AT\textsubscript{1} Blockade Prevents Glucose-Induced Cardiac Dysfunction in Ventricular Myocytes

Role of the AT\textsubscript{1} Receptor and NADPH Oxidase

Jamie R. Privratsky, Loren E. Wold, James R. Sowers, Mark T. Quinn, Jun Ren

Abstract—Enhanced tissue angiotensin (Ang) II levels have been reported in diabetes and might lead to cardiac dysfunction through oxidative stress. This study examined the effect of blocking the Ang II type 1 (AT\textsubscript{1}) receptor on high glucose–induced cardiac contractile dysfunction. Rat ventricular myocytes were maintained in normal- (NG, 5.5 mmol/L) or high- (HG, 25.5 mmol/L) glucose medium for 24 hours. Mechanical and intracellular Ca\textsuperscript{2+} properties were assessed as peak shortening (PS), time to PS (TPS), time to 90% relengthening (TR\textsubscript{90}), maximal velocity of shortening/relengthening (±dL/dt\textsubscript{max}), and intracellular Ca\textsuperscript{2+} decay (τ). HG myocytes exhibited normal PS; decreased ±dL/dt; and prolonged TPS, TR\textsubscript{90}, and τ. Interestingly, the HG-induced abnormalities were prevented with the AT\textsubscript{1} blocker L-158,809 (10 to 1000 nmol/L) but not the Janus kinase-2 (JAK2) inhibitor AG-490 (10 to 100 μmol/L). The only effect of AT\textsubscript{1} blockade on NG myocytes was enhanced PS at 1000 nmol/L. AT\textsubscript{1} antagonist–elicited cardiac protection against HG was nullified by the NADPH oxidase activator sodium dodecyl sulfate (80 μmol/L) and mimicked by the NADPH oxidase inhibitors diphenyleneiodonium (10 μmol/L) or apocynin (100 μmol/L). Western blot analysis confirmed that the protein abundance of NADPH oxidase subunit p47\textsubscript{phox} and the AT\textsubscript{1} but not the AT\textsubscript{2} receptor was enhanced in HG myocytes. In addition, the HG-induced increase of p47\textsubscript{phox} was prevented by L-158,809. Enhanced reactive oxygen species production observed in HG myocytes was prevented by AT\textsubscript{1} blockade or NADPH oxidase inhibition. Collectively, our data suggest that local Ang II, acting via AT\textsubscript{1}, receptor–mediated NADPH oxidase activation, is involved in hyperglycemia-induced cardiomyocyte dysfunction, which might play a role in diabetic cardiomyopathy. (Hypertension. 2003;42:206-212.)

Key Words: glucose ■ cardiac function ■ cardiomyopathy ■ oxidases ■ angiotensin II

Diabetic cardiomyopathy, one of the leading causes of increased morbidity and mortality in the diabetic population, is defined as a phenotypic change(s) in cardiac muscle, independent of micro- and macrovascular disease, coronary artery disease, and hypertension.\textsuperscript{1-3} It is characterized by prolongation of action potential duration, delayed cytosolic Ca\textsuperscript{2+} clearance, and impaired ventricular function, especially during diastole.\textsuperscript{4-6} Several factors have been postulated to contribute to the pathogenesis of diabetic cardiomyopathy, including hyperglycemia, insulin resistance, and free-radical damage,\textsuperscript{3,4} among which hyperglycemia is considered one of the most important factors in the onset of diabetic cardiomyopathy.\textsuperscript{4,7}

The renin-angiotensin system (RAS) plays a major role in the regulation of blood pressure and other cardiovascular functions. Enhanced RAS activity has been demonstrated in several cardiovascular diseases, such as diabetes and hypertension, and might play a role in the pathogenesis of congestive heart failure, coronary insufficiency, and hypertensive cardiomyopathy.\textsuperscript{8} The hypertrophic and growth-promoting effects of angiotensin (Ang) II are mediated primarily through its type I receptor (AT\textsubscript{1}).\textsuperscript{8} Enhanced tissue RAS action is thought to contribute to cardiovascular disease in diabetes, including diabetic cardiomyopathy.\textsuperscript{9} In addition, AT\textsubscript{1} receptor stimulation induces the generation of oxygen-derived free radicals, which can have detrimental effects.\textsuperscript{9,10} Several postreceptor signaling pathways have been demonstrated to be coupled to the AT\textsubscript{1} receptor, including Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and NADPH oxidase.\textsuperscript{11,12} The JAK/STAT proteins might transmit intracellular signals elicited by various kinds of cytokines and growth factors in a wide variety of cell types.\textsuperscript{13} On the other hand, NADPH oxidase is a membrane-bound enzyme that catalyzes the electron reduction of oxygen, with NADH or NADPH as electron donors.\textsuperscript{9,14,15} Nevertheless, the precise role of Ang II and its downstream effects in the pathogenesis of diabetic cardiomyopathy remains unclear.
signaling in the onset of cardiac dysfunction, especially in diabetes, is poorly understood. We have established an in vitro model of diabetic cardiomyopathy by culturing ventricular myocytes in a high-glucose (HG) medium for 24 hours. The aim of the present study was to determine the effect of AT1 receptor blockade with the AT1 receptor–specific antagonist L-158,809 on glucose toxicity–induced cardiac mechanical dysfunction and the potential signaling pathways involved.

**Methods**

**Isolation and Culture of Ventricular Myocytes**

The experimental procedures were approved by the institutional Animal Care and Use Committee of the University of North Dakota. The experimental procedures were approved by the institutional Animal Care and Use Committee of the University of North Dakota.

Ventricular myocytes were isolated from adult, male Sprague-Dawley rats (200 to 250 g; Harlan, Indianapolis, Ind) under sterile conditions by collagenase (176 U/mL) and hyaluronidase (0.1 mg/mL) perfusion through the coronaries and were further digested by trypsin (0.02 mg/mL) after the tissue was minced. Isolated myocytes were plated on glass coverslips precoated with laminin (10 mg/mL) and maintained for 24 hours in a defined medium consisting of medium 199 with Earle’s salts containing 25 mmol/L HEPES and NaHCO3 supplemented with albumin (2 mg/mL), L-carnitine (2 mmol/L), creatine (5 mmol/L), taurine (5 mmol/L), insulin (0.1 µmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL), and gentamicin (100 µg/mL). This medium also contained either normal-glucose (NG, 5.5 mmol/L) or HG (25.5 mmol/L) concentrations. The HG concentration is comparable to serum glucose levels in severely diabetic rats. Subsets of each medium were also supplemented with the AT1 antagonist L-158,809 (10 to 1000 nmol/L) or apocynin (100 µmol/L), the NADPH oxidase activator sodium dodecyl sulfate (SDS, 80 µmol/L), the NADPH oxidase substrate NADPH (100 µmol/L), or the NADPH inhibitors diphenyleneiodonium (DPI, 10 µmol/L) or apocynin (10 µmol/L). The myocytes in either NG or HG medium were incubated overnight at 37°C under 100% humidity and 5% CO2.

**Intracellular Ca2+ Fluorescence Measurements**

A separate cohort of myocytes was loaded with fura 2-AM (0.5 µmol/L) for 10 minutes, and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix, MyoCam system, IonOptix Corp). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70) and superfused with a buffer containing (in mmol/L) 131 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES, at pH 7.4. Cells were field-stimulated with a suprathreshold voltage at a frequency of 0.5 Hz. The myocytes being studied were displayed on the computer monitor with the IonOptix MyoCam camera. SoftEdge software (IonOptix) was used to capture changes in cell length during shortening and relengthening.

**Cell Shortening and Relengthening**

Mechanical properties of ventricular myocytes were assessed with the use of a commercially available system (IonOptix, MyoCam system, IonOptix Corp). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70) and superfused with a buffer containing (in mmol/L) 131 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES, at pH 7.4. Cells were field-stimulated with a suprathreshold voltage at a frequency of 0.5 Hz. The myocytes being studied were displayed on the computer monitor with the IonOptix MyoCam camera. SoftEdge software (IonOptix) was used to capture changes in cell length during shortening and relengthening.

**Western Blot Analysis**

Membrane proteins from NG- or HG-cultured myocytes were extracted as described. Myocytes were collected and sonicated, and the supernatants were centrifuged at 7000g for 30 minutes at 4°C. Total cell homogenates from the pellets were used for immunoblotting of the NADPH oxidase subunit p47phox and AT1 receptors. We confirmed that these membrane fractions did not contain any detectable collagens. Membrane proteins (50 µg/lane) were separated on 10% (AT1 and AT2) or 15% (p47phox) SDS-poly-
acrylamide gels in a Mini gel apparatus (Mini-Protein II, Bio-Rad) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA with Tris-buffered saline-Tween-20 solution for 60 minutes and then incubated overnight with anti-p47phox (1:1000), anti-AT1 (1:500), or anti-AT2 (1:500) antibodies. Monoclonal antibody recognizing the NADPH oxidase subunit p47phox has been previously described. Anti-AT1, (monoclonal) and anti-AT2, (polyclonal) antibodies were obtained from Abcam Limited and Santa Cruz Biotechnology, respectively. The antigens were detected by the luminescence method with a commercially available substrate (Supersignal West Dura extended duration substrate, Pierce Co). After immunoblotting, the film was scanned and the intensity of immunoblot bands was detected with a densitometer (Bio-Rad model GS-800).

Analysis of ROS Production by Myocytes
Production of cellular reactive oxygen species (ROS) was evaluated by analyzing the changes in fluorescence intensity resulting from oxidation of the intracellular fluoroprobe 5-(6)-chloromethyl-2,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). In brief, isolated myocytes from each group were loaded with 1 μmol/L of the nonfluorescent dye H2DCFDA (Molecular Probes) at 37°C for 30 minutes. The myocytes were rinsed, and the fluorescence intensity was then measured with a fluorescence microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Molecular Devices). Untreated cells showed no fluorescence and were used to determine background fluorescence, which was subtracted from the treated samples. The final fluorescence intensity was normalized to the protein content in each myocyte group and was expressed as the percentage of the fluorescence intensity of the NG group.

Data Analysis
For each experimental series, data are presented as mean±SEM. Statistical significance (P<0.05) for each variable was estimated by ANOVA. A Dunnett test was used for post hoc analysis when required.

Results
Effect of L-158,809 on Mechanical and Fluorescence Properties of NG and HG Myocytes
As previously reported, maintaining myocytes for 24 hours in NG or HG medium had no significant effects on cell phenotype (data not shown). Myocyte shape, resting cell length, and the presence of distinct, cellular striations were comparable between NG and HG myocytes. Myocytes from the HG group exhibited significantly depressed maximal velocities of shortening/relengthening (dL/dt) and prolonged duration of shortening and relengthening (time to peak shortening [TPS] and time to 90% relengthening [TR90]) associated with comparable peak shortening (PS), consistent with previous observations. Cotreatment with the AT1 receptor antagonist L-158,809 (10 to 1000 nmol/L) significantly attenuated the HG-induced decreases in dL/dt and the prolongation of TPS and TR90 (Figure 1). Low levels of L-158,809 (10 and 100 nmol/L) exerted no effect on PS, whereas the highest level of L-158,809 examined (1000 nmol/L) significantly enhanced PS (Figure 1). Figure 2 shows that myocytes maintained in HG medium displayed similar baseline intracellular Ca2+ fluorescence intensity (FFI) and electrically stimulated increases in FFI (ΔFFI) associated with slowed intracellular Ca2+ decay compared with myocytes maintained in NG medium. L-158,809 (10 nmol/L) exerted no effect on baseline FFI and ΔFFI but prevented the HG-induced prolongation of intracellular Ca2+ decay.

Effect of JAK2 and NADPH Oxidase Inhibition on HG-Induced Mechanical Dysfunction
To evaluate the signaling involved in L-158,809-inhibited protection from HG-induced cardiac mechanical dysfunction, myocytes were coincubated in NG or HG medium with the JAK2 inhibitor AG-490 (10 and 100 μmol/L) and the NADPH oxidase inhibitors DPI (10 μmol/L) or apocynin (100 μmol/L). The selectivity of apocynin, a methoxy-substituted catechol, on NADPH oxidase has been well documented. It impedes the assembly of the p47phox and p67phox subunits within the membrane NADPH oxidase complex. As shown in Figure 3, neither concentration of AG-490 significantly affected mechanical function in myocytes maintained in either NG or HG medium. However, both DPI and apocynin prevented the HG-induced prolongation of TPS and...
TR$_{90}$, suggesting that activation of an NADPH oxidase might be involved in HG-induced cardiac contractile dysfunction.

**Effect of NADPH Oxidase Activation on Myocyte Mechanics**

To further evaluate the role of NADPH oxidase in HG-induced cardiac mechanical dysfunction, SDS (80 \mu mol/L), an activator of NADPH oxidase,$^{21}$ was coincubated with the NG or HG myocytes. SDS itself prolonged TPS and TR$_{90}$ in NG myocytes, which was not prevented by coincubation with the AT$_1$ antagonist L-158,809 (Figure 4). Consistently, short-term (30-minute) incubation with the NADPH oxidase donor/substrate NADPH (100 \mu mol/L) significantly prolonged both TPS (NG, 141±8 vs NG+NADPH, 170±7 ms; n=19 cells/group, $P<0.05$) and TR$_{90}$ (NG, 301±20 vs NG+NADPH, 432±39 ms; n=19 cells/group, $P<0.05$). These results suggest that NADPH oxidase activation might play a key role in cardiac myocyte contractile dysfunction, reminiscent of that triggered by the HG culture.

**Immunoblot Analysis of AT$_1$/AT$_2$ Receptors and NADPH Oxidase Subunit p47$^{phox}$**

Western blot analysis indicated that maintaining myocytes in HG medium for 24 hours directly enhanced expression of the NADPH oxidase subunit p47$^{phox}$ as well as the AT$_1$ receptor. In contrast, expression of the AT$_2$ receptor was unaffected. Interestingly, the HG-induced elevation in p47$^{phox}$ expression was blocked by L-158,809 and DPI but was enhanced by SDS (Figure 4). SDS itself also induced an increased expression of the AT$_1$ receptor. These data are consistent with the stimulatory and inhibitory effects of the NADPH oxidase activator and inhibitor and strongly suggest a role for NADPH oxidase activity in HG-induced cardiac myocyte contractile dysfunction.

**ROS Production in NG and HG Myocytes in the Presence of L-158,809, SDS, or Apocynin**

As shown in Figure 5, ROS production was enhanced in 24-hour HG-cultured myocytes, which was blocked by the AT$_1$ antagonist L-158,809 (10 nmol/L) and the NADPH oxidase inhibitor apocynin (100 \mu mol/L). In comparison, ROS production was stimulated by SDS (80 \mu mol/L). These data suggest that the HG-induced cardiac mechanical dysfunction might be mediated, at least in part, by HG-induced production of ROS in ventricular myocytes. The observation that the HG-induced elevation in ROS generation was abolished by L-158,809 and apocynin further indicates that the
AT1 receptor and NADPH oxidase might play significant roles in HG-induced cardiac dysfunction.

Discussion

Novel results from this study indicated a potential role of Ang II via the AT1 receptor in the glucose toxicity-induced cardiac mechanical and intracellular Ca2+ dysfunction, simulating in vivo diabetic cardiomyopathy. These data further suggest that upregulation of AT1 but not the AT2 receptor, local activation of NADPH oxidase but not JAK/STAT, and generation of ROS might be involved in HG-induced cardiomyocyte dysfunction. Although the expression and function of NADPH oxidase have been documented in peripheral vascular tissue,9,12,14 its function in the heart remains poorly understood. Our current study provides compelling evidence that both the AT1 receptor and NADPH oxidase are involved in glucose toxicity-induced cardiac contractile dysfunction.

Diabetic cardiomyopathy is characterized by a prolonged duration of contraction/relaxation and intracellular Ca2+ clearing.4,6 Findings from our current study revealed that prolonged TPS and TR90 were associated with slowed intracellular Ca2+ clearance in myocytes maintained in HG medium for 24 hours. These data are reminiscent of in vivo diabetes and are consistent with our earlier observations.16 More important, the HG-induced cardiomyocyte dysfunction was abrogated by coincubation with the AT1 antagonist L-158,809 and NADPH oxidase inhibitors DPI and apocynin. Ang II is well known to cause myocyte hypertrophy and apoptosis, both in vitro and in vivo. Current data suggest that the hyperglycemia-induced cardiac myopathies might be mediated via the AT1 receptor, consequently causing NADPH oxidase activation and ROS generation. A functional role for NADPH oxidase in the oxidant response to Ang II was suggested by our observation that the NADPH oxidase activator SDS and the NADPH oxidase donor/substrate NADPH mimicked the effect of HG in myocytes maintained in NG medium.

Although the mechanism(s) of action underlying the role of the RAS and AT1 in HG-induced cardiomyocyte dysfunction is not fully understood at this time, a plausible explanation is that hyperglycemia promotes ROS generation (as shown in our study) and myocyte apoptosis through AT1-mediated
NADPH oxidase activation induced by the local RAS.22,23 The AT1 receptor is a G protein–coupled receptor that mediates most of the known biologic effects of Ang II. Both local production of Ang II and AT1 receptor expression are significantly increased in cardiac myocytes and vessels in streptozotocin-induced diabetic rats24 and in vascular smooth muscle cells exposed to high glucose levels,25 which are consistent with our findings. Activation of the AT1 receptor might “turn on” NADPH oxidase and consequently enhance ROS, such as superoxide anion, which can react with nitric oxide, leading to its inactivation by producing peroxynitrite.10 Peroxynitrite is known to directly oxidize membrane components such as arachidonic acid, thus altering membrane integrity and cardiac function.26 JAK and STAT proteins have been demonstrated to be directly coupled to the AT1 receptor, and Ang II is known to directly stimulate tyrosine phosphorylation and activation of JAK2 and the STAT family.26 However, the lack of effect of the JAK2-specific inhibitor AG-490 on HG-induced cardiomyocyte dysfunction does not favor any involvement of JAK/STAT in HG-induced cardiac dysfunction, at least in our current experimental setting. It is rather surprising that inhibition of NADPH oxidase activity with DPI abolished the HG-induced upregulation of p47phox protein expression itself. The mechanism of action is unknown, although a possible “feedback regulatory mechanism” of NADPH oxidase might be postulated for this observation.

We are currently investigating the underlying cellular mechanisms associated with HG-induced cardiomyocyte dysfunction and the signaling mechanisms contributing to these changes. To date, few studies have used a cell-culture system to address the pathogenesis of diabetes-related cardiac dysfunction. Our in vitro “diabetic” model allows us to explore the direct actions of HG, independent of other complications associated with diabetes, such as hyperlipidemia, hyper- or hypoinsulinemia, and hypothyroidism that might also contribute to depressed myocardial function. This work provides evidence that Ang II and NADPH oxidase might be considered important targets for the prevention and treatment of diabetic cardiomyopathy.

**Perspectives**

The findings from our present study indicate that the Ang II system (especially its membrane receptors) and postreceptor signaling pathway NADPH oxidase might play critical roles in the onset of hyperglycemia-induced oxidative stress and contractile dysfunction in the heart. These results should have a significant clinical implication in the use of AT1 receptor–specific antagonists for the prevention and treatment of diabetic cardiomyopathy.

**Acknowledgments**

This work was supported in part by grants from the American Diabetes Association and the Max Baer Heart Fund (to J.R.) and National Institutes of Health grants NIH R01 HL65575 (to M.T.Q.) and NIH R01-HL63904-01 (to J.R.S.). L-158,809 was generously provided by Merck & Co, Inc, Rahway, NJ. The authors would like to thank Jinhong Duan and Nicholas Aberle II for technical assistance.

**References**


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Hypertension. 2003;42:206-212; originally published online July 7, 2003;
doi: 10.1161/01.HYP.0000082814.62655.85
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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