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RESEARCH PAPER

Increased availability of angiotensin AT_1 receptors leads to sustained arterial constriction to angiotensin II in diabetes – role for Rho-kinase activation

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BACKGROUND AND PURPOSE

Antagonists of angiotensin AT_1 receptors elicit beneficial vascular effects in diabetes mellitus. We hypothesized that diabetes induces sustained availability of AT_1 receptors, causing enhanced arterial constriction to angiotensin II.

EXPERIMENTAL APPROACH

To assess functional availability of AT_1 receptors, constrictions to successive applications of angiotensin II were measured in isolated skeletal muscle resistance arteries (~150 μm) of Zucker diabetic fatty (ZDF) rats and of their controls (+/Fa), exposed acutely to high glucose concentrations (HG, 25 mM, 1 h). AT_1 receptors on cell membrane surface were measured by immunofluorescence.

KEY RESULTS

Angiotensin II-induced constrictions to first applications were greater in arteries of ZDF rats (maximum: 82 ± 3% original diameter) than in those from +/Fa rats (61 ± 5%). Constrictions to repeated angiotensin II administration were decreased in +/Fa arteries (20 ± 6%), but were maintained in ZDF arteries (67 ± 4%) and in +/Fa arteries vessels exposed to HG (65 ± 6%). In ZDF arteries and in HG-exposed +/Fa arteries, Rho-kinase activities were enhanced. The Rho-kinase inhibitor, Y27632 inhibited sustained constrictions to angiotensin II in ZDF arteries and in +/Fa arteries exposed to HG. Levels of surface AT_1 receptors on cultured vascular smooth muscle cells (VSMCs) were decreased by angiotensin II but were maintained in VSMCs exposed to HG. In VSMCs exposed to HG and treated with Y27632, angiotensin II decreased surface AT_1 receptors.

CONCLUSIONS AND IMPLICATIONS

In diabetes, elevated glucose concentrations activate Rho-kinase which inhibits internalization or facilitates recycling of AT_1 receptors, leading to increased functional availability of AT_1 receptors and sustained angiotensin II-induced arterial constriction.

Abbreviations

HG, high glucose concentration; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PKC, protein kinase C; RAS, renin–angiotensin system; VSMCs, vascular smooth muscle cells; ZDF, Zucker diabetic fatty

Introduction

The systemic and vascular renin–angiotensin systems (RAS) play important roles in regulating vasomotor tone and arterial blood pressure. Several earlier clinical studies demonstrated the effectiveness of antagonists of angiotensin AT_1 receptors (receptor nomenclature follows Alexander *et al.*, 2009) in the treatment and/or prevention of vascular

complications related to diabetes mellitus (Mathur *et al.*, 2007). Enhanced AT_1 receptor signalling elicits increased vasomotor tone and peripheral vascular resistance and, in chronic conditions, it could contribute to diabetes-related vascular remodelling, pathological alterations and cardiovascular morbidity. Thus, it is possible that diabetes alters the function of AT_1 receptors in the vessel wall, although the exact mechanisms are not entirely understood.

In this context, an augmented angiotensin II-induced vascular contractility has been reported in arteries of animals with type 1 and type 2 experimental diabetes. For example, in streptozotocin-induced diabetic rats an increased contraction of the thoracic aorta to angiotensin II has been demonstrated by Arun *et al.* (2005). Similarly, others have found an enhanced angiotensin II-induced contraction in the aorta of obese Zucker rats (Nishimatsu *et al.*, 2005; Siddiqui and Hussain, 2007) and also in db/db mice, known experimental models of type 2 diabetes (Guo *et al.*, 2005). It has been proposed that alterations in downstream cell signalling mechanisms, such as increased calcium sensitivity of vascular smooth muscle cells (VSMCs), may be responsible for the enhanced vascular contraction to angiotensin II (Nishimatsu *et al.*, 2005). Other studies demonstrated that augmented release of constrictor prostanoids (Guo *et al.*, 2005) and/or an excess production of reactive oxygen species in VSMCs (Viswanad *et al.*, 2006) play a key role in the augmented, angiotensin II-induced contractility. Mechanism(s) by which diabetes alters the function of AT_1 receptors have received less attention and remained unclear.

It has been suggested that the augmented angiotensin II-mediated vascular responsiveness is due to increased expression of AT_1 receptors in the aorta of diabetic rats (Siddiqui and Hussain, 2007). However, a study by Nishimatsu *et al.* has found no significant changes in AT_1 receptor expression in the aorta of diabetic rats, although an enhanced angiotensin II-induced vascular contractility was still detected (Nishimatsu *et al.*, 2005). To solve this discrepancy, in this study we have raised the hypothesis that, without changes in its expression level, an increased availability of AT_1 receptors on the cell surface plays the major role in the enhanced angiotensin II-induced vasoconstriction in diabetes. It is known that upon stimulation by angiotensin II, AT_1 receptors are rapidly desensitized and then internalized, becoming unavailable for further stimulation (Hunyady *et al.*, 2000). This negative-feedback regulation of AT_1 receptor availability is an important physiological mechanism to prevent prolonged effects of angiotensin II. This mechanism can be followed on the functional level. On first application, angiotensin II, via the AT_1 receptors, elicits pronounced vasoconstriction, which however is markedly diminished upon repeated application. This phenomenon is known as tachyphylaxis to angiotensin II (Juul *et al.*, 1987; Vicaut *et al.*, 1989). This normal vasoregulatory mechanism could be altered in pathological conditions, such as diabetes. Our recent study demonstrated that even a short-term (30 min) exposure of arteries to hydrogen peroxide, elicited sustained angiotensin II-induced arterial constriction, without changing AT_1 receptor expression and also without affecting downstream contractile signalling, such as calcium sensitization (Bagi *et al.*, 2008). Because we have previously demonstrated that diabetes (Bagi and Koller, 2003; Bagi *et al.*, 2003; 2004a) and acute elevation in glucose concentration (Bagi *et al.*, 2004b) are both accompanied by enhanced vascular production of reactive oxygen species, we have proposed that high glucose concentration alters the functional availability of the AT_1 receptors and reduces angiotensin II tachyphylaxis in diabetes.

Furthermore, it has been suggested that activation of Rho-kinase is involved in the augmented agonist-induced vasoconstriction in obese Zucker rats (Naik *et al.*, 2006). It is known that various Rho family members are involved in the regulation of intracellular vesicle trafficking (Schmalzing *et al.*, 1995; Symons and Rusk, 2003). Because Rho-kinase has found to be directly activated by high glucose concentrations (Naik *et al.*, 2006), we have also raised the possibility that Rho-kinase signalling plays an important role in the altered regulation of AT_1 receptor function.

However, the potential impact of Rho-kinase on trafficking of AT_1 receptors has not yet been demonstrated. Elucidating the underlying mechanism(s) that are responsible for augmented angiotensin II-induced vasoconstriction and also leads to enhanced surface availability of AT_1 receptors is important, because these alterations could contribute not only to the enhanced vasomotor tone but other, angiotensin II-mediated pathological abnormalities in the vasculature in diabetes mellitus.

Methods

Isolation of skeletal muscle arteries and experimental protocols

All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee at New York Medical College, Valhalla. We used 12-week-old hyperglycaemic Zucker diabetic fatty (ZDF) and normoglycaemic control +/Fa rats (n = 15 of each strain). The ZDF rats exhibit homozygous mutation in the leptin receptor gene and develop hyperlipidaemia, hyperglycaemia and diabetes by 12 weeks, while their heterozygous controls, the +/Fa rats show normal phenotype. Rats were anesthetized with pentobarbital sodium (50 mg·kg^{-1}, i.p.). Under anaesthesia, the gracilis muscles were excised and placed in ice-cold, oxygenated Krebs solution. Animals were killed with additional pentobarbital sodium (150 mg·kg^{-1}, i.p.). With the use of microsurgical instruments and an operating microscope, the third-order branches of femoral artery (~1.5 mm in length and ~150 μm in internal diameter) of rats were isolated and cannulated, as described previously (Huang *et al.*, 1993; Koller and Huang, 1994; 1999; Huang and Koller, 1997). The cannulated small arteries were connected with silicone tubing (filled with normal Krebs solution) to a pressure servo control system (Living Systems Instrumentation, VT, USA) in order to set intraluminal pressure to 80 mmHg, at zero intraluminal flow rate. The whole preparation was continuously superfused with Krebs solution with a flow rate of 10 mL·min^{-1} and the temperature was maintained at 37°C by a circulating bath temperature controller (Cole Parmer, USA). The internal diameter at the midpoint of the isolated arteries was continuously measured with videomicroscopy.

In the first series of experiments cumulative concentrations of angiotensin II (10 pM–10 nM; Sigma) and

noradrenaline (1–100 nM; Sigma) were administered to the superfusion solution (final concentrations are reported) and changes in diameter of arteries from control, +/Fa and ZDF rats were continuously measured. Thirty minutes after washout, agonist-induced responses were repeated (second applications). In selected protocols, after the second application, angiotensin II was applied again in a similar fashion (third application) to reveal if further applications cause changes in the vasomotor responsiveness.

In separate protocols, repeated agonist (angiotensin II and noradrenaline)-induced responses were also obtained in vessels from +/Fa rats, which were exposed to high glucose concentration (25 mM glucose in the superfusion solution for 1 h) and vessels were kept in this high glucose condition during the repeated administration of agonists. To reveal whether the effect of high glucose on angiotensin II-induced responses were reversible we performed additional protocols, in which after the 1 h high glucose exposure, superfusion solution was changed back to normal, 5.5 mM glucose containing physiological salt solution for an additional 1 h and repeated angiotensin II-induced vascular responses were obtained.

In other sets of experiments, repeated agonist-induced vasomotor responses were obtained in the presence of Y27632 (1 μM, for 30 min; Sigma), a known inhibitor of Rho-kinase (Naik *et al.*, 2006) both in vessels of ZDF and in those from normal rats, exposed to high glucose.

Rho-kinase activation assay

Assay of Rho-kinase was performed as described previously (Galaria *et al.*, 2004; Lee and Ragolia, 2006) using the Cyclex Rho-kinase activity assay as instructed by the manufacturer (Cyclex Co. Ltd). Briefly, first branches of the femoral artery were homogenized in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.25% deoxycholic acid, 1% NP-40 and 1 mM EDTA) enriched with 1 mM phenyl methyl sulphonyl fluoride, 1 $\mu g \cdot mL^{-1}$ leupeptin, 1 $\mu g \cdot mL^{-1}$ aprotonin, 1 $\mu g \cdot mL^{-1}$ pepstatin and 1 mM Na_3VO_4. Protein concentration was determined (Pierce Chemical, Rockford, IL, USA). Rho-kinase activity was measured based on the phosphorylation of threonine-695 of MYPT1 with tissue lysates. Equal amounts of arterial lysates were incubated with each substrate precoated on 96-well plates. Phosphospecific antibody labelled with horseradish peroxidase was then incubated for 1 h before the addition of 3,3′,5,5′-tetramethylbenzidine substrate. The developed colour was quantified by spectrophotometry and represented the relative amount of Rho-kinase activity in the sample. The Rho-kinase inhibitor, Y27632 (10 μM) was used to inhibit Rho-kinase activity in each experiment.

Assessment of cell surface AT_1 receptors in cultured VSMCs

The VSMCs (SV40LT-transfected cells, purchased from American Cell Type Culture Collection) were cultured under condition indicated by the provider. After passage 4, cells were cultured in six-well plates in normal (5 mM) or high glucose (25 mM) containing media for an additional 24 h. Cells were exposed to angiotensin II (100 nM) for 10 min, washed in ice-cold phosphate buffer solution and fixed with 5% formaldehyde. Surface AT_1 receptors were labelled with an anti-AT_1 receptor antibody that binds only to the extracellular region of the receptor (1:100, BML-SA608, Biomol, USA). Immunofluorescent detection was performed with Alexa488-labelled secondary antibody (Invitrogen, Carlsbad, CA, USA). For non-specific binding, the primary antibody was omitted. Fluorescence was directly measured with an electron multiplying charge-coupled device camera (Luca^{EM}-S, Andor, UK) connected to an Olympus BX61 microscope. Y27632 (10 μM) was used to inhibit Rho-kinase activity in VSMC cultures.

Data analysis

Data are expressed as means ± SEM Agonist-induced constrictions were expressed as percent changes of the initial arterial diameter at 80 mmHg pressure. Myogenic tone of arteries was calculated from the active diameter (in Ca^{2+}-containing Krebs solution), expressed as percent of the corresponding passive diameter (in Ca^{2+}-free Krebs solution). Statistical analyses were performed using GraphPad Prism 5 Software (San Diego, CA, USA) by repeated measures ANOVA followed by Tukey's *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Repeated administration of angiotensin II to assess the functional availability of AT_1 receptors in isolated arteries

In this study, we first demonstrated that sequential administration of cumulative concentrations of angiotensin II (two applications 30 min apart) resulted in reduced constrictions of arteries in the +/Fa rats (Figure 1), whereas constrictions to two successive applications of noradrenaline showed no tachyphylaxis (Table 1). There was no further reduction to the third application of angiotensin II (maximum constrictions to first, second and third applications of angiotensin II were: 59 ± 4%, 23 ± 5% and 27 ± 5% respectively). Removal of endothelium did not significantly affect the repeated vasoconstrictions to angiotensin II or noradrenaline (data not shown).

Augmented and sustained angiotensin II-induced arterial constrictions in diabetes

In this study, we have used a known experimental model of diabetes, the ZDF rats, which have hyperglycaemia. In non-fasted 12-week-old ZDF rats, there was a fourfold increase in glucose levels compared with normoglycaemic, control, non-fasted +/Fa rats (32.4 ± 3.1 mM vs. 7.6 ± 1.1 mM respectively). In isolated skeletal muscle arteries from ZDF rats, a spontaneous tone developed in response to 80 mmHg intraluminal pressure. The magnitude of pressure-induced myogenic tone was significantly greater in ZDF arteries (at 80 mmHg: 42 ± 2% of passive diameter), compared with control, +/Fa arteries (31 ± 6%).

In arteries from ZDF rats, constrictions to the first application of cumulative concentrations of angiotensin II were greater than those in arteries of control +/Fa rats (Figure 1). Moreover, compared with the arteries of +/Fa rats, angiotensin II-induced constrictions in ZDF arteries were maintained on the second application of angiotensin II (Figure 1).

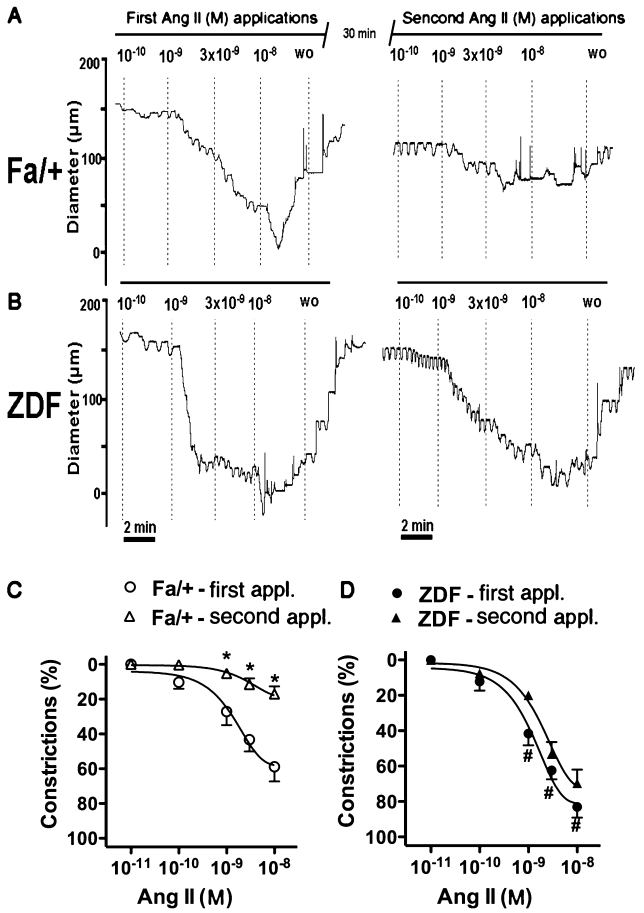

Figure 1

Original records (A,B) and summary data (C,D) of constrictions (%) to repeated applications of angiotensin II (Ang II; 10 pM–10 nM, $n = 9$) in skeletal muscle arteries from control heterozygous (+/Fa, $n = 7$) or Zucker diabetic fatty (ZDF, $n = 7$) rats. Data are means ± SEM. *$P < 0.05$, significantly different from first application. # indicate differences from control (+/Fa).

Arterial constrictions to noradrenaline were not significantly increased in arteries from ZDF rats nor was there any tachyphylaxis (Table 1).

Effects of high glucose concentration on angiotensin II-induced arterial constrictions

To test the hypothesis that, in diabetes the high concentration of plasma glucose is the underlying cause, leading to the augmentation of vascular constrictions to angiotensin II, arteries from normal +/Fa rats were exposed to high glucose concentration (25 mM for 1 h). After this incubation and in the presence of high glucose solutions, arterial constrictions to the first application of angiotensin II were augmented at the highest angiotensin II dose applied (Figure 2). Importantly, these constrictions were maintained in response to the second (Figure 2) and third applications of angiotensin II (maximum constriction: 67 ± 6%, $n = 5$, not significantly different from that of first response), compared with arteries exposed to normal level of glucose. In contrast, noradrenaline-induced constrictions were not significantly affected by high glucose concentrations (Table 1).

In additional protocols, we have also tested the reversibility of the effect of high glucose on the sustained angiotensin II response. To this end, after 1 h of exposure to high glucose, the arteries were returned to normal (5.5 mM) glucose solutions for another hour and then the effects of repeated doses of angiotensin II tested. Interestingly, even after this time at normal glucose concentrations, constrictions were still maintained to repeated applications of angiotensin II (maximum constrictions to first and second applications of angiotensin II: 65 ± 7% and 54 ± 5%, respectively, $n = 4$, $P > 0.05$).

Role of Rho-kinase activation in the enhanced and sustained angiotensin II-induced vasoconstrictions in diabetes

First, the level of the Rho-kinase activation was assessed in homogenates of branches of femoral arteries of +/Fa and ZDF rats and in arteries from +/Fa rats, exposed *in vitro* to high glucose concentration (25 mM for 1 h) by Rho-kinase assay (Cyclex). Samples from ZDF rats and those from normal arteries treated with high glucose concentrations exhibited increased Rho-kinase activity (Figure 3), compared with samples from normal arteries in normal glucose solutions. Rho-kinase activity in arteries from ZDF rats and in those of high glucose exposed normal vessels was fully inhibited by the selective Rho-kinase inhibitor, Y27632 (10 µM) (Naik *et al.*, 2006; data not shown).

To assess a functional role for enhanced Rho-kinase in augmenting the vascular availability of AT_1 receptors, angiotensin II-induced constrictions were measured in the presence of Y27632, Incubation of arteries with Y27632 (1 µM) reduced the pressure-induced myogenic tone in both ZDF (to 28 ± 4%) and Fa/+ rats (to 16 ± 5%), but these reduced levels of myogenic tone remained significantly different between the two groups.

In arteries of control, +/Fa rats, incubation with Y27632 did not affect angiotensin II-induced constrictions either to the first or second applications (Figure 4). In arteries from ZDF rats, angiotensin II-induced constrictions were, however, reduced in the presence of Y27632 (1 µM) on the first application and were almost completely lost to the second application of angiotensin II (Figure 4). Exposure to Y27632 did not affect constrictions to noradrenaline in arteries from ZDF rats (maximum constrictions: 57 ± 13%) or from +/Fa rats (65 ± 10%) (Table 2) and the lack of tachyphylaxis was similarly unaffected by Y27632 (Table 2).

In arteries of control, +/Fa rats exposed to high glucose concentration (25 mM, for 1 h), incubation with Y27632 also prevented the augmentation in angiotensin II-induced constrictions to the first application (Figure 4). Importantly, in the presence of Y27632, angiotensin II-induced constrictions to the second application were markedly reduced in the presence of high glucose (Figure 4).

Sustained surface availability of AT_1 receptors in VSMCs exposed to high glucose

As we had found in this study that endothelium removal had no effect on angiotensin II-induced constrictions and on the magnitude of response upon repeated application, we inves-

Table 1

Constrictions (%) to repeated applications of NA (1–100 nM) in arteries of +/Fa and ZDF rats and in arteries of +/Fa rats exposed HG (25 mM)

	+/Fa (n = 7)		ZDF (n = 7)		+/Fa + HG (n = 7)	
	First appl.	Second appl.	First appl.	Second appl.	First appl.	Second appl.
NA (1 nM)	3 ± 2	3 ± 2	8 ± 4	7 ± 4	7 ± 2	4 ± 1
NA (10 nM)	25 ± 7	30 ± 6	38 ± 9	26 ± 8	32 ± 4	30 ± 3
NA (100 nM)	76 ± 7	81 ± 8	86 ± 10	74 ± 10	87 ± 2	76 ± 5

Noradrenaline-induced constrictions are expressed as percent changes of the initial diameter. Values are means ± SEM.
HG, high glucose; NA, noradrenaline; ZDF, Zucker diabetic fatty.

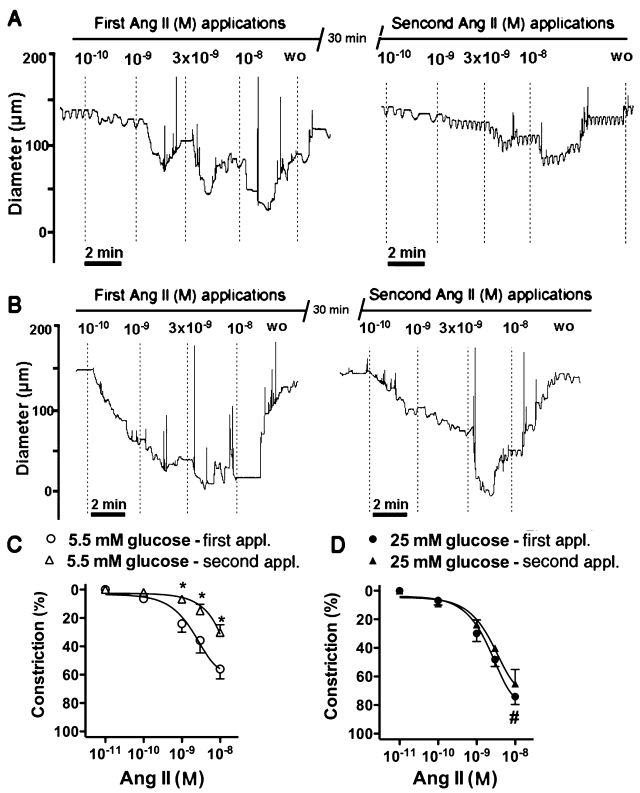

Figure 2

Original records (A,B) and summary data (C,D) of constrictions (%) to repeated applications of angiotensin II (Ang II; 10 pM–10 nM, n = 9) in skeletal muscle arteries from control heterozygous (+/Fa) rats in the presence of normal (5.5 mM, n = 5) or high glucose (25 mM, n = 5) concentration. Data are means ± SEM. *P < 0.05, significantly different from first application. # indicates difference from control (+/Fa).

tigated the effect of high glucose concentrations on the AT_1 receptors on the cell surface of VSMCs, in culture. AT_1 receptors were assayed with a specific antibody recognizing one of the extracellular loops of these receptors (BML-SA608, Biomol), before and after application of angiotensin II (100 nM) and also in the absence or presence of the Rho-kinase inhibitor, Y27632 (10 µM). Under normal glucose conditions, AT_1 receptor immunofluorescence was significantly reduced by angiotensin II and this reduction was not affected by Y27632 (Figure 5). Compared with controls, high glucose-treated VSMCs, however, exhibited a sustained AT_1 receptor immunofluorescence, which did not significantly decrease upon angiotensin II, but was reduced after pretreatment with Y27632 (Figure 5).

Discussion

The novel findings of the present study are that hyperglycaemia in diabetes or high glucose concentration *in vitro* leads not only to an enhanced but also a sustained arterial constriction to angiotensin II. These vasomotor changes are likely to be due to increased activation of vascular Rho-kinase elicited by high glucose, which results in a sustained vascular availability of AT_1 angiotensin II receptors. These conclusions are supported by our finding that constriction to repeated angiotensin II administration was decreased in isolated arteries of normoglycaemic control (+/Fa) rats, but remained sustained in vessels of hyperglycaemic diabetic (ZDF) rats, and also in normal rat arteries exposed acutely to high glucose concentrations. Also the levels of AT_1 receptors on the cell surface of VSMCs assessed by immunofluorescence were decreased after application of angiotensin II to VSMC cultures, but these levels were maintained if cells were also exposed to high glucose concentration. Further, these vasoconstrictions to repeated angiotensin II applications in arteries from diabetic rats and normal arteries exposed to high glucose were no longer maintained after treatment with the Rho-kinase inhibitor, Y27632. Finally, treatment with Y27632 decreased the surface level of AT_1 receptors after angiotensin II administration to VSMCs exposed to high glucose concentrations.

Augmented vasoconstrictor action of angiotensin II may contribute to enhanced peripheral vascular resistance in various forms of hypertension (Cai and Harrison, 2000; Ungvari *et al.*, 2004; Mehta and Griendling, 2007). In addition, several studies have demonstrated that angiotensin II, via activation of AT_1 receptors elicits enhanced constriction of both conduit and resistance-sized arteries obtained from animals with experimental diabetes (Zhang *et al.*, 2005; Viswanad *et al.*, 2006; Siddiqui and Hussain, 2007). In this study, we have tested the hypothesis that the augmented angiotensin II-induced arterial constriction is primarily due to the increased and sustained functional availability of AT_1 receptors in arteries elicited by a high glucose environment. It

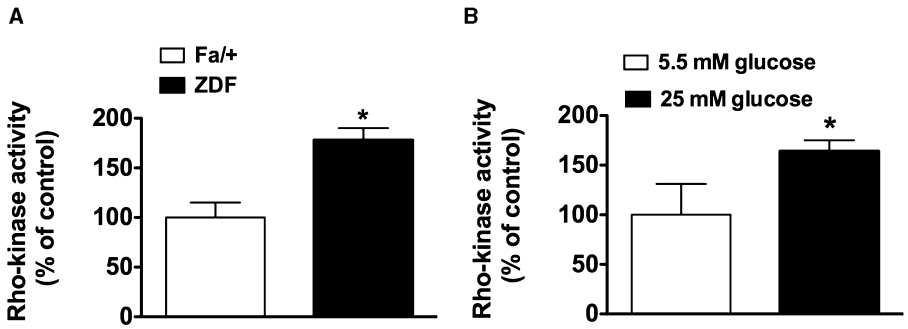

Figure 3

Rho-kinase activity in skeletal muscle arteries obtained from control heterozygous (+/Fa, $n = 4$) or Zucker diabetic fatty (ZDF, $n = 4$) rats, or in arteries obtained from +/Fa rats in the presence of normal (5.5 mM, $n = 4$) or high glucose (25 mM, $n = 4$) concentration. Data are means ± SEM. *$P < 0.05$, significantly different from (A) Fa/+ or from (B) 5.5 mM glucose.

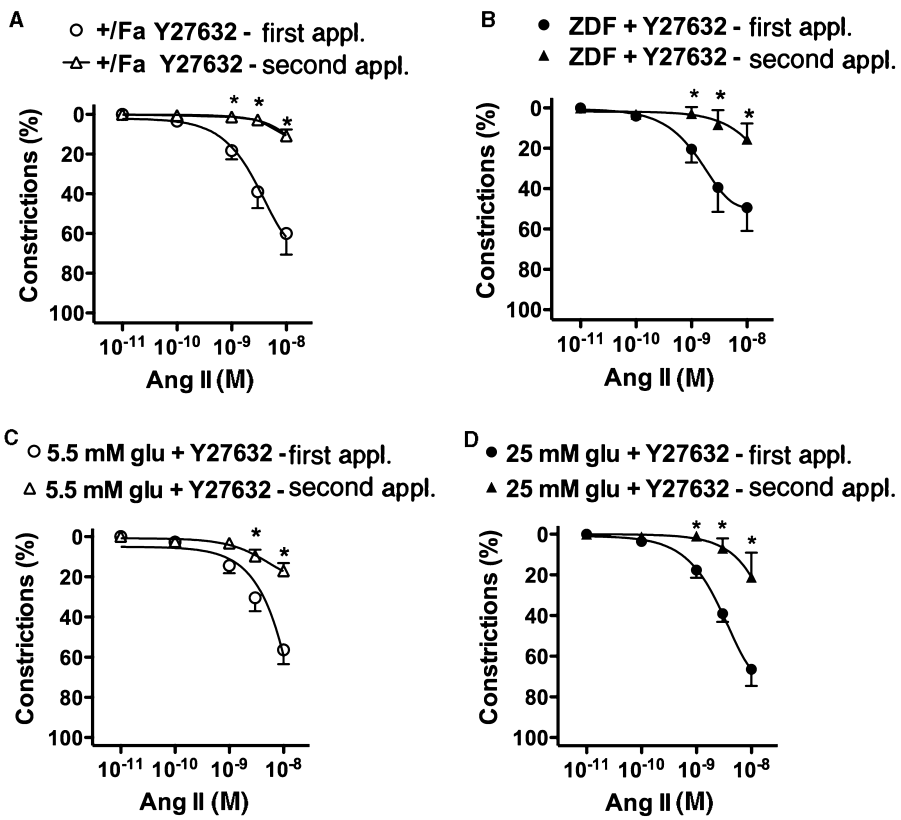

Figure 4

Summary data of constrictions (%) to repeated applications of angiotensin II (Ang II; 10 pM–10 nM, $n = 9$) in skeletal muscle arteries from +/Fa ($n = 5$) or Zucker diabetic fatty (ZDF, $n = 5$) rats (A,B) or arteries from +/Fa rats ($n = 5$) exposed to normal (5.5 mM glu) and high glucose (25 mM glu) concentration (C,D) in the presence of the Rho-kinase inhibitor, Y27632 (1 μM). Data are means ± SEM. *$P < 0.05$, significantly different from first application. Asterisks indicate significant differences ($P < 0.05$).

is known that AT_1 receptors continuously cycle between the cell surface membrane and endoplasmic reticulum (Hunyady *et al.*, 2000). This process sets the number of active AT_1 receptors available for further stimulation and provides a (negative feedback) regulation of the availability of AT_1 receptors (Hunyady *et al.*, 2000). We hypothesized that the normal regulation of AT_1 receptor trafficking is altered in diabetes leading to sustained availability of AT_1 receptors and thus sustained vasoconstrictions.

To assess the functionally available, 'active' AT_1 receptors in intact arteries we administered successive doses of angiotensin II. Under normal condition, repeated administration

Table 2

Constrictions (%) to repeated applications of NA (1–100 nM) in arteries of +/Fa and ZDF rats and in arteries of rats +/Fa exposed HG (25 mM) in the presence of Rho-kinase inhibitor, Y27632

	+/Fa (n = 7)		ZDF (n = 7)		+/Fa + HG (n = 7)	
	First appl.	Second appl.	First appl.	Second appl.	First appl.	Second appl.
NA (1 nM)	1 ± 1	2 ± 1	1 ± 4	2 ± 3	3 ± 1	4 ± 1
NA (10 nM)	25 ± 3	41 ± 11	18 ± 3	24 ± 8	17 ± 1	20 ± 5
NA (100 nM)	65 ± 10	78 ± 8	57 ± 13	64 ± 7	62 ± 8	56 ± 10

Noradrenaline-induced constrictions are expressed as percent changes of the initial diameter. Values are means ± SEM.
HG, high glucose; NA, noradrenaline; ZDF, Zucker diabetic fatty.

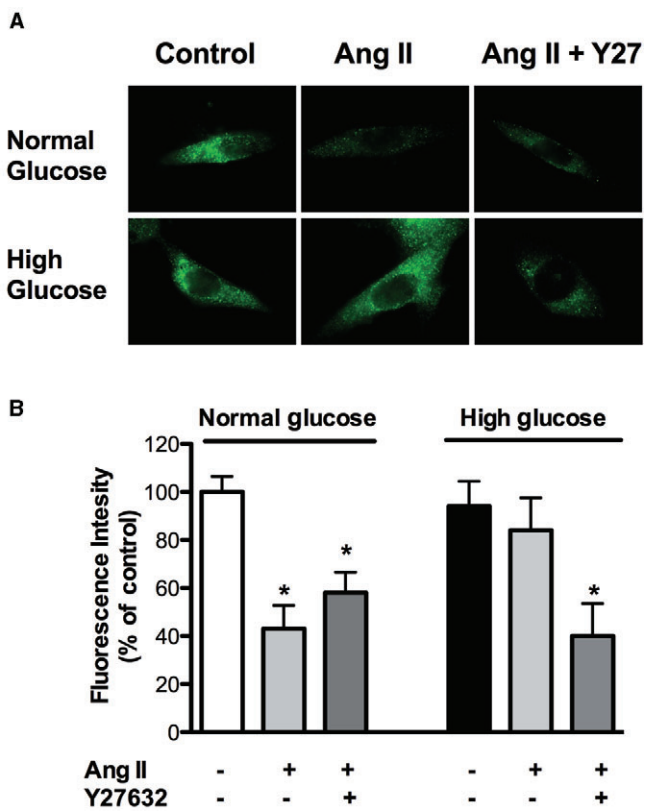

Figure 5

Detection of surface AT_1 receptors in vascular smooth muscle cells (VSMCs). Representative images (A) and summary data (B) show changes in extracellular AT_1 receptor immunofluorescence of VSMCs exposed to normal (5 mM) or high glucose (25 mM) concentrations, before and after stimulation with angiotensin II (Ang II; 100 nM, for 10 min) and in the absence and presence of Rho-kinase inhibitor, Y27632 (Y27; 10 μM). Images and data represent three separate experiments. Data are means ± SEM. *$P < 0.05$, significantly different from control (100%).

of angiotensin II induces tachyphylaxis (Juul *et al.*, 1987; Vicaut *et al.*, 1989). This is due to the angiotensin II-induced desensitization and consequent internalization of AT_1 receptors (Thomas *et al.*, 1996; Hunyady *et al.*, 2000). Interestingly, our present study revealed that, in arteries of diabetic rats or in normal arteries exposed to high glucose concentration (25 mM, a concentration similar to those seen in ZDF rats *in vivo*) angiotensin II-induced constrictions remained sustained upon repeated applications, that is, there was no tachyphylaxis. We have also found that the effect of chronic hyperglycaemia (in the ZDF rat) or acute high glucose exposure on sustained angiotensin II-induced arterial constriction were not reversible by normalizing the glucose concentrations *in vitro*, suggesting a long-lasting effect on angiotensin II tachyphylaxis. As there was no tachyphylaxis to noradrenaline in arteries of diabetic rats or in high glucose-exposed normal vessels, alterations in the downstream contractile function of VSMCs, such as increased calcium sensitivity, seem unlikely to play a role in the sustained response to angiotensin II. Even a short-term (1 h) exposure to high glucose concentration elicited greater and maintained angiotensin II-induced constrictions in normal arteries implying that changes in the expression of AT_1 receptors were also unlikely to be responsible for this phenomenon. We then hypothesized that, under normal glucose conditions, the tachyphylaxis to angiotensin II was due to the reduced number of functionally available AT_1 receptors on the surface of smooth muscle cells. On the other hand, the augmented and maintained constriction to angiotensin II in high glucose exposed arteries (*in vivo* or *in vitro*) would be probably due to the sustained surface availability of active AT_1 receptors on the smooth muscle cells. To test this hypothesis we have used a model system to detect the level of surface AT_1 receptors in VSMCs, in the presence of normal and high glucose conditions. Under normal glucose condition, surface AT_1 receptor immunofluorescence was significantly reduced by angiotensin II application, suggesting rapid internalization of AT_1 receptors. In contrast, high glucose-treated VSMCs exhibited a sustained AT_1 receptor immunofluorescence after angiotensin II stimulation, suggesting sustained surface presence of AT_1 receptors in high glucose environment. These experiments provided evidence of the effect of glucose concentrations on surface level of AT_1 receptors, although the mechanism(s) involved in the increased surface availability of vascular AT_1 receptors in diabetes has not been established.

It has been already demonstrated that activation of Rho-kinase is involved in the augmented α_1-adrenoceptor agonist-induced vasoconstriction in obese Zucker rats (Naik *et al.*, 2006). In addition, in a recent study Kizub *et al.* (2010) have found that Rho-kinase and protein kinase C (PKC) activation

play an important role in the augmented phenylephrine-induced arterial contraction in streptozotocin diabetic rats a model for type 1 diabetes. In the present study, the arterial constrictions to noradrenaline were not significantly increased in vessels from ZDF rats. However, comparable with the observations of Naik *et al*. and also Kizub *et al*. we have found that the contribution of Rho-kinase to the vasomotor response to a α_1-adrenoceptor agonist was greater in the ZDF rat. In this study, we have also demonstrated that the pressure-induced myogenic tone is enhanced in arteries of ZDF rats. We found that the Rho-kinase inhibitor, Y27632 reduced myogenic tone in both in ZDF and Fa/+ rats; however, the difference in the magnitude of myogenic tone remained significant between the two groups. These findings suggested that Rho-kinase contributes to the development of myogenic tone of skeletal muscle resistance arteries, as previously shown in mesenteric arteries (Dubroca *et al*., 2007). However, based on the present observations, it is likely that the enhanced myogenic tone in arteries of diabetic animals can be attributed to other mechanisms, such as increased production of constrictor prostanoids (Bagi *et al*., 2005) and/or activation of PKC (Ungvari *et al*., 1999).

Collectively, these data suggested that diabetes is associated with an increased activation of Rho-kinase pathway, although the potential impact of this pathway on AT_1 receptor function is entirely unknown. Small GTPases mediate the assembly of actin stress fibres (contractile actomyosin filaments) and regulate a wide range of fundamental biological functions, such as cell contraction and motility (Noma *et al*., 2006). Various Rho family members are also involved in the regulation of intracellular vesicle trafficking in eukaryotic cells (Symons and Rusk, 2003). In particular, RhoA is reported to be an essential component of a constitutive, PKC-dependent, endocytotic pathway in *Xenopus* oocytes (Schmalzing *et al*., 1995). These and our present functional findings suggest that the RhoA/Rho-kinase pathway was involved in the regulation of AT_1 receptor function. Because Rho-kinase has found to be directly activated by high glucose concentration (Naik *et al*., 2006), a finding, which was confirmed in this study, we have formulated the novel hypothesis that Rho-kinase signalling is involved in the regulation of AT_1 receptor trafficking, thereby contributing to the sustained arteriolar constriction to angiotensin II in diabetes. To demonstrate the functional consequence of Rho-kinase activation in this process, angiotensin II-induced responses were measured after Rho-kinase inhibition. Under normal glucose conditions and in the presence of the Rho-kinase inhibitor, Y27632, angiotensin II elicited substantial constrictions to the first application, which became reduced only to the second application. This suggests only a minor contribution of Rho-kinase in mediating angiotensin II constrictions of skeletal muscle resistance arteries of the rat under normal conditions. Importantly, in arteries from diabetic rats and also in those exposed to high glucose concentration the Rho-kinase inhibitor diminished the sustained angiotensin II-induced constriction upon repeated applications. Correspondingly, the surface level of AT_1 receptors, as detected by immunofluorescence labelling, was reduced by Rho-kinase inhibition in VSMCs exposed to high glucose, after angiotensin II application, to level similar to those found in normal glucose conditions. These findings suggest that in arteries, activation of Rho-kinase leads to sustained surface availability of AT_1 receptors and consequently augmented arterial constriction to angiotensin II in diabetes mellitus and also in acute hyperglycaemia.

Several mechanisms could be involved in the enhanced surface availability of vascular AT_1 receptors. It is known that the surface level of AT_1 receptors is regulated primarily by internalization and recycling, which are governed by complex subcellular mechanisms (Hein *et al*., 1997; Hunyady *et al*., 2000). AT_1 receptor internalization has been shown to involve both clathrin-dependent and -independent pathways (Hunyady *et al*., 2000) and it requires initial phosphorylation of the AT_1 receptors by G-protein-coupled receptor kinases (Lefkowitz, 1998; Anborgh *et al*., 2000). It is also known that receptor internalization directs the AT_1 receptors into the endosomes, where they are de-phosphorylated by protein phosphatases and recycled to the cell surface (Lefkowitz, 1998; Anborgh *et al*., 2000). It is possible that high glucose environment may interfere with various mechanisms involved in either the internalization or the recycling of AT_1 receptors, including those mediated by Rho-kinase activation. Molecular evidence supporting the key role of Rho-kinase pathway affecting AT_1 receptor internalization and recycling needs to be obtained in future studies.

It seems well established that in human diabetes AT_1 receptor antagonists not only reduce systemic blood pressure, but also prevent the development of the functional and morphological alterations of arteries (Pahor *et al*., 2000; Cooper, 2004). Our present findings provide experimental evidence and rationale for these clinical observations. Moreover, in recent studies, a possible role for 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, has been proposed to exert their vasculoprotective effects via interfering with the RhoA pathway in several cardiovascular diseases (Rikitake and Liao, 2005). Clinical studies claim that in diabetes, vascular complications can be effectively treated with statins, although the underlying mechanisms are incompletely understood (Garcia and Spellman, 2006). Thus, it seems plausible that in diabetes, statins interfere with the regulation of functional availability of AT_1 receptors, via inhibiting the RhoA/Rho-kinase pathway, a potential mechanism, which has not yet been addressed.

In conclusion, we propose that hyperglycaemia or high glucose environments lead to enhanced and sustained arterial constrictions to angiotensin II. These vasomotor changes are likely to be due to increased activation of vascular Rho-kinase elicited by high glucose, resulting in sustained functional availability of AT_1 receptors in the smooth muscle cells of arteries. These findings suggest a novel role for Rho-kinase, in augmenting the surface availability of vascular AT_1 receptors in diabetes. Thus, in diabetes, interfering with the vascular Rho-kinase signalling may prevent or delay the development of vasomotor dysfunction of resistance arteries, which is associated with augmented AT_1 receptor-mediated signalling.

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Conflict of interest

For all authors, no conflict of interest is declared.

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