Metformin, an antidiabetic agent, potentiates insulin action and reduces insulin resistance. We examined the antihypertensive effects and vascular effects of metformin in spontaneously hypertensive rats (SHR). Wistar-Kyoto normotensive (WKY) and SHR were injected with metformin (100 mg/kg) or saline subcutaneously twice daily for 4 weeks. Blood pressure was recorded by a tail-cuff plethysmographic method. Metformin treatment significantly attenuated (P < .05) the increase in blood pressure in metformin treated SHR versus untreated control SHR. At the end of the experimental period of 4 weeks, metformin-treated SHR had a mean blood pressure that was 34 mm lower than that of untreated SHR. Metformin treatment had no significant effect on blood pressure in WKY rats. Treatment of SHR aortic smooth muscle (SM) cells with metformin (2 μg/mL) for 24 h significantly decreased (P < .05) arginine vasopressin- and thrombin-stimulated increase in 

\[Ca^{2+}\],. However, metformin treatment did not have a significant effect on the basal \[Ca^{2+}\]. Incubation of SHR aortic SM cells with OH-L-arginine (25 to 100 μmol/L) for 24 h increased nitrite production in a dose dependent manner. Metformin (5 μg/mL) treatment of SM cells increased nitrite production at all concentrations of OH-L-arginine; however, differences were significant (P < .05) only at 25 and 50 μmol/L OH-L-arginine. These results suggest that metformin may be decreasing arterial pressure in the SHR, at least in part, by attenuating the agonist-stimulated \[Ca^{2+}\] response in SHR vascular smooth muscle cells. Am J Hypertens 1996;9:570–576

**KEY WORDS:** Metformin, spontaneously hypertensive rat, blood pressure, cytosolic calcium, image analysis, vascular smooth muscle cells, nitric oxide, Wistar-Kyoto rat.

Epidemiological and clinical evidence support the concept that there is a strong association between insulin insensitivity, obesity, and hypertension and that there is a direct corre-
has been shown to exert direct effects on isolated arterial segments by attenuating the contractile response to hormone stimulation and membrane depolarization.12-14 Insulin has been shown to attenuate agonist-stimulated increase in cytosolic calcium concentration ([Ca^{2+}]_{i}) in isolated vascular smooth muscle (VSM) cells.15-18 These observations suggest that in insulin-insensitive hypertensive patients, an increase in VSM cell [Ca^{2+}], may be a contributing factor for the increase in blood pressure.19 Indeed, an increase in [Ca^{2+}]_{i} has been observed in erythrocytes of both normotensive and hypertensive subjects with non-insulin dependent diabetes mellitus (NIDDM) compared to the normal subjects.20 The effects of insulin on agonist-stimulated [Ca^{2+}]_{i} are well documented. However, the effects of antidiabetic agents like metformin on the regulation of agonist-stimulated [Ca^{2+}], need to be investigated.

Locally synthesized and secreted nitric oxide (NO) in the blood vessel acts as a potent vasodilator and inhibitor of platelet aggregation.21 NO mediates vasodilatation by increasing intracellular cGMP levels via activation of soluble guanylate cyclase.22 An increase in cGMP levels in VSM cells has been shown to attenuate agonist-stimulated [Ca^{2+}].23 Thus, it is possible that metformin may attenuate agonist-stimulated [Ca^{2+}], either by direct interference with hormone receptor-effector coupling or by lowering [Ca^{2+}], by augmenting VSM cell NO levels. Any one of these mechanisms may, at least in part, explain the antihypertensive actions of metformin.

In this study, we have examined the effects of daily injections of metformin for 4 weeks on blood pressure in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. We have also tested whether metformin has direct effects on VSM cells by measuring the effect of metformin on agonist-stimulated [Ca^{2+}], and OH-L-arginine dependent nitrite production in SHR VSM cells. Our results demonstrate that metformin attenuated the increase in blood pressure in SHR while it had no effect in WKY rats. Our results also indicate that metformin directly interacts with VSM cells to decrease agonist-stimulated [Ca^{2+}], and increase nitrite production in the SHR VSM cells incubated with OH-L-arginine. It is possible that metformin may be producing antihypertensive effects in SHR, at least in part, by attenuating agonist-stimulated [Ca^{2+}], with a concomitant decrease in peripheral vascular resistance.

**MATERIALS AND METHODS**

**Animals and Cultured Arterial Smooth Muscle Cells**

Age matched male SHR and WKY rats (6 to 8 weeks old) were obtained from Harlan (Indianapolis, IN) at least 1 week prior to the experiment, and were fed a standard pellet diet and water ad libitum. SHR and WKY rats were injected with metformin (100 mg/kg) or saline subcutaneously twice a day for 4 weeks. Systolic pressure of control and metformin treated WKY and SHR was determined in the unanesthetized state by the tail plethysmographic method using an automated cuff inflator pulse reading system that utilizes a photoelectric sensor (ITTC, Lending, NJ).24 Prior to recording the blood pressure, rats were accustomed to confinement by putting them in appropriate sized rat holders for 3 consecutive days for 30 min each under a heat lamp. After this conditioning period, the rats were put into holders for 20 to 30 min before blood pressures were measured for both control and metformin treated rats under identical conditions. Systolic pressure was read from the chart readout as the level at which pulsations reappeared during the gradual deflation of the cuff and an average of three to five recordings were taken per rat on a given day. It has been demonstrated that systolic blood pressure measured by a photoelectric sensor is in very close agreement with blood pressure readings taken concurrently from femoral catheters.25

Smooth muscle cells from the thoracic aorta were cultured essentially as described previously.26-27 Cells were subcultured every week. In the studies described in this article, we used cells between the second and ninth passages. The purity of VSM cells was confirmed by immunocytochemical localization of smooth muscle specific actin using monoclonal antibodies raised against the NH2-terminal decapeptide of a smooth muscle α-actin. Using these procedures, it was ascertained that all the cultured cells had actin stress fibers throughout the cytosol suggesting that the procedures used to isolate and culture cells yielded VSM cells free from contamination with endothelial cells and fibroblasts.28-29

**Calcium Imaging in Individual VSM Cells**

Cytosolic free calcium concentration ([Ca^{2+}]_{i}) was measured using Fura 2 and a video microscopic digital image analysis system (Photon Technology International, South Brunswick, NJ) to analyze the calcium responsiveness of individual cells.26-28 Semiconfluent VSM cells grown on 25-mm coverslips were loaded with calcium specific dye Fura 2 by incubating with Fura 2-acetoxyethyl ester (10 μmol/L) in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% bovine serum albumin (BSA) and 0.02% pluronic F-127 for 45 to 50 min at 37°C. After washing once with DMEM-BSA, cells were reincubated in DMEM-BSA for 30 min at 37°C to allow complete hydrolysis of Fura 2-acetoxyethyl ester to Fura 2 inside the cell. Fura 2 loaded cells displayed stable and bright fluorescence at 340-, 360-, and 380-nm excitation, whereas unloaded cells had no detectable autofluorescence. Photo bleaching of Fura 2 loaded cells was kept to a minimum by using low intensity excitation light and a computer controlled...
shutter that allowed exposure of cells to light only when images were collected. Intracellular calcium concentration was measured at 37°C in N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-buffered (25 mmol/L, pH 7.4) Eagle's salt solution containing 0.1% BSA and 10 mmol/L sodium bicarbonate.

Agonists were added as twice concentrated solutions to yield the desired final concentration. To test the effect of metformin, cells were pretreated with indicated concentrations of metformin 24 h before the calcium measurements. Metformin was also present during all subsequent steps of Fura 2 loading and [Ca²⁺], measurement. [Ca²⁺] changes in response to agonists were computed from recorded images using a defined mask and the average [Ca²⁺], computed in the 50 × 50 pixel area in the center of the cell.

Nitrite Assay For nitrite production assays, SHR VSM cells were grown to confluence in 12-well culture dishes. Treatments were carried out for 24 h in phenol red free and arginine-free media (Gibco, Grand Island, NY; DrK: phenol red free MEM select amine kit) supplemented with metformin (5 μg/mL) and/or OH-L-arginine (25 to 100 μmol/L). Nitrite was measured spectrophotometrically using Griess reagent and expressed as μmol/L NO₂⁻/mg protein. Briefly, 500 μL of culture media was mixed with 50 μL each of 5 mmol/L sulfanilamide, 2 N HCl, and 6 mmol/L N-(1-naphthyl)-ethylenediamine, incubated at room temperature for 10 min, and the absorbance of the resulting color product read at 546 nm. After removing the media for the nitrite assay, the cells were washed once each with chilled H₂O and 70% ethanol and allowed to air dry. Proteins were solubilized by adding 500 μL of 2 N NaOH into each well and incubating the culture dishes overnight at room temperature with constant shaking. Proteins were assayed in microtiter plates using BioRad (Richmond, CA) protein reagent and reading absorbance in a microtiter plate reader at 595 nm.

Statistical analysis Statistical analysis was carried out using Student's t test or one-way analysis of variance (ANOVA). Differences between the treatment groups were considered significant at P < .05.

RESULTS

Over the 4 weeks of the study, the body weight of metformin treated and metformin untreated control rats did not differ either for SHR (control = 339 ± 3 g, n = 11; treated = 333 ± 3 g, n = 10) or WKY (control = 532 ± 8 g, n = 12; treated = 515 ± 8 g, n = 11) rats. The blood pressure of metformin-treated SHR remained steady between 165 and 170 mmHg after the start of treatment with metformin, whereas the blood pressure of untreated SHR rose to 200 mmHg from the starting blood pressure of 175 mmHg (Figure 1). This pattern of change in blood pressure with increasing time of treatment between control and metformin treated SHR was significantly different (P = .02). Moreover, the pattern of change in blood pressure with time in control and metformin treated SHR was also significantly different (P = .005) as compared to control and metformin treated WKY rats. Metformin treatment apparently decreased (P < .05) the blood pressure in SHR within 1 week as compared to untreated SHR; however, with repeated measures analysis of variance, the divergence was significant at 2 weeks and thereafter (P < .05, with Bonferroni adjusted alpha) (Figure 1). At the end of 4 weeks, metformin-treated SHR had a mean blood pressure that was 34 mm lower than that of untreated SHR. Metformin treatment had no significant sustained effect on blood pressure in WKY rats (Figure 1).

Resting [Ca²⁺], was not significantly altered in cells treated with 2 μg/ml. metformin compared to the untreated cells (Figure 2). In response to stimulation with arginine vasopressin (AVP) or thrombin there was a significant increase (P < .05) in [Ca²⁺], compared to the resting [Ca²⁺]. The spatial cellular distri-
bution of the agonist-stimulated [Ca^{2+}] \_i did not differ between control and metformin-treated cells judged by pseudocolor presentation. However, quantitative differences were noted in the responsiveness of cells to the agonist stimulation after treatment with metformin (Figure 2). Peak [Ca^{2+}] \_i was taken as the maximum increase in [Ca^{2+}] \_i, after addition of the agonist, independent of time. Treatment of cells with metformin (2 \mu g/mL) significantly attenuated (P < .05) peak [Ca^{2+}] \_i in response to AVP and thrombin stimulation (Figure 2). Delta change in [Ca^{2+}] \_i was calculated for each cell after subtracting resting [Ca^{2+}] \_i from the peak [Ca^{2+}] \_i. Delta change in [Ca^{2+}] \_i was also significantly decreased (P < .05) in metformin-treated cells compared to the untreated control cells (Figure 2). These results demonstrate that metformin significantly decreases agonist-stimulated [Ca^{2+}] \_i, in SHR VSM cells. In addition, metformin treatment significantly decreased (P < .05, Fisher exact test) the number of responsive cells to AVP and thrombin compared to the control group (72% vs 93% for AVP and 86% vs 99% for thrombin). The cells were considered responsive if a spike of 20 nmol/L [Ca^{2+}] \_i was observed after treatment with the agonist. This criteria is important in discriminating a slow drift in [Ca^{2+}] \_i (2 to 3 nmol/L between consecutive measurements) which is not due to agonist addition.

Incubation of SHR VSM cells with different concentrations of OH-t-arginine for 24 h increased nitrite production in a concentration dependent manner (Figure 3). The addition of metformin (5 \mu g/mL) along with OH-t-arginine further increased nitrite production at all concentrations of OH-t-arginine tested and the differences were significant (P < .05) at 25 and 50 \mu mol/L OH-t-arginine (Figure 3).

**DISCUSSION**

The results presented in this article demonstrate that the administration of metformin for 4 weeks significantly decreased (P < .05) blood pressure in SHR but not in WKY normotensive rats. The cardiovascular effects of metformin have been amply described in the literature. Stern et al showed that the injection of 50 and 100 mg/kg metformin in dogs produced a very rapid hypotension that reached its maximum within 30 min. The hypotensive action of metformin was not blocked by atropine, and metformin enhanced the hypotension produced by acetylcholine. These observations suggest a direct effect of metformin on the blood vessel wall independent of the endothelium. Further, metformin pretreatment attenuated the hypertensive action of epinephrine and hampered the rise in blood pressure produced by bilateral coronary artery occlusion. Similar to our findings, metformin administration for 1 week has been shown to lower blood pressure in spontaneously hypertensive rats but not in WKY normotensive rats. Chan et al have shown that in 12 normotensive NIDDM patients, metformin treatment for 4 weeks resulted in a reduction of body weight, plasma cholesterol concentration, and erect diastolic blood pressure. Similarly, Landin-Wilhelmsen, in a pilot study, has shown that metformin treatment of nonobese, nondiabetic, hypertensive patients improved insulin sensitivity, decreased plasma insulin, serum cholesterol, and triglycerides, and mildly decreased blood pressure. However, these findings in hypertensive patients have yet to be confirmed by other investigators. In addition, metformin was shown to improve cardiac performance in streptozotocin diabetic rat, by improving the relaxation rate at increasing preload. All these observations taken together contribute to the concept that metformin improves cardiovascular abnormalities associated with NIDDM and hypertension that could be due to its improved cardiac performance in streptozotocin diabetic rat.
on 25 May 2018
by guest
Downloaded from https://academic.oup.com/ajh/article-abstract/9/6/570/194908
direct action on arterial smooth muscle. However, recently Zhang et al have shown that while metformin improves glucose tolerance, it has no significant effect on blood pressure or glucose clearance rate in either Dahl S or one kidney, one clip hypertensive rats.32

Our results have also demonstrated that metformin significantly attenuated AVP- and thrombin-stimulated increase in [Ca2+], in SHR aortic smooth muscle cells. These results suggest that metformin interacts directly with VSM cells to produce vascular responses. The concentration at which metformin was effective in inhibiting agonist-stimulated calcium transients was similar to that found in the serum of diabetic patients treated with metformin.33 We have shown that both basal and agonist-stimulated [Ca2+], is increased in SHR VSM cells compared to WKY cells.34 Similarly, other investigators have reported an increased basal [Ca2+], in SHR VSM cells compared to WKY.35 Thus, it is possible that changes in both basal and agonist-stimulated [Ca2+], may play an important role in the increased vascular reactivity and peripheral resistance in SHR. However, in the present study, we did not find a decrease in basal [Ca2+], after metformin treatment, while agonist-stimulated [Ca2+], was significantly attenuated. Lack of metformin effect on basal [Ca2+], would suggest that similar to insulin, metformin only attenuates agonist-stimulated [Ca2+],. Recently, it has been shown that insulin attenuation of agonist-stimulated [Ca2+], is decreased in primary cultures of SHR VSM cells suggesting insulin resistance of VSM cells.36 Attenuation of agonist stimulated [Ca2+], by metformin supports the idea of insulin resistance of SHR VSM cells and would suggest that metformin may be improving insulin sensitivity of VSM cells and that it may also mimic insulin action. It is well accepted that insulin inhibits VSM contractions possibly by attenuating agonist-stimulated increase in [Ca2+],.37 Our data suggest that the vascular effects of metformin are similar in nature to insulin and are possibly mediated by the attenuation of agonist-stimulated [Ca2+], in VSM cells. However, there may not be a tight one to one correlation between changes in VSM [Ca2+], NO production, and blood pressure regulation by metformin.

The mechanisms by which metformin attenuates agonist-stimulated [Ca2+], in SHR VSM cells are not fully understood. We have observed that metformin increases NO production from OH-L-arginine in VSM cells. It has been shown that OH-L-arginine is converted to NO and citrulline in VSM cells by cytochrome P-450 dependent pathway in the absence of NO synthase activity.39 Our results demonstrate that OH-L-arginine can enter the vascular smooth muscle cells where it is oxidized to NO and nitrite is produced, and that this phenomenon is augmented by metformin. Thus, OH-L-arginine released from the endothelial cells may exert effects on the vascular smooth muscle cells by paracrine mechanisms. Whether metformin increases the transport of OH-L-arginine or increases the activity of cytochrome P-450 needs to be further examined. The increased NO production has been shown to decrease [Ca2+], via cGMP pathway in VSM cells.33 It is possible that metformin-mediated increase in NO production may result, at least in part, in a decrease in [Ca2+],. Alternately, it is possible that metformin may inhibit the receptor effector coupling or downstream receptor signaling events resulting in a decrease in [Ca2+], and that the effects of metformin on agonist-stimulated [Ca2+], and NO production from OH-L-arginine may be coincidental findings that may or may not be related to each other.

Another class of antidiabetic drugs, thiazolidinediones, has been shown to decrease blood pressure in genetically hypertensive rats36,37 and to decrease cytosolic calcium in VSM cells. Thus, ciglitazone, which lowers blood pressure in the spontaneously hypertensive rat, also decreases PDGF-stimulated increase in [Ca2+], in the a75 aortic smooth muscle cell line.37 More recently, pioglitazone has been shown to inhibit L-type calcium channels in the a75 cell line, as well as in freshly isolated rat caudal artery smooth muscle cells.38 These findings are consistent with the notion that insulin and insulin sensitizing drugs may regulate arterial pressure via inhibiting agonist-stimulated calcium transients in VSM cells.

In summary, data presented in this article show that metformin at therapeutic concentrations attenuates the increase in blood pressure in spontaneously hypertensive

FIGURE 3. Effect of metformin on nitrite production from OH-L-arginine in SHR aortic smooth muscle cells. VSM cells were incubated with the indicated concentrations of OH-L-arginine in the presence or absence of metformin (5 μg/mL) for 24 h. Nitrite levels in the media were then determined as described in the Materials and Methods section. Data are given as the mean ± SEM of the number given in parentheses. Statistical analysis of data was done using one-way ANOVA. * denotes significant increase (P < .05) in nitrite levels in metformin treated cells compared to the untreated control cells.
rats. To explain the blood pressure lowering effects of metformin in spontaneously hypertensive rats, we would like to suggest that metformin directly interacts with VSM cells to lower agonist-stimulated [Ca$^{2+}$]. Metformin also augments NO production from OH-L-arginine in SHR VSM cells. These data would suggest that the observed hypotensive effects of metformin in NIDDM hypertensive patients may be due, at least in part, to an inhibition of agonist-stimulated [Ca$^{2+}$], in VSM cells.

ACKNOWLEDGEMENTS
We wish to thank Mr. Kice Brown, Division of Biostatistics, Department of Preventive Medicine and Environmental Health for assistance with statistical analysis of the data.

REFERENCES