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Aldose reductase decreases endoplasmic reticulum stress in ischemic hearts

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Abstract

Aldose reductase (AR) is a multi-functional AKR (AKR1B1) that catalyzes the reduction of a wide range of endogenous and xenobiotic aldehydes and their glutathione conjugates with high efficiency. Previous studies from our laboratory show that AR protects against myocardial ischemia-reperfusion injury, however, the mechanisms by which it confers cardioprotection remain unknown. Because AR metabolizes aldehydes generated from lipid peroxidation, we tested the hypothesis that it protects against ischemic injury by preventing ER stress induced by excessive accumulation of aldehyde-modified proteins in the ischemic heart. In cell culture experiments, exposure to model lipid peroxidation aldehydes – 4-hydroxy *trans*-2-nonenal (HNE), 1-palmitoyl-2-oxovaleroyl phosphatidylcholine (POVPC) or acrolein led to an increase in the phosphorylation of ER stress markers PERK and eIF2- α and an increase in ATF3. The reduced metabolite of POVPC 1-palmitoyl-2-hydroxyvaleroyl phosphatidylcholine (PHVPC) was unable to stimulate JNK phosphorylation. No increase in phospho-eIF2- α , ATF3 or phospho-PERK was observed in cells treated with the reduced HNE metabolite 1,4-dihydroxynonenol (DHN). Lysates prepared from isolated perfused mouse hearts subjected to 15 min of global ischemia followed by 30 min of reperfusion *ex vivo* showed greater phosphorylation of PERK and eIF2- α than hearts subjected to aerobic perfusion alone. Ischemia-induced increases in phospho-PERK and phospho-eIF2- α were diminished in the hearts of cardiomyocyte-specific transgenic mice overexpressing the AR transgene. These observations support the notion that by removing aldehydic products of lipid peroxidation, AR decreases ischemia-reperfusion injury by diminishing ER stress.

Keywords

aldose reductase; ischemia; ER stress; unfolded protein response; mouse

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1. Introduction

Acute myocardial infarction (MI) is the leading cause of death in the US and Europe [1;2]. Clinical symptoms of MI appear upon abrupt interruption of blood flow due to plaque rupture and thrombotic occlusion of major coronary arteries. Sudden interruption of blood flow to myocardial tissue establishes an energy deficit and prevents removal of metabolic waste. These changes result in several pathophysiological alterations that increase $[Ca^{2+}]_i$ and decrease intracellular pH. Ischemic injury is further exacerbated by redox changes. Interruption of blood flow due to vessel occlusion decreases oxidative phosphorylation leading to mitochondrial leakage, which results in an increased production of reactive oxygen species (ROS) [3]. If reperfusion occurs, ROS production is increased further, leading to extensive reperfusion injury. Several studies have shown that inhibition of ROS production or an increase in antioxidant defense decreases reperfusion injury and increases myocardial salvage after ischemia-reperfusion [3–5].

Although highly reactive and toxic, most ROS are short-lived and it has been suggested that part of the tissue injury induced by ROS may be mediated by secondary products of lipid peroxidation [6]. Oxidation of unsaturated lipids in lipoproteins and membrane phospholipids generates a variety of toxic species, of which unsaturated aldehydes such as 4-hydroxy-*trans*-2-nonenal (HNE) are reactive [6] and are generated in high abundance in the ischemic heart [7;8]. Additionally, in isolated myocytes HNE has been shown to cause ATP depletion, changes in sodium and potassium current and to induce cell death [9]. Despite these findings, the contribution of HNE and related products of lipid peroxidation products to myocardial ischemia-reperfusion injury are unknown.

To assess the contribution of lipid-derived aldehydes to myocardial ischemia-reperfusion injury, we studied biochemical pathways that metabolize and detoxify unsaturated aldehydes in the heart. These studies showed that HNE is metabolized via three major pathways catalyzed by aldose reductase (AR; AKR1B) [10;11], aldehyde dehydrogenase (ALDH) [12] and glutathione-S-transferases (GSTs) [13;14]. The GSTs catalyze the conjugation of unsaturated aldehydes with reduced glutathione, whereas ALDH oxidizes the aldehyde using NAD^+ as a cofactor. AR catalyzes the reduction of both the free aldehyde as well as the aldehyde-glutathione conjugate. In the myocardial metabolism of HNE, AR appears to be a critical component because as shown by our previous work inhibition of AR increases the accumulation of HNE in the heart [15]. AR also abolishes the late phase of ischemic preconditioning in rabbit heart [15] and increases infarct size in rat hearts subjected to coronary ligation and reperfusion [16]. Although these data are consistent with the view that AR plays an important, non-redundant role in myocardial protection against ischemia-reperfusion, specific mechanisms by which AR protects against myocardial injury induced by HNE and related aldehydes remain unclear.

Unsaturated aldehydes such as HNE are strong electrophiles that react readily with reduced glutathione [6], nucleophilic centers of proteins [17], DNA [18] and phospholipids [19]. Although several studies have shown that during ischemia and reperfusion, proteins covalently adducted to HNE accumulate in the heart [7], the pathophysiological significance of protein-HNE adducts formation has not been assessed. Modified or misfolded proteins in the heart are removed by proteolysis [20] and excessive accumulation of undegraded proteins results in ER stress. Indeed, previous studies have shown that myocardial ischemia induces ER stress [21] and triggers the unfolded protein response [22;23].

Proteins are normally folded in the ER with the assistance of ER chaperones such as GRP78, before being transported to cellular locations specified by protein localization signals. Increased protein adducts in the ER or a decrease in the ability to properly fold proteins

disrupts ER homeostasis and induces ER stress [24;25]. To decrease this stress, cells trigger the unfolded protein response (UPR), which is an attempt to restore homeostasis [24]. During UPR protein synthesis is decreased, expression of ER chaperones is increased in an attempt to refold proteins, and misfolded proteins are removed by proteolysis [24;26;27]. Unresolved ER stress, however, triggers apoptosis [24;27]. In view of the evidence showing excessive accumulation of HNE-adducted proteins and ER stress in the ischemic heart, we tested the hypothesis that removal of HNE by AR prevents ischemic injury by diminishing ER stress. Our results show that HNE is a potent agent of ER stress and that overexpression of AR in the heart decreases ischemia-induced ER stress. These observations support the notion that ER stress in the ischemic heart is in part mediated by the accumulation of proteins modified by aldehydic products of lipid peroxidation.

2. Materials and Methods

2.1. Chemicals and reagents

Reagent HNE, DHN, POVPC and PHVPC were synthesized as described previously [10;28]. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Supplies for electrophoresis and Western blot analysis were purchased from BioRad (Hercules, CA, USA). Primary antibodies against eIF2- α and JNK as well as antibodies recognizing the phosphorylated proteins and secondary HRP-conjugated antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against ATF3 and the phosphorylated and non-phosphorylated form of PERK were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies recognizing actin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and antibodies against AR were raised in house as described before.

2.2. Animals

Wild-type (WT) C57BL/6 mice were purchased from Jackson laboratories and cardio-specific AR overexpressing transgenic (TG) mice were raised under standard conditions within our animal facility. The transgene was constructed by inserting a 0.95-kb rat AR cDNA next to the mouse α -myosin heavy chain (α -MHC) promoter in the multiple cloning site of the Myo vector. These mice were bred on to a C57 background to have only cardiac specific overexpression of AR. These animals breed and grow normally. The care and use of all animals was approved by the University of Louisville IACUC.

2.3. Cell culture and treatment of VSMC and HUVEC

Vascular smooth muscle cells (VSMC) obtained from Sprague-Dawley rat aortic explants were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin purchased from Invitrogen (Carlsbad, CA, USA). Human umbilical vein endothelial cells (HUVEC), obtained from PromoCell (Heidelberg, Germany), were cultured in endothelial basal medium (EBM, Clonetics/Lonza, Walkersville, MD, USA) containing human endothelial growth factor (hEGF), hydrocortisone, gentamicin/amphotericin B (GA), bovine brain extract (BBE) and 2% fetal bovine serum (FBS, EGM SingleQuots[®], Clonetics/Lonza, Walkersville, MD, USA). Cells were cultured under standard cell culture conditions (37°C, 5% CO₂).

For indicated treatments, the cells were grown to a confluence of 90–95%. Serum-starved VSMC or HUVEC (20 h, 0.1% EBM-media) were incubated as indicated with HNE (25 or 50 μ M), DHN (25 μ M), POVPC (25 or 50 μ M), PHVPC (25 μ M) or acrolein (50 μ M). To avoid reaction between the aldehydes and constituents of the culture medium, the cells were incubated with aldehydes for 30 or 60 min in Hanks' balanced salt solution (HBSS) containing 20 mM HEPES, 135 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl₂, 2.0 mM CaCl₂,

2.0 mM NaH₂PO₄, 5.5 mM glucose, pH 7.4. Long-term incubation (6 h total) required to measure changes in protein expression of ATF3, HBSS was replaced by complete media after 2h and the cells were incubated for an additional 4 h in the complete medium. Cells treated with thapsigargin (1 μ M Th) were used as positive control. After treatment, the cells were washed three times with ice-cold PBS (Invitrogen, Carlsbad, CA, USA) and processed for Western analysis.

2.4. Isolation and treatment of cardiomyocytes

Cardiomyocytes were isolated from mice (10–12 weeks) as described previously [29]. Briefly, the hearts were excised from mice, cannulated and perfused with oxygenated (95% O₂ and 5% CO₂) Tyrode solution containing 126 mM NaCl, 4.4 mM KCl, 1 mM MgCl₂·6H₂O, 18 mM NaHCO₃, 11 mM glucose, 4 mM HEPES, 20 mM 2,3 butanedione monoxime (BDM), and 30 mM taurine, pH 7.4. The extracellular matrix was digested by perfusing the heart for 12 min with Tyrode solution containing 0.6 units/mL Liberase IV (Roche, Basel, Switzerland). The heart was minced in Tyrode solution supplemented with BSA and Liberase IV and cells were allowed to settle down. Ca²⁺ was reintroduced in 5 steps. After 2 h incubation in DMEM (Invitrogen, Carlsbad, CA, USA) containing 20 mM BDM, cells were incubated in fresh media and allowed to attach overnight on laminin coated dishes. For treatment, the medium was removed and cells were incubated for 30 min with 25 or 50 μ M HNE or 300 nM thapsigargin in HBSS.

2.5. Echocardiography

Cardiovascular function of WT and AR overexpressing mice was analyzed by baseline two-dimensional echocardiography (Toshiba T380 Powervision) performed as previously described [30].

2.6. Global myocardial ischemia/reperfusion ex vivo

Murine hearts were excised, cannulated and perfused with Krebs Henseleit buffer by a standard Langendorf retrograde procedure as described previously [31]. Briefly, the hearts were cannulated and perfused at a constant pressure of 80 mm Hg with KHB supplemented with glucose (11mM) and CaCl₂ (2.5mM) at 37°C oxygenated with 95% O₂ and 5% CO₂. Hearts were equilibrated for 10 min and subjected to 3 treatment groups: perfusion alone (15 or 30 min), no-flow global ischemia (I, 15 or 30 min), no-flow global ischemia followed by reperfusion (I/R 15/ 15 or 30 min). After treatment the hearts were removed from cannulation and snap frozen in liquid N₂.

2.7. In situ ischemia

The murine model of myocardial ischemia and reperfusion has been described in detail [32]. Myocardial infarction was produced by a 15 min coronary occlusion followed by 15 min of reperfusion. The ischemic zone (IZ) was identified and segmented from the non-ischemic zone (NIZ) for analysis.

2.8. Western Analysis

Protein lysates were prepared from pulverized hearts, cardiomyocytes, HUVEC, or VSMC using a lysis buffer (25 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1% NP-40 and 0.1 % SDS, pH 7.0) supplemented with protease inhibitor cocktail (1:100 Sigma-Aldrich) and phosphatase inhibitor cocktail (1:100, Pierce, Rockland, IL, USA). After lysis and sonication, the lysates were centrifuged for 15 min at 4°C at 4000xg (for myocardial tissue) or 12000xg (for cell extracts). Total protein concentration was measured using a commercial kit (Bradford, Bio-Rad, Hercules, CA, USA).

SDS/PAGE and western blotting were performed as previously described [33]. Western blots were developed using ECL[®] plus reagent (Amersham Biosciences, Piscataway, NJ, USA) and were detected with a Typhoon 9400 variable mode imager (Amersham Biosciences, Piscataway, NJ, USA). Band intensity was quantified using the Image Quant TL software (Amersham Biosciences, Piscataway, NJ, USA).

2.9. AR activity and sorbitol measurements

AR activity was determined as previously described [31]. Briefly left ventricular tissue was homogenized, centrifuged and the supernatant was collected for activity measurements. Enzyme activity was measured at 25 °C, 1–1.5 mg of protein, 100 mM potassium phosphate (pH 6.0), 15 mM NADPH, and 10 mM glyceraldehyde. The reaction was monitored by the rate of oxidation of NADPH at 340 nm for 3 min. For sorbitol measurements, left ventricular tissue 0.1–0.2 g pulverized, homogenized then centrifuged was mixed with the protein-free supernatant (1.0 ml), and 0.5 ml of glycine buffer (pH 9.4) containing 3.6 mM NAD⁺ and 6 units of sorbitol dehydrogenase. The sorbitol levels were measured spectrofluorometrically, as described previously [34].

2.10. Statistical analysis

Data are presented as mean $\pm$ S.E.M and paired student *t*-test or one way ANOVA (LSD, Posthoc) was used for comparison between groups.

3. Results

3.1. Aldehydes derived from lipid peroxidation induce ER stress

The ER stress triggered UPR is characterized by an alarm and an adaptive phase involving the activation of multiple signaling cascades to halt protein synthesis and increase protein folding [27]. In this response, phosphorylation of PERK represents a major limb of the alarm phase. This leads to the phosphorylation of eIF2- α . Accordingly, we investigated whether aldehydes generated from oxidized lipids increase phosphorylation of these kinases. For this isolated mouse cardiac myocytes were treated with 25 or 50 μ M HNE for 30 min and changes in PERK and eIF2- α phosphorylation were measured by Western analysis. As shown in Fig. 1, treatment with HNE led to an increase in the phosphorylation of both PERK and eIF2- α . The extent of increase in phosphorylation of these proteins with 50 μ M HNE was comparable to that obtained with thapsigargin, indicating that the HNE-induced response was as robust as thapsigargin in stimulating the PERK pathway of UPR.

To examine whether lipid-derived aldehydes other than HNE also trigger UPR, we examined the effects of POVPC and acrolein. POVPC is one the most abundant oxidation product of 1-palmitoyl-2-arachidonyl-phosphatidylcholine (PAPC) and has been shown to increase endothelial adhesion and cytokine production [35]. Acrolein is also generated by oxidized lipids. It is also produced by myeloperoxidase-catalyzed oxidation of threonine [36]. Exposure of VSMC to POVPC (25 μ M) or acrolein (25 μ M) for 30 min led an increase in PERK phosphorylation. The extent of increase in PERK phosphorylation was most robust with acrolein, while HNE and POVPC were equally effective. Based on these data we conclude that the major aldehydes generated by lipid peroxidation trigger UPR in both cardiac myocytes as well as vascular smooth muscle cells.

3.2. Reduction decreases the ability of aldehydes to trigger UPR

Our previous work shows that AR catalyzes the reduction of POVPC [28], HNE [37] and acrolein [38]. Because reduction decreases carbonyl reactivity, we assumed it represents a mechanism for detoxification, which diminish the biological activities of the parent aldehyde. To test this assumption, we synthesized reduced metabolites of HNE and POVPC

i.e. DHN and PHVPC. To test the relative efficacies of POVPC and PHVPC, we examined changes in JNK. Previous studies show that induction of ER stress and activation of the UPR results in increased JNK phosphorylation [27]. As shown in Fig. 2A treatment of HUVECs with 25 μ M POVPC increased phosphorylation of p46, although the levels of total JNK were not affected. In total an approximately 4-fold increase in JNK phosphorylation was observed. Cells treated with PHVPC show a minimal increase in phospho-JNK, indicating clearly that POVPC, but not its reduced analog PHVPC, stimulates JNK phosphorylation.

To examine the relative efficacies of HNE and DHN, we measured changes in ATF3 and phospho-eIF2- α . Serum-starved HUVEC treated with HNE for 6h showed a significant increase in ATF3 expression, which was nearly undetectable in untreated cells. In contrast to HNE treatment, no increase in ATF3 was observed in cells treated with DHN. Because ATF3 is downstream to the PERK-eIF2- α pathway, we also examined the efficacy of DHN in stimulating the phosphorylation of eIF2- α . As shown in Fig. 2B, treatment with HNE led to robust phosphorylation of eIF2- α within 30 min of treatment. This increase in phosphorylation was still evident 60 min after treatment. No increase in phospho-eIF2- α was observed in cells treated with DHN either 30 or 60 min of treatment. To examine how DHN affects UPR in cardiac myocytes, the cells were treated with equal concentrations of either HNE or DHN. Western blots of lysates prepared from these cells showed that although treatment with HNE for 30 min led to a strong increase in phospho-PERK, no increase was observed in cells treated with DHN. Based on these observations we conclude that aldehydes generated from lipid peroxidation, but not their corresponding alcohols trigger UPR. This finding is consistent with the view that reduction by AR decreases aldehyde toxicity and therefore the catalytic activity of AR *in situ* is likely to prevent the induction of stress responses.

3.3. Myocyte-specific AR-transgenic mice

To study the role of AR we had previously used pharmacological inhibitors; however, the non-specific effects of chemical inhibitors could not be rigorously ruled out. Hence to minimize such off-target effects, we designed gain-of-function experiments to assess whether an increase in AR activity would prevent ischemia induced ER stress. As described under Materials and Methods, AR-TG mice were generated on a C57 background. These mice were phenotypically normal and bred as well as their WT littermates. Cardiac extracts from these mice showed a large increase in AR protein (Fig. 3A). Because it was attached to a His-tag, the product of the transgene could be readily distinguished from the native protein. The increase in AR protein was accompanied by an increase in enzyme activity. Both lines showed a 2–4 fold increase in enzyme activity determined in cardiac lysates with saturating concentrations of substrates. In contrast, there was a <2-fold increase in sorbitol accumulation, indicating that despite high levels of expression, the overall activity of the enzyme was not drastically increased and there was no osmotic overload imposed by overexpression of AR. Because the S 137 strain showed maximal activity, it was used for all further experiments. Table 1 summarizes parameters of cardiac contractility. No changes in body weight, heart rate, heart/body weight, or LV/body weight ratios were observed. Significantly, echocardiographic measurements of ejection fraction and fractional shortening in the TG mice were similar to those obtained with their WT littermates, indicating that overexpression of AR did not affect cardiac contractility or induce cardiac hypertrophy. These observations suggest that transgenic overexpression of AR does not affect basal function in mouse hearts.

3.4. Overexpression of AR prevents ischemic activation of ER stress in the heart

To examine ischemia-induced changes hearts from WT and TG mice were perfused and subjected to global ischemia and reperfusion *ex vivo*. As shown in Fig. 4A, hearts subjected to 30 min of ischemia displayed an increase in PERK phosphorylation. A similar increase in phospho-PERK was also observed in the ischemic zone of hearts subjected to 30 min of coronary occlusion *in situ* (Fig. 4B), indicating that the increase in PERK phosphorylation was dependent on *ex vivo* perfusion, but was also induced *in situ*. Global ischemia in isolated hearts was also associated with an increase in the phosphorylation of eIF2- α . The phosphorylation of eIF2- α was increased further upon reperfusion (Fig. 4A). After 30 min of reperfusion, the increase in phospho-eIF2- α was more than 2-fold higher than hearts that were perfused with the aerobic buffer alone. In contrast, no increases in the phospho-PERK or phospho-eIF2- α were observed in transgenic heart subjected to 30 min of ischemia or 30 min of ischemia followed by 30 min of reperfusion. From these data we conclude that an increase in AR activity prevents ER stress induced in hearts subjected to ischemia-reperfusion.

4. Discussion

The major findings of this paper are that aldehydes generated from lipid peroxidation trigger the unfolded protein response (UPR) and that increased metabolism of these aldehydes during myocardial ischemia and reperfusion via aldose reductase (AR) prevents ischemia-induced ER stress. These findings support the view that ER stress is a significant component of myocardial ischemia-reperfusion injury and that it is mediated in part by the aldehyde products of lipid peroxidation.

Aldehydes are the major end products of lipid peroxidation reactions that take place in membrane proteins and lipoproteins. Several aldehydes are generated in oxidized lipids. Aldehydes such as POVPC are formed due to fragmentation of bis-allylic double bonds of unsaturated fatty acids esterified to a phospholipid backbone. Free aldehydes such as HNE are generated from ω -6 polyunsaturated fatty acids and are one of the most reactive products of lipid peroxidation [39]. Under some conditions, HNE represents 95 % of the total unsaturated aldehyde production. Normal cellular levels of HNE range from 0.8 to 2.8 μ M but up to 10–100 mM HNE has been measured in oxidizing microsomes [40;41]. Several conditions associated with oxidative stress leads to increased accumulation of proteins covalently adducted to lipid-derived aldehydes. In particular, cardiovascular oxidative stress associated with vasculitis [42], myocardial hypertrophy [43], heart failure [44], and ischemic hearts [7;45–47] are associated with an increase in the accumulation of protein-aldehyde adducts. Nonetheless, in most cases, the functional significance of such adduct formation is unknown. Hence we tested the hypothesis that accumulation of aldehyde-modified proteins in the heart induces ER stress and triggers the unfolded protein response (UPR), which is a significant cause of myocardial ischemia-reperfusion injury.

Our results show that both free (HNE) and esterified (POVPC) aldehydes are potent UPR triggers. Treatment of isolated cardiac myocytes, vascular smooth muscle cells or endothelial cells resulted in increased phosphorylation of PERK and eIF2- α . Both kinases play a central role in orchestrating UPR in response to ER stress. The mammalian UPR is mediated by 3 distinct pathways due to IRE-1, PERK and ATF6 activation following their dissociation from GRP78 (Bip) [27]. Not all forms and inducers of ER stress trigger identical UPR which is not an all-or-none response. In neonatal cardiac myocytes, simulated ischemia induces all three arms of UPR (PERK, ATF6, and IRE-1) [22;48;49] and their downstream targets (CHOP, procaspase-12 cleavage) [50], however, in a model of global brain ischemia, PERK-eIF2- α were activated, but ATF6, XBP-1, CHOP, and ATF4 were not [51]. Hence our studies showing that aldehydes stimulate the phosphorylation of both PERK

and eIF2- α provide strong evidence that lipid-derived aldehydes cause ER stress. This notion is further supported by the robust activation of ATF3 by HNE in endothelial cells. Although it has been shown before that HNE upregulates ER stress genes such as ATF3, HERP and Gadd34 [52], the significance of these changes is not clear. Therefore, our observation that ATF3 is induced by HNE after activation of PERK and eIF2- α supports the idea that induction of ATF3 may be in response to ER stress.

Treatment with POVPC was also found to increase JNK phosphorylation. While JNK is considered to a classical stress-activated kinase, recent studies suggest that its phosphorylation is enhanced by UPR signaling activated by ER stress [27]. Activation of JNK by ER stress has been shown to be downstream of IRE1 activation [53], and phosphorylation of JNK is also impaired in PERK-null fibroblasts [54]. Although mechanisms by which aldehydes-induced JNK activation were not examined, phosphorylation of this stress kinase with ER stress markers suggests that as described in other cells, JNK activation may be related to ER stress. More importantly, phosphorylation of JNK was stimulated by POVPC but not by PHVPC, indicating that unlike the parent aldehydes, alcohols cannot induce ER stress. A similar conclusion is supported by the observations that DHN was unable to induce eIF2- α phosphorylation in endothelial cells and PERK phosphorylation in cardiac myocytes. Taken together, these observations support the concept that metabolism of aldehydes to alcohols in the heart abolishes their ability to induce ER stress and to induce stress-kinase signaling.

Our previous work shows that reduction of aldehydes in the heart is catalyzed by AR. Extensive studies show that AR catalyzes the reduction of structurally diverse range of aldehydes. The enzyme is particularly efficient in reducing medium to long-chain saturated and unsaturated aldehydes [55]. AR also catalyzes the reduction of aldehyde-glutathione conjugates; in many cases with efficiency higher than that of the parent aldehyde [56]. In addition, our studies show that reduction of HNE and GS-HNE is prevented in hearts pretreated with AR inhibitors [10] and that inhibition of AR results in the accumulation of HNE in the heart [15]. Inhibition of AR has also been shown to increase HNE during vascular inflammation [42]. AR is upregulated upon exposure to oxidative stress specifically in the areas of high HNE formation [42]. Furthermore, our recent studies show that AR is activated in the ischemic heart [31] and that pharmacological inhibition of the enzyme increases myocardial ischemia-reperfusion injury [16]. In extension of this work, data presented here show that transgenic overexpression of AR in cardiac myocytes prevented ischemia-reperfusion induced phosphorylation of eIF2- α . These data are consistent with our hypothesis (Fig. 5) that AR protects the heart by preventing the accumulation of lipid-peroxidation derived aldehydes, which if left unmetabolized could contribute to ischemic injury by inducing ER stress.

Results from several recent studies suggest that ER stress may be an important consequence of ischemia and a significant mediator of ischemia-reperfusion injury. It has been shown that brief ischemic episodes increase the expression of GRP94, GRP78 [22;48;57] and that prolonged ischemia leads to the activation of UPR components CHOP and caspase-12 that can trigger cell death. Induction of GRP78 and GRP94 has also been reported in mouse hearts subjected to I/R *ex vivo* [57] and elevated levels of GRP78 have been reported in the peri-infarct zone of the heart [22]. That UPR triggered by ER stress is a significant cause of myocardial I/R injury is suggested by studies reporting that overexpression of GRP94 [25] or pre-induction of UPR prevents hypoxic cardiac myocyte cell death [58]. Conversely, cardiomyocyte death due to simulated I/R is increased by dominant negative XBP-1 [22] and anti-sense GRP78 could abolish the protective effects of early preconditioning [59]. Activated AFT6 has been recently reported to protect the heart from I/R injury [57] indicating that enhancing the expression of ER chaperones could prevent ischemic injury. In

agreement with these observations we found that ischemia lead to an increase in eIF2- α and PERK phosphorylation. Similar results were obtained in hearts subjected to global ischemia-reperfusion ex vivo and in hearts subjected to coronary occlusion and reperfusion in situ, indicating model-independent induction of ER stress in the ischemic heart. Significantly, eIF2- α phosphorylation was attenuated in AR-TG hearts, suggesting that AR is a significant regulator of ischemia-induced ER stress in the heart.

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Fig. 1. Aldehydes-derived from lipid peroxidation induce ER stress

(A) Representative Western blot of lysates prepared from isolated mouse cardiomyocytes incubated with either 25 or 50 μM HNE or thapsigargin for 30 min. The blots were developed with antibodies raised against phospho-PERK, PERK, and phospho-eIF2- α . (B) Western blots from lysates of VSMC incubated with 50 μM POVPC, 50 μM HNE or 50 μM acrolein. The blots were developed using antibodies that recognize phospho-PERK and total PERK. After band quantification, the extent of phosphorylation was calculated from the ratio of the band intensities of the phospho-PERK normalized to total PERK. Data are shown as mean $\pm$ S.E.M. * $P < 0.01$ ($n = 3-4$).

Fig. 2. Reduction decreases the ability of HNE to induce stress responses

(A) Representative Western blots of lysates from HUVECs incubated with POVPC (25 μ M) or PHVPC (25 μ M) developed using antibodies directed against phospho-JNK or total JNK. Quantification performed by densitometric analysis of phospho-JNK normalized to total JNK is shown in lower panel. (B) Western blots of lysates from HUVECs incubated with 25 μ M HNE or DHN. The levels of ATF3 and phospho-eIF2- α were measured as indicated after 6 h or 30 min and 60 min, respectively. (C) Stimulation of PERK phosphorylation in isolated cardiomyocytes. Western blots were performed with lysates of cells treated with 25 μ M HNE or 25 μ M DHN for 30 min and developed using an anti-phospho-PERK antibody. Data are shown as mean $\pm$ S.E.M. In panel A, * $P < 0.01$; control (untreated cells) versus

POVPC; and # versus. PHVPC (n = 4). In panel B, * $P < 0.01$ HNE-treated versus untreated cells and # versus DHN (n=3).

Fig. 3. Cardiac-myocyte specific overexpression of aldose reductase in mouse heart

The AR-transgenic (TG) mice were generated in our laboratory as described under Materials and Methods using the α -myosin heavy chain (α -MHC) promoter to drive the expression of the transgene. (A) Representative Western blots of myocardial lysates from wild type (WT) and two TG lines –S 137 and S 138– developed with anti-AR antibody. The additional band in TG hearts with the slightly higher molecular weight (due to His-Tag) was ascribed to the transgene. For quantification, the intensities of both bands were summed and normalized to GAPDH. (B) AR activity measured in whole hearts lysates from WT and TG mice. Enzyme activity was measured using 10 mM glyceraldehyde and 0.1 mM NADPH. Sorbitol content was determined enzymatically by using sorbitol dehydrogenase. Data are presented as mean $\pm$ S.E.M; * $P < 0.01$ ($n = 3-4$).

Fig. 4. Overexpression of AR decrease ischemia-induced ER stress in the heart

(A) Representative Western blots of lysates from perfused hearts which were either subjected to perfusion (P) alone, 30 min of ischemia (I), or 30 min ischemia followed by 30 min of reperfusion (I/R) ex vivo. The blots were developed using anti-phospho-PERK, anti-phospho-eIF2-α or anti-eIF2-α antibodies. Quantification for WT and TG hearts are shown in a bar diagram. (B). Wild type mouse hearts were subjected to 15 min coronary ligation and 15 min reperfusion in vivo. Whole hearts were lysed and representative western blots developed with anti-phospho-PERK compared the phosphorylation of PERK in ischemic (IZ) and non-ischemic (NIZ) zones. Data are presented as mean ± S.E.M. * $P < 0.01$, ($n = 3 - 4$).

Scheme I. ER stress in the ischemic heart

Myocardial ischemia leads to an increase in ROS generation which results in an increase in the formation of aldehydes derived from oxidized lipids. These aldehydes are reduced by AR to alcohols, which are inactive. Aldehydes that escape AR reduction form covalent adducts with proteins which induce ER stress. Modified proteins induce a dissociation of GRP78 from PERK leading to PERK phosphorylation, which in turn phosphorylates eIF2- α resulting in the suppression of protein translation. Although not studied here, ER stress also results in the activation of IRE-1 α -XBP-1 pathway inducing the transcription of inflammatory genes as well as genes encoding ER chaperones and ERAD components. The transcription of ER chaperones is enhanced to increase the protein capacity of the cell. These changes are prevented in hearts in which overexpression of AR prevents the accumulation of lipid peroxidation-derived aldehydes.

Table 1

Cardiac parameters in AR TG and WT mice

M-mode Echocardiogram	WT	AR-TG
Body Weight	35 ± 0.76	31.28 ± 1.4
HR (beats/min)	527 ± 24	537 ± 14
Heart/Body Weight	5.0± 0.18	5.8 ± 0.30
LV/Body Weight	3.6± 0.14	4.08 ±0.27
Ejection Fraction	0.67 ± 0.01	0.68 ± 0.01
Fractional shortening	0.71 ± 0.01	0.68 ± 0.01
RWT	0.34± 0.01	0.33 ±0.01