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Role of metabolic environment on nitric oxide mediated inhibition of neointimal hyperplasia in type 1 and type 2 diabetes

Monica P. Rodriguez [1], Zachary M. Emond [1], Zheng Wang, Janet Martinez, Qun Jiang, Melina R. Kibbe *

Division of Vascular Surgery, and Institute for BioNanotechnology in Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, United States
Jesse Brown Veterans Affairs Medical Center, Chicago, IL, United States

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ABSTRACT

Nitric oxide (NO) is well known to inhibit neointimal hyperplasia following arterial injury. Previously, we reported that NO was more effective at inhibiting neointimal hyperplasia in a type 2 diabetic environment than control. We also found that NO was ineffective in an uncontrolled type 1 diabetic environment; however, insulin restored the efficacy of NO. Thus, the goal of this study was to more closely evaluate the effect of insulin and glucose on the efficacy of NO at inhibiting neointimal hyperplasia in both type 1 and type 2 diabetic environments using different doses of insulin as well as pioglitazone. Type 1 diabetes was induced in male lean Zucker (LZ) rats with streptozotocin (60 mg/kg IP). Groups included control, moderate glucose control, and tight glucose control. Zucker diabetic fatty (ZDF) rats fed Purina 5008 chow were used as a type 2 diabetic model. Groups included no therapy, insulin therapy, or pioglitazone therapy. After 4 weeks of maintaining group assignments, the carotid artery injury model was performed. Treatment groups included: control, injury and injury plus NO. 2 weeks following arterial injury, in the type 1 diabetic rats, NO most effectively reduced the neointimal area in the moderate and tightly controlled groups (81% and 88% vs. 33%, respectively, $p = 0.01$). In type 2 diabetic rats, the metabolic environment had no impact on the efficacy of NO (81–82% reduction for all groups). Thus, in this study, we show NO is effective at inhibiting neointimal hyperplasia in both type 1 and type 2 diabetic environments. A greater understanding of how the metabolic environment may impact the efficacy of NO may lead to the development of more effective NO-based therapies for patients with diabetes.

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Introduction

Diabetes continues to be a major public health problem in the United States. In 2010, the Centers for Disease Control estimated that 26.9% of Americans over the age of 65 have diabetes [1]. Diabetes is the leading cause of nontraumatic lower limb amputation in the United States [1]. Significant strides have been made in the reduction of macrovascular complications of the disease, but unfortunately, patients with type 1 and 2 diabetes suffer worse prognosis for the treatment of macrovascular disease, such as coronary artery, cerebrovascular and peripheral vascular interventions, due to a greater rate of restenosis [2–6].

Nitric oxide (NO) is a small, naturally occurring molecule that has many beneficial effects in the vasculature, such as inhibition of vascular smooth muscle cell migration and proliferation, endothelial cell apoptosis and leukocyte chemotaxis [7,8]. We and others have shown that NO effectively inhibits the formation of neointimal hyperplasia [8–19]. We have also shown that NO is more effective in uncontrolled type 2 compared to type 1 diabetic and control rats [20]. However, with the addition of insulin, the efficacy of NO was restored in type 1 diabetic rats [21]. Yet, with these studies, the effect of NO in a controlled type 1 or type 2 diabetic environment was not determined. It is unclear whether the hyperinsulinemia and/or hyperglycemia are responsible for regulating the efficacy of NO in these diabetic environments. Also, the effect of pioglitazone, an insulin sensitizer that is known to regulate the proliferation and migration of vascular smooth muscle cells, improve endothelial cell function and reduce inflammation, on the efficacy of NO has not been determined [22–24]. Thus, the goal of this study is to more closely evaluate the effect of insulin and glucose on the efficacy of NO at inhibiting neointimal hyperplasia in both type 1 and type 2 diabetic environments through the use of sustained release insulin pellets, and pioglitazone therapy. This study differs from our earlier work as our goal here is to produce metabolic environments with more controlled insulin and glucose levels, to discern the individual contribution of each on the efficacy of NO. With our prior work, the animals remained hyperglycemic, making it impossible to discern if the restored efficacy was due to the insulin therapy, or the lower glucose level. We

* Corresponding author. Address: Division of Vascular Surgery, 676 N. St. Clair Street, #650, Chicago, IL 60611, United States. Fax: +1 (312) 503 1222.
E-mail address: mkibbe@nmh.org (M.R. Kibbe).
[1] These authors contributed equally to this manuscript.

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hypothesized that NO is more effective in a normoglycemic, hyperinsulinemic environment in both type 1 and type 2 diabetic rats *in vivo*.

Materials and methods

Type 1 diabetic animal model

11-Week-old male lean Zucker (LZ) rats were obtained from Charles Rivers Laboratories (Wilmington, MA). Type 1 diabetes was induced in LZ rats with a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg). Daily serum glucose levels were assessed with a glucometer via tail vein puncture and animals with glucose concentrations of 300 mg/dL or above were considered diabetic and included in the study. Blood glucose concentrations were maintained by the use of subcutaneous insulin pellets (Linplant, Canada) which deliver 2U insulin every 24 h for up to 40 days. Three subsets of type 1 diabetic rats were created (Fig. 1). The first subset, consisting of uncontrolled type 1 diabetic rats that received no insulin therapy, was referred to as "STZ No Control". The second subset, which received subcutaneous insulin pellets to maintain blood glucose concentrations between 200 and 300 mg/dL, was referred to as "STZ moderate control". The third subset, which received subcutaneous insulin pellets to maintain blood glucose levels of less than 200 mg/dL was referred to as "STZ Tight Control". Insulin therapy commenced approximately 7 days after STZ injection, when the hyperglycemia was detected and stable for several days. Insulin therapy continued for 21 days before and 14 days after surgery was performed. The non-fasting daily blood glucose concentration was recorded.

Type 2 diabetic animal model

Zucker diabetic fatty (ZDF) rats were obtained from Charles Rivers Laboratories. The ZDF strain has a homozygous leptin receptor mutation pre-disposing the rats to type 2 diabetes. When the inbred ZDF males were fed the Purina 5008 diet, which is manufactured high in carbohydrates and fats, they exhibited hyperinsulinemia, hyperglycemia, hypercholesterolemia and hypertriglyceridemia, mimicking a type 2 diabetic state. Three subsets of type 2 diabetic rats were created (Fig. 1). The first group received no supplemental insulin therapy and was referred to as "ZDF no control". The second subset received subcutaneously implanted insulin pellets to maintain insulin levels below 300 mg/dL and was referred to as "ZDF insulin-treated". The third subset received pioglitazone-treated chow obtained from Science Diets (New Brunswick, NJ) and was referred to as "ZDF pioglitazone-treated". The pioglitazone dose delivered per rat was determined by the average amount of chow consumed by a 400 g rat, and was estimated to be 10 mg/kg per day. Rats were kept in their respective group assignments for 3 weeks prior to and 2 weeks following surgery.

Animal surgery

All animal procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, 1996) and approved by the Northwestern University Animal Care and Use Committee. Rats were anesthetized with inhaled isoflurane (0.5–3%). Atropine was administered subcutaneously (0.1 mg/kg) to decrease airway secretions. Weight was documented and blood glucose was measured daily upon administration of STZ. The neck was shaved and prepped with betadine and alcohol (70%). Following a midline neck incision, the rat carotid artery balloon injury model was performed using a 2F Fogarty catheter (generously provided by Edwards Lifesciences) as previously described [8,20]. After injury and restoration of blood flow, 10 mg of the diazeniumdiolate NO donor disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) was applied evenly to the external surface of the injured common carotid artery of rats in the treatment group and the neck incision was closed. This is the same dose of PROLI/NO that was used in our prior studies [20,21]. Treatment groups for all STZ and ZDF group assignments included: (1) injury and (2) injury + PROLI/NO (n = 6–7/treatment group). Carotid arteries were harvested 14 days after injury for morphometric analysis. Blood was collected to measure insulin and glucose levels. Insulin levels were determined using an ELISA-based insulin assay kit (SPI-Bio, Bertin Pharma, France). PROLI/NO was used as the diazeniumdiolate for the *in vivo* experiments as it is the diazeniumdiolate NO donor that we have consistently demonstrated to be superior at inhibiting neointimal hyperplasia compared to other diazeniumdiolates, and is the NO donor used in our prior studies on diabetes [20,21].

Tissue processing

Carotid arteries were harvested following *in situ* perfusion-fixation with cold PBS (250 mL) and 2% paraformaldehyde (500 mL). Vessels were placed in paraformaldehyde at 4 °C for 1 h, then cryoprotected in 30% sucrose at 4 °C overnight. The tissue was quick-

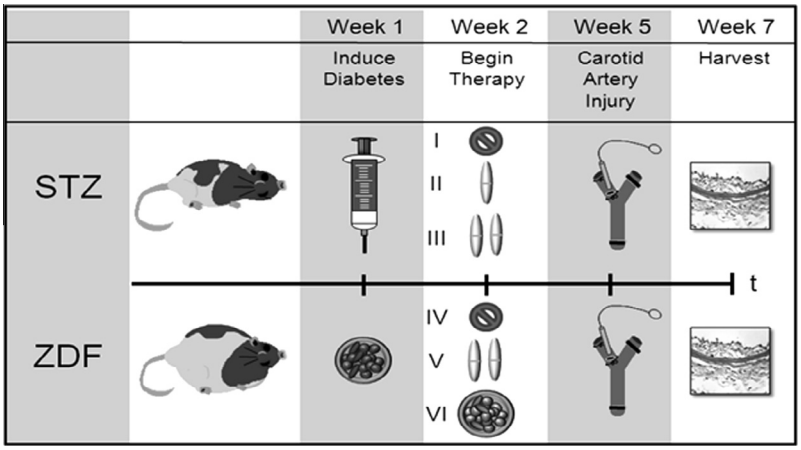

Fig. 1. Study timeline. Type 1 diabetes was induced in LZ rats with a single injection of streptozotocin (STZ). Group assignments included: I – STZ No control; II – STZ moderate control; and III – STZ tight control. Type 2 diabetes was induced in ZDF rats fed Purina 5008 chow. Group assignments included: IV – ZDF no control; V – ZDF insulin-treated; and VI – ZDF pioglitazone-treated.