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

Danna M. Breen, Kalam K. Chan, Jiwanjeet K. Dhaliwall, Michael R. Ward, Nael Al Koudsi, Loretta Lam, Melissa De Souza, Husam Ghanim, Paresh Dandona, Duncan J. Stewart, Michelle P. Bendeck, Adria Giacca

**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

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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-inflammatory 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 or had no effect on atherosclerosis in cholesterol-fed rabbits but oral insulin administration decreased atherosclerosis in apoE-deficient 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,20 as can adventitial cells.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 rats22 and also in noninsulin resistant rats23 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 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.24

Intimal SMC proliferation was measured 7, 14, and 28 days after carotid injury with an anti-BrdU antibody (Dako).24 Intimal SMC apoptosis was measured 14 days after injury with the terminal dUTP nick-end labeling (TUNEL) method (Apoptag 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.25 Protein (25 μg) was separated on a 10% SDS-PAGE gel and analyzed with antimyosin heavy chain (SM-MHC; Santa Cruz Biotechnology sc-6956), antismooth muscle 22 alpha (SM22α; Santa Cruz Biotechnology sc-18513), and anti-SMC α-actin (Sigma A2547) antibodies, all specific for rat.

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

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<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>
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<td>4.8±0.1 (n=23)</td>
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<td>5.0±0.1 (n=24)</td>
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<td>1577±294‡ (n=4)</td>
<td>55±11 (n=7)</td>
<td>833±198‡ (n=8)</td>
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<td>718±70‡ (n=13)</td>
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<td>Fed glucose, mmol/L†</td>
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<td>Fed insulin, pmol/L*</td>
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<td>185±13 (n=12)</td>
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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.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 \((C=5347\pm233 \text{ cells/mm}^2, I=6108\pm468 \text{ cells/mm}^2)\). 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\% and significantly decreased elastin accumulation \((P<0.05)\).

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\% \((P<0.01; \text{Figure 3A and 3B}).\)

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. \(^{26,29}\) 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 hematopoetic cell marker) with no difference between treatments (data not shown).

Because in vitro studies have shown that insulin promotes SMC differentiation, \(^{30}\) 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 \((P<0.05)\), SM22\(\alpha\) \((P<0.05)\), and SMC \(\alpha\)-actin \((P<0.01)\) expression with insulin. Furthermore, 7 days after injury there was also an increase in SMC \(\alpha\)-actin–positive area with insulin treatment (Figure 4G and 4H).

Because recruitment of circulating progenitor cells contributes to neointima formation, \(^{30}\) we used flow cytometry to detect circulating progenitor cells, which were defined by sca-1 or c-kit positivity. \(^{26,29}\) Insulin increased the number of circulating cells positive for sca-1 \((P<0.05; \text{Figure 5A})\) 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 aortas 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 \((P<0.01; \text{Figure 5D}).\)

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. \(^{31,32}\) 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. \(^{34}\) 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, \(^{35}\) 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. \(^{6,7}\) At higher plasma concentrations \((\approx 2000 \text{ pmol/L})\) we previously found that insulin does increase intimal cell proliferation despite decreasing neointimal growth. \(^{36}\) Taken

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Representative Western blot showing SM-MHC (A), SM22\(\alpha\) (C), and SMC \(\alpha\)-actin (E). Densitometric analysis of Western blots to detect SM-MHC (B), SM22\(\alpha\) (D), and SMC \(\alpha\)-actin (F) expression 7 days (control: \(n=8\); insulin; \(n=6\)) after vessel injury. Representative photomicrographs showing SMC \(\alpha\)-actin staining on a vessel cross-section at 7 days after carotid injury \((\times 40\); G). Quantitative analysis of percent area stained positive for SMC \(\alpha\)-actin (control: \(n=7\); insulin: \(n=7\); H). Values are means\(\pm\)SEM \(*P<0.05\), \(\dagger P<0.01\), insulin vs control.
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.

For example, a recent study reported that low-dose IGF-1 decreased restenosis after PCI because of the reduction of neointimal thickness after arterial injury. These results provide evidence for inhibition of the growth response to vessel injury by insulin in vivo, suggest that insulin-resistant states may result in loss of vascular protection, and warrant further investigation of the potential cardiovascular benefit of insulin therapy.

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References


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Supplemental Material

METHODS

Animal Models. Male Sprague-Dawley rats (Charles River) weighing 400-450 g and fed rat chow (Purina 5001) received either subcutaneous blank or porcine insulin (3U/day) capsule (LinShin Inc.) implantation under light isoflurane anesthesia. The implants allowed continuous insulin delivery. Analgesic (Buprenorphine) was given at the end of each surgical procedure. Insulin-treated rats were given 40% glucose water to prevent hypoglycemia. The Animal Care Committee of the University of Toronto approved all procedures.

Balloon Catheter Injury of the Carotid Artery. Two days after implant procedure, the rats were anesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg/ml, 1 μl/g body wt) and the left common carotid artery was injured with a 2F balloon catheter (Baxter) which was inflated and withdrawn 4 times. After balloon injury, an i.p. catheter was also inserted for infusion of 25% glucose (saline in controls) to maintain normoglycemia during the recovery period after surgery (when rats do not eat or drink). The rate of infusion was adjusted according to frequent glycemic determination (every 2 h). On the fourth day, rats were removed from the infusions, and blood glucose was maintained only by feeding the rats glucose in water (40%). Fed blood glucose was monitored daily throughout the treatment. To obtain fasting blood glucose levels, rats were fasted for ~16h (drinking water/oral glucose was given without food) once per week. Both fasting and fed blood glucose were measured at the same time of the day (10 a.m.) using a glucometer (Glucometer Elite, Bayer Inc.). Values were converted to plasma glucose assuming a normal hematocrit.

Blood and Vessel Sample Collection. The rats were sacrificed at various time points after injury, based on previous studies investigating the kinetics of vascular injury. To label
proliferating SMCs, rats were injected i.p. with 25 mg/kg 5-bromo-2’-deoxyuridine (BrdU; Sigma) at 17, 9 and 1 hour before sacrifice. Rats were sacrificed either in the fed or fasting state to obtain blood samples in different metabolic states. We had previously shown that the fasting or fed state at the time of sacrifice had no effect on the morphologic parameters\(^2\) and no effect was seen in this study. Blood samples were collected by cardiac puncture under general anesthesia. Thereafter, rats were sacrificed via anesthetic overdose and the carotids were perfusion-fixed for 4 minutes with 4% buffered paraformaldehyde at physiologic pressure (110 mmHg). The carotids were removed, immersed in 4% paraformaldehyde for >48h, embedded in paraffin, and divided into two blocks by cutting the midsection of the artery. Cross-sections were taken from the midsection.

**Metabolic Parameters.** Plasma insulin was determined by radioimmunoassay (Linco) using kits specific for rat insulin and with 100% cross-reactivity with porcine insulin (used for treatment). Plasma FFA was measured using a colorimetric kit from Wako. Plasma triglycerides were also measured using colorimetric kits from Boehringer. Plasma inflammatory markers were run on Lincoplex.

**Histomorphometry (details).** Lumen area was calculated by tracing the inside edge of the vessel to determine the circumference and assuming a circular geometry of the vessel. This method avoids artifacts due to paraffin embedding. Intimal area was measured as the area between the internal elastic lamina and lumen. Medial area was measured as the area between the external elastic lamina and internal elastic lamina. Intimal SMC density was calculated by dividing the total number of intimal nuclei by the intimal area.

To measure migration into the intima, carotids were collected 4 days after injury and intimal cells on the lumen surface were immunostained with an antibody against histone H1
(MAB1276; Chemicon) which does not permeate the internal elastic lamina. The entire luminal surface of the vessel was counted with a minimum of 12 fields taken at a magnification of 20X. Each nucleus that was oriented parallel to the long axis of the vessel was counted, whereas medial cells were perpendicular. A blinded coding system was used to avoid experimenter bias. This migration assay takes advantage of the fact that the first cells appear in the intima 3 to 4 days after injury and do not complete a round of replication until 24h later.

**Immunohistochemistry (details).** Immunohistochemical staining for rat c-kit (Dako), sca-1 (Cell Signalling), SMC α- actin (Sigma), CD68 (Serotec antibody), and CD45 (BD Pharmingen antibody) was carried out on carotid cross-sections of 7 day treated rats. Rat small intestine (c-kit, sca-1, SMC α-actin) and rat spleen (CD68, CD45) tissue were used as positive controls for staining. 4μ formalin-fixed paraffin-embedded sections were dewaxed in 5 changes of xylene and brought down to water through graded alcohols. Antigen retrieval or unmasking procedures were applied, if necessary (see below). Endogenous peroxidase and biotin activities were blocked respectively using 3% hydrogen peroxide and avidin/ biotin blocking kit (Lab Vision Cat# TA-015-BB). After blocking for 15 min with 10% normal serum from the species where the secondary antibody is obtained, sections were incubated accordingly at room temperature with the appropriate primary antibodies using conditions (see below) previously optimized. This was followed with a biotinylated secondary (Vector labs) for 30 min and horseradish peroxidase-conjugated ultrastreptavidin labeling reagent (ID labs.) for 30 min. After washing well in PBS, color development was done with freshly prepared NovaRed solution (Vector labs. Cat# SK-4800). Finally, sections were counterstained lightly with Mayer’s hematoxylin, dehydrated in alcohols, cleared in xylene and mounted in Permount (Fisher, cat# SP15-500).
H.I.E.R.: Heat Induced Epitope Retrieval refers to microwaving tissue sections in a medium for antigen retrieval. 10 mM citrate buffer at pH 6.0 is used and the solution and tissue sections are heated up inside a microwavable pressure cooker. After the pressure is built up inside the cooker (exact time will depend on the actual set-up), boiling is maintained for another 3 min with a lower setting. The cooker is then removed from the microwave oven and allowed to cool off on the bench for 20 min. Sections are then removed from the hot buffer into warm water and then rinsed in PBS. Pepsin: 1% pepsin (Sigma: P7125) in 0.01 N HCl (pH 2.0), 15 min at 37°C. Trypsin: 0.1% trypsin (Difco) in PBS (pH 7.6), 15 min at 37°C.

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**Flow Cytometry.** Blood was obtained at baseline and 3 and 7 days after carotid injury in separate rats by cardiac puncture and drawn into Sodium Citrate (9:1 v/v) Vacutainer tubes (BD Biosciences). For each staining sample, 200 µl of blood was mixed with 1.0 mL RBC lysis buffer (eBioscience) and incubated for 10 min at room temperature in the dark. Tubes were centrifuged and the supernatant discarded. The remaining cells (pellet) were suspended in 100 µl of PBE staining buffer, and appropriate primary antibody (mouse anti-rat VEGFR2 (Novus Biologicals), rabbit anti-rat c-kit (Neuromics), and rabbit anti-rat sca-1 (Novus Biologicals) was
incubated with the cells at room temperature in the dark for 30 minutes. Following washing (2x 1.0 ml PBE buffer) and centrifugation, cells were exposed to secondary antibody (FITC donkey anti-rabbit IgG) or Straptavidin-APC for 30 minutes in the dark. Following two additional wash and centrifugations steps, cells were suspended in PBS. The number of gated cells positive for the three markers was quantified by flow cytometry (Beckman Coulter Cytomics FC500) using FL1 (FITC for c-kit and sca-1) and FL4 (for VEGFR2) filters.

**Balloon Catheter Injury of the Aorta and Re-endothelialization Measurements (details).** In a separate group of animals used for the re-endothelialization measurements, the aorta was injured with a 2F balloon catheter (Baxter) which was inflated and withdrawn 3 times. The re-endothelialized area was measured at 14 days after aortic balloon injury, 1.0 ml of 2.5% Evans blue solution was injected into rats via the abdominal artery. Ten minutes after the injection, rats were sacrificed via anesthetic overdose and the aorta was perfusion-fixed for 15 minutes with 4% buffered formalin at physiologic pressure (110 mmHg). The thoracic aorta was dissected out and opened longitudinally to observe Evans Blue uptake macroscopically. The re-endothelialized area was defined as the area not stained with Evans Blue.
REFERENCES


FIGURE LEGENDS

Figure I. Representative photomicrographs showing sca-1 staining on a vessel cross-section at 7 days after carotid injury (x 40) (A). Quantitative analysis of percent area stained positive for sca-1 (Control: \( n=7 \); Insulin: \( n=7 \)) (B). Values are means ± SEM. No significant differences were found.
Table I. Daily food, fluid and total caloric intake, and final weight gain over the treatment periods.
C=Control; I=Insulin-treated. Data are expressed as means ± SEM. *P<0.05, I vs. C.

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<td>I (n=26)</td>
<td>I (n=26)</td>
</tr>
<tr>
<td></td>
<td>C (n=24)</td>
<td>I (n=24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>I (n=23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/day)</td>
<td>17.6±2.9</td>
<td>11.8±1.3</td>
<td>26.5±2.1</td>
<td>13.8±0.9*</td>
</tr>
<tr>
<td></td>
<td>30.7±2.1</td>
<td>16.8±0.8*</td>
<td>32.6±0.9</td>
<td>16.9±0.8*</td>
</tr>
<tr>
<td>Fluid Intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/day)</td>
<td>29.8±14.7</td>
<td>25.1±10.4</td>
<td>40.7±4.9</td>
<td>37.9±4.5</td>
</tr>
<tr>
<td></td>
<td>46.8±1.9</td>
<td>52.2±2.3</td>
<td>50.8±1.8</td>
<td>51.2±2.4</td>
</tr>
<tr>
<td>Total Caloric</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(kCal/g/day)</td>
<td>58.2±9.6</td>
<td>78.9±6.3</td>
<td>87.5±7.1</td>
<td>106.2±6.8</td>
</tr>
<tr>
<td></td>
<td>101.4±6.8</td>
<td>139.0±4.3*</td>
<td>107.7±2.9</td>
<td>137.8±5.4*</td>
</tr>
<tr>
<td>Weight Gain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g)</td>
<td>-2.0±1.4</td>
<td>-0.1±2.1</td>
<td>-8.5±5.4</td>
<td>2.4±4.3</td>
</tr>
<tr>
<td></td>
<td>44.6±3.3</td>
<td>55.9±4.0</td>
<td>89.5±4.7</td>
<td>92.8±6.0</td>
</tr>
</tbody>
</table>
Table II. Fasting and fed plasma levels of insulin, triglycerides and free fatty acids.

C=Control; I=Insulin-treated. Data are expressed as means ± SEM and represent values taken at sacrifice. *P<0.05, I vs. C.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>4 days</th>
<th>7 days</th>
<th>14 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C (n=4)</td>
<td>I (n=4)</td>
<td>C (n=7)</td>
<td>I (n=8)</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.71±0.25</td>
<td>1.27±0.23</td>
<td>0.50±0.19</td>
<td>0.59±0.13</td>
</tr>
<tr>
<td>Free Fatty Acids (μEq/l)</td>
<td>285±51</td>
<td>81±5*</td>
<td>288±16</td>
<td>157±32*</td>
</tr>
<tr>
<td></td>
<td>C (n=6)</td>
<td>I (n=5)</td>
<td>C (n=5)</td>
<td>I (n=6)</td>
</tr>
<tr>
<td></td>
<td>C (n=11)</td>
<td>I (n=13)</td>
<td>C (n=12)</td>
<td>I (n=11)</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.28±0.19</td>
<td>1.01±0.22</td>
<td>1.06±0.18</td>
<td>0.99±0.15</td>
</tr>
<tr>
<td>Free Fatty Acids (μEq/l)</td>
<td>173±32</td>
<td>132±10</td>
<td>188±49</td>
<td>128±17</td>
</tr>
<tr>
<td></td>
<td>C (n=12)</td>
<td>I (n=12)</td>
<td>C (n=12)</td>
<td>I (n=12)</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.32±0.11*</td>
<td>0.97±0.10</td>
<td>1.37±0.15*</td>
<td></td>
</tr>
<tr>
<td>Free Fatty Acids (μEq/l)</td>
<td>182±34</td>
<td>140±14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table III. Number of gated cells stained positive for c-kit, sca-1, and VEGFR2.
C=Control; I=Insulin-treated. Data are expressed as means ± SEM.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Basal</th>
<th>3 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated/ Uninjured (n=5)</td>
<td>C (n=7)</td>
<td>I (n=7)</td>
</tr>
<tr>
<td>c-kit*</td>
<td>4.77 ± 0.57</td>
<td>3.59 ± 0.29</td>
<td>4.41 ± 0.50</td>
</tr>
<tr>
<td>VEGFR2*</td>
<td>0.068 ± 0.008</td>
<td>0.049 ± 0.005</td>
<td>0.053 ± 0.006</td>
</tr>
<tr>
<td>c-kit/ VEGFR2*</td>
<td>0.002 ± 0.002</td>
<td>0.003 ± 0.002</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>sca-1/ VEGFR2†</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Expressed as % of gated cells stained positive.
†Note: In the Fadini et al[^1] paper sca-1° cells separated by fluorescence-activated cell sorting also showed very little positivity for VEGFR2.
Fig. I

(A) Control and Insulin treated tissues.

(B) Bar graph showing % Area sca-1 Positive over 7 days.