Insulin Increases Reendothelialization and Inhibits Cell Migration and Neointimal Growth After Arterial Injury

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Objective—Insulin has both growth-promoting and protective vascular effects in vitro, however the predominant effect in vivo is unclear. We investigated the effects of insulin in vivo on neointimal growth after arterial injury.

Methods and Results—Rats were given subcutaneous control (C) or insulin implants (3U/d;I) 3 days before arterial (carotid or aortic) balloon catheter injury. Normoglycemia was maintained by oral glucose and, after surgery, by intraperitoneal glucose infusion (saline in C). Insulin decreased intimal area (P<0.01) but did not change intimal cell proliferation or apoptosis. However, insulin inhibited cell migration into the intima (P<0.01) and increased expression of smooth muscle cell (SMC) differentiation markers (P<0.05). Insulin also increased reendothelialization (P<0.01) and the number of circulating progenitor cells (P<0.05).

Conclusions—These results are the first demonstration that insulin has a protective effect on both SMC and endothelium in vivo, resulting in inhibition of neointimal growth after vessel injury. (Arterioscler Thromb Vasc Biol. 2009;29:1060-1066.)

Key Words: insulin ■ vascular smooth muscle cell ■ migration ■ neointima ■ angioplasty ■ reendothelialization

Insulin resistance, defined as impairment of the metabolic actions of insulin, is characteristic of the metabolic syndrome, which is present in 25% of the population, is associated with obesity, and often precedes the onset of type 2 diabetes. Both type 2 diabetes and metabolic syndrome are risk factors for cardiovascular disease (CVD), partly because of associated abnormalities such as hypertension, dyslipidemia, glucose intolerance, or overt hyperglycemia, and partly because of the atherogenic risk of insulin resistance or hyperinsulinemia.

It is well established that hyperglycemia correlates linearly with CVD mortality, even in the range of values found in patients with insulin resistance or metabolic syndrome. However metabolic syndrome confers an additional risk, which could be related to insulin resistance and the associated proinflammatory state or compensatory hyperinsulinemia. There is evidence for both growth-promoting and vasculo-protective effects of insulin. For example, insulin increases proliferation of vascular smooth muscle cells (SMCs) in vitro at pharmacological concentrations. However, insulin also stimulates endothelial cell production of nitric oxide (NO) and induces endothelial NO synthase (eNOS). NO inhibits SMC growth and migration and has antiinflammatory effects. In vivo, chronic insulin treatment aggravated atherosclerosis in cholesterol-fed rabbits but oral insulin administration decreased atherosclerosis in apoE−/− mice, whereas insulin receptor substrate-2 (IRS-2) knockout increased it. Recent articles have reported diametrically opposed results after knocking down the insulin receptor or IRS-2 in myeloid cells in LDLR−/− and ApoE−/− mice.

In addition to the increased risk for atherosclerosis, patients with diabetes and metabolic syndrome also have an increased risk for restenosis after percutaneous transluminal coronary angioplasty and stenting. After balloon angioplasty, intimal injury results in endothelial denudation, followed by platelet activation and secretion of growth factors from platelets and damaged SMCs. Subsequently, there is SMC migration and proliferation, and matrix deposition resulting in neointimal growth. The time-course of neointimal growth has been well established after balloon angioplasty of the carotid artery in the rat. Medial SMC proliferation peaks 2 days after angioplasty, followed by a peak in SMC migration from the media to the intima at 4 days. Vascular SMCs replicate in the neointima and reach maximum proliferation by 7 days. Neointimal growth continues through 14 days after injury and peaks and plateaus at 28 days. In addition to SMC migration and proliferation, bone marrow–derived...
smooth muscle precursors can also contribute to neointimal growth,\textsuperscript{20} as can adventitial cells.\textsuperscript{21}

The objective of this study is to determine the effect of insulin on SMC growth kinetics over the time-course of neointimal formation after arterial injury in rats. Moderately elevated levels of insulin, comparable to the levels found in insulin resistant rats\textsuperscript{22} and also in noninsulin resistant rats after oral glucose loading,\textsuperscript{23} were attained by insulin treatment of normal rats whereas the insulin-induced decrease in plasma glucose levels was prevented by oral glucose.

### Methods

For detailed methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

### Animals

Two groups of male Sprague-Dawley (Charles River, Montreal, Quebec, Canada) rats were studied: control (blank capsule) and insulin-treated (3 U/d s.c. via implanted capsule; LinShin Inc). Two groups of male Sprague-Dawley (Charles River, Montreal, Quebec, Canada) rats were studied: control (blank capsule) and insulin-treated (3 U/d s.c. via implanted capsule; LinShin Inc). Two days after treatment, the left common carotid artery was injured with a balloon catheter, followed by the insertion of an i.p. catheter for infusion of 25% glucose (saline in controls) to maintain normoglycemia. Four days later, when rats resumed drinking after surgery, infusions were stopped and blood glucose was maintained only with oral glucose in water (40%). Rats were euthanized at various time points after injury, based on previous studies investigating the kinetics of vascular injury. In a separate group of animals used for the reendothelialization measurements, the aorta was injured with a balloon catheter. Carotids and aortas were perfusion-fixed with buffered paraformaldehyde, removed, and embedded in paraffin.

### Histomorphometry/Matrix Staining

Cell migration into the intima was measured 4 days after carotid injury by immunostaining intimal cells on the lumen surface with an antibody against histone H1 (MAB1276; Chemicon), which does not permeate the internal elastic lamina.\textsuperscript{24}

Intimal SMC proliferation was measured 7, 14, and 28 days after carotid injury with an anti-BrdU antibody (Dako).\textsuperscript{24} Intimal SMC apoptosis was measured 14 days after injury with the terminal dUTP nick-end labeling (TUNEL) method (Aptopag Kit, Intergen). Cross-sections taken 28 days after injury were also stained with Movat pentachrome or picrosirius red (PSR) for elastin and collagen. Cross-sectional areas were measured on hematoxylin and eosin stained slides at 14 and 28 days after injury. Images were analyzed using a computer-assisted morphometric system (Simple PCI, Compix Inc).

### Immunohistochemistry

Immunohistochemical staining for rat c-kit, sca-1, SMC α-actin, CD68, and CD45 was performed on carotid cross-sections of 7 day treated rats as described in the supplemental materials.

### Western Blot

Carotids were removed 7 days after injury, homogenized, and processed as previously described.\textsuperscript{25} Protein (25 μg) was separated on a 10% SDS-PAGE gel and analyzed with antimyosin heavy chain (Promega H9251), anti-actin, CD68, and CD45 was performed on carotid cross-sections of 7 day treated rats as described in the supplemental materials.

### Table. Fasting and Fed Plasma Levels of Glucose and Insulin

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>C</th>
<th>I</th>
<th>C</th>
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<th>C</th>
<th>I</th>
<th>C</th>
<th>I</th>
</tr>
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<tbody>
<tr>
<td>Fasting glucose, mmol/L†</td>
<td>5.3±0.3 (n=8)</td>
<td>5.0±0.3 (n=8)</td>
<td>5.5±0.2 (n=8)</td>
<td>5.2±0.3 (n=12)</td>
<td>4.8±0.1 (n=23)</td>
<td>5.3±0.5 (n=26)</td>
<td>5.0±0.1 (n=24)</td>
<td>5.5±0.3 (n=23)</td>
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<tr>
<td>Fasting insulin, pmol/L*</td>
<td>46±7 (n=4)</td>
<td>1577±294† (n=4)</td>
<td>55±11 (n=7)</td>
<td>833±198† (n=8)</td>
<td>43±9 (n=12)</td>
<td>718±70† (n=13)</td>
<td>61±7 (n=12)</td>
<td>768±104† (n=11)</td>
</tr>
<tr>
<td>Fed glucose, mmol/L†</td>
<td>6.8±0.1 (n=8)</td>
<td>6.8±0.4 (n=8)</td>
<td>6.2±0.5 (n=8)</td>
<td>5.7±0.3 (n=12)</td>
<td>6.3±0.1 (n=23)</td>
<td>5.8±0.3 (n=26)</td>
<td>6.2±0.1 (n=24)</td>
<td>5.9±0.1 (n=23)</td>
</tr>
<tr>
<td>Fed insulin, pmol/L*</td>
<td>106±17 (n=6)</td>
<td>1406±127† (n=5)</td>
<td>186±31 (n=5)</td>
<td>1059±375† (n=6)</td>
<td>149±16 (n=11)</td>
<td>918±100† (n=13)</td>
<td>185±13 (n=12)</td>
<td>819±131† (n=12)</td>
</tr>
</tbody>
</table>

C indicates control; I, insulin-treated.

*Data are expressed as means±SEM and represent values taken at sacrifice.

†Data are means±SEM of individual average glucose levels taken over the treatment period.

§P<0.05, I vs C.
Flow Cytometry
Rat blood was obtained 3 and 7 days after carotid injury in separate rats by cardiac puncture and flow cytometry was performed as in Fadini et al. Experiments were performed as in Fadini et al.26 (see also supplemental materials).

Evans Blue Staining
The reendothelialized area was measured with Evans blue dye at 14 days after aortic injury.27 Cross-sectional areas were measured as described in Histomorphometry/Matrix Staining above.

Statistics
Values are mean±SEM. Means for control versus insulin treatment at each time-point were compared by the 2-tailed Student t test. Within each treatment, means between groups at different time-points were compared using 1-way ANOVA followed by Tukey t test. Calculations were performed using SAS (Statistical Analysis System).

Results
Food intake was less for insulin-treated (I) rats because of the 40% oral glucose, however total caloric intake was greater compared to controls (C) at 14 and 28 days (supplemental Table I). Weight gain tended to be greater in I than C, however the difference was not significant (supplemental Table I). Both fasting and fed plasma glucose levels taken over the treatment period were similar in I and C (Table). Plasma hormone and metabolites were taken at sacrifice. Fasting plasma insulin was elevated by 10- to 14-fold in I and fed insulin was also greater (Table). Fasting plasma triglycerides were not significantly different but fed triglycerides were elevated at 14 and 28 days in I (supplemental Table II).

Fasting but not fed plasma FFA levels were lower in I than C in accordance with the antilipolytic effect of insulin (supplemental Table II).

Insulin exerts antiinflammatory effects in vitro and in vivo.28 To address the systemic antiinflammatory effect of insulin we measured a panel of cytokines and chemokines. We did not find any significant differences in plasma IL-6, IL-12, IL-18, MCP-1, and MIP-1α at 28 days (data not shown).

Treatment with insulin resulted in decreased neointimal formation at 28 days (Figure 1A and 1B). There was no change in medial area between the groups (14 days: C=0.16±0.01 mm², I=0.15±0.01 mm²; 28 days: C=0.15±0.01 mm², I=0.16±0.01 mm²). Lumen area was greater in I at 28 days (Figure 1C). There were no differences in external elastic lamina perimeter at 14 (C=3.0±0.1 mm, I=2.9±0.1 mm) and 28 days (C=2.8±0.1 mm, I=3.0±0.1 mm), indicating that outward remodeling did not contribute to the increase in lumen area with insulin treatment.

There was no correlation between average plasma glucose throughout treatment and neointimal area in C and I (Pearson’s r=-0.232 in C and -0.172 in I) and in both groups combined (r=0.135). However, this may only indicate that the spread of glucose levels was minimal.
There was no difference in intimal cell proliferation between C and I (Figure 2A). The total number of intimal cells was decreased (P<0.05) in I at 28 days (Figure 2B). There was no significant difference in the percentage of apoptotic cells in the intima 14 days after vessel injury (Figure 2C). Intimal cell density (the total number of cells divided by the intimal cross-sectional area) was not affected by insulin at 14 days, however intimal cell density tended to be greater in I at 28 days (C=5347±233 cells/mm², I=6108±468 cells/mm²). This suggests a decrease in matrix accumulation with insulin treatment. To assess the matrix, vessel cross-sections were stained with PSR for collagen or Movat pentachrome for elastin. Insulin tended to decrease collagen accumulation by 14.7±4.6% and significantly decreased elastin accumulation by 21.5±5.6% (P<0.05).

To investigate whether insulin decreased the intimal cell number by inhibiting migration, migration to the intima was measured 4 days after vessel injury. Insulin markedly inhibited migration by 56% (P<0.01; Figure 3A and 3B).

Because the migration assay cannot distinguish between cell origin or type, and because circulating, medial, and adventitial precursors contribute to neointimal growth, we performed immunostaining of the neointimal cross-sections for precursor markers c-kit and sca-1. Both c-kit (Figure 3C and 3D) and sca-1 (supplemental Figure IA and IB) staining at 7 days after injury were decreased in I, however the decrease in sca-1 staining did not reach significance. We found very few cells positive for CD68 (macrophage marker) or CD45 (general hematopoietic cell marker) with no difference between treatments (data not shown).

Because in vitro studies have shown that insulin promotes SMC differentiation, we investigated the in vivo effect of insulin on the SMC phenotype by measuring 3 differentiation markers 7 days after injury (Figure 4A through 4F). Western blots revealed an increase in SM-MHC (P<0.05), SM22α (P<0.05), and SMC α-actin (P<0.01) expression with insulin. Furthermore, 7 days after injury there was also an increase in SMC α-actin–positive area with insulin treatment (Figure 4G and 4H).

Because recruitment of circulating progenitor cells contributes to neointima formation, we used flow cytometry to detect circulating progenitor cells, which were defined by sca-1 or c-kit positivity. Insulin increased the number of circulating cells positive for sca-1 (P<0.05; Figure 5A) and tended to increase the number of c-kit–positive cells (supplemental Table III). Circulating precursors also contribute to reendothelialization, and it is possible that neointimal formation is reduced by accelerated reendothelialization. To address this, we used a model of aortic injury where reendothelialization is more consistent and complete. Analysis of rat aorta at 14 days showed that insulin accelerated reendothelialization by 24% (P<0.01) (Figure 5B and 5C). This was accompanied by a significant reduction in neointimal thickening in I (P<0.01; Figure 5D).

**Discussion**

We herein focused on the in vivo effect of insulin on the kinetics of neointimal growth in the rat arterial injury model. Moderate hyperinsulinemia was achieved while normal plasma glucose was maintained. Euglycemic hyperinsulinemia decreased neointimal growth. Our results are in contrast with other studies showing that hyperinsulinemia obtained via islet transplantation or s.c. delivery increased neointimal growth 14 days after balloon injury in nondiabetic rats. However, these authors did not measure intimal area at the time of maximum intimal thickening, ie, 28 days after injury, and the hyperinsulinemic group had lower blood glucose. Additionally, chronic hyperinsulinemia was achieved long before arterial injury, which may have entailed some insulin resistance. Another study found increased neointimal thickness in rats treated with s.c. insulin for only 2 days before injury as in our model, however these rats were fed oral sucrose, which contains fructose, a known inducer of insulin resistance. Our findings are in accordance with those of a recent study in mice with disrupted insulin signaling because of IRS-1 or IRS-2 deficiency. In these mice neointimal growth after cuff-injury was increased compared to wild-type mice, however it is not clear whether this was attributable to decreased insulin action on the vessel or to the abnormal metabolic milieu.

We found no change in intimal cell proliferation after insulin treatment. Previous studies showing insulin to be a growth-promoting hormone were almost exclusively in vitro studies in which very high concentrations of insulin were used. At higher plasma concentrations (~2000 pmol/L) we previously found that insulin does increase intimal cell proliferation despite decreasing neointimal growth.
Insulin increases SMC differentiation as indicated by increased SMC differentiation markers. As mentioned above, medial SMCs contribute to neointimal growth by dedifferentiation and migration to the intima. Insulin could have affected SMC migration via an effect on cell motility or through the regulation of the medial SMC phenotype, or both. In vitro studies have implicated a PI3K- and NO-dependent pathway, and insulin-induced upregulation of iNOS or GMP in migration, and a PI3K-dependent pathway in the maintenance of a quiescent phenotype.

However, it is clear that insulin exerts additional effects in addition to that on migration because inhibition of SMC migration does not necessarily result in decreased neointimal growth. We noted a decrease in matrix accumulation after insulin treatment, which also contributed to the decrease in neointimal area. Furthermore, decreased matrix accumulation is consistent with insulin preventing the SMC switch from a contractile to synthetic phenotype.

It is possible that reduced medial SMC dedifferentiation and migration and increased redifferentiation of precursors into intimal SMC are secondary to the acceleration of the reendothelialization of insulin. Reendothelialization occurs starting from the endothelium of the margin of the denuded area and is eNOS-dependent. Insulin is known to activate and upregulate eNOS via PI3K. Reendothelialization also occurs from circulating precursors, and the fact that insulin increased the circulating precursors, despite decreasing the precursor-positive intimal area, suggests that insulin stimulated the differentiation of the precursors into endothelium rather than SMCs.

These possible effects of insulin are all in keeping with the antiinflammatory effects of insulin shown on endothelium and monocytes. In the present study, plasma concentration of cytokines and chemokines were not affected by insulin. However, insulin may have exerted antiinflammatory effects at the vessel level. Insulin infusion lowering glucose has important antiinflammatory effects, which improve clinical outcome in critically ill patients. These antiinflammatory effects of insulin are partly independent of glucose lowering. In diabetic patients, hyperglycemia at the time of percutaneous intervention (PCI) predicts restenosis. Intervention studies are needed to demonstrate that insulin therapy decreases restenosis after PCI because of the reduction of hyperglycemia and perhaps also because of a direct vasculo-protective effect.

In conclusion, we have shown for the first time that insulin decreases restenosis after PCI by its inhibition of SMC migration and increases redifferentiation of precursors into the intima. This effect is consistent with the findings of others in different models and is a potential mechanism by which insulin could improve cardiovascular outcomes.

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Disclosure

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