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Metformin treatment decreases nitroxidative stress, restores nitric oxide bioavailability and endothelial function beyond glucose control

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ABSTRACT

Reduction of nitric oxide (NO), a potent vasodilator, and an increase in cytotoxic peroxynitrite ($ONOO^-$) may be associated with the uncoupling of NO synthase (eNOS) and endothelial cell (EC) dysfunction. In addition to its effect on glucose control, metformin, may also directly benefit in the restoration of the function of eNOS and EC. Obese Zucker rats were administered vehicle or 300 mg/kg/day metformin for 4 weeks. NO concentration [NO] and $ONOO^-$ concentration [$ONOO^-$] were measured in aortic and glomerular endothelial cells from Zucker rats in vitro. Compared with controls, aortic and glomerular endothelial [NO] was reduced by 32% and 41%, while [$ONOO^-$] release increased 79% and 69%, respectively. Metformin treatment increased aortic and glomerular endothelial [NO] by 37% and 57%, respectively, while decreasing [$ONOO^-$] by 32% and 34%, compared with vehicle-treated animals. Treatment with metformin significantly restored the balance in the [NO]/[$ONOO^-$] ratio with 101% and 138% increase for aortic and glomerular endothelial cells, respectively. Fasting glucose levels were not significantly changed. These findings indicate that metformin therapy has a direct and beneficial effect on arterial and renal EC function in obese rats, including enhanced NO release and reduced nitroxidative stress, beyond any effects on fasting glucose levels.

1. Introduction

Patients with type 2 diabetes mellitus (DM) are at high risk for cardiovascular events [1]. Metformin is well established as first line therapy for type 2 DM [2,3]. The benefits of metformin include improved insulin sensitivity and reduced gluconeogenesis [4].

In the United Kingdom Prospective Diabetes Study (UKPDS), treatment with metformin therapy was shown to reduce myocardial infarction in overweight DM patients [5]. The beneficial effects of metformin in the intensive treatment group persisted in the 10-year follow-up study of UKPDS [6]. Additionally, an observational study using the REACH Registry showed metformin therapy was associated with a decrease in mortality among DM patients with atherosclerosis [7].

One of the hallmark features of DM is endothelial dysfunction associated with hyperglycemia and insulin resistance [8]. The healthy endothelium plays an essential role in maintaining vascular integrity, nutrient distribution, platelet and leukocyte adherence, fibrinolysis, coagulation, and inflammation [9]. Endothelial cells produce a number of regulatory mediators crucial to sustaining proper endothelium function, including nitric oxide (NO), endothelin-1, angiotensin II, prostanoids, and cytokines [10]. NO, a potent vasodilator, is produced by the enzymatic conversion of L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS) [11]. In healthy patients, NO maintains vascular function and regulates blood pressure, but in patients with type 2 DM, the bioavailability of NO is significantly lower [12,13]. Although it has been well established that metformin has direct effects on glucose availability in patients with DM, there is evidence that it could improve endothelial function in a glucose-independent manner, as observed in prediabetic populations [14]. The mechanisms for metformin's glucose-independent effects on endothelial dysfunction are undefined, but some data exist. There is evidence that suggests metformin may increase AMP-activated protein kinase-dependent activation of eNOS, which in turn may be linked to its cardiovascular benefits [15–17]. In the present study, we hypothesized that metformin increases NO bioavailability while simultaneously decreasing cytotoxic peroxynitrite ($ONOO^-$), a well-known vasoconstrictor and the main component of nitroxidative stress. We further hypothesize that the effect of metformin cannot be predicted by glucose changes alone. To test this hypothesis, we measured NO and $ONOO^-$ concentrations in aortic and glomerular endothelium using nanosensors in obese Zucker rats maintained on a high-fat diet following treatment with metformin.

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2. Materials and methods

2.1. Animals and materials

All procedures were approved and conducted in accordance with standard Institutional Animal Care and Use Committee guidelines of Ohio University and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Four-week old Zucker rats were obtained from Harlan Laboratories (Indianapolis, IN). The animals were homozygous (*fa/fa*, obese) for the autosomal recessive mutation (*fa*) in the leptin receptor gene on chromosome 5. All animals were housed in a controlled environment that provided food and water ad libitum. Food was provided in the form of a high-fat diet (16% protein, 40% fat, 44% carbohydrates) obtained from Purina TestDiet, Richmond, IN (AIN-76A Purified Diet and AIN-76A Western Diet, respectively). Superoxide dismutase-polyethylene glycol (PEG-SOD); 3-Benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo(4,5-6k) pyrimidine (VAS2870); calcium ionophores (CaI, A23187),) were purchased from Sigma-Aldrich (St. Louis, MO). Metformin was purchased from Toronto Research Chemicals Inc (North York, Ontario, Canada).

2.2. Treatment groups

Male, Zucker lean obese (*fa/fa*) rats were separated into three groups (n = 8 in each group): [1] rats not given treatment and assessed for control measurements; [2] rats on a high-fat diet and treated with vehicle (sterile saline solution) for four weeks; [3] rats on a high-fat diet and treated with metformin (300 mg/kg/day) for four weeks. All rats were anesthetized with ketamine (150 mg/kg, IM), and xylazine (5 mg/kg, IM). Vehicle and metformin were administered orally by gavage. At the end of trial period, animals were euthanized with pentothal (150 mg/kg, IP) and tissue samples obtained immediately for further experimentation. Prior to measuring NO and $ONOO^-$ from aortic and glomerular endothelial cells, the tissue is washed and any residual metformin is removed. This, tissues obtained from both the vehicle and metformin treated animals are tested for NO and $ONOO^-$ release under identical drug-free conditions. Thus, any difference in NO and $ONOO^-$ release is due to the effects of treatment over the study period and not due to acute administration or presence of metformin at the time of measurements.

2.3. Oral glucose tolerance test

Glucose levels were measured using an Accu-Check Compact Plus glucometer (Sanmina-SCI, San Jose, CA). All glucose measurements were preceded by a 6–8 hr fasting period. Test agents (metformin, and vehicle) were administered perorally 4 hr prior to starting the glucose tolerance test. Glucose was administered perorally at 2.0 g/kg. Plasma glucose levels were measured in blood samples collected by tail snip before glucose challenge (0 min) and at 15, 30, 45, 60, 75, 90, 120 and 180 min following glucose administration.

2.4. NO and $ONOO^-$ nanosensors

Concurrent measurements of NO and $ONOO^-$ were performed with tandem electrochemical nanosensors combined into one working unit with a total diameter of 200–400 nm. Their design was based on previously developed and chemically modified carbon-fiber technology [18,19]. Each of the nanosensors was made by depositing a sensing material on the active tip of the carbon fiber (length 4–5 μm, diameter 100–200 nm). The fibers were sealed with nonconductive epoxy and electrically connected to copper wires with conductive silver epoxy. The exposed active tip of fibers were coated with conductive films of polymeric Ni (II) tetrakis (3-methoxy-4-hydroxy-phenyl) porphyrin Mn (III) [2.2] paracyclo-phanylporphyrin (Frontier Scientific) for the NO and $ONOO^-$ sensors, respectively. The polymeric porphyrin was then covered with Nafion for the NO sensor and with poly(4-vinylpyridine) for the $ONOO^-$ sensor.

The amperometric method (with a response time of 10 μs) provided a quantitative signal (current) that was directly proportional to changes (from basal levels) in NO or $ONOO^-$ concentration with a detection limit of about 1×10^{-9} M. The amperometric measurements of NO and $ONOO^-$ were carried out by a three-electrode system, where NO and $ONOO^-$ nanosensors served as working electrodes, a platinum wire (diameter 0.1 mm) as a counter electrode, and a silver/silver chloride (Ag/AgCl) as a reference electrode. The amperometric measurements were performed at 0.65 V for NO and −0.35 V for $ONOO^-$, using a computer-based Gamry VFP600 multichannel potentiostate. Basal NO or $ONOO^-$ levels were measured by differential pulse voltammetry in separate experiments. The differential pulse voltammetry current at the peak potential for NO and $ONOO^-$ was directly proportional to the local concentrations of these compounds in the immediate vicinity of the sensor. For each set of analyses, linear calibration curves (current versus concentration) were constructed for each sensor from 10 nM to 1 μM before and after measurements with aliquots of NO and $ONOO^-$ standard solutions.

2.5. Aortic NO and $ONOO^-$ measurements

Rats were euthanized with pentothal (150 mg/kg, IP) and the aortae removed and placed in modified Hanks' balanced salt solution (4 °C, pH 7.4) containing 0.1 mM L-arginine. All measurements of NO and $ONOO^-$ were performed on intact endothelial cells. Aortic ring segments were isolated and immobilized on Sylgord film in an organ chamber containing fresh, oxygenated Hanks' balanced salt solution at 37 °C. The NO/$ONOO^-$ nanosensor tandem module was positioned near to the surface of a single endothelial cell using computerized remote-controlled micromanipulator (Sensapex SMX, Finland) with x, y, and z space resolution of 1 μm, and a microscope fitted with a CCD camera (AmScope, Irvine, CA).

The measurement of each analyte was performed with a peak current from amperograms and standard calibration curves. After establishing a background current, CaI (1 μM) was injected into the organ chamber using a nanoinjector (World Precision Instruments, Berlin Germany). Rapid changes in current (proportional to the molar concentrations of NO or $ONOO^-$ released) were observed after injecting calcium ionophore A23187. We have previously measured the changes in NO and $ONOO^-$ release in isolated ECs and in animal models of cardiovascular risk [20–22]. We examined the direct effect of the eNOS substrate, L-arginine, superoxide dismutase mimetic, PEG-SOD, and the NAD(P)H oxidase inhibitors, VAS2870, on NO and $ONOO^-$ release from aortic endothelial cells isolated from obese Zucker rats maintained on a high-fat diet for 4 weeks treated with vehicle or metformin. Aortic cells were incubated with these agents for 15 min at 5 mM, 100 U/mL, 10 μM (L-arginine, PEG-SOD, and VAS2870, respectively) followed by CaI stimulation and concurrent measurements of [NO] and [$ONOO^-$].

2.6. Glomerular NO and $ONOO^-$ measurements

Immediately after sacrificing animals as described above, the kidneys were removed, cut into 100 μm sections, and transferred to an organ chamber containing Dulbecco's phosphate buffered saline and 5.6 M glucose at pH 7.4. The NO and $ONOO^-$ nanosensor module was positioned 5 ± 2 μM from the surface of a glomerular EC (cortical zone). All other aspects of NO and $ONOO^-$ measurements were performed as described for aortic endothelial cells above. In a set of separate experiments [NO] and [$ONOO^-$] were measured in the presence of elevated concentrations of L-arginine (5mM), membrane permeable PEG-SOD (100 U/ml) and NADPH inhibitor, VAS 2870 (10 μM).