Central role for aldose reductase pathway in myocardial ischemic injury

YUYING C. HWANG,* MICHIYO KANEKO,* SOLIMAN BAKR,* HUI LIAO,* YAN LU,* ERIN R. LEWIS,† SHIDU YAN,* SETSUKO II,§ MITSUO ITAKURA,§ LIU RUI, HAL SKOPICKI,† SHUNICHI HOMMA,† ANN MARIE SCHMIDT,* PETER J. OATES,‖ MATTHIAS SZABOLCS,‡ AND RAVICHANDRAN RAMASAMY*,[1]

Departments of *Surgery, †Medicine, and ‡Pathology, College of Physicians and Surgeons, Columbia University, New York, New York, USA; §University of Tokushima, Japan; and ‖Pfizer Global Research and Development, Groton, Connecticut, USA

ABSTRACT **Aldose reductase (AR), a member of the aldo-keto reductase family, has been implicated in the development of vascular and neurological complications of diabetes. Recently, we demonstrated that aldose reductase is a component of myocardial ischemic injury and that inhibitors of this enzyme protect rat hearts from ischemia-reperfusion injury. To rigorously test the effect of aldose reductase on myocardial ischemia-reperfusion injury, we used transgenic mice broadly overexpressing human aldose reductase (ARTg) driven by the major histocompatibility complex I promoter. Hearts from these ARTg or littermate mice (WT) (n=6 in each group) were isolated, perfused under normoxic conditions, then subjected to 50 min of severe low flow ischemia followed by 60 min of reperfusion. Creatine kinase (CK) release (a marker of ischemic injury) was measured during reperfusion; left ventricular developed pressure (LVDP), end diastolic pressure (EDP), and ATP were measured throughout the protocol. CK release was significantly greater in ARTg mice compared with the WT mice. LVDP recovery was significantly reduced in ARTg mice compared with the WT mice. Furthermore, ATP content was higher in WT mice compared with ARTg mice during ischemia and reperfusion. Infarct size measured by staining techniques and myocardial damage evaluated histologically were also significantly worse in ARTg mice hearts than in controls. Pharmacological inhibition of aldose reductase significantly reduced ischemic injury and improved functional recovery in ARTg mice. These data strongly support key roles for AR in ischemic injury and impairment of functional and metabolic recovery after ischemia. We propose that interventions targeting AR may provide a novel adjunctive approach to protect ischemic myocardium.—Hwang, Y. C., Kaneko, M., Bakr, S., Liao, H., Lu, Y., Lewis, E. R., Yan, S., Ii, S., Itakura, M., Rui, L., Skopicki, H., Homma, S., Schmidt, A. M., Oates, P. J., Szabolcs, M., Ramasamy, R. Central role for aldose reductase pathway in myocardial ischemic injury. *FASEB J.* 18, 1192–1199 (2004)**

Key Words: cytosolic redox state · glucose metabolism · ischemia-reperfusion injury

ALDOSE REDUCTASE (AR), a monomeric, NADPH-dependent enzyme and member of the aldo-keto reductase family, catalyzes the reduction of aldo sugars and other saturated and unsaturated aldehydes (1–5). This enzyme constitutes the first step of polyol pathway or AR pathway. It has been shown that AR uses a broad array of substrates, including hydroxy-nonenals and catecholamines (6–8). Activation of AR, linked to the development of vascular and neurological complications in diabetes (9–13), often results in impaired substrate metabolism and function (1, 9–15). Consistent with these concepts, amelioration of vascular and other complications of diabetes was observed upon treatment with AR inhibitors (1, 14, 15).

Recently, it was demonstrated that inhibition of AR protects rat hearts from ischemic injury and improves functional recovery upon reperfusion (16–18). To demonstrate unambiguously that AR is indeed a central mediator of ischemic injury, we subjected transgenic mice broadly overexpressing human AR to ischemia-reperfusion. Compared with human hearts, mice have very low levels of AR activity; hence, overexpression of this enzyme provides an attractive opportunity to test our hypothesis. The data demonstrate increased cardiac ischemic injury and poor functional recovery in human AR-overexpressing mice and reveal potential mechanisms by which AR mediates ischemic injury.

MATERIALS AND METHODS

All studies were performed with the approval of the Institutional Animal Care and Use Committee at Columbia University, New York. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication no. 85-23, 1996).

[1] Correspondence: Division of Surgical Science (P&S 17-410), College of Physicians and Surgeons, Columbia University, 630 West 168th St., New York, NY 10032, USA. E-mail: rr260@columbia.edu

doi: 10.1096/fj.03-1400com

0892-6638/04/0018-1192 © FASEB

Animals

Mice transgenic for human aldose reductase (ARTg) were obtained from Dr. Mitsuo Itakura (University of Tokushima, Japan) and a colony was established at Columbia University. Briefly, these transgenic mice were developed by injecting full-length hAR cDNA (19) with a mouse major histocompatibility antigen class I promoter (20). These hAR transgenic mice (ARTg) have been backcrossed 10 generations to obtain the mice in the C57BL6 background and used in our studies. The litters were examined for hAR transgene expression with the polymerase chain reaction (PCR) using a set of transgene specific primers. Sequences for the primers were upstream primer 5′-CTGCTAACCATGTTCATGCC-3′ and downstream primer 5′-TTCACGGCCTCAGTCACCT-3′. PCR was performed with 30 cycles through the following temperature sequence: 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The PCR reaction mixture consisted of 50 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.4, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 2.5 units of *Taq* polymerase, 100 ng of DNA, and 10 pmol/L of each primer. DNA was recovered from the tail biopsy as described previously (19, 20). Mice negative for transgenic expression were used as wild-type (Wt) littermate controls.

Isolated perfused heart preparation

Experiments were performed using an isovolumic isolated heart preparation as published (21–23) and modified for the use in mice hearts. Mice were anesthetized using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). After deep anesthesia was achieved, hearts were rapidly excised, placed into iced saline, and retrogradely perfused at 37°C in a nonrecirculating mode through the aorta at a rate of 2.5 mL/min. Hearts were perfused with modified Krebs-Henseleit buffer containing (in mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 5, palmitate 0.4, BSA 0.4, and 70 mU/L insulin. The perfusate was equilibrated with a mixture of 95% O_2-5% CO_2, which maintained perfusate $Po_2 > 600$ mmHg. Left ventricular developed pressure (LVDP) and left ventricular end diastolic pressure (LVEDP) were measured using a latex balloon in the left ventricle. LVDP, heart rate, and coronary perfusion pressure were monitored continuously on a 4-channel Gould recorder.

Ischemia/reperfusion protocol

Hemodynamic function was monitored throughout the protocol. All mice hearts were paced at 420 beats/min with the use of pacing electrodes placed on the right atrium. Hearts from Wt and AR transgenic mice were subjected to 50 min of low flow ischemia (flow reduced from 2.5 mL/min to 0.04 mL/min) and 60 min of reperfusion. Perfusate temperature was maintained at 37°C at all times during the protocol (i.e., during baseline, ischemia, and reperfusion). After an equilibration period of 30 min, both groups of hearts were perfused with modified Krebs-Henseleit buffer throughout ischemia and reperfusion.

To determine the mechanisms by which flux via aldose reductase affects ischemic injury, experiments were performed in the presence of inhibitors of AR(ARI) or sorbitol dehydrogenase (SDI) or niacin. Studies involving the use of SDI were to establish whether the flux via SDH and accompanying increases in cytosolic $NADH/NAD^+$ is an important event by which increases in aldose reductase mediate ischemic injury. The use of niacin helped to determine whether lowering cytosolic $NADH/NAD^+$ independent of aldose reductase pathway reduces ischemic injury in aldose reductase transgenic mice. This would help identify changes in cytosolic $NADH/NAD^+$ as a key component by which aldose reductase pathway affects myocardial ischemic injury. After the equilibration period of 30 min, hearts (n=6) from Wt and transgenic mice were perfused with modified Krebs-Henseleit buffer containing 1 μM ARI zopolrestat, 200 nM SDI CP-470,711 (22), or 10 μM niacin (23) starting 10 min before ischemia and continued throughout ischemia and reperfusion.

Induction of ischemia/reperfusion in vivo

Ischemia/reperfusion (I/R) was induced in Wt and AR transgenic mice by ligature of the left anterior descending coronary artery for 30 min, followed by reperfusion.

On the day of study, mice were anesthetized and intubated to maintain mechanical ventilation. A left lateral thoracotomy was performed and the heart was displaced to the left of the chest cavity. The left anterior descending coronary artery was tied with a suture (7-0 silk); the effectiveness of this strategy was monitored by observing myocardial blanching. Ischemia was induced for 30 min, then the ligature was released. The chest cavity was closed and animals gradually weaned from the respirator. Animals were maintained for 2 days after induction of ischemia to allow for adequate reperfusion in the injured heart.

On the day before surgery and on day 2, immediately before sacrifice for performance of molecular studies, all animals were subjected to echocardiography while awake since anesthetic agents are known to influence echocardiographic parameters (24, 25). Two-dimensional echocardiographic assessment of cardiac function was performed using PHILIPS 5500 ultrasound system (Philips Medical, Andover, MA, USA) with a 15 MHz transducer. Echocardiographic images of the short axis view at midpapillary muscle level were obtained at a frame rate of 120 Hz and digitally stored for later analysis. Endocardial border from image at end diastole and systole were traced in order to obtain percent of fractional area change (%FAC):

$$\%FAC = (EDA\text{-}ESA)/EDA \cdot 100$$

where EDA = end diastolic internal area at midpapillary muscle level in short axis view and ESA = end systolic internal area at midpapillary muscle level in short axis view. Five consecutive cardiac cycles were used for each measurement. This technique has been before used to assess the function of murine hearts (24, 25).

Western blot and immunohistochemical studies

SDS-PAGE was performed on a 12% acrylamide gel using NOVEX XCell II electrophoresis apparatus (NOVEX, San Diego, CA, USA). Proteins were stained with Coomassie blue (Bio-Rad Laboratories, Hercules, CA, USA).

For immunoblot, after electrophoresis the proteins were transferred onto the nitrocellulose membrane Protran™ (Schleicher and Schuell, Keene, NH, USA) in Tris-glycine buffer, pH 8.3, containing 20% (v/v) methanol at 25 mV for 90 min using NOVEX XCell II Blot Module (NOVEX). Nonspecific binding of the membrane was blocked with 5% non-fat dry milk (Bio-Rad). The membrane was incubated in PBS containing antibody against rat lens AR at 4°C overnight, then further incubated in PBS containing anti-goat IgG coupled with horseradish peroxidase. The immunostaining of AR was visualized with 4 CN as substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA).

Immunostaining was performed on 10% formalin-embedded sections (6 μm) and visualized using 3,3′ diaminobenzi-

dine with goat anti-AR IgG (1:100) (gift from Dr. P. Kador, NEI, Bethesda, MD, USA), followed by rabbit anti-goat peroxidase conjugated IgG (1:200) (Sigma, St. Louis, MO, USA).

Measurements of glucose uptake, glycolysis, glucose oxidation, and palmitate oxidation

The effect of AR overexpression on myocardial glucose uptake was measured by perfusing mice hearts with [1-^{3}H]2-deoxyglucose as published earlier (18, 21, 23, 26); its effect on myocardial glucose oxidation was measured by perfusing hearts with [U-^{14}C]glucose in the recirculating mode and analyzing the perfusate for oxidation product $^{14}CO_2$ (18, 21, 23, 26).

Biochemical assay for lactate and pyruvate

Cardiac AR activity in homogenates was spectrophotometrically assayed as published earlier (18, 23). Lactate, pyruvate, sorbitol, fructose, and glycogen in hearts extracts were measured using standard biochemical assays published in the literature (18, 23, 27–29).

Histology

Cross sections of mice hearts were fixed in 10% buffered formalin, paraffin embedded, and serial 4 μm-thick sections were cut and stained for hematoxylin and eosin (H&E) and Masson's trichrome technique.

Statistical methods

Data were analyzed using INSTAT (GraphPad, San Diego, CA, USA) software operating on an IBM compatible personal computer. Differences between different groups were assessed using ANOVA for repeated measures, with subsequent Student-Newman-Keuls multiple comparisons post tests if the *P* value for ANOVA was significant. All data are expressed as mean ± SD.

RESULTS

AR expression

Myocardial levels of total AR (mice+human) were determined using ELISA and found be 377 ± 57 ng/mg total protein in ARTg mice vs. 90 ± 14 ng/mg total protein in nontransgenic littermates (**Table 1**). Consistent with increased expression of ARTg, the transgenic mice hearts had higher levels of sorbitol (3.81±0.14 nmol/g wet wt in ArTg vs. 0.91±0.12 nmol/g wet wt in Wt, $P<0.05$) and fructose (4.56±0.52 nmol/g wet wt in ArTg vs. 0.72±21 in Wt, $P<0.05$). The presence of higher levels of AR in transgenic mice hearts were further confirmed by Western blot studies (**Fig. 1*A***). Both Wt and transgenic mice hearts clearly displayed the immunostaining that corresponded to the purified recombinant AR. Hearts from mice overexpressing human AR had significantly higher levels of the enzyme than the Wt. In ARTg mice, higher AR levels were also observed in kidney, nerve, and liver compared with these organs from Wt mice. Immunohistologic analysis (Fig. 1) demonstrated widespread expression of hAR in the Tg mouse heart, including endothelial cells, as indicated by colocalization studies using anti-CD31 IgG (Fig. 1*B*). Increased AR antigen was observed in myocytes of ARTg mice compared with Wt (Fig. 1*C*). In addition, thioglycollate-elicited macrophages from the peritoneal cavity of Tg mice displayed increased hAR antigen vs. wild-type MP, as shown in the Western blots (Fig. 1*D*).

AR and myocardial ischemia

Consistent with the immunostaining data, ELISA measurements (Table 1) showed increased levels of AR in both transgenic mice. Myocardial AR activity was significantly greater in both transgenic mice compared with that in Wt mice (Table 1).

Low flow ischemia resulted in greater ischemic injury in ARTg mice hearts than in Wt. **Figure 2*A*** shows that creatine kinase release during reperfusion after ischemia, a marker of myocardial ischemic injury, was ~threefold higher in ARTg mice compared with Wt. Pharmacological inhibition of AR in ARTg significantly reduced ischemic injury compared with ARTg mice hearts (Fig. 2*A*). Inhibition of AR in Wt mice also reduced ischemic injury. Ischemia increased AR activity by ~three- to fourfold (Table 1) in ARTg and Wt mice hearts. Inhibition of AR by zopolrestat reduced enzyme activity ~97 ± 3% in ARTg and Wt mice hearts under all conditions (data not shown). These data indicate that increased expression and activity of AR was associated with increased ischemic injury and that pharmacological agents that inhibit AR reduce ischemic injury.

AR overexpression and cardiac function during ischemia-reperfusion

LVDP was similar in all groups under baseline conditions, with mean values of 76 ±12 mm Hg in ARTg vs.

TABLE 1. *Aldose reductase levels and activity in mice hearts*[a]

Group	Aldose reductase protein (ng/mg protein)	Aldose reductase activity (nmol NADPH · min^{-1} · mg protein^{-1})	
		Normoxic hearts	Ischemic hearts
Wild-type (n=5)	81 ± 14	0.8 ± 0.04	2.2 ± 0.4*
ARTg (n=6)	377 ± 54[#]	5.2 ± 0.7[#]	18.9 ± 1.7[#]

[a] Values are means ± SD. * $P = 0.02$ vs. normoxic. [#] $P = 0.01$ vs. wild-type.

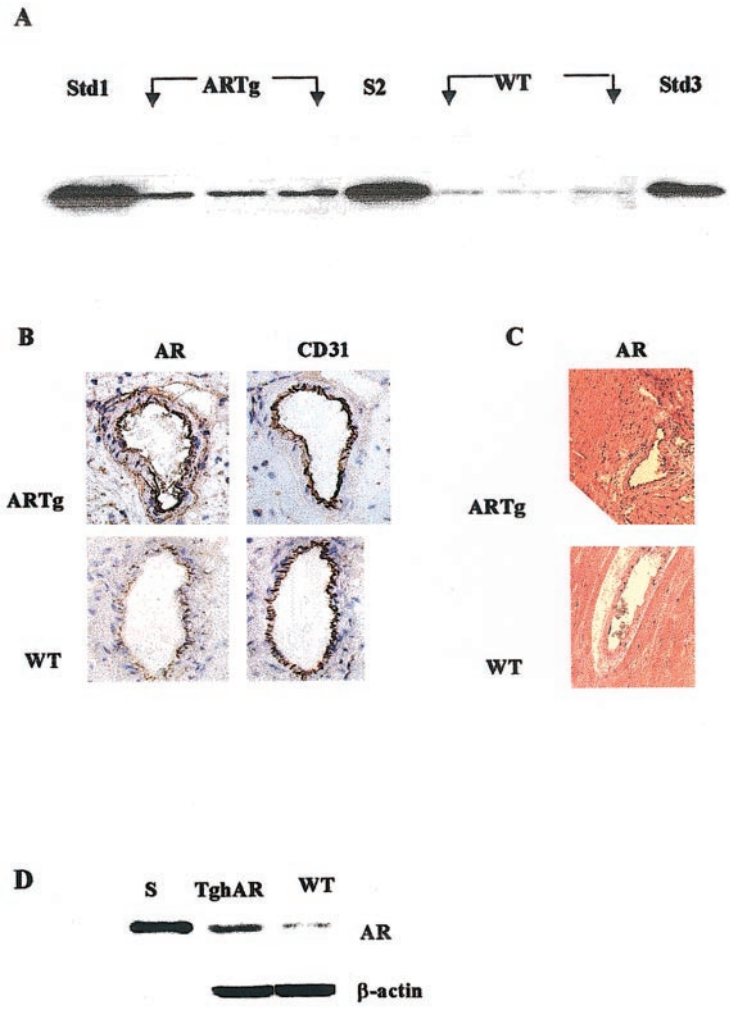

Figure 1. *A)* Western blots of aldose reductase in mice hearts. Lanes Std1, Std2, and Std3 represent 50, 25, and 12.5 ng of recombinant human aldose reductase, AR lanes represent aldose reductase in ARTg hearts; WT lanes represent aldose reductase in Wt mice hearts. *B)* Immunostaining using anti-AR IgG demonstrated increased endothelial AR in ARTg vs. wild-type, as demonstrated by colocalization using anti-CD31 IgG. *C)* Immunostaining of AR in myocytes demonstrates increased ARTg vs. wild-type. *D)* Macrophages were prepared from the peritoneum of ARTTg vs. wild-type mice. Equal amounts of protein were subjected to Western blot using anti-AR IgG; blots were then stripped and reprobed using anti-β-actin IgG.

81 ± 16 mmHg in Wt mice hearts. Upon reperfusion after low flow ischemia, ARTg mice hearts exhibited significantly poor LVDP recovery than the Wt (Fig. 2*B*). AR inhibition improved LVDP recovery in reperfused hearts from ARTg and Wt mice (Fig. 2*B*). Myocardial oxygen consumption was similar in all groups under baseline, ischemic, and reperfusion conditions and was unaffected by AR overexpression.

AR and energy metabolism

To determine whether myocardial energy metabolism is affected during ischemia, ATP levels in ARTg mice were measured and compared with those in Wt mice hearts. Myocardial ATP levels were significantly lower in ARTg mice during ischemia and during reperfusion than in Wt (**Fig. 3**). Inhibition of AR improved ATP levels during ischemia and reperfusion in ARTg and Wt hearts (Fig. 3). Baseline ATP levels were unaffected by AR inhibition in all hearts.

To determine whether glucose metabolism is affected due to AR overexpression, rates of glucose uptake and oxidation were measured in ARTg and Wt mice hearts. Glucose uptake, as measured by 2-[^{3}H]deoxyglucose uptake, was similar in ARTg and Wt mice hearts (**Fig. 4*A***). However, glucose oxidation was significantly lower in ARTg mice hearts compared with Wt (Fig. 4*B*). Since glucose uptake is similar in both mice hearts, the data are indicative of bottlenecks at glycolytic or downstream steps of glucose oxidation in ARTg mice hearts. A similar reduction in glucose oxidation was observed during reperfusion in ARTg mice hearts and normalization upon treatment with an ARI. Glucose oxidation rates ($^{14}CO_2$ production in nmol · min^{-1} · g dry wt^{-1}) during reperfusion were 1166 ± 207 in ARTg hearts vs. 1982 ± 322 in Wt hearts ($P<0.04$), whereas glucose oxidation rates were normalized in ARTg hearts upon treatment with an ARI (oxidation rates were 2316±298).

To determine whether glycogen metabolism could contribute to the differences in glucose metabolism between ARTg and Wt mice hearts, glycogen content was measured before ischemia and after reperfusion. Glycogen content, expressed as μmol glucose/g dry wt, was similar in ARTg and Wt mice hearts before ischemia (21.9±5.4 in ARTg vs. 19.7±3.2 in Wt). The glycogen content at reperfusion was reduced by similar amounts in ARTg and Wt mice hearts (7.1±2.9 in ARTg vs. 5.9±1.8 in Wt). These data indicate that the contribution of glycogen breakdown to glucose metabolism was similar in ARTg and Wt mice hearts.

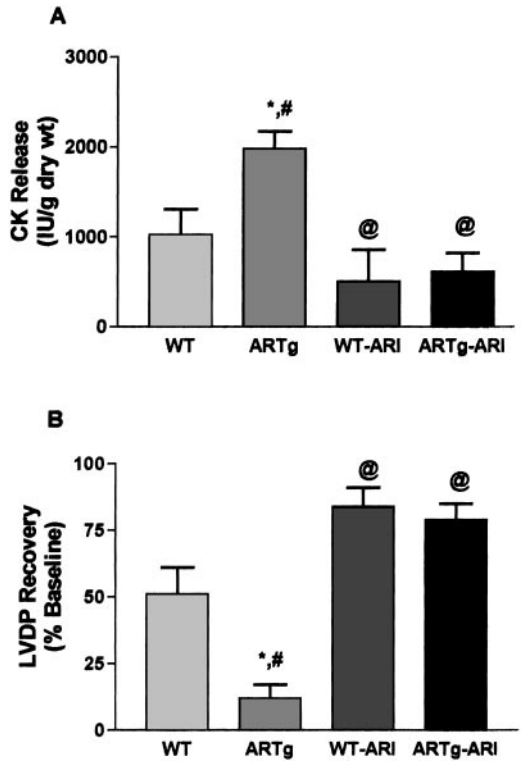

Figure 2. *A)* Creatine kinase (CK) release from reperfused hearts after severe low flow ischemia. CK release was measured during the entire 60 min of reperfusion in wild-type (WT), AR transgenic (ARTg), aldose reductase inhibitor (ARI) -treated WT-ARI, and ARI-treated ARTg (ARTg-ARI) hearts. $^{*}P < 0.03$ vs. WT, $^{\#}P < 0.01$ vs. WT-ARI, ARTg-ARI, $^{@}P < 0.05$ vs. WT. *B)* Left ventricular developed pressure (LVDP) recovery expressed as % of baseline in WT, ARTG, WT-ARI, and ARTg-ARI hearts. $^{*}P < 0.01$ vs. WT, $^{\#}P < 0.005$ vs. WT-ARI, ARTg-ARI, $^{@}P < 0.04$ vs. WT.

To determine whether the altered ATP levels and glucose metabolism were associated with changes in cytosolic NADH/NAD^+, tissue lactate/pyruvate ratio was measured in all hearts. The L/P ratio was increased in ARTg mice hearts under all perfusion conditions compared with Wt mice. Though L/P ratio increased in Wt hearts during ischemia, the increases seen ARTg hearts were far greater during the same period (**Table 2**). Tissue lactate and pyruvate levels were significantly different in ARTg and Wt mice hearts. Tissue lactate levels (μmol/g dry wt) were 138.2 ± 6.1 in ARTg vs. 117.1 ± 5.3 in Wt mice hearts ($P<0.05$). The tissue pyruvate levels (μmol/g dry wt) were 0.27 ± 0.02 in ARTg vs. 0.38 ± 0.03 in Wt mice hearts ($P<0.05$). Overall, these data indicate increases in L/P ratio, hence NADH/NAD^+, due to increased flux via AR.

Protection of AR transgenic mice by lowering NADH/NAD^+ ratio

To determine whether the rise in cytosolic NADH/NAD^+ ratio is a critical component of AR-mediated ischemic injury, ARTg hearts were subjected to ischemia-reperfusion in the presence of an SDH inhibitor or niacin. Flux via AR and sorbitol dehydrogenase results in use of NAD^+, influencing the cytosolic NADH/NAD^+ ratio; hence, inhibition of sorbitol dehydrogenase would limit such changes. Niacin is known to increase cytosolic NAD^+ independent of flux via AR pathway, thus affecting the cytosolic NADH/NAD^+ ratio. **Figure 5*A*** demonstrates that the cytosolic L/P ratio was reduced in ARTg mice by inhibiting AR or sorbitol dehydrogenase. Similar reductions in the L/P ratio was also observed in ARTg mice hearts perfused with niacin. Figure 5*B* demonstrates that ARTg mice treated with Ari or SDI or niacin had reduced ischemic injury compared with untreated ARTg mice hearts. The data demonstrate that sorbitol dehyrogenase inhibition or niacin treatment reduced ischemic injury and improved functional recovery upon reperfusion in ARTg mice hearts, demonstrating that NADH/NAD^+ changes are essential in AR-mediated ischemic injury.

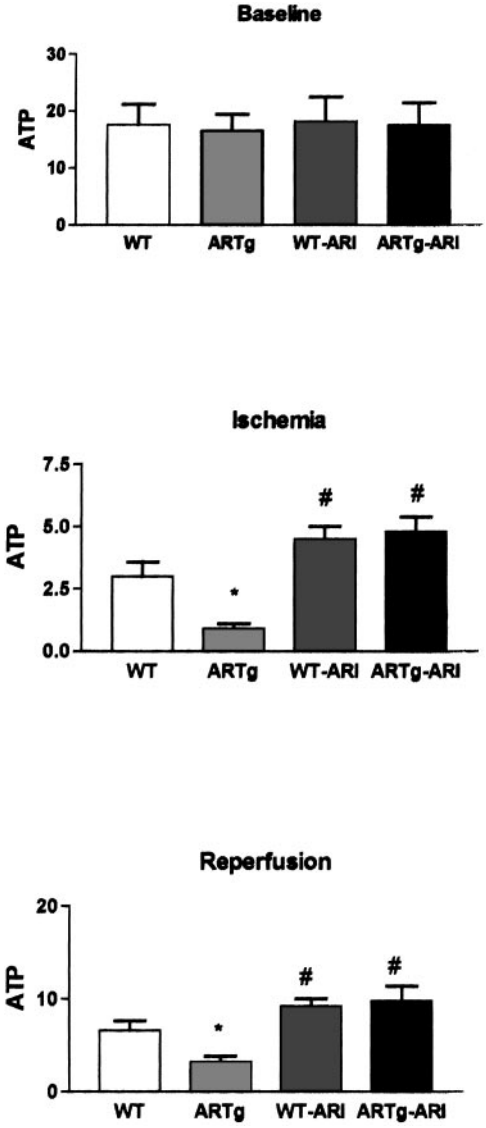

Figure 3. ATP, expressed as μmol/g dry wt, in WT and ARTg hearts at baseline, the end of ischemia, and the end of reperfusion. Six hearts were studied per group. $^{*}P < 0.03$ vs. ATP in WT group; $^{\#}P < 0.05$ vs. WT and ARTg groups.

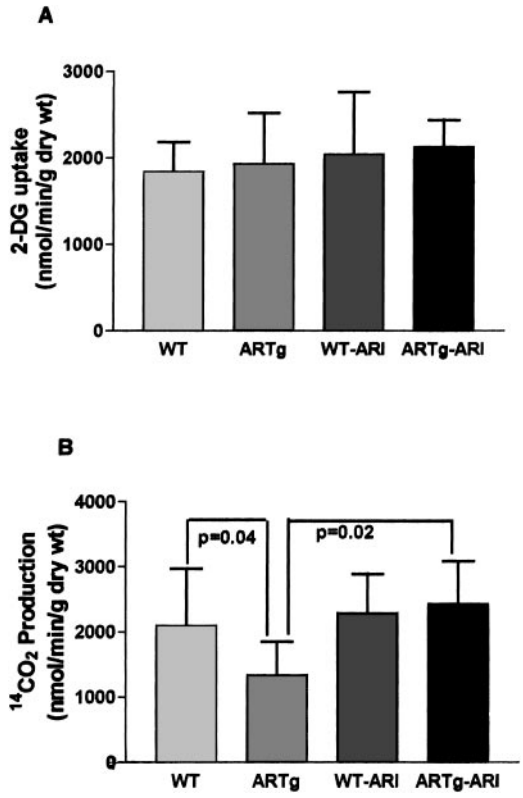

Figure 4. *A)* Glucose uptake as measured by 2-[^{3}H]deoxyglucose and *B)* glucose oxidation as measured by release of $^{14}CO_2$ from [U-^{14}C]glucose in WT, ARTg, WT-ARI, and ARTg-ARI hearts perfused under normoxic conditions. Glucose uptake is expressed as nmol · min^{-1} · g · dry wt^{-1}.

In vivo ischemia-reperfusion

In vivo ischemia-reperfusion studies further confirmed that injury is greater in ARTg mice than in Wt mice . Histologic evaluation with H&E and trichrome of reperfused ARTg hearts revealed large areas of necrosis, severe edema, and inflammation, whereas little edema or inflammation was observed in Wt hearts (**Fig. 6*A***). The area of necrosis was considerably smaller in Wt mice hearts than in ARTg hearts (Fig. 6*A*). Echo cardiographic studies revealed that the fractional area change during reperfusion was significantly altered in ARTg hearts compared with Wt (Fig. 6*B*).

TABLE 2. *Lactate/pyruvate ratios in wild-type (WT) and aldose reductase overexpressing (ARTg) mice hearts under various conditions (n=5) in each group)*[a]

		Lactate/pyruvate ratio	
Group	Baseline	End of ischemia	Reperfusion
WT	12.6 ± 3.7	298.5 ± 56.3	16.4 ± 2.1
ARTg	26.9 ± 5.4*	498.8 ± 69.2*	36.3 ± 6.8*

[a] Lactate/pyruvate ratios were measured under baseline conditions, at the end of 30 min of global ischemia, and after 30 min of reperfusion. * $P < 0.05$ vs. wild-type.

DISCUSSION

AR is a member of the aldo-keto reductase family with remarkably broad substrate specificity. Earlier studies from our laboratory demonstrated that pharmacological inhibition of AR protects perfused rat hearts from ischemic injury. In this study, we demonstrate that hearts from human AR-overexpressing mice exhibit increased ischemic injury and poor functional recovery upon reperfusion. These data clearly establish that increased AR activity is detrimental under ischemic conditions and impedes functional and metabolic recovery during reperfusion.

The amount of AR overexpression obtained in transgenic mice was similar to that seen in humans (30). It is evident from the present study that at the levels seen in humans, AR has an adverse effect on the ability of myocardium to recover from ischemic insult. Even in nontransgenic mice, ischemia increased AR activity by ~threefold. Inhibition of such increases in AR activity reduced ischemic injury and improved functional recovery. In vivo ischemia reperfusion studies indicated increases in myocyte necrosis, inflammation, and edema in ARTg mice hearts compared with Wt. Clearly, increased expression and activity of AR were associated

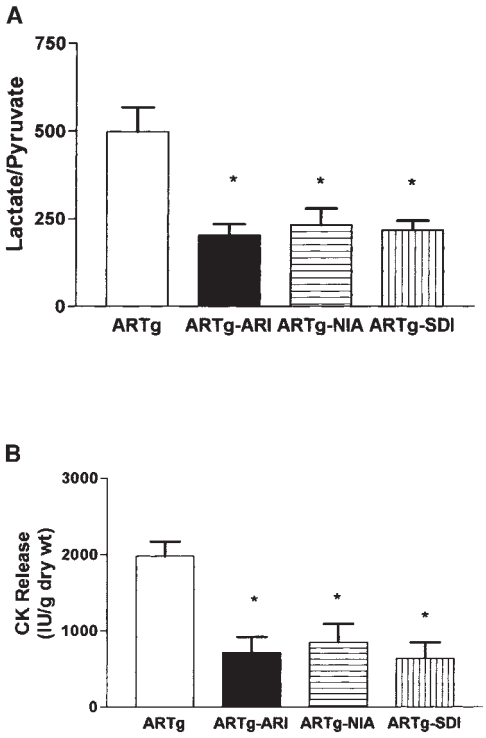

Figure 5. *A)* CK release from reperfused hearts after severe low flow ischemia. CK release was measured during the entire 60 min of reperfusion in AR transgenic (ARTg), aldose reductase inhibitor (ARI) -treated ARTg (ARTg-ARI), niacin-treated ARTg (ARTg-NIA), and sorbitol dehydrogenase inhibitor-treated ARTG (ARTg-SDI) hearts. *$P < 0.05$ vs. ARTg group. *B)* Myocardial lactate/pyruvate ratio, a measure of cytosolic NADH/NAD$^+$, at the end of ischemia in ARTg, ARTg-ARI, ARTg-NIA, and ARTg-SDI hearts. *$P < 0.05$ vs. ARTg group.

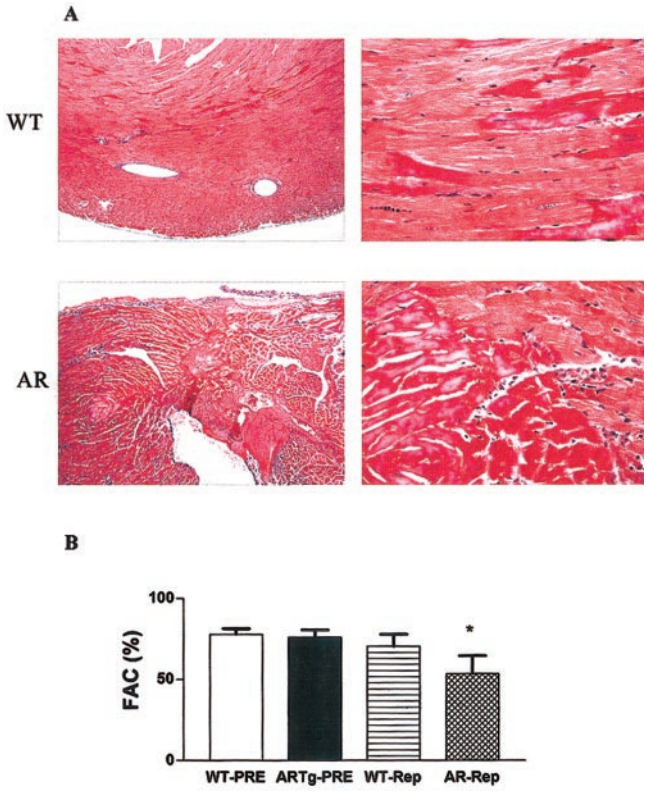

Figure 6. *A)* Masson's trichrome staining method was used to determine myocardial injury. Wild-type and ARTg mice were subjected to 30 min ischemia (produced by occlusion of the left anterior descending coronary artery) and 2 days of reperfusion. In wild-type mice, only scattered myocytes show increased red staining representing ischemic damage. No myocyte dropout and formation of granulation tissue were noted. Conversely, hARTg mice hearts exhibited large continuous areas of hyperfuthsinophilic myocytes (i.e., necrosis) associated with inflammation and early stages of repair. 3 hearts/group were studied. *B)* Echo cardiographic measurements of fractional area change (FAC%) in wild-type and ARTg mice hearts before ischemia and after 2 days of reperfusion. 6 hearts per group were studied by ECHO cardiography. $*P < 0.05$ vs. all other groups

with increased markers of injury due to ischemia-reperfusion.

A potential mechanism by which AR influences ischemic injury involves energy metabolism and associated events. Ischemia results in an increased flux via the AR pathway. Increased flux via this pathway leads to utilization of NADPH by AR and NAD^+ by sorbitol dehydrogenase. This flux contributes to increases in the cytosolic $NADH/NAD^+$ ratio. Lactate/pyruvate ratio, a measure of the cytosolic $NADH/NAD^+$ ratio, was significantly higher in ARTg mice hearts than in Wt under normoxic and ischemic conditions. As shown before, sorbitol dehydrogenase in this pathway competes with glycolytic enzyme GAPDH for cytosolic NAD+, increasing $NADH/NAD^+$ ratio and impairing glycolysis and glucose use (18, 23, 31–33). Studies have shown that AR inhibition or sorbitol dehydrogenase inhibition normalizes glucose metabolism in hearts (18, 22). In the present study, increases in cytosolic $NADH/NAD^+$ ratio were attenuated in ARTg mice hearts by treating them with AR inhibitor or sorbitol dehydrogenase inhibitor or by niacin. Ischemic injury was reduced in ARTg mice hearts by inhibiting AR, sorbitol dehydrogenase, and niacin, all of which reduced the cytosolic $NADH/NAD^+$ ratio. All three interventions improved functional recovery upon reperfusion. Further support for lower $NADH/NAD^+$ as an important factor for reducing ischemic injury comes from rat studies using interventions such as niacin and pyruvate (22, 32), which have been shown to lower cytosolic $NADH/NAD^+$ ratios independent of polyol pathway and to reduce ischemic injury These data demonstrate that increases in cytosolic $NADH/NAD^+$ ratio is an important step in AR-mediated increases in ischemic injury.

Inhibition of the AR pathway improved glucose metabolism in ARTg hearts. Although glucose uptake was similar in all hearts, AR inhibition improved glucose oxidation in ARTg hearts. Studies have shown that inhibition of AR or sorbitol dehydrogenase improves myocardial glycolysis and glucose oxidation in rats and that these changes were also associated with attenuation of cytosolic $NADH/NAD^+$ ratio and relieving the bottleneck at GAPDH (18, 23, 31). The present study showing decreased glucose oxidation and increased cytosolic $NADH/NAD^+$ ratio is consistent with a bottleneck at glycolysis being responsible for decreased glucose oxidation in ARTg hearts.

Improving glucose use is critical for rescuing ischemic myocardium (34, 35). Myocardium is primarily dependent on glycolysis to meet its energy demands during ischemia. Blocking of glycolytic flux due to competition between GAPDH and sorbitol dehydrogenase for NAD^+ limits ATP generation and lowers the cytosolic $NADH/NAD^+$ ratio under ischemic conditions (18, 22, 33). Ischemia ATP levels were significantly lower and the cytosolic $NADH/NAD^+$ ratio was higher in ARTg mice hearts compared with Wt. AR inhibition improved ATP levels and lowered the $NADH/NAD^+$ ratio in ARTg mice during ischemia. These data indicate that decreased ATP levels in ARTg mice are due, in part, to impaired glycolysis.

This study has provided data supporting the changes in cytosolic $NADH/NAD^+$ ratio, impaired glucose metabolism, and ATP levels as one primary mechanism by which ischemic injury is increased in ARTg mice hearts. However, the influence of the AR pathway in affecting intracellular sodium and calcium homeostasis (36), oxidative stress (37, 38, 39), and PKC-mediated signaling (1, 4) events cannot be ruled out. The current study indicates that AR plays a central role in mediating myocardial ischemic injury and provides a foundation for evaluating AR inhibitors as potential therapeutic adjuncts in treating patients with myocardial infarction. FJ

This work was supported by grants from National Institutes of Health (RO1HL 61783, RO1HL68954) and Juvenile Diabetes Research Foundation. R.R. is the recipient of an Established Investigator Award from the American Heart Association (0040152N).

REFERENCES

1. Williamson, J. R., Chang, K., Frangos, M., Hasan, K. S., Ido, Y., Kawamura, T., Nyengaard, J. R., Van den Enden, M., Kilo, C., and Tilton, R. G. (1993) Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* **42,** 801–813
2. Busik, J. V., Hootman, S. R., Greenidge, C. A., and Henry, D. N. (1997) Glucose-specific regulation of aldose reductase in Capan-1 human pancreatic duct cells in vitro. *J. Clin. Invest.* **100,** 1685–1692
3. Srivastava, S. K., Ansari, N. H., Hair, G. A., Awasthi, S., and Das, B. (1986) Activation of human erythrocyte, brain, aorta, muscle, and ocular tissue aldose reductase. *Metabolism* **35,** 114–118
4. Keogh, R. J., Dunlop, M. E., and Larkins, R. G. (1997) Effect of inhibition of aldose reductase on glucose flux, diacylglycerol formation, protein kinase C, and phospholipase A_2 activation. *Metabolism* **46,** 41–47
5. Harrison, D. H., Bohren, K. M., Ringe, D., Petsko, G. A., and Gabbay, K. H. (1994) An anion binding site in human aldose reductase: mechanistic implications for the binding of citrate, cacodylate, and glucose-6-phosphate. *Biochemistry* **33,** 2011–2020
6. Bhatnagar, A., Srivastava, S., Wang, L. F., Chandra, A., Ansari, N. H., and Srivastava, S. K. (1999) Cardiac metabolism of enals. *Adv. Exp. Med. Biol.* **463**, 223–229
7. Srivastava, S., Watowich, S. J., Petrash, J. M., Srivastava, S. K., and Bhatnagar, A. (1999) Structural and kinetic determinants of aldehyde reduction by aldose reductase. *Biochemistry* **38,** 42–54
8. Srivastava, S., Chandra, A., Wang, L. F., Seifert, W. E. J., DaGue, B. B., Ansari, N. H., Srivastava, S. K., and Bhatnagar, A. (1998) Metabolism of the lipid peroxidation product, 4-hydroxy-trans-2-nonenal, in isolated perfused rat heart. *J. Biol. Chem.* **273,** 10893–10900
9. Kador, P. F., and Kinoshita, J. H. (1985) Role of aldose reductase in the development of diabetes associated complications. *Am. J. Med.* **79**, 8–12
10. Kador, P. F. (1988) The role of aldose reductase in the development of diabetic complications. *Med. Res. Rev.* **8,** 325–352
11. Gabbay, K. H. (1973) The polyol pathway and the complications of diabetes. *N. Engl. J. Med.* **288,** 831–836
12. Kinoshita, J. H., and Nishimura, C. (1988) The involvement of aldose reductase in diabetic complications. *Diabetes Metab. Rev.* **4,** 323–337
13. Greene, D. A., Lattimer, S. A., and Sima, A. A. F. (1987) Sorbitol, phosphoinolitides and sodium-potassium ATPase in the pathogenesis of diabetic complications. *N. Engl. J. Med.* **316,** 599–606
14. Greene, D. A., Sima, A. A. F., Stevens, M. J., Feldman, E. L., Killen, P. D., Henry, D. N., Thomas, T., Dananberg, J., and Lattimer, S. A. (1993) Aldose reductase inhibitors: an approach to the treatment of diabetic nerve damage. *Diabetes Metab. Rev.* **9,** 189–217
15. Greene, D. A., Lattimer, S. A., Ulbrecht, J., and Carrol, P. (1988) Glucose-induced alterations in nerve metabolism: current perspective on the pathogenesis of diabetic neuropathy and future directions for research and therapy. *Diabetes Care* **8,** 290–299
16. Ramasamy, R., Oates, P. J., and Schaefer, S. (1997) Aldose reductase inhibition protects diabetic and non-diabetic rat hearts from ischemic injury. *Diabetes* **46,** 292–300
17. Tracey, W. R., Magee, W. P., Ellery, C. A., MacAndrew, J. T., Smith, A. H., Knight, D. R., and Oates, P. J. (2000) Aldose reductase inhibition alone or combined with an adenosine A(3) agonist reduces ischemic myocardial injury. *Am. J. Physiol.* **279,** H1447–H1452
18. Hwang, Y. C., Sato, S., Tsai, J. Y., Bakr, S., Yan, S. D., Oates, P. J., and Ramasamy, R. (2002) Aldose reductase activation is a key component of myocardial response to ischemia. *FASEB J.* **16,** 243–245
19. Nishimura, C., Matsuura, Y., Kokai, Y., Akera, T., Carper, D. A., Lyons, C., and Flynn, T. G. (1990) Cloning and expression of human aldose reductase. *J. Biol. Chem.* **265,** 9788–9792
20. Yamaoka, T., Nishimura, C., Yamashita, K., Itakura, M., Yamada, T., Fujimoto, J., and Kokai, Y. (1995) Acute onset of diabetic pathological changes in transgenic mice with human aldose reductase cDNA. *Diabetologia* **38,** 255–261
21. Ramasamy, R., Hwang, Y. C., Whang, J., and Bergmann, S. R. (2001) Protection of ischemic hearts by high glucose is mediated by the glucose transporter, GLUT-4. *Am. J. Physiol.* **281,** H290–H297
22. Hwang, Y. C., Bakr, S., Ellery, C. R., Oates, P. J., and Ramasamy R. (2003) Sorbitol dehydrogenase: a novel target for adjunctive protection of ischemic myocardium. *FASEB J.* **17,** 2331–2333
23. Trueblood, N. A., Ramasamy, R., Wang, L. F., and Schaefer, S. (2000) Niacin protects the isolated perfused heart from ischemia/reperfusion injury: the role of lactate/proton co-transport. *Am. J. Physiol.* **279,** H764–H77
24. Takuma, S., Suehiro, K., Cardinale, C., Yano, H., Shimizu, J., Mullis-Jansson, S., Sciacca, R., Wang, J., Burkhoff, D., Di Tullio, M. R., et al. (2001) Anesthetic inhibition in ischemic and non-ischemic murine heart: comparison with conscious echocardiographic approach for functional assessment. *Am. J. Physiol.* **280,** H2364–H2370
25. Kocher, A. A., Schuster, M. D., Szabolcs, M. J., Takuma, S., Burkhoff, D., Wang, J. F., Homma, S., Edwards, N. M., and Itescu, S. (2001) Neoangiogenesis of ischemic myocardium by human adult bone marrow-derived angioblasts prevents postinfarction remodeling and improves cardiac function. *Nat. Med.* **7,** 430–436
26. Broderick, T. L., Quinney, H. A., Barker, C. C., and Lopaschuk, G. D. (1993) Beneficial effect of carnitine on mechanical recovery of rat hearts reperfused after a transient period of global ischemia is accompanied by a stimulation of glucose oxidation. *Circulation* **87,** 972–981
27. Bergmeyer, H. U., Bergmeyer, J., and Grabl, M. (1995) *Carbohydrates, VI, Metabolites,* Verlag Chemie, Boca Raton, FL, pp. 570–587
28. Siegel, T. W., Smith, S. R., Ellery, C. A., Williamson, J. R., and Oates, P. J. (2000) An enzymatic fluorimetric assay for fructose. *Anal. Biochem.* **280,** 329–331
29. Schaefer, S., and Ramasamy, R. (1997) Glycogen utilization and ischemic injury in the isolated rat heart. *Cardiovasc. Res.* **35,** 90–98
30. Tanimoto, T., Maekawa, K., Okada, S., and Yabe-Nishimura, C. (1998)) Clinical analysis of aldose reductase for differential diagnosis of the pathogenesis of diabetic complications. *Anal. Chim. Acta* **365,** 285–292
31. Trueblood, N. A., and Ramasamy, R. (1998) Aldose reductase inhibition improves the altered glucose metabolism of isolated diabetic rat hearts. *Am. J. Physiol.* **275,** H75–H83
32. Park, J.-W., Chun, Y.-S., Kim, M.-S., Park, Y.-C., Kwak, S. J., and Park, S. C. (1998) Metabolic modulation of cellular redox potential can improve cardiac recovery from ischemia-reperfusion injury. *Int. J. Cardiol.* **65,** 139–147
33. Ramasamy, R., Trueblood, N. A., and Schaefer, S. (1998) Metabolic effects of aldose reductase inhibition during low-flow ischemia and reperfusion. *Am. J. Physiol.* **275,** H195–H203
34. Stanley, W. C., Lopaschuk, G. D., Hall, J. L., and McCormack, J. G. (1997) Regulation of myocardial carbohydrate metabolism under normal and ischemic conditions. Potential for pharmacological interventions. *Cardiovasc. Res.* **33,** 243–257
35. Taegtmeyer, H., King, L. M., and Jones, B. E. (1998) Energy substrate metabolism, myocardial ischemia, and targets for pharmacotherapy. *Am. J. Cardiol.* **82,** 54K–60K
36. Ramasamy, R., Liu, H., Oates, P. J., and Schaefer, S. (1999) Attenuation of ischemia induced increases in sodium and calcium by an aldose reductase inhibitor zopolrestat. *Cardiovasc. Res.* **42,** 130–139
37. Obrosova, I. G., Van Huysen, C., Fathallah, L., Cao, X. C., Greene, D. A., and Stevens, M. J. (2002) An aldose reductase inhibitor reverses early diabetes-induced changes in peripheral nerve function, metabolism, and antioxidative defense. *FASEB J.* **16,** 123–125
38. Chung, S. S., Ho, E. C., Lam, K. S., and Chung, S. K. (2003) Contribution of polyol pathway to diabetes-induced oxidative stress. *J. Am. Soc. Nephrol.* **14 (Suppl. 3),** S233–S236
39. Amano, Y., Yamagishi, S., Kato, N., Inagaki, Y., Okamoto, T., Makino, M., Taniko, K., Hirooka, H., Jomori, T., and Takeuchi, M. (2002) Sorbitol dehydrogenase overexpression potentiates glucose toxicity to cultured retinal pericytes. *Biochem. Biophys. Res. Commun.* **299,** 183–188

Received for publication December 18, 2003.
Accepted for publication April 14, 2004.