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ALDOSE REDUCTASE PATHWAY CONTRIBUTES TO VULNERABILITY OF AGING MYOCARDIUM TO ISCHEMIC INJURY

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Abstract

Aging men and women display both increased incidence of cardiovascular disease and complications of myocardial infarction and heart failure. We hypothesized that altered glucose metabolism, in particular, flux of glucose via the polyol pathway (PP) may be responsible, in part, for the enhanced vulnerability of aging myocardium to ischemic injury, even in the absence of superimposed disease processes linked to PP flux, such as diabetes. To test our hypothesis, we determined the expression and products of PP enzymes aldose reductase (AR) and sorbitol dehydrogenase (SDH) in hearts from Fischer 344 aged (26 month) and young (4 month) rats subjected to global ischemia followed by reperfusion in the presence or absence of blockers of PP and the measures of ischemic injury and functional recovery were determined. Expression and activities of AR and SDH were significantly higher in aged vs. young hearts, and induction of ischemia further increased AR and SDH activity in the aged hearts. Myocardial ischemic injury was significantly greater in aged vs. young hearts, and blockade of AR reduced ischemic injury and improved cardiac functional recovery on reperfusion in aged hearts. These data indicate that innate increases in activity of the PP enzymes augment myocardial vulnerability to I/R injury in aging, and that blockers of PP protect the vulnerable aging hearts.

Keywords

ldose reductase; polyol pathway; myocardial ischemic injury; aging hearts; cardiac function

1. INTRODUCTION

Lakatta and Levy summarized the complexities of understanding aging and the risk of cardiovascular disease, and concluded that the major factor underlying the propensity of cardiovascular diseases in human subjects is advancing age itself, even after taking into account aging-associated diseases, such as diabetes, sedentary life style, and genetic predisposition (Lakatta et al., 2003a; Lakatta et al., 2003b; Lakatta et al, 2003c) Innate

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dysfunction of the aging cardiovascular system in the vascular arterial structures and myocardium, in part, primes aging subjects for increased vulnerability to superimposed stresses. In this work, we focused on one such stress, ischemia/reperfusion (I/R) injury (Wei et al., 1992). Studies in aging animals have demonstrated increased cell death and poor survival after cardiac I/R injury compared to young animals (Azhar et al., 1999a; Liu et al., 2002; Isoyama et al., 2002; Azhar et al., 1999b). Although multiple pathways contribute to the above findings, we have focused on the role of glucose metabolizing pathways in aging and ischemic stress.

A number of mechanisms with diverse etiologies have been postulated to contribute to myocardial damage due to ischemia in young animals. Although reperfusion, especially early after the onset of occlusion, has provided immense benefit during acute myocardial ischemia, recovery of function after ischemia is predicated not only on restoration of nutritive perfusion but also on the ability of the myocyte to maintain sufficient substrate metabolism during ischemia. Numerous studies (Camici et al., 1989; Stanley et al., 1997; Taegtmeier et al., 1998; Young et al., 1997; Ramasamy et al., 2001; Vanoverschelde et al., 1994; Lopaschuk et al., 1997; Lopaschuk et al., 1988; Neely et al., 1974; Schaefer et al., 1995; Steenbergen et al., 1990; Steenbergen et al., 1993), have shown that the recovery of function after ischemia is enhanced by interventions that (a) maintain tissue ATP availability, and (b) limit derangements in ionic homeostasis. The presence of inherent metabolic alterations seen in aging animals and humans (Hall et al., 1994; Cartee, 1993; McMillin et al., 1993; Lesnefsky et al., 1994) restricts the availability of therapeutic interventions to protect ischemic myocardium in aging subjects. Impaired metabolism and energy homeostasis have been associated with poor metabolic and functional recovery after I/R in hearts (Kates et al., 2003; Misare et al., 1992; Headrick et al., 1998). In this context, the relationship between altered glucose metabolism and response to I/R is poorly understood in hearts from aging subjects. Studies by us and others have demonstrated that increased substrate flux via aldose reductase impairs and impedes recovery of myocardium after ischemic insult in young animals (Ramasamy et al., 1997; Ramasamy et al., 1998; Hwang et al., 2002; Hwang et al., 2004; Tracey et al., 2000). In this study, we investigated if aging impacts flux via the aldose reductase pathway and as a consequence increases vulnerability of hearts to I/R injury.

2. MATERIALS AND METHODS

All animal studies were performed with the approval of the Institutional Animal Care and Use Committee at Columbia University, New York and New York University School of Medicine. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, 1996). Fischer 344 young (4 months) and aged (24 months) rats were purchased from NIA colony (Bethesda, MD). Transgenic mice expressing human aldose reductase driven by the major histocompatibility type 1 promoter and nontransgenic littermates in the C57BL/6 background, young (4 months) and old (24 months), were obtained from our established breeding colony. (Hwang et al., 2004).

2.1. Isolated Perfused Heart Preparation

Experiments were performed using an isovolumic isolated rat/mice heart preparation as published earlier (Ramasamy et al., 2001; Hwang et al., 2004). After deep anesthesia was achieved, hearts were rapidly excised, placed into iced saline, and retrogradely perfused at 37°C in a non-recirculating mode through the aorta. Hearts were perfused with modified Krebs-Henseleit buffer containing (in mM) NaCl 118, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 25, glucose 5, palmitate 0.4, bovine serum albumin 0.4, and 70 mU/L insulin. The perfusate was equilibrated with a mixture of 95% O₂-5% CO₂, which maintained perfusate

PO₂ > 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 an ADI recorder. All rat hearts were subjected to 20 min of zero-flow ischemia and 60 min of reperfusion (I/R). All mice hearts were subjected to 30 min of zero-flow ischemia and 60 min of reperfusion.

In studies involving the use of inhibitors, hearts were perfused with modified Krebs-Henseleit buffer containing enzyme specific inhibitors of AR (ARI-gift from Pfizer, Groton, CT) 1 μ M unbound zopolrestat, or SDH (SDI-gift from Pfizer, Groton, CT) 200 nM CP-407,711, 10 min prior to ischemia and was continued throughout the perfusion protocol. The specificity of ARI and SDI used in this study has been well established (Mylari et al., 1991; Mylari et al., 2003). Hearts were freeze-clamped after baseline, ischemia, and reperfusion periods for enzyme activity and expression studies. These hearts were stored frozen at -80°C until the analyses were performed.

2.2 Biochemical assays and western blot measurements

Aldose reductase and sorbitol dehydrogenase activities were measured in the heart homogenates using spectrophotometric techniques (Ramasamy et al., 1997; Ramasamy et al., 1998; Hwang et al., 2002). ATP was measured in the neutralized perchloric acid extracts of hearts using HPLC methods according to previously-published procedures (Ramasamy et al., 1997; Ramasamy et al., 1998; Hwang et al., 2002). Creatine kinase (CK) release, a marker of myocardial I/R injury, was measured as published earlier (Ramasamy et al., 1997; Ramasamy et al., 1998; Hwang et al., 2002). For western blot studies, equal amounts of proteins were separated on 4–20% SDS –PAGE and transferred on to PVDF membrane (Invitrogen), blocked with 5% nonfat dry milk for 1.5 hrs and probed with primary antibody overnight at 4°C . Polyclonal rabbit anti-AR IgG; and anti-SDH IgG at 1:500 or 1:1000 dilutions were used. After washing the blots three times in TBS-Tween (TBST), they were incubated in secondary antibody conjugated with HRP, followed by washing in TBST and signals were detected using ECL reagent (Amersham-Pharmacia). Signal intensities were quantified using software Alpha-ease (Kodak). All blots were normalized by reprobing the blots with antibody specific for β -actin or GAPDH.

2.3. Statistical methods

Data was analyzed using INSTAT (GraphPad, San Diego, CA) 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 $\pm$ standard deviation.

3. RESULTS & DISCUSSION

3.1. AR pathway and myocardial I/R injury in aging

To determine if aging alters the activities of aldose reductase (AR) and sorbitol dehydrogenase (SDH), hearts from young (4 months) and old (26 months) Fischer 344 rats were isolated, perfused and freeze-clamped and the activities of these enzymes and their products (sorbitol, fructose) were measured using biochemical assays, and the expression levels assessed using western blots. Figure 1 a,b,c indicate that in the absence of I/R injury, aging hearts displayed higher myocardial AR and SDH activities along with their respective products sorbitol and fructose, and protein expression compared to young hearts. These data indicate for the first time that aging is linked to increases in the AR pathway expression and activity in the Fischer 344 rat heart.

To investigate if increases in the AR pathway impact on the ability of aging rat hearts to recover from I/R injury, isolated perfused hearts were subjected to I/R and the measures of injury and cardiac function monitored. Creatine kinase (CK) release during reperfusion, a marker of ischemic injury, was greater in old hearts than in young rat hearts (Fig 2a). Pharmacological blockade of AR or SDH reduced CK release during reperfusion in old hearts after ischemia. Left ventricular developed pressure recovery was lower in old hearts compared to the young hearts after I/R (Fig 2b), indicating impaired functional recovery in old hearts. ARI or SDI improved cardiac functional recovery on reperfusion in aging hearts. ATP is a critical determinant of the ability of myocardium to recover from ischemic injury. In order to determine if ATP levels are differentially influenced in aging vs. young myocardium, ATP measurements were made during baseline, ischemia, and reperfusion using HPLC methods. Although there were no significant differences in ATP levels in the myocardium at baseline, ATP levels were much less conserved in old hearts vs. young during ischemia and reperfusion (Fig. 2c). Inhibition of AR or SDH improved ATP levels in aging hearts during ischemia and reperfusion. Together, these data indicate that interventions that block AR and SDH protect aging hearts from I/R injury.

While most studies have demonstrated that increased flux via AR is detrimental during ischemia-reperfusion in hearts (Ramasamy et al., 1997; Ramasamy et al., 1998; Hwang et al., 2002; Hwang et al., 2004; Tracey et al., 2000; Ananthakrishnan et al., 2009; Iwata et al., 2006; Tang et al., 2008; Li et al., 2008), some studies have shown contrary results (Shinmura et al., 2002; Kaiserova et al., 2008). Though others (Kaiserova et al., 2008) showed increases in AR activity during ischemia consistent with our earlier publication (Hwang et al., 2002), they were unable to demonstrate cardioprotection with ARIs in a glucose perfused isolated rat heart I/R model. Reasons for these contrasting findings in young animals could, in part, be due to model-dependent variations and substrate availability. Our findings here in aging rats indicate that increased flux via AR leads to increased injury during I/R in the heart.

3.2. Metabolic consequences of AR pathway increases in aging

Several studies have shown that increases in flux via the AR pathway lead to increases in cytosolic NADH/NAD⁺ ratio (Williamson *et al.* 1993; Trueblood & Ramasamy (1998). Furthermore, it has also been shown that tissue lactate/pyruvate (L/P) ratio reflects the cytosolic ratio of NADH/NAD⁺ (Williamson *et al.* 1993; Trueblood & Ramasamy (1998). To determine if increases in AR and SDH seen in aging hearts impact on cytosolic NADH/NAD⁺, L/P ratios were measured in freeze-clamped heart tissue extracts. As shown in Table 1, the baseline L/P ratios were significantly higher in old hearts than in young hearts. Pharmacological blockade of AR or SDH significantly lowered L/P ratio in both old and young rat hearts. 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 cytosolic NADH/NAD⁺ ratio. As shown previously, increasing flux via the AR pathway increases L/P ratio, impairs glycolysis, and decreases ATP levels in ischemic hearts (Ramasamy et al., 1997; Ramasamy et al., 1998; Hwang et al., 2002; Hwang et al., 2004; Tracey et al., 2000; Ananthakrishnan et al., 2009; Iwata et al., 2006; Tang et al., 2008; Li et al., 2008; Williamson et al., 1993; Trueblood et al., 1998; Hwang et al., 2003). In the present study increases in L/P ratio were attenuated; ATP decline was reversed, and ischemic injury was reduced in aged hearts by treatment with an AR or SDH inhibitor. Inhibition of AR or SDH also resulted in improved functional recovery on reperfusion in aging hearts. These data demonstrate that increased L/P ratio, hence cytosolic NADH/NAD⁺ ratio, is an important event in AR pathway mediated increases in ischemic injury in aging.

3.3. Aldose reductase in aging mice

To determine if the AR pathway plays a critical role in increasing the vulnerability of aging hearts to I/R injury, we investigated the impact of I/R stress and changes in AR activity and content in young and aging C57BL/6 mice hearts and compared them with those in young and old rat hearts. Data in the C57BL/6 mice hearts reveal that wild-type mice of this strain do not fully recapitulate the enhanced vulnerability to I/R stress noted in rats (Figure 3). In the isolated perfused heart, aged vs. young C57BL/6 mice did not display significant increases in release of CK nor LVDP recovery – in contrast to data in the rat, as shown above in figure 2. Similar findings in the C57BL/6 strain in aged mice (release of LDH upon I/R for example in aged vs. young C57BL/6 mice) have been demonstrated (Willems *et al.* 2005). Despite lack of differences in markers of injury in aged mice hearts, Willems *et al.* (2005) did observe poor functional recovery. In our studies we did not observe differences in functional recovery after I/R in aged vs young mice. As reviewed by Boengler *et al.* (2009), the precise manner of induced injury in the model may impact on the changes in injury and functional recovery after I/R.

To address the differences in ischemic injury response in rats (Figure 2) vs. mice (Figure 3), we investigated if differences in AR activity and expression in rats vs. mice might, in part, be responsible, given the therapeutic benefit of ARI in the aged rat heart in I/R. Table 2 illustrates the levels and activities of AR in rats and mice. Rats display significantly higher cardiac AR protein and activity compared to mice of the same age. Aging increased the AR protein level and activity in rat hearts, whereas the activity of cardiac AR and levels in aged C57BL/6 mice did not change with age. In humans, the levels of AR protein in heart are 300–800 ng/mg total protein in non-diabetic subjects (Hwang *et al.* 2004). The AR protein levels in the mouse heart are several fold lower than those observed in humans and rats. These results prompted us to employ transgenic mice expressing human AR to restore human and rat-relevant levels of AR in the mouse; we hypothesized that transgenic expression of AR would better model the human aging heart. Note that transgenic hAR mice (a) display human-relevant levels of AR; and (b) the levels of sorbitol and fructose, and AR activity increase with aging, unlike the C57BL/6 mouse (Figure 3, Table 2). Thus, although mice may display many of the features of aging, their inconsistent enhanced vulnerability to I/R stress in the isolated perfused heart, at least in C57BL/6 strain, suggest that the transgenic hAR mouse model offers distinct advantages to probe our hypotheses in aging.

Consistent with the hypothesis that restoration of human relevant levels of AR in the heart will drive increased I/R injury in the mouse, aged transgenic hAR mice (24 months) were subjected to identical degrees of I/R as that induced in the rats as shown in figure 2 above. Aged transgenic hAR mice hearts had similar wet and dry weight, total body weight, and physical appearance as the aged non-transgenic littermates. Data from these aged transgenic hAR mice and aged non-transgenic littermate mice are presented in Figure 3. Transgenic hAR mice exhibited increases in AR activity and polyol content (sorbitol and fructose with aging (fig 3a & b). Thus, increases in AR expression were observed in aging rats and aging hAR mice hearts. Of note, in the transgenic hAR mice, these observed increases in AR expression with aging are likely due, at least in part, to significant increases in human AR transgene transcription. Indeed, studies by others have clearly shown that pathological insults can specifically lead to increases in expression of transgene-driven hAR gene expression in mice (Vikramadithyan *et al.* 2005 and Dan *et al.* 2004). In this context, while the precise mechanisms by which AR expression increases in the aging hAR mice and rats is unclear, it is conceivable that increases in reactive oxygen species and AGEs may play an important role in directly mediating increases in AR expression (Dan *et al.* 2004, Nakamura *et al.* 2000).

Aged transgenic hAR mice hearts exhibited increased ischemic injury (CK release, fig. 3c) and poorer LVDP recovery (fig. 3d) after I/R than young transgenic hAR mice hearts. Inhibition of AR with ARI in aged hAR mice reduced ischemic injury (fig 3c) and improved functional recovery (fig 3d) after I/R. It is evident from the present study that in aging hearts, basal increases in AR expression and activity may exert an adverse impact on the ability of myocardium to recover from an added stress such as ischemia. Inhibition of such increases in AR activity reduced ischemic injury and improved functional recovery. Clearly, increased expression and activity of AR was associated with increased markers of injury due to I/R. Furthermore, mice expressing human AR recapitulated the increased vulnerability to I/R stress that were observed in aging Fischer 344 rat hearts. Consistent with earlier findings, we showed that AR pathway inhibition lowered L/P ratio and improved ATP levels in aging hearts (Ramasamy et al., 1997; Ramasamy et al., 1998; Hwang et al., 2002; Hwang et al., 2004; Tracey et al., 2000; Ananthakrishnan et al., 2009; Iwata et al., 2006; Tang et al., 2008; Li et al., 2008; Williamson et al., 1993; Trueblood et al., 1998; Hwang et al., 2003). However, the influence of the AR pathway in impacting intracellular sodium and calcium homeostasis (Ramasamy et al., 1999), oxidative stress (Ananthakrishnan et al., 2009; Tang et al., 2008; Ho et al., 2006) and PKC mediated signaling in aging (Hwang et al., 2005; Ramana et al., 2006) events needs to be investigated. The current studies indicate 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 myocardial infarction in aging.

Research Highlights

1. Aging increases flux via PP enzymes AR and SDH;
2. Increases in PP activity are linked to increased injury and poorer functional recovery after I/R in aging;
3. Increases in PP flux linked to reduced levels of ATP during I/R

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Figure 1.

(A) Aldose reductase (AR) (nmols/min/mg prot) and sorbitol dehydrogenase (SDH) (IU/mg prot) activity; (B) Protein expression of AR and SDH; and (C) concentration of polyol pathway products sorbitol and fructose in perfused hearts from young (n=6) and old (n=6) Fischer 344 rat hearts subjected to normoxic perfusion (4 mos vs. 24 mos of age, respectively).

Figure 2.

(A) Creatine kinase (CK) release during 60 min of reperfusion, a marker of myocardial I/R injury in hearts from young, old, Old-ARI treated (Old-ARI) or Old-SDI treated (Old-SDI) Fischer 344 rats. Pre-ischemic CK release data for young (Pre-Isch-Y) and old (Pre-Isch-O) presented reveal no basal differences in aged vs. young rat hearts. (B) Left ventricular developed pressure (LVDP) recovery in the above hearts. (C) Myocardial ATP content in young, old, old-ARI treated (Old-ARI) or old-SDI treated (old-SDI) Fischer 344 rats. * $p < 0.05$ vs. young in their respective ischemia or reperfusion group. All hearts were subjected to global ischemia followed by reperfusion. ARI = zopolrestat; 1 μ M and SDH = CP-470,711, 200 nM. $n = 6$ per group

Figure 3. Cardiac tissue levels of sorbitol (A) and fructose in young, hAR transgenic young (ARTg-Young), Old, and hAR transgenic old (ARTg-Old) subjected to normoxic perfusion. (n=6 per group). (C) Creatine kinase (CK) release during 60 min of reperfusion, a marker of myocardial I/R injury in hearts from young non-transgenic (n=6), hAR transgenic young (n=6), old non-transgenic (n=6), hAR transgenic old (n=6) mice. (D) Left ventricular developed pressure (LVDP) recovery during reperfusion in young and old non-transgenic and hAR transgenic mice.

Table 1

Lactate:pyruvate ratios in young and old Fisher 344 rat hearts

Group	n	Lactate/Pyruvate ratio
Young	12	16.8 ± 2.2
Young-ARI	9	6.8 ± 2.8 [#]
Old	6	36.2 ± 8.8 [*]
Old-ARI	5	7.2 ± 3.1 [#]
Old-SDI	5	8.9 ± 4.4 [#]

^{*} p < 0.05 Old vs Young,

[#] p < 0.05 Old-ARI, Old-SDI vs Old, young-ARI vs Young. The lactate/pyruvate ratios were measured after perfusion for one hour under normoxic conditions.

Table 2

Aldose reductase levels and activity in rat and mice hearts

Group	AR protein (ng/mg total prot)	AR activity (nmols NADPH/min/mg prot)
Fisher 344 RATS		
Young rats (n=5)	256 ± 51	2.66 ± 0.32
Old rats (n=5)	540 ± 62 *	5.21 ± 0.61 [#]
C57BL6 MICE		
Wild Type mice (Young n=8)	98 ± 21	0.66 ± 0.23
Wild Type mice (Old,n=6)	122 ± 52	1.21 ± 0.33
TghAR mice (Young,n=9)	456 ± 49 [@]	4.12 ± 0.81 ^{\$}
TghAR mice (Old mice, n=5)	598 ± 25 ^{**}	6.89 ± 0.31 ^{**}

*
p<0.03 vs. young rats#
p<0.04 vs. young rats@
p<0.05 vs. young mice\$
p<0.05 vs. young and old mice**
p<0.05 vs. young TghAR mice