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The effect of insulin to decrease neointimal growth after arterial injury is endothelial nitric oxide synthase-dependent

June Guo [a, 1], Danna M. Breen [a, 1], Troy J. Pereira [b], Prasad S. Dalvi [a, h], Hangjun Zhang [a], Yusaku Mori [a, c], Husam Ghanim [d], Laura Tumiati [e], I. George Fantus [a, f], Michelle P. Bendeck [f, g], Paresh Dandona [d], Vivek Rao [e], Vernon W. Dolinsky [b], Scott P. Heximer [a], Adria Giacca [a, f, *]

[a] Department of Physiology, University of Toronto, Toronto, ON M5S 1A8, Canada
[b] Children's Hospital Research Institute of Manitoba, Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, MB R3E 3P4, Canada
[c] Division of Diabetes, Metabolism and Endocrinology, Showa University, Shinagawa, Tokyo 142-0064, Japan
[d] Division of Endocrinology, Diabetes and Metabolism, State University of New York at Buffalo, Kaleida Health, Buffalo, NY 14209, USA
[e] Division of Cardiovascular Surgery, Toronto General Hospital, University of Toronto, Toronto, ON M5G 2C4, Canada
[f] Department of Medicine, University of Toronto, Toronto, ON M5S 1A8, Canada
[g] Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON M5S 1A8, Canada
[h] Division of Physiology and Experimental Medicine, The Hospital for Sick Children, Toronto, ON M5G 1X8, Canada

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ABSTRACT

In vitro, insulin has mitogenic effects on vascular smooth muscle cells (VSMC) but also has protective effects on endothelial cells by stimulating nitric oxide (NO) production and endothelial nitric oxide synthase (eNOS) expression. Furthermore, NOS inhibition attenuates the effect of insulin to inhibit VSMC migration *in vitro*. Using an *in vivo* model, we have previously shown that insulin decreases neointimal growth and cell migration and increases re-endothelialization after arterial injury in normal rats. Since insulin can stimulate NOS, and NO can decrease neointimal growth, we hypothesized that NOS, and more specifically eNOS was required for the effects of insulin *in vivo*.

Rats were given subcutaneous insulin implants (3 U/day) alone or with the NOS inhibitor L-NAME (2 mg kg^{-1} day^{-1}) 3 days before arterial (carotid or aortic) balloon catheter injury. Insulin decreased both neointimal area ($P < 0.01$) and cell migration ($P < 0.01$), and increased re-endothelialization ($P < 0.05$). All of these effects were prevented by the co-administration of L-NAME. Insulin was found to decrease inducible NOS expression ($P < 0.05$) but increase eNOS phosphorylation ($P < 0.05$). These changes were also translated at the functional level where insulin improved endothelial-dependent vasorelaxation. To further study the NOS isoform involved in insulin action, s.c. insulin (0.1 U/day) was given to wild-type and eNOS knockout mice. We found that insulin was effective at decreasing neointimal formation in wild-type mice after wire injury of the femoral artery, whereas this effect of insulin was absent in eNOS knockout mice. These results show that the vasculoprotective effect of insulin after arterial injury is mediated by an eNOS-dependent mechanism.

1. Introduction

Type 2 diabetes and the metabolic syndrome are well known risk factors for atherosclerotic cardiovascular disease in part due to insulin resistance and/or hyperinsulinemia [1]. Insulin has both growth promoting and vasculoprotective effects. For example, insulin increases the proliferation of vascular smooth muscle cells (VSMC) *in vitro* [1]. On the other hand, insulin stimulates

* Corresponding author. University of Toronto, Department of Physiology, Medical Science Building, 1 King's College Circle, Room 3336, Toronto, ON M5S 1A8, Canada.
E-mail address: adria.giacca@utoronto.ca (A. Giacca).
[1] Equally contributed to work.

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endothelial cell production of nitric oxide (NO) [1], which inhibits VSMC growth and migration, and has anti-inflammatory effects [2].

In addition to the increased risk for atherosclerosis, patients with diabetes and metabolic syndrome are also at an increased risk for the development of restenosis after percutaneous transluminal coronary angioplasty and stenting [3,4]. The effect of insulin on restenosis is controversial. Insulin treatment via islet transplantation increased neointimal growth after balloon injury in rats [5]. In agreement, Foster et al. [6] and Pu et al. [7] have shown that subcutaneous insulin also increases neointimal formation. In contrast to these studies, Kubota et al. [8] have shown that neointimal growth after cuff injury was greater in mice with a defect in insulin signaling (insulin receptor substrate (IRS)-2 null mice). Furthermore, we and Murthy et al. [9] have demonstrated that subcutaneous insulin as well as insulin analogs decreased neointimal formation after carotid balloon injury in normal rats [10,11] and Zucker fatty rats [9]. The discrepancies between these studies may relate to the chronicity of insulin administration before arterial injury and to the administration of sucrose to maintain euglycemia [11], factors that induce insulin resistance of the phosphatidylinositol 3-kinase (PI3K)/Akt/NO pathway while leaving the growth-promoting mitogen activated protein kinase (MAPK) pathway intact. In addition, we have shown that euglycemic hyperinsulinemia (3 U/day; s.c.) decreases cell migration and accelerates re-endothelialization, resulting in decreased neointimal area [10].

Studies in endothelial cells have shown that insulin activates and upregulates endothelial NO synthase (eNOS) via PI3K and Akt [12]. eNOS signaling is important in endothelial barrier function [13], and furthermore, deficient eNOS phosphorylation may be a mechanism of diabetic vascular dysfunction [14–16]. In VSMC, acute insulin treatment has been reported to upregulate while chronic insulin treatment downregulates inducible nitric oxide synthase (iNOS) [17]. Insulin can also enhance NO-stimulated guanylate cyclase activity in VSMC [18]. Furthermore, insulin's inhibitory effect on VSMC migration *in vitro* was abolished by NOS inhibitors [19]. Thus, NOS activation represents a likely mechanism for insulin to exert its inhibition of neointimal formation. Therefore, the objective of our study was to investigate the consequences of NOS inhibition on the *in vivo* effects of insulin.

2. Methods

2.1. Animal models

Studies were conducted according to protocols approved by the Animal Care Committee of the University of Toronto.

2.1.1. Rat studies

Four groups of male Sprague–Dawley rats (Charles River, Sherbrooke, QC) weighing 400–450 g and fed normal chow were studied: 1) control (C); 2) insulin (I, 3 U/day; s.c. capsule (bovine insulin, LinShin Inc., Toronto, ON)-treatment as in our previous study [10]; 3) insulin + the NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME, Sigma, Oakville, ON) (I + L, 2 mg kg^{-1} day^{-1}, s.c. via osmotic pumps, as we previously used [20]; and 4) control + L-NAME (C + L). The pellets/osmotic pump implantation were performed under isoflurane anesthesia 3 days before carotid balloon injury. S.c. analgesic (Buprenorphine) was given intraoperatively at each surgery. To maintain normoglycemia, all insulin-treated rats were given 40% glucose in drinking water, which we have previously found to have no effect on neointimal formation [11].

2.1.2. Mouse studies

Four groups of male mice (7 weeks old, Jackson Laboratories, Bar Harbor, Maine) were studied: 1) control wild-type (C57BL/6J) (WT); 2) insulin-treated (I, 0.1 U/day; s.c. pellet (bovine insulin)) (LinShin Inc., Toronto, ON); 3) eNOS knockout mice treated with insulin (eNOS-KO + I); and 4) eNOS knockout (eNOS-KO) mice. Insulin pellets were implanted 3 days before femoral arterial injury as described above. All mice treated with insulin were also treated with 40% glucose in drinking water.

2.2. Surgery

2.2.1. Rat arterial injury

Three days after treatment initiation, injury to the left common carotid artery and/or the thoracic aorta (re-endothelialization and functional test) was induced by a 2F Fogarty balloon catheter (Edwards Lifesciences, Irvine, CA) as previously described [10].

2.2.2. Mouse arterial injury

Three days after treatment initiation, the left femoral artery was injured with a straight guide wire (0.38 mm in diameter, C-SF-IS-IS Cook Medical, Bloomington, IL) as previously described [20].

2.3. Blood and vessel collection

The rats were sacrificed at various time points after injury, based on previous studies that established the kinetics of neointimal formation after vascular injury [21]. The mice were sacrificed at 28 days after injury. Rats/mice were sacrificed in the fed state. Blood samples were collected by cardiac puncture under general anesthesia. Thereafter, animals were euthanized by anesthetic overdose of a mixture of ketamine:xylazine:acepromazine (87:1.7:0.4 mg/ml; 1 ml/rat, 0.2 ml/mouse, i.p.), and after heart beating had stopped the carotids and femoral arteries were perfusion-fixed for 4 min (rats) or 15 min (mice), respectively, with 10% neutral buffered formalin at physiologic pressure (110–120 mmHg). The vessels were removed, immersed in formalin for >24 h, embedded in paraffin, and divided into two blocks by cutting the midsection of the artery. Cross-sections were taken from the midsection. For assessment of endothelial-dependent vasorelaxation, two segment of 5 mm aorta were used. The carotids used for RT-PCR or Western blotting were snap frozen in liquid nitrogen and stored at −80 °C.

2.4. Metabolic parameters

Fed blood glucose was monitored throughout the treatment using a glucometer (Glucometer Elite, Bayer Inc.). Plasma insulin was determined by radioimmunoassay (Linco Research Inc, St. Charles, MO). Plasma free fatty acids (FFA) (Wako Pure Chemical Industries, Osaka, Japan) and plasma triglycerides (Roche Diagnostics, Indianapolis, IN) were measured using colorimetric kits.

2.5. Histomorphometry/matrix staining

A blinded coding system was used. Cell migration into the neointima was measured 4 days after injury as described in [10]. In brief, it has been shown that intimal cells start to appear at 3–4 days after injury and do not complete a round of replication until 24 h later [10]. Cells on the luminal surface were immunostained with an antibody against histone H1 (MAB1276; Chemicon, Temecula, CA), which does not permeate the internal elastic lamina. As a result only intimal cell nuclei were stained [10].