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ORIGINAL RESEARCH COMMUNICATION

Endogenous Adenosine Selectively Modulates Oxidant Stress *via* the A_1 Receptor in Ischemic Hearts

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Abstract

We tested the impact of A_1 adenosine receptor (AR) deletion on injury and oxidant damage in mouse hearts subjected to 25-min ischemia/45-min reperfusion (I/R). Wild-type hearts recovered ~50% of contractile function and released 8.2 ± 0.7 IU/g of lactate dehydrogenase (LDH). A_1AR deletion worsened dysfunction and LDH efflux (15.2 ± 2.6 IU/g). Tissue cholesterol and native cholesteryl esters were unchanged, whereas cholesteryl ester–derived lipid hydroperoxides and hydroxides (CE-O(O)H; a marker of lipid oxidation) increased threefold, and α-tocopherylquinone [α-TQ; oxidation product of α-tocopherol (α-TOH)] increased sixfold. Elevations in α-TQ were augmented by two- to threefold by A_1AR deletion, whereas CE-O(O)H was unaltered. A_1AR deletion also decreased glutathione redox status ([GSH]/[GSSG + GSH]) and enhanced expression of the antioxidant response element heme oxygenase-1 (HO-1) during I/R: fourfold elevations in HO-1 mRNA and activity were doubled by A_1AR deletion. Broad-spectrum AR agonism (10 μM 2-chloroadenosine; 2-CAD) countered effects of A_1AR deletion on oxidant damage, HO-1, and tissue injury, indicating that additional ARs (A_{2A}, A_{2B}, and/or A_3) can mediate similar actions. These data reveal that local adenosine engages A_1ARs during I/R to limit oxidant damage and enhance outcome selectively. Control of α-TOH/α-TQ levels may contribute to A_1AR-dependent cardioprotection. *Antioxid. Redox Signal.* 11, 2641–2650.

Introduction

MYOCARDIAL DAMAGE during ischemia–reperfusion (I/R) is sensitive to receptor-dependent and -independent actions of adenosine (29, 35, 40), and endogenous adenosine may be an intrinsic determinant of I/R tolerance (18, 36, 40). Adenosine also triggers benefit with pre- and postconditioning (17, 18, 23, 24, 46). However, the mechanistic basis of protection is unclear. Multiple AR subtypes may contribute; responses involve different protein kinase paths and ATP-sensitive K^+ (K_{ATP}) channel types (18, 36), and protection occurs not only in myocytes but also within the vasculature (9, 64). A chief unanswered question is how cell function and viability are preserved by receptor-coupled signaling involving so-called RISK-pathway components (17). Current dogma is that signaling activated by adenosine or other receptors (*e.g.*, opioid, bradykinin) converges on mitochondrial K_{ATP} channels and the mitochondrial permeability transition pore (MPTP) as key targets (14, 16, 30). The MPTP may be inhibited through control of protein phosphorylation (together with effects of K_{ATP} opening), or by inhibition of cellular oxidative stress and subsequent MPTP thiol modification (14).

The cardiac AR system may thus protect by regulating antioxidant status (18, 38) and oxidant generation (22, 31, 51), and we have shown that exogenous adenosine can limit oxidant stress during I/R, including modulation of α-TOH oxidation (13). The latter may reflect a critical link to cardioprotection, because relative levels of α-TOH and its oxidation product α-TQ are important in limiting the MPTP and cell death in other tissues (12, 61). However, whether the antioxidant effects of adenosine are receptor mediated [*vs.* direct metabolic effects (18, 35)], which ARs might be involved, and whether endogenous rather than exogenous adenosine can trigger these effects, are all unknown. We therefore set out to establish the role of endogenous adenosine in modulating I/R oxidant stress through the A_1AR, the principal subtype thought to have antioxidant properties (22, 31, 37, 51).

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Cellular targets for I/R-dependent oxidative damage include polyunsaturated lipids, proteins, reduced thiols, and antioxidants such as ascorbate and α-TOH (15). Prior studies almost exclusively report indirect and less-informative measures of oxidative damage, such as MDA (39, 62, 63)—this marker may yield spurious measures of oxidant stress (53). Because one-electron (or radical) and two-electron (non-radical) oxidants generate different products, we assess markers of both processes, including myocardial lipid hydro (per)oxides [CE-O(O)H] and oxidized glutathione (GSSG), both features of damage typically initiated by one-electron oxidants; and α-TQ, typically derived from α-TOH oxidation with two-electron oxidants such as hypochlorous acid or peroxynitrite (50). We also assessed the impact of I/R and intrinsic A_1AR activity on induction of HO-1, a powerful antioxidant/antiinflammatory induced in response to reactive oxygen species (ROS) to enhance cellular tolerance.

Materials and Methods

Perfused heart preparation

Investigations conformed 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, revised 1996). Local ethics approval was obtained before experimentation. Hearts were isolated from 2- to 4-month-old male mice (mean body weight, 23 ± 4 g) anesthetized with 50 mg/kg sodium pentobarbitone administered intraperitoneally. Mice lacking functional A_1ARs and their wild-type littermates were from a mixed 129sv/C57BL/6J background (40). A thoracotomy was performed, and hearts were excised into ice-cold perfusion fluid. The aorta was cannulated and perfused at a constant pressure of 80 mm Hg with modified Krebs bicarbonate buffer containing (in m*M*): NaCl, 118; $NaHCO_3$, 25; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; EDTA, 0.6; and 11 m*M* glucose plus 2 m*M* pyruvate as carbon substrates. This perfusion fluid was equilibrated with 95% O_2, 5% CO_2 at 37°C to give a pH of 7.4 and a Po_2 of >550 mm Hg at the aortic cannula. The left ventricle was vented with a small polyethylene apical drain to prevent fluid accumulation. Ventricular contractile function was monitored with a fluid-filled balloon introduced into the left ventricle, as described previously (19, 34, 35, 40). Coronary flow was monitored by an ultrasonic flow-probe in the aortic perfusion line, connected to a T106 flow meter (Transonic Systems, Inc., Ithaca, NY). Functional data were recorded at 1 KHz on a four-channel MacLab (AD Instruments, Castle Hill, Australia). The left ventricular pressure signal was digitally processed to yield systolic pressure, end-diastolic pressure (LVEDP), developed pressure (LVDP), +dP/dt (reflecting inotropic state), −dP/dt (reflecting lusitropic state), and heart rate (19, 20, 34, 35, 40). All hearts were introduced into a water-jacketed chamber continuously superfused with buffer at 37°C.

Experimental protocol

After 20 min, hearts were switched to electrical pacing at 400 beats/min to normalize heart rate, and thus its influence on rate-dependent measures of contractility, between groups (19, 20, 35, 40, 55). After a further 10 min, baseline measurements were acquired before subjecting hearts to 25-min global normothermic ischemia [an insult that we show induces significant cell death and impairs function by ~50% (19, 40, 55)] followed by 45 min of aerobic reperfusion. Pacing was stopped during ischemia and resumed at 2 min of reperfusion (19, 40, 55). Responses to I/R were assessed in untreated hearts from wild-type mice ($n = 8$) and mice lacking functional A_1ARs ($n = 7$). Broad-spectrum AR agonism with 10 μ*M* 2-chloroadenosine (2-CAD) (40) was initiated in hearts lacking A_1ARs 10 min before ischemia and maintained for 15 min of reperfusion ($n = 7$). This concentration will near-maximally activate different AR subtypes (40), and in this way, we test the ability of exogenous activation of other AR subtypes (A_{2A}, A_{2B}, A_3) to trigger similar antioxidant/protective effects in hearts lacking A_1ARs. A control normoxic group was also studied, with wild-type hearts subjected to 70 min of aerobic perfusion (paced at 400 beats/min) after stabilization ($n = 8$). On completion of experiments, individual hearts were snap-frozen in liquid N_2 and stored at −80°C for analyses of native and oxidized lipid (unesterified and esterified cholesterol), and α-TQ (the two-electron oxidation product of α-TOH), as described previously (13, 48).

Preparation of cardiac tissue for biochemical analyses

In brief, hearts were thawed, cut into small pieces, and suspended in 2 ml of Dulbecco's phosphate-buffered saline containing 5 μ*M* butylated hydroxytoluene and 2 m*M* ethylenediaminetetraacetic acid (Buffer A) supplemented with 2.5 μ*M* α-tocotrienol (as an internal standard). The tissue was degassed with carbon monoxide gas, and gas-soaked tissue transferred to a glass tube and homogenized (13, 25). Aliquots of homogenate (50 μl) were frozen for subsequent protein determination, and the remainder extracted into methanol and hexane (5:1 vol/vol), the hexane fraction dried under vacuum, and the lipid-soluble residue resuspended in isopropyl alcohol for analyses. Retrieval of the internal standard from the homogenate was determined to be 92 ± 4%, indicating a high recovery of lipid-soluble components.

Lipid analysis

Reversed-phase high-performance liquid chromatography (HPLC) was used to determine cardiac contents of α-TQ (absolute concentration and fraction relative to its parent α-TOH), unesterified cholesterol (C), cholesteryl esters (cholesteryl linoleate, C18:2; and cholesteryl arachidonate, C20:4, together referred to as CE), and CE-derived lipid hydroperoxides and hydroxides [CE-OH + CE-OOH monitored at $A_{234\,nm}$ and referred to as CE-O(O)H] (13, 45, 56). Lipid-soluble analytes were quantified by peak-area comparison with authentic standards under identical conditions and finally normalized against total homogenate protein.

Electronic spectroscopy

Absorbance spectroscopy was performed with a Victor III multiwell plate reader (Perkin Elmer, Australia). Myocardial content of glutathione (GSH) and its one-electron oxidation product glutathione disulfide (GSSG) were determined with a commercial kit (Cayman Chemicals, MI), whereas total protein was determined with the BCA assay (Sigma, Australia). All biochemical data were normalized against corresponding protein levels.

Quantitative PCR analysis

Analyses of HO-1 mRNA were performed as described previously (57). Where required, cardiac homogenates were thawed, total RNA extracted with a commercial kit (Aurum; Bio-Rad, Gladesville, Australia), and the corresponding cDNA reversed-transcribed with oligo-dT priming (Invitrogen, Sydney, Australia). Real-time PCRs were performed with a Rotor-Gene 2000 (Corbett Research, Sydney, Australia) and a SYBR Greener Supermix (Invitrogen). Nontemplate controls were used to assess baseline noise. Threshold levels were set during the linear phase of gene amplification, and cycle threshold (C_T) values were determined by using standard Rotor-Gene 2000 software v4.2. Expression of *Hmox1* was determined relative to glyceraldehyde phosphate dehydrogenase (GAPDH), by using the comparative C_T method (26). The C_T values for cardiac GAPDH showed little variation across all samples tested (range, 17.5–18.9 cycles), indicating that the ratio defined by C_T values for HO-1 relative to GAPDH are dependent on the concentration of HO-1 mRNA. Sense and anti-sense primers: HO-1, 5′-GAGATTGAGCGC AACAAGGA-3′ and 5′-AGCGGTAGAGCTGCTTGAACT-3′; and for GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

Assessment of myocardial HO activity

Myocardial HO activity was determined with a reaction mixture containing microsomes prepared from homogenized myocardial tissues (43). Bilirubin production was determined with reversed-phase gradient liquid chromatography, with concentrations determined with peak-area comparison with authentic standards (6). In some reactions, fresh preparations of tin [SnPPX(IX)] (Alexis Biochemicals, Plymouth Meeting, PA) dissolved in 0.01N NaOH were added to reaction mixtures at ratios of hemin:metalloporphyrin of 1:1 mol/mol as positive controls, because SnPPX(IX) is a potent inhibitor of HO activity. Addition of vehicle alone (0.01N NaOH) did not affect the pH (not shown).

Purine metabolism and LDH release

To assess the impact of both I/R and A_1AR deficiency on adenosine release, coronary venous effluent was collected on ice and frozen at −80°C until analysis with HPLC, as detailed previously (20, 55). Adenosine concentrations were determined immediately before ischemia, and in venous effluent collected over the initial 10 min of reperfusion. To assess extent of cell death, effluent samples also were analyzed for lactate dehydrogenase (LDH) activity in a spectrophotometric enzymatic assay described previously (34, 40). We established that LDH efflux accurately estimates necrosis in this murine heart model (34).

Statistical analysis

Data are presented as mean ± SEM. Differences in functional outcomes, LDH efflux, or postischemic oxidative markers between experimental groups were assessed with one-way ANOVA. When significant differences were detected, a Newman–Keuls *post hoc* test was used for specific comparisons. A value of $p < 0.05$ was considered indicative of significance in all tests.

Results

Cardiac response to I/R and A_1AR deletion

Baseline contractile function was comparable in all groups, although coronary flow was increased by 2-CAD (Table 1). Postischemic recovery of ventricular function was incomplete in all groups (Fig. 1): LVEDP remained elevated above preischemic levels, whereas LVDP was significantly impaired (Fig. 1A). Coronary flow recovered to ~70% of preischemic levels in all groups (Fig. 1A). Inotropic and lusitropic states, indicated by recoveries for +dP/dt and −dP/dt, respectively, were also depressed after ischemia (Fig. 1B). Deletion of the A_1AR further reduced recovery of LVDP, +dP/dt, and −dP/dt, without significantly altering LVEDP or coronary flow (Fig. 1). Treatment with 2-CAD in hearts lacking A_1ARs enhanced recovery, improving LVDP (Fig. 1A) and both +dP/dt and −dP/dt (Fig. 1B). Necrosis estimated from postischemic LDH washout (34) was significantly exaggerated by A_1AR deletion (Fig. 1C), an effect countered by treatment with 2-CAD. Ischemia also increased extracellular accumulation of adenosine and its catabolites (Fig. 2). Release of purine metabolites was not significantly modified by A_1AR deletion.

Effects of I/R and A_1AR deletion on oxidant stress

Cardiac concentrations of native (C) and (CE) did not differ between groups (Fig. 3). Tissue content of CE-O(O)H (Fig. 3C) and α-TQ (Fig. 4) were significantly elevated by I/R. Although the elevation in CE-O(O)H was insensitive to A_1AR deletion, accumulation of α-TQ (absolute or relative to α-TOH) increased significantly in hearts lacking A_1ARs, reaching two- to threefold higher levels than those in corresponding controls (Fig. 4). Treatment with 2-CAD limited the impact of A_1AR deletion on α-TQ accumulation (Fig. 4) without modifying

Table 1. Baseline Functional Properties of Perfused Hearts

Group	*LVEDP (mm Hg)*	*LVDP (mm Hg)*	*+dP/dt (mm Hg/s)*	*−dP/dt (mm Hg/s)*	*Coronary flow (ml/min/g)*
Wild-type ($n = 8$)	4 ± 1	133 ± 5	6,654 ± 295	4,542 ± 201	22.0 ± 1.4
A_1AR KO ($n = 7$)	3 ± 2	129 ± 5	6,383 ± 224	4,130 ± 284	22.4 ± 1.3
A_1AR KO + 2-CAD ($n = 7$)	3 ± 1	143 ± 6	6,980 ± 306	4,645 ± 197	30.2 ± 1.8*

2-CAD, 2-chloroadenosine; KO, knockout; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure.
All values represent mean ± SEM. Parameters were measured after 30-min aerobic perfusion (before induction of ischemia). Function did not differ between wild-type and A_1AR-deficient hearts. *$p < 0.05$ for A_1AR KO + 2-CAD *vs.* A_1AR KO alone.

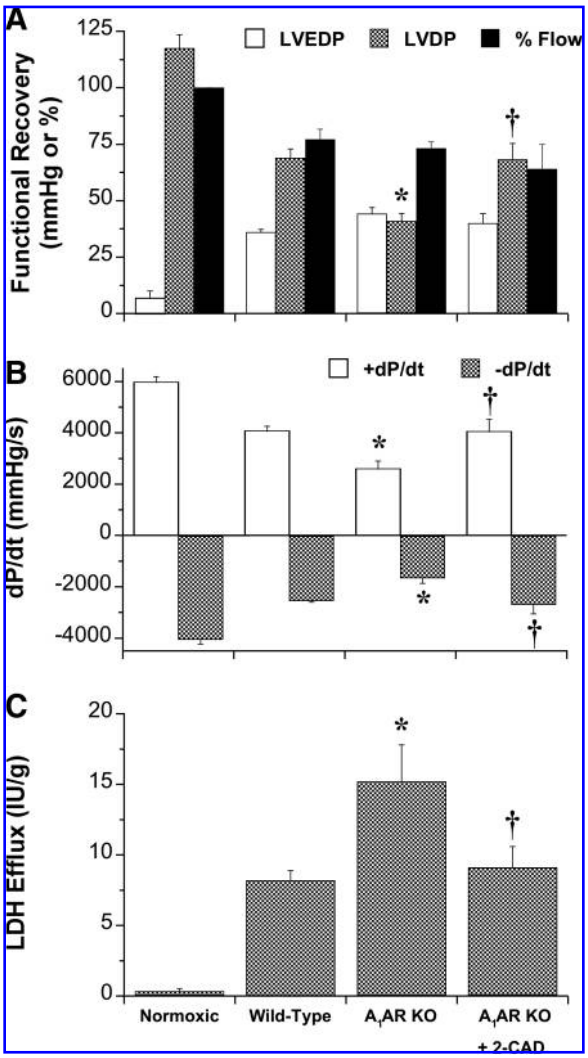

FIG. 1. Effects of I/R and A_1AR deletion on myocardial function and LDH efflux. Hearts from wild-type mice and mice lacking functional A_1ARs were subjected to 25-min ischemia and 45-min reperfusion. Data are shown for (**A**) absolute recoveries for end-diastolic pressure (LVEDP; *open bars*) and developed pressure (LVDP; *grey bars*), and percentage recovery of coronary flow (% Flow; *black bars*); (**B**) absolute recoveries for +dP/dt and −dP/dt; and (**C**) total LDH release during reperfusion (units/g of heart). Shown for comparison are functional values and LDH efflux for normoxic controls. All values are expressed as mean ± SEM (for *n* values, refer to Table 1). Note: all postischemic measures of function and LDH efflux differed significantly from preischemic values and corresponding values for normoxic wild-type hearts. $^*p < 0.05$ *vs.* Wild-Type; $^\dagger p < 0.05$ for A_1AR KO + 2-CAD *vs.* A_1AR KO alone.

other markers (Fig. 3), suggesting that additional AR subtypes can also be harnessed to limit oxidant damage. Consistent with this, the glutathione redox status (defined as [GSH]/[GSH + GSSG]) decreased significantly in postischemic hearts lacking A_1ARs, with 2-CAD treatment countering this effect (Fig. 5). Together these data indicate that oxidant stress is substantially increased in hearts lacking A_1ARs, whereas nonspecific AR agonism can ameliorate the effects of oxidative stress.

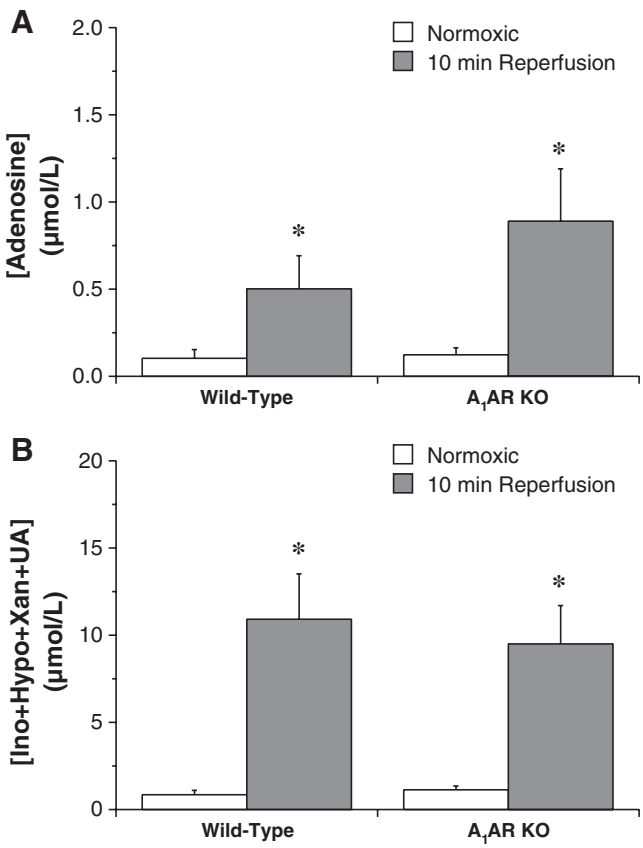

FIG. 2. Effects of I/R and A_1AR deletion on coronary venous adenosine and purine metabolites. Data are shown for extracellular concentrations of (**A**) adenosine; and (**B**) inosine + hypoxanthine + xanthine + uric acid (Ino + Hypo + Xan + UA) under normoxic (*open bars*) and postischemic conditions (*grey bars*). All values are expressed as mean ± SEM (for *n* values, refer to Table 1). $^*p < 0.05$ *vs.* Normoxic. None of the baseline parameters in A_1AR-deficient hearts differed from corresponding values in wild-type hearts.

Effects of I/R and A_1AR deletion on HO-1 induction

Under normoxic conditions, expression of HO-1 mRNA was relatively low, and this was enhanced about fourfold by I/R (Fig. 6A). Deletion of the A_1AR approximately doubled HO-1 gene expression in response to I/R insult. Pretreatment with 2-CAD in hearts lacking A_1ARs significantly diminished HO-1 induction, although gene expression remained above that for normoxic tissue (Fig. 6A). Changes in gene expression were generally mirrored at the level of HO enzyme activity (Fig. 6B), although the trend to reduced HO activity after 2-CAD treatment did not achieve significance (Fig. 6B). As anticipated, the addition of SnPPx(IX) markedly reduced HO activity in homogenates from A_1AR-deficient hearts.

Discussion

We recently demonstrated potent antioxidant effects of exogenous adenosine during I/R (13), which may underlie the protective actions of this signal molecule. Here we show that A_1AR-dependent changes in cardiac I/R tolerance are

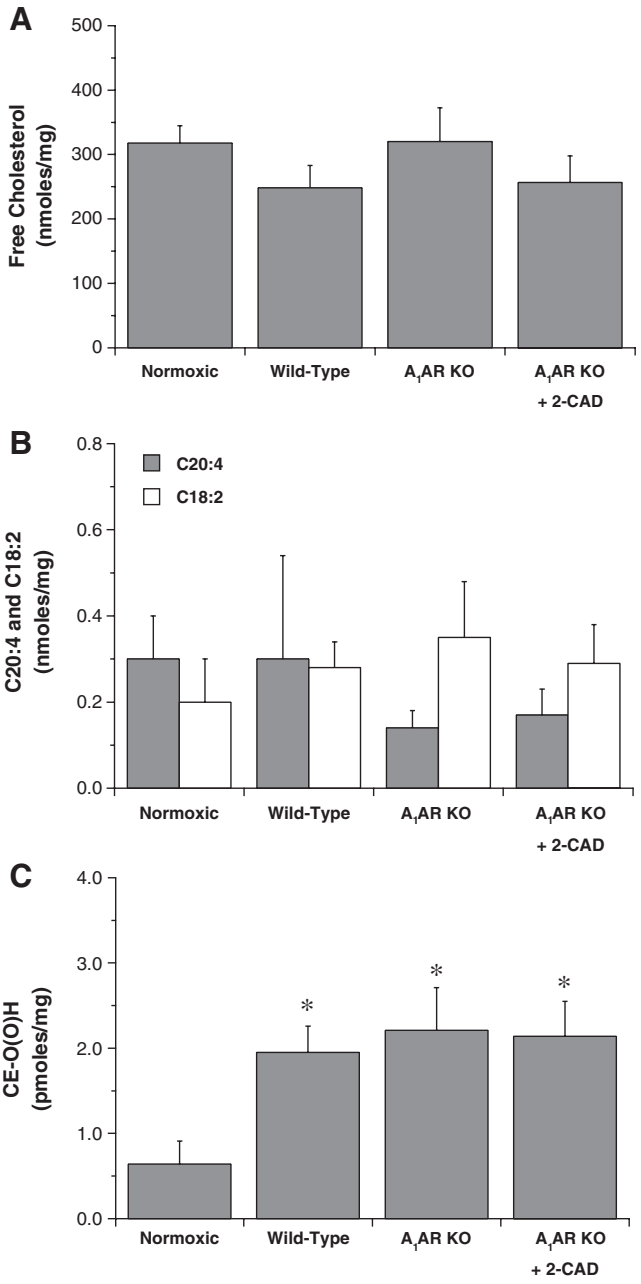

FIG. 3. Effects of I/R and A_1AR deletion on myocardial lipids. Data represent protein normalized values for (**A**) unesterified cholesterol; (**B**) cholesteryl linoleate (C18:2; *open bars*) and arachidonate (C20:4; *grey bars*); and (**C**) cholesteryl ester–derived lipid hydroperoxides and hydroxides [CE-O(O)H] in hearts exposed to ischemia–reperfusion. All values are expressed as mean ± SEM and are expressed as either nmol or pmol/mg protein (for *n* values, refer to Table 1). $^*p < 0.05$ *vs.* Normoxic.

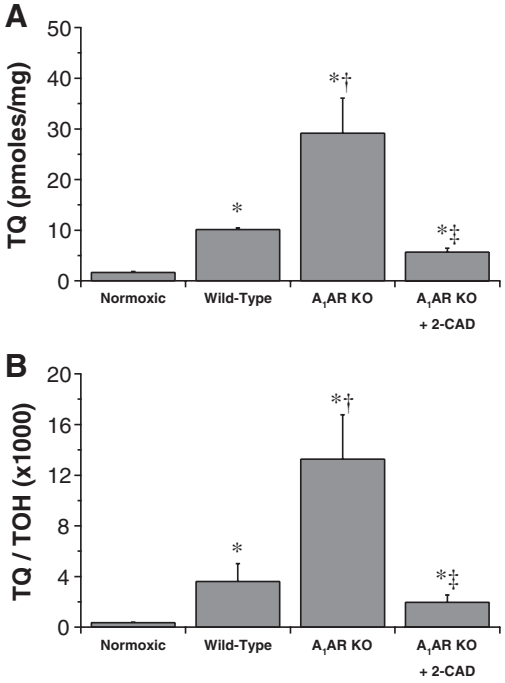

FIG. 4. Effects of I/R and A_1AR deletion on myocardial α-TQ content. Myocardial tissue content of (**A**) α-TQ; and (**B**) α-TQ expressed as a fraction of the parent α-TOH in the myocardial tissue. All values represent mean ± SEM and are expressed as either nmol or pmol/mg protein (for *n* values, refer to Table 1). $^*p < 0.05$ *vs.* Normoxic; $^\dagger p < 0.05$ *vs.* Wild-Type; $^\ddagger p < 0.05$ for A_1AR KO + 2-CAD *vs.* A_1AR KO alone.

associated with modulation of oxidant stress that may differentially affect specific cell compartments: in the absence of A_1ARs, hearts accumulate higher α-TQ and GSSG [but not CEO-(O)H] in association with exaggerated contractile dysfunction and cell death. Because the majority of cellular vitamin E exists within membrane fractions, these findings suggest that intrinsic A_1AR activity protects membranous α-TOH from two-electron oxidants without modifying lipid oxidation in the same compartment. Our findings support a nonredundant and selective antioxidant effect of intrinsically activated A_1ARs.

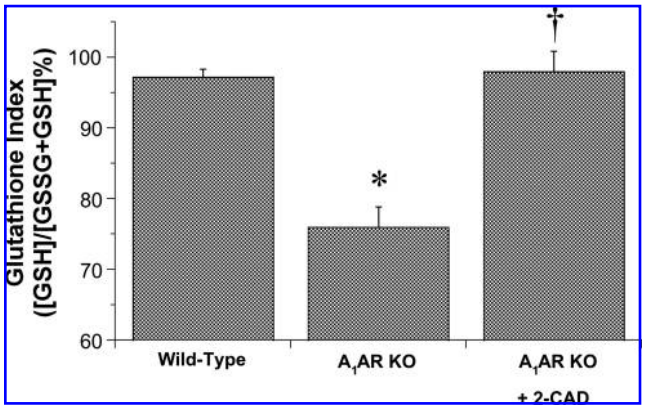

FIG. 5. Effects of A_1AR deletion on I/R-dependent changes in the myocardial [GSH] redox ratio. Before analysis of GSSG and GSH with a commercial kit, hearts were thawed and homogenized, as described in the Methods section. Percentage expression for [GSH]/[GSH + GSSG] is a surrogate for myocardial glutathione redox status; a decrease in the ratio is indicative of enhanced oxidative stress. All values are expressed as mean ± SEM (for *n* values, refer to Table 1). $^*p < 0.05$ *vs.* Wild-Type; $^\dagger p < 0.05$ for A_1AR KO + 2-CAD *vs.* A_1AR KO alone.

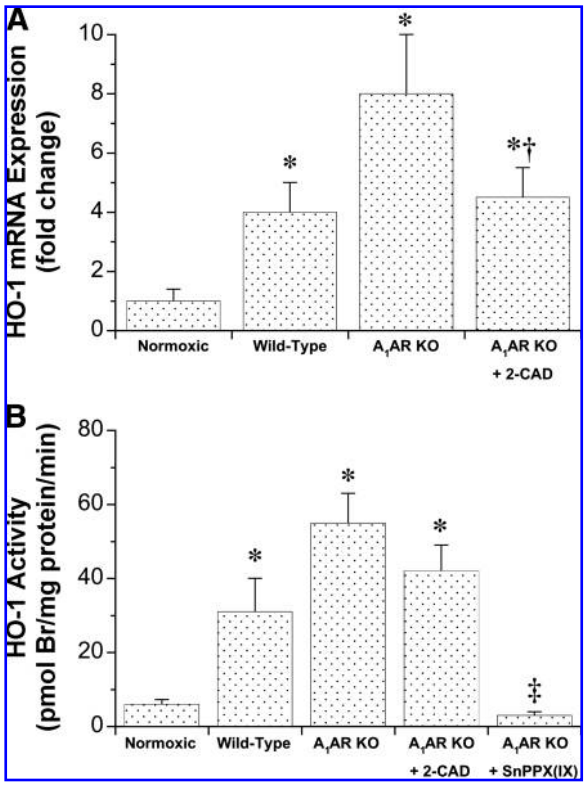

FIG. 6. Effects of I/R and A_1AR deletion on myocardial HO-1 expression and activity. Data represent (**A**) quantitative RT-PCR analysis of HO-1 gene expression in cardiac homogenates; and (**B**) corresponding total HO enzyme activity. Pretreatment of the homogenate from A_1AR KO hearts with 25 μM SnPPx(IX) inhibited the increase in HO activity. All values are expressed as mean ± SEM ($n = 4$ per group). *$p < 0.05$ *vs.* Normoxic; †$p < 0.05$ for A_1AR KO + 2-CAD *vs.* A_1AR KO alone; ‡$p < 0.05$ for A_1AR KO + SnPPx(IX) *vs.* A_1AR KO alone.

Intrinsic protection via *the A_1AR*

In agreement with some prior work (29, 40), deletion of A_1ARs limited cardiac tolerance to I/R (Fig. 1). However, this has not been consistently observed across studies (24, 46). Conversely, deletion of A_1ARs consistently eliminates protection with other stimuli, including local (24) or remote preconditioning (46) and phosphodiesterase inhibition (44). Our data (Fig. 1) and prior studies thus confirm a role for intrinsically activated A_1ARs in mediating protection, although underlying mechanisms remain ill defined.

Because protection with adenosine may be countered by associated changes in xanthine oxidase–derived radical formation (55), we measured accumulation of adenosine and the catabolites that provide a substrate for this reaction (hypoxanthine, xanthine). Despite worsened recovery from I/R, A_1AR deletion did not alter levels of these metabolites (Fig. 2). Thus, enhanced xanthine oxidase–derived radical generation does not contribute to worsened I/R outcomes with A_1AR deletion. Moreover, shifts in extracellular agonist levels cannot be involved. Lack of effect of A_1AR deletion on purine outflow, despite exaggerated injury, suggests that adenosine generation is more dependent on the ischemic insult itself (which is unaltered by A_1AR deletion), rather than on the level of subsequent tissue injury.

Antioxidant actions of intrinsically activated A_1ARs

The basis of adenosinergic protection is not known, although investigations into cardioprotection unmask the involvement of protein kinase signaling (18, 36) and mitochondrial targeting of K_{ATP} channels and the MPTP (14, 16, 30, 36). How the MPTP is inhibited, to limit mitochondrial depolarization, disruption, and cell death, is also debated. Two possibilities are presented: protein kinase–dependent phosphorylation of regulatory proteins *versus* inhibition of cellular oxidative stress to limit modification of MPTP thiol groups (thus desensitizing the pore to Ca^{2+}) (14, 17, 30). Clarke *et al.* (2) recently provided support for the latter model, identifying consistent relations between tissue injury and mitochondrial protein oxidation rather than phosphorylation.

Whether intrinsically activated A_1ARs limit oxidant generation or oxidant damage or both has not been previously established, although prior studies support the notion. Adenosinergic and ischemic preconditioning decrease interstitial hydroxyl radical levels (37), and augmented adenosine in preconditioned hearts inhibits H_2O_2-mediated dysfunction in an A_1AR/K_{ATP}-dependent manner (11). Xu *et al.* (58) reported that mixed A_1/A_2AR agonism limits myocyte ROS generation during I/R, and Narayan *et al.* (31) showed that A_1AR agonism inhibits ROS formation and contractile dysfunction in reoxygenated myocytes, an effect dependent on K_{ATP} channels and mimicked by a free radical scavenger. Additionally, A_1ARs (and not A_{2A} or A_3ARs) limit oxidant-mediated myocyte Ca^{2+} entry *via* L-type channels (51). Preconditioning and postconditioning responses, known to involve ARs, also limit oxidant accumulation and oxidative stress (37, 49, 52).

Here we advance these prior studies, which report on abilities of exogenous AR agonism to protect against damage with exogenously applied oxidants (11, 51) or to limit ROS generation after ischemic/hypoxic insult (31, 52, 58), and test whether endogenously generated adenosine can achieve such effects by engagement of the A_1AR.

One prior report supports an antioxidant function for intrinsically activated ARs in cardiovascular tissue (albeit venous endothelium), demonstrating that A_1 and A_2AR antagonism amplifies posthypoxic generation of superoxide radical anion in a NO-dependent manner (47). We show that deletion of the A_1AR augments changes in cardiac α-TQ (Fig. 4) and glutathione status (Fig. 5), without modifying markers of lipid oxidation (Fig. 3). This selective modulation is unlikely to reflect nonspecific effects of adenosine on prooxidant processes such as Ca^{2+} overload or mitochondrial electron-transport disruption. Adenosine can limit Ca^{2+} accumulation during I/R (10), and A_1AR-mediated inhibition of contracture (41) probably reflects such an effect. By inhibiting catecholamine release (42) and adrenergic activation of the heart (8), A_1ARs can inhibit oxidant generation, ATP consumption, and mitochondrial dysfunction. Nonetheless, such processes are nonselective in terms of oxidant generation and injury, which is difficult to reconcile with the specific impact of A_1AR deletion (Figs. 3–5).

Changes in HO-1 support exaggeration of oxidant generation with A_1AR deletion (Fig. 6). Cardiac HO-1 is induced early with ischemia/hypoxia in response to ROS accumulation (28). Adenosine and preconditioning stimuli may trigger delayed protection through HO-1 induction (21), and other

protective responses may harness HO-1 (1, 5, 63). We find that I/R rapidly induces HO-1 mRNA and tissue activity (within 45 min), an effect exaggerated by A_1AR deletion (Fig. 6). Given the induction of HO-1 by ROS (28), this pattern is consistent with exaggerated oxidant accumulation in response to A_1AR deficiency, augmenting adaptive HO-1 induction.

The selective regulation of α-TOH oxidation and α-TQ accumulation by A_1ARs may be highly relevant to protection. Recent evidence reveals a role for α-TOH in inhibiting MPTP opening (61) and limiting oxidant cytotoxicity in noncardiac cells (7), whereas age-related shifts in α-TOH may contribute to changes in mitochondria-dependent cell-death pathways (12). Thus, α-TOH levels (and conversely, the proportion oxidized to α-TQ) are important determinants of MPTP function. Increased α-TQ (at the expense of vitamin E) in hearts lacking A_1ARs (Fig. 4) will favor MPTP formation and cell death, whereas intrinsic A_1AR activity may normally facilitate α-TOH inhibition of the MPTP to promote cell survival. Consistent modulation of α-TOH/α-TQ with exogenous (13) or endogenous adenosine (Fig. 4) warrants further investigation as a basis for MPTP control and tissue protection.

Effects of other AR subtypes

Generally, only the A_1AR has been shown to contribute to intrinsic I/R tolerance, with antagonism or deletion enhancing I/R injury (18, 29, 40). Nonetheless, other ARs may improve I/R tolerance when exogenously activated (18, 36). We therefore used 2-CAD, a broad-spectrum AR agonist, to test for potential antioxidant effects of other AR subtypes in A_1AR-deficient hearts. Interestingly, 2-CAD reduced α-TQ accumulation to maintain the α-TQ/α-TOH ratio (Fig. 4) and prevented the decline in glutathione redox status (Fig. 5). These changes were associated with repression of the HO-1 gene response (Fig. 6) and improved postischemic outcomes (Fig. 1). These data are consistent with effects of exogenous adenosine (13) and reveal a similar beneficial action of other AR subtypes (albeit in response to exogenous agonism). Because 10 μM 2-CAD will activate A_{2A} and A_3ARs, both with protective properties (18, 36), they may contribute to this response. However, A_{2A}ARs protect indirectly, by modulating inflammatory processes in circulating and invading leukocytes and lymphocytes (18, 36), and are unlikely to be important in this *ex vivo* model. This leaves the A_3AR as a likely mediator of benefit with 2-CAD in hearts lacking A_1ARs. Interestingly, Park *et al.* (33) provided recent support for A_3AR-mediated protection through MPTP inhibition. The A_{2B}AR could also contribute, because these low-sensitivity ARs can be sensitized by PKC, allowing them to contribute to protection on reperfusion (23).

Study limitations

Three limitations are worth noting. First, because our initial investigation focused on exogenous adenosine (18, 35), which may act through receptor-dependent or -independent processes (18, 35), or through direct interactions with oxidants (32), we chose to adopt a genetic approach to negate intrinsic A_1AR responses. A potential pitfall to this approach is expression of compensatory changes that may mask the phenotypic outcome. Although we have shown that A_1AR deletion does not modify the sensitivity of other AR subtypes (40), and herein shows a lack of effect on extracellular agonist levels (Fig. 2), we cannot eliminate the possibility of unpredicted shifts in phenotype compensating for deletion of the A_1AR.

A second consideration regards use of isolated hearts. Although this facilitates analysis of cardiac-specific responses to the A_1AR, it precludes analysis of the effects of A_1AR deletion on *in vivo* inflammatory responses. Whereas adenosinergic inhibition of neutrophil-mediated cardiac injury is A_2/A_3AR dependent (18, 36), some evidence indicates that A_1ARs may paradoxically stimulate neutrophil adhesion (3, 4), which could exacerbate injury *in vivo*. However, *in vivo* studies confirm that A_1AR activation confers protection in excess of potentially deleterious effects associated with any proinflammatory capacity (27, 59, 60). That said, we note that extrinsic inflammatory responses are absent from this exsanguinous model and can influence injury and outcomes *in vivo*.

Finally, as with prior work linking inhibition of oxidant stress to tissue protection with preconditioning (37, 52), postconditioning (49), or adenosine (11, 13, 31, 37, 58), causality is not strictly established. As in earlier studies, we interpret inhibition of oxidant damage with protective stimuli as evidence that these stimuli act by limiting oxidative stress. Nonetheless, an alternate explanation is that protection does not mechanistically involve shifts in oxidant damage, but simply leads to reductions in markers of oxidant stress (*i.e.*, reductions in primary injury processes and tissue injury subsequently limit markers of oxidant damage). Although we cannot entirely exclude this possibility, our data argue against it. First, effects of exogenous adenosine (13) and endogenous adenosine acting at A_1ARs (shown here) are selective: only α-TOH/α-TQ and glutathione redox status respond to A_1AR deletion or AR agonism, whereas markers of lipid damage remain unaltered. Second, HO-1 induction is augmented in hearts deficient in A_1ARs, reflecting an impact of this receptor on the primary promoter of HO-1 induction: ROS levels rather than I/R injury *per se* (28).

Finally, a body of evidence supports a primary role for oxidants in mediating rather than simply evidencing tissue injury, although this admittedly remains contentious. We argue, as have prior investigations (11, 31, 37, 51, 58), that protective stimuli (in this case, A_1ARs) protect at least in part through limitation of oxidant-mediated injury. We present a potentially selective process whereby protection might be achieved, bringing together current ideas on the importance of the MPTP to tissue survival (2, 14, 16, 30), and the influence of α-TOH on mitochondrial injury and MPTP function (7, 12, 61). Receptor-mediated control of α-TOH/α-TQ may be critical in limiting mitochondrial injury and MPTP opening.

Conclusions

This study demonstrates that endogenous adenosine engages the A_1AR to limit oxidative stress selectively in reperfused myocardium. Specific effects of A_1AR deficiency on α-TQ (and the GSH redox ratio) may reflect A_1AR-dependent modulation of two- *versus* one-electron oxidant processes in different cell compartments. These effects parallel shifts in postischemic outcome, and other AR sub-types may be exogenously harnessed to mediate similar antioxidant actions. Our data lead us to hypothesize that A_1AR-dependent inhibition of α-TOH oxidation may limit MPTP opening to

promote cell survival during I/R, a possibility that warrants more direct interrogation.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

A_1AR = A_1 adenosine receptor
C = unesterified cholesterol
C18:2 = cholesteryl linoleate
C20:4 = cholesteryl arachidonate
2-CAD = 2-chloroadenosine
CE = cholesteryl esters
CE-O(O)H = cholesteryl ester–derived lipid hydroperoxides and hydroxides
dP/dt = differential of ventricular pressure change with time
GAPDH = glyceraldehyde phosphate dehydrogenase
GSH = native glutathione
GSSG = oxidized glutathione
HO-1 = heme oxygenase-1
I/R = ischemia–reperfusion
K_{ATP} = ATP-sensitive K^+ channels
KO = knockout
LDH = lactate dehydrogenase
LVDP = left ventricular developed pressure
LVEDP = left ventricular end-diastolic pressure
MDA = malondialdehyde
MPTP = mitochondrial permeability transition pore
PI3-K = phosphatidylinositol 3-kinase
PKC = protein kinase C
RISK = reperfusion injury salvage kinase
ROS = reactive oxygen species
α-TOH = α-tocopherol
α-TQ = α-tocopherylquinone

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