insulin per se on the early proliferative response to deendothelialization and to clarify the relations between plasma insulin and glucose concentrations on this process. Simultaneous infusion of insulin (10 units/day) and glucose at a flow rate of 17 μl/min enabled us to produce marked hyperinsulinemia and to maintain euglycemia throughout the infusion period. Using deendothelialization to provoke SMC proliferation, it was previously shown that DNA synthesis was maximal 2 days after the injury and decreased thereafter to stabilize at levels that were close to the low basal activity after 14 days. Therefore, to test the effects of hyperinsulinemia on the early steps of SMC proliferation, insulin infusion was induced immediately after injury and was maintained for 2 or 4 days after formation of the lesion.

In control rats the kinetics of aortic SMCs followed its usual pattern, peaking on day 2 and declining on day 4 after the injury; in Hi-Eu rats, a similar evolution was
observed without a significant difference on day 2 as well as on day 4 compared with control rats. Therefore, elevated hyperinsulinemia even in the presence of euglycemia had no influence on the acute mitotic response of SMCs to deendothelialization.

The mitogenic effect of insulin on arterial SMCs has been demonstrated in vitro with aortic cells of primate,\textsuperscript{15} rat,\textsuperscript{3,15} and human\textsuperscript{17} origin. However, these data remain controversial. Indeed, although a small mitogenic effect has been demonstrated at physiological concentrations of insulin by some authors,\textsuperscript{15,17} it was, as a general rule, maximal at supraphysiological concentrations. In other studies,\textsuperscript{18,19} even high concentrations of insulin had no mitogenic effect. Interest has recently been focused on the involvement of insulin-like growth factor (IGF-1) on the regulation of SMC growth. Based on cell culture studies, it is perhaps more likely that IGF-1 rather than insulin would influence SMC proliferation.\textsuperscript{20,21} In particular, in vitro studies indicate that SMCs have substantially more high-affinity binding sites.

FIGURE 3. Line plot showing evolution of plasma insulin concentrations in control (○—○), euglycemic-hyperinsulinemic (●—●), hypoglycemic-hyperinsulinemic (○—○), and hyperglycemic-hyperinsulinemic (●—●) rats during 2 days of simultaneous glucose and insulin infusion. Values are mean±SEM of 30 rats in the control group and 9–11 rats in hyperinsulinemic groups. *Significantly different from control rats at p=0.0001. I.R.I., immunoreactive insulin.

FIGURE 4. Line plot showing evolution of plasma insulin concentrations in control (○—○), euglycemic-hyperinsulinemic (●—●), and hyperglycemic-hyperinsulinemic (●—●) rats during 4 days of simultaneous glucose and insulin infusion. Values are mean±SEM of 30 rats in the control group and 8–10 rats in hyperinsulinemic groups. *Significantly different from control rats at p=0.0001. I.R.I., immunoreactive insulin.
for IGF-1 than for insulin. Moreover, it has been suggested that insulin and IGF-1 have no additive effects on SMC growth when added at maximal concentrations, suggesting that they are probably acting via the same receptor, i.e., the IGF-1 receptor. On the other hand, it has recently been shown that insulin enhances mRNA IGFI-1 expression in the rat aorta.

On the basis of our in vivo study, insulin per se is unlikely to promote the early steps of SMC proliferation after endothelial injury. However, variations in blood glucose concentrations might act together with insulin to modulate SMC proliferation.

On one hand, it has previously been observed that the mitotic reaction of SMCs was increased in experimental diabetic rats treated with high-dose insulin compared with untreated diabetic rats. On the other, injection of insulin into nondiabetic rats that resulted in sustained hypoglycemia strongly decreased DNA synthesis. This suggests that insulin may have long-term effects on this process or favor atherogenesis via other metabolic effects. A recent study indicates that atherosclerosis-like lesions could be induced by long-term insulin injection. In humans the role of prolonged hyperinsulinemia in atherosclerosis remains controversial. Although some suggestive data are now available, a link between hyperinsulinemia and macrovascular disease has not been clearly demonstrated (for review, see Reference 1).

In conclusion, the acute mitotic response of SMCs to arterial injury is not influenced by insulin in vivo in the nondiabetic rat. However, we cannot exclude the possibility that insulin may have long-term effects on this process or favor atherogenesis via other metabolic effects. A recent study indicates that atherosclerosis-like lesions could be induced by long-term insulin injection. In humans the role of prolonged hyperinsulinemia in atherosclerosis remains controversial. Although some suggestive data are now available, a link between hyperinsulinemia and macrovascular disease has not been clearly demonstrated (for review, see Reference 1).

Another main finding of our study is the lower SMC proliferation in HO-Hi rats, in agreement with previous studies. Therefore, taken together these data suggest that the early steps of SMC proliferation are depressed when energetic fuels, especially glucose, are not available, thus underlining the possible important role played by metabolic factors, especially energy balance, in the proliferative response to endothelial injury.

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