Metformin Improves Vascular Function in Insulin-Resistant Rats

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Abstract—This study assessed the effect of metformin treatment on insulin, mean arterial pressure (MAP), and endothelial function in insulin-resistant (IR) rats. In addition, we assessed the direct effect of metformin in vitro. Sprague-Dawley rats were randomized to control (n=28) or IR (n=28) groups. Rats were further randomized to receive metformin (300 mg/kg) or placebo for 2 weeks. MAP and insulin were measured. Subsequently, a third-order branch of the superior mesenteric artery was isolated, and endothelial function was assessed. Specifically, dose-response experiments of acetylcholine (ACh) with or without N-nitro- L-arginine (LNNA) were performed. For in vitro experiments, mesenteric arteries were removed from untreated control and IR rats and treated with metformin (100 μmol/L) before ACh±LNNA. MAP and insulin levels were improved in IR-metformin compared with IR-placebo rats. Maximal relaxation (Eₘ₉) to ACh was enhanced in IR-metformin (92±2%) compared with IR-placebo rats (44±4%) (P<0.05). Relaxation in response to ACh+LNNA was greater in IR-metformin (33±4%) than in IR-placebo rats (12±4%) but remained depressed compared with control rats (Eₘ₉=68±5%). The control group was not affected by metformin. In vitro treatment of arteries with metformin in response to ACh produced results similar to those in the experiments with metformin-treated rats. Although metformin improves metabolic abnormality in IR rats, this action does not appear to mediate its effect on vascular function. Both in vivo and in vitro metformin improved ACh-induced relaxation in IR rats to control levels, apparently through nitric oxide–dependent relaxation. These data suggest that metformin improves vascular function through a direct mechanism rather than by improving metabolic abnormalities. (Hypertension. 2000;35:108-112.)

Key Words: insulin resistance • relaxation • metformin • nitric oxide • blood pressure

Excess cardiovascular morbidity and mortality in patients with type 2 diabetes mellitus (non–insulin-dependent diabetes mellitus) is not explained by the presence of traditional cardiovascular risk factors. In addition, glucose control with traditional agents, such as sulfonylureas or insulin, does not alter the risk of macrovascular complications. Epidemiological and observational studies suggest that insulin resistance (IR) may play a role in the development of vascular disease in type 2 diabetes mellitus. Moreover, a recent study has shown that treatment of obese type 2 diabetes mellitus patients with metformin, an insulin-sensitizing agent, improves cardiovascular sequelae. Taken together, these data suggest that IR is an important risk factor for the development of cardiovascular disease. Unfortunately, the underlying mechanism(s) by which IR promotes vascular disease remains unknown. Previous data from our laboratory and others suggest endothelial dysfunction as a possible mechanism linking IR to vascular disease. Specifically, we have shown that endothelium-dependent relaxation is impaired in IR rats because of a defect in nitric oxide (NO)/prostanoid–independent relaxation.

Metformin improves insulin sensitivity, decreases insulin levels, and controls hyperglycemia. In addition, metformin improves lipid profiles and lowers blood pressure in both patients and animal models with impaired glucose tolerance and type 2 diabetes mellitus. In addition to its insulin-sensitizing effects, metformin has also been shown to have direct vascular effects. Thus, it is currently unclear whether the hypotensive effect of metformin is due to a direct vascular effect, its ability to improve insulin sensitivity, its ability to improve lipids, or a combination of mechanisms.

The purpose of the present study was to assess the effect of chronic metformin on insulin, mean arterial pressure (MAP), lipid profile, and endothelial function in IR rats. Second, the acute (in vitro) vascular effects of metformin were compared with chronic feeding to determine whether reversing the metabolic abnormalities induced by IR versus a direct effect of metformin is the principal mechanism of reversing vascular dysfunction.

Methods

The animal care committees at the Medical College of Georgia and the Augusta VA Medical Center approved this protocol. Male Sprague-Dawley rats were randomized to control or IR (n=28) groups. Rats were further randomized to receive metformin (300 mg/kg) or placebo for 2 weeks. MAP and insulin were measured. Subsequently, a third-order branch of the superior mesenteric artery was isolated, and endothelial function was assessed. Specifically, dose-response experiments of acetylcholine (ACh) with or without N-nitro- L-arginine (LNNA) were performed. For in vitro experiments, mesenteric arteries were removed from untreated control and IR rats and treated with metformin (100 μmol/L) before ACh±LNNA. MAP and insulin levels were improved in IR-metformin compared with IR-placebo rats. Maximal relaxation (Eₘ₉) to ACh was enhanced in IR-metformin (92±2%) compared with IR-placebo rats (44±4%) (P<0.05). Relaxation in response to ACh+LNNA was greater in IR-metformin (33±4%) than in IR-placebo rats (12±4%) but remained depressed compared with control rats (Eₘ₉=68±5%). The control group was not affected by metformin. In vitro treatment of arteries with metformin in response to ACh produced results similar to those in the experiments with metformin-treated rats. Although metformin improves metabolic abnormality in IR rats, this action does not appear to mediate its effect on vascular function. Both in vivo and in vitro metformin improved ACh-induced relaxation in IR rats to control levels, apparently through nitric oxide–dependent relaxation. These data suggest that metformin improves vascular function through a direct mechanism rather than by improving metabolic abnormalities.
Sprague-Dawley rats were randomized to control (n=28) or IR (n=28) groups. IR rats were fed a fructose-rich diet starting at 6 weeks of age (Teklad Labs), while control animals received rat chow.

In Vivo Effects of Metformin
After 2 weeks of diet, 16 rats in both the IR and control groups were randomized to receive metformin treatment or placebo in their drinking water. The first 5 days of metformin were used as a titration period in which the dose was started on day 1 as 50 mg/kg and increased each day by 50 mg/kg until it reached a dose of 300 mg/kg per day. This maintenance dose was continued for 14 days. This regimen was based on data in IR rats in which a decrease in blood pressure was observed. Of note, there was no difference in water intake between groups after the first 2 days of treatment. After the 2-week treatment period, rats were sedated with pentobarbital (30 mg/kg IP), and a cannula was placed in the femoral artery and externalized to the back of the neck. After a 24-hour recovery, the cannula was aligned to a transducer (CPXL-23, Statham) for measurement of MAP.

The following day, rats (in a fasting state) were anesthetized (pentobarbital 50 mg/kg IP) and anticoagulated with (heparin 500 U IP). Blood was removed from the left ventricle for biochemical measurements, and a section of small intestine was removed and placed in chilled oxygenated buffer (mmol/L concentration: NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO 4 1.2, KH 2 PO 4 1.2, NaHCO 3 25, dextrose 11.1). A third-order branch of the superior mesenteric artery was isolated and removed. Intraluminal diameter of arteries was measured with nitroprusside.

In Vitro Effects of Metformin
Six control and 6 IR rats were used to evaluate the in vitro effects of metformin on isolated mesenteric arteries. After 4 weeks of diet, mesenteric arteries were removed, and arterial function was assessed as previously described. To evaluate the role of metformin in endothelial-dependent relaxation, mesenteric arteries were incubated in metformin (100 μmol/L) for 30 minutes followed by ACh (10^-6 to 3×10^-4 mol/L) in the presence or absence of LNNA. In addition, a dose-response experiment of metformin (10^-7 to 3×10^-4 mol/L) with or without endothelium was also performed. Endothelial denudation was performed by placing an air bubble in the lumen followed by rolling vessels on a glass pipette. Endothelial disruption was verified by the absence of response to ACh, and viability was tested by nitroprusside.

Biochemical Measurements
Plasma insulin was assayed with the use of a dextran-coated charcoal immunoassay with rat antibody. Glucose concentrations were measured with a Glucose Trinder Kit (Sigma Chemical Co). Lipid concentrations were measured with the Dimension Clinical Chemistry System.

Chemicals
All chemicals except metformin were obtained from Sigma Chemical Co. Metformin was graciously donated by Bristol-Myers Squibb Laboratories.

Data Analysis
Statistical differences for MAP and biochemical measurements were calculated with an unpaired t test. Statistical comparisons for concentration-response experiments were performed by repeated-measures ANCOVA followed by a Fisher’s pairwise least significant difference test for multiple comparisons. Data are reported as mean±SEM. The criterion for significance was P<0.05.

Results
In Vivo Effects of Metformin
MAP, fasting insulin, and triglycerides were significantly elevated in IR-placebo compared with control-placebo rats, whereas HDL was significantly lower (Table). Metformin treatment of IR rats decreased MAP and fasting insulin compared with IR-placebo rats, but these remained elevated compared with control. In contrast, metformin treatment modified both the triglyceride and HDL concentrations to control levels. Metformin had no effect on control rats. Metformin serum concentrations were not measured in the present study; however, the concentration of metformin achieved from this dose is ~3 times greater than the upper limit of the therapeutic range in humans.

Percent arterial constriction was similar between groups (43±2% for control and 42±2% for IR rats). ACh-induced relaxation was similar between arteries in metformin-treated or placebo-treated control animals (Figure 1). Similarly, ACh-induced relaxation after LNNA pretreatment did not differ between the control groups (Figure 2). In contrast, in vivo metformin treatment markedly enhanced relaxation to ACh in the IR-metformin group compared with the IR-placebo group (Figure 1). Relaxation after LNNA was also enhanced in the IR-metformin group, but this was only significant at the highest concentration (33±4% in IR-metformin group versus 12±4% in IR-placebo group;
P<0.05) (Figure 2). Of note, ACh-induced relaxation with LNNA in the IR-metformin group remained depressed compared with control with LNNA (Figure 2).

**In Vitro Effects of Metformin**

The in vitro metformin concentration (100 μmol/L) was based on approximate levels we achieved in vivo. Pretreatment with metformin (100 μmol/L) did not alter basal arterial diameter in either group. Percent arterial constriction was similar between groups (41±3% for control and 41±2% for IR groups). Relaxation in response to ACh was similar between control groups with or without metformin (100 μmol/L) (Figure 3). Likewise, relaxation in response to ACh with LNNA pretreatment was similar between control groups with or without metformin (100 μmol/L) (Figure 4). In contrast, metformin (100 μmol/L) pretreatment of IR arteries enhanced ACh-induced relaxation compared with IR arteries without metformin (Figure 3). However, ACh-induced relaxation after LNNA pretreatment was similar between IR groups either with or without metformin (100 μmol/L) (Figure 4).

The dose-response experiments with metformin (10^-7 to 3×10^-3 mol/L) did not differ between control and IR groups (Figure 5). In addition, removal of the endothelium did not change the concentration response to metformin in either group (Figure 5).

**Discussion**

In the present study, we hypothesized that metformin treatment would decrease insulin, lower MAP, and improve endothelial function. Indeed, we have shown that fasting insulin, lipid profile, and MAP were improved after 2 weeks of metformin treatment in IR rats. Moreover, ACh-induced relaxation in mesenteric arteries from IR rats was restored to control levels after a 2-week treatment with metformin. In contrast, NO-independent relaxation remained impaired compared with control, suggesting that the improvement in endothelium-dependent relaxation was due to an increase in NO. This is of interest because the mechanism for impaired endothelial function in this arterial bed is due to an NO-independent factor. In a separate set of experiments, we determined the in vitro effect of metformin on endothelial...
function by incubating arteries from untreated animals with metformin before ACh. Again, we found that the endothelium-dependent relaxation was reversed, but NO-independent relaxation was not affected by in vitro metformin. Finally, we performed a dose-response experiment with metformin and found that at very high concentrations, metformin induces vascular smooth muscle (VSM) relaxation independent of the endothelium. Thus, the similar effect of metformin between in vivo and in vitro administration suggests that the positive effects of metformin on vascular function are due to direct effects on endothelial and VSM function.

Consistent with other studies, we have shown that metformin improves metabolic disorder in IR rats by decreasing insulin and improving the lipid profile. Likewise, we have shown that it has no metabolic effects on control animals. It is likely that these effects on metabolic function are due to the insulin-sensitizing effect of metformin, although enhancing NO may also affect these indices. Metformin treatment also decreased MAP in IR but not in control rats. The mechanism of this effect is unclear; however, because MAP was not affected in control rats and both chronic and acute metformin improved endothelial function, the ability of metformin to reduce MAP may be associated with a direct effect on the endothelium. Conversely, the ability of metformin to lower MAP may be associated with its insulin-sensitizing effect. This hypothesis is supported by the study of Verma et al., in which the ability of metformin to lower blood pressure in IR rats was impaired by insulin administration, suggesting that hyperinsulinemia drives MAP in this model.

We have shown that impaired ACh-induced relaxation in mesenteric arteries from IR rats was reversed to control levels after 2 weeks of in vivo metformin treatment. However, we believe that this is due to a direct vascular effect of metformin since we were able to demonstrate the same result after incubation of the artery with metformin in vitro. In addition, both in vivo and in vitro metformin experiments appear to enhance endothelium-dependent relaxation as a result of an increase in NO-dependent relaxation. The mechanism by which metformin directly enhances NO-induced relaxation is unclear. It is not likely due to a direct effect on the VSM because incubation of the artery with metformin before ACh did not cause relaxation. In addition, in dose-response experiments with metformin, vasodilation only occurred at very high concentrations. Thus, we believe that metformin enhances agonist-stimulated NO production. This is supported by data from Marfella et al., in which metformin pretreatment enhanced L-arginine–induced increases in forearm blood flow in patients with type 2 diabetes mellitus but had no effect when given alone. This may be due to a direct effect of metformin to enhance endothelial intracellular calcium, but this is speculation.

Finally, we showed that high concentrations of metformin can induce direct VSM relaxation. It is likely that this effect is through its effects on calcium handling. Previous experiments have demonstrated that in rat tail artery, high concentrations of metformin (>10 mmol/L) induced vascular relaxation and decreased intracellular calcium.

Thus, it appears from the present study that metformin predominantly has a direct effect on vascular function. Although at higher concentrations metformin directly induces VSM relaxation, at clinically relevant concentrations metformin appears to directly enhance agonist-induced NO-mediated relaxation.

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References

11. Fanghanel G, Sanchez-Reys L, Trujillo C, Sotres D, Espinosa-Campos J. Metformin’s effects on glucose and lipid metabolism in patients


