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Ischaemic tolerance in aged mouse myocardium: the role of adenosine and effects of A_1 adenosine receptor overexpression

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The genesis of the ischaemia intolerant phenotype in aged myocardium is poorly understood. We tested the hypothesis that impaired adenosine-mediated protection contributes to ischaemic intolerance, and examined whether this is countered by A_1 adenosine receptor (A_1AR) overexpression. Responses to 20 min ischaemia and 45 min reperfusion were assessed in perfused hearts from young (2–4 months) and moderately aged (16–18 months) mice. Post-ischaemic contractility was impaired by ageing with elevated ventricular diastolic (32 ± 2 *vs.* 18 ± 2 mmHg in young) and reduced developed (37 ± 3 *vs.* 83 ± 6 mmHg in young) pressures. Lactate dehydrogenase (LDH) loss was exaggerated (27 ± 2 *vs.* 16 ± 2 IU g^{-1} in young) whereas the incidence of tachyarrhythmias was similar in young (15 ± 1 %) and aged hearts (16 ± 1 %). Functional analysis confirmed equipotent effects of 50 μM adenosine at A_1 and A_2 receptors in young and aged hearts. Nonetheless, while 50 μM adenosine improved diastolic (5 ± 1 mmHg) and developed pressures (134 ± 7 mmHg) and LDH loss (6 ± 2 IU g^{-1}) in young hearts, it did not alter these variables in the aged group. Adenosine did attenuate arrhythmogenesis for both ages (to ~10 %). In contrast to adenosine, 50 μM diazoxide reduced ischaemic damage and arrhythmogenesis for both ages. Contractile and anti-necrotic effects of adenosine were limited by 100 μM 5-hydroxydecanoate (5-HD) and 3 μM chelerythrine. Anti-arrhythmic effects were limited by 5-HD but not chelerythrine. Non-selective (100 μM 8-sulfophenyltheophylline) and A_1-selective (150 nM 8-cyclopentyl-1,3-dipropylxanthine) adenosine receptor antagonism impaired ischaemic tolerance in young but not aged hearts. Quantitative real-time PCR and radioligand analysis indicated that impaired protection is unrelated to changes in A_1AR mRNA transcription, or receptor density (~8 fmol mg^{-1} protein in both age groups). However, A_1AR overexpression improved tolerance for both ages, restoring adenosine-mediated protection. These data reveal impaired protection via exogenous and endogenous adenosine contributes to ischaemic intolerance with ageing. This is independent of A_1AR expression, and involves ineffective activation of a 5-HD-/diazoxide-sensitive process. The effects of A_1AR overexpression indicate that the age-related failure in signalling can be overcome.

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There is increasing evidence of a decline in myocardial tolerance to injury with ageing. A reduction in the 'intrinsic' tolerance to ischaemic insult is supported by data from animal models (Pahor *et al.* 1985; Frolkis *et al.* 1991; Misare *et al.* 1992; Lesnefsky *et al.* 1994; Tani *et al.* 1997; Headrick, 1998; Abete *et al.* 1999; Rosenfeldt *et al.* 2002) and humans (Mariani *et al.* 2000; Rosenfeldt *et al.* 2002). The molecular basis of this intolerant phenotype is unclear, but it may involve multiple alterations including mitochondrial abnormalities (Lesnefsky *et al.* 2001), impaired anti-oxidant responses (Boucher *et al.* 1998; Coombes *et al.* 2000; Lesnefsky *et al.* 2001), loss of proteasome function (Bulteau *et al.* 2002) and modified Ca^{2+} handling (Cain *et al.* 1998). One possibility receiving increased attention is impairment of intrinsic cardioprotective responses (Abete *et al.* 1996; Gao *et al.* 2000; Schulman *et al.* 2001; Lee *et al.* 2002). This may be a particularly important factor since it may impact directly on the therapeutic approach to ischaemic injury in aged hearts. It is increasingly evident that conventional therapeutic strategies developed through findings in young tissues and subjects may not be relevant in aged subjects (Rosenfeldt *et al.* 2002).

Adenosine is an important determinant of ischaemic (Zhao *et al.* 1993, 1994; Peart & Headrick, 2000) or hypoxic tolerance (Matherne *et al.* 1996). We previously acquired evidence that altered adenosine handling might contribute to impaired ischaemic tolerance with age (Headrick, 1998), and more recent evidence supports an age-related decline in adenosine-mediated protection (Gao *et al.* 2000). However, this latter study failed to establish whether functionally equipotent levels of adenosine were effective in different

ages. The aims of the current study were to characterise age-related changes in ischaemic tolerance in mouse heart, to examine the efficacy of exogenous and endogenous adenosine in protecting against ischaemic injury, and to test the hypothesis that enhanced expression of protective A_1 adenosine receptors (A_1ARs) will reverse the detrimental effects of ageing on ischaemic tolerance.

METHODS

Perfused mouse heart model

Investigations conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Hearts were acquired from young (2–4 months, 23.3 ± 2.4 g body weight) and aged (16–18 months, 38.1 ± 2.0 g body weight) male and female wild-type C57/Bl6 mice, and mice overexpressing cardiac A_1ARs. Details of the generation and characterisation of transgenic mice have previously been provided (Matherne *et al.* 1997; Gauthier *et al.* 1998). Mice were anaesthetised with 50 mg kg^{-1} sodium pentobarbitone, a thoracotomy was performed and hearts were rapidly excised into ice-cold perfusion fluid. The aorta was immediately cannulated and perfused at a pressure of 80 mmHg with modified Krebs bicarbonate buffer containing (mM): NaCl, 118; $NaHCO_3$, 25; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; glucose, 11; and EDTA, 0.6. Perfusate was equilibrated with 95 % O_2, 5 % CO_2 at 37 °C to give a pH of 7.4 and a P_{O_2} of > 550 mmHg at the aortic cannula. The perfusate was passed through an in-line 0.45 μm Sterivex-HV filter cartridge (Millipore, Bedford, MA, USA). The left ventricle was vented with an apical drain and hearts were instrumented for functional assessment. Hearts were placed in a water-jacketed chamber continuously superfused with warmed buffer maintained at 37 °C.

Contractile function was assessed via an intra-ventricular balloon, as described previously (Peart & Headrick, 2000; Headrick *et al.* 2001). Coronary flow was monitored using an ultrasonic flow-probe in the aortic perfusion line connected to a T106 flowmeter (Transonic Systems Inc, Ithaca, NY, USA). Functional data were recorded at 1 kHz on a 4-channel MacLab (ADInstruments, Castle Hill, Australia). The ventricular pressure signal was digitally processed to give systolic and diastolic pressures, +dP/dt (reflecting inotropic state), −dP/dt (reflecting lusitropic state) and heart rate (Peart & Headrick, 2000; Headrick *et al.* 2001).

Experimental protocol

After 20 min stabilisation hearts were switched to pacing at 400 beats min^{-1} (silver electrodes, pacing 20 % above threshold, 1 ms square pulses; Peart & Headrick, 2000; Headrick *et al.* 2001) and permitted to stabilise for a further 10 min. Hearts were excluded if they met one of the following criteria: (i) left ventricular systolic pressure below 100 mmHg; (ii) coronary flow equal to or exceeding 5 ml min^{-1} (maximal dilatation or aortic tear); (iii) unstable (fluctuating) contractile function; or (iv) significant arrhythmias. This amounted to less than 3 % of hearts perfused. Baseline measurements were made and hearts were subjected to 20 min global normothermic ischaemia and 45 min aerobic reperfusion. Pacing was stopped on induction of ischaemia and resumed after 2 min reperfusion (Peart & Headrick, 2000; Headrick *et al.* 2001). Functional parameters were assessed throughout, and coronary venous effluent collected on ice for analysis of LDH efflux using a commercially available kit (Sigma Chemical Co., St Louis, MO, USA; Headrick *et al.* 2001). Efflux is expressed as units per gram wet weight. The degree of ectopy (tachyarrhythmias) during the initial 10 min reperfusion was calculated as the sum of premature and tachycardic beats divided by the total number of beats during this period, as described previously (Headrick, 1998):

$$\text{Percentage ectopy} = (\text{abnormal beats/total beats}) \times 100.$$

The protocol was performed in young (n = 11) and aged wild-type hearts (n = 10) and young (n = 9) and aged transgenic hearts (n = 10) overexpressing A_1ARs. Adenosine-mediated cardioprotection was studied after first verifying that 50 μM adenosine was functionally equipotent in young and aged hearts. Adenosine was infused incrementally at concentrations of 1, 50 and 100 μM in young (n = 8) and aged (n = 6) hearts, and A_1AR-mediated bradycardia and A_2 adenosine receptor-mediated dilatation measured at each concentration. For adenosine-mediated cardioprotection, 50 μM adenosine was infused 10 min prior to ischaemia and re-instated upon reperfusion. The effects of adenosine were measured in young (n = 9) and aged wild-type hearts (n = 10) and young (n = 9) and aged transgenic hearts (n = 9).

To identify the role of endogenously released adenosine at A_1ARs and other receptors, we studied the effects of treatment with 150 nM of the selective and potent A_1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or 100 μM of the non-selective antagonist 8-ρ-sulfophenyltheophylline (8-SPT) in ischaemic–reperfused hearts from young wild-type (n = 8 for DPCPX, n = 9 for 8-SPT) and aged wild-type mice (n = 9 for DPCPX, n = 8 for 8-SPT). Infusion of antagonist was initiated 10 min prior to ischaemia and was re-instated upon reperfusion since we have shown protective effects of adenosine pre- and post-ischaemia (Peart & Headrick, 2000).

The protective effects of the putative mito K_{ATP} channel activator diazoxide (50 μM) were assessed in young (n = 9) and aged wild-type (n = 6) hearts. Diazoxide was infused as for adenosine. To assess the contributions of mito K_{ATP} channels and protein kinase C (PKC) in adenosine and diazoxide-mediated protection, young wild-type hearts were treated with 100 μM 5-hydroxydecanoic acid (5-HD), a mito K_{ATP} channel inhibitor, or 3 μM chelerythrine, a non-isoform-specific PKC inhibitor. Inhibitors were infused for 15 min prior to ischaemia, and re-instated during reperfusion. This was performed in untreated hearts (n = 7 for 5-HD, n = 8 for chelerythrine), 50 μM adenosine-treated hearts (n = 8 for 5-HD, n = 8 for chelerythrine) and 50 μM diazoxide-treated hearts (n = 8 for 5-HD, n = 7 for chelerythrine). These experiments were undertaken in young hearts since adenosine failed to modify ischaemic tolerance in the aged group.

Analysis of A_1AR transcription and expression

The effects of age on A_1AR gene transcription and protein expression were assessed in normoxic myocardium. Wild-type and transgenic hearts were perfused for 30 min under normoxic conditions and snap-frozen in aluminium tongs cooled in liquid N_2. For analysis of A_1AR transcription, samples of ventricular myocardium were homogenised and total RNA was extracted using standard TRIzol and DNase treatment with subsequent spin column purification (Qiagen, Hilden, Germany). RNA integrity was verified by formaldehyde agarose gel electrophoresis and total RNA stored at −80 °C until analysed. Expression of mRNA for the A_1AR was assessed via quantitative real-time PCR using the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). 18S ribosomal RNA was used as an

Table 1. Baseline functional parameters in untreated and adenosine-treated hearts from young and aged wild-type and transgenic mice

Group	Treatment	LVDP (mmHg)	+dP/dt (mmHg s^{-1})	−dP/dt (mmHg s^{-1})	Heart rate (beats min^{-1})	Flow (ml min g^{-1})
Young wild-type (n = 11)	Untreated	159 ± 6	6869 ± 360	4511 ± 242	381 ± 9	28 ± 2
Young wild-type (n = 9)	Adenosine (50 μM)	169 ± 7	7084 ± 291	4100 ± 228	—	35 ± 2 †
Aged wild-type (n = 10)	Untreated	160 ± 8	6762 ± 240	4882 ± 185	350 ± 10 *	23 ± 2 *
Aged wild-type (n = 10)	Adenosine (50 μM)	165 ± 9	7147 ± 427	4568 ± 246	—	33 ± 2 †
Young transgenic (n = 9)	Untreated	167 ± 6	6575 ± 210	4390 ± 163	343 ± 11 *‡	27 ± 1
Young transgenic (n = 9)	Adenosine (50 μM)	179 ± 9	6989 ± 384	4658 ± 243	—	33 ± 1 †
Aged transgenic (n = 10)	Untreated	162 ± 9	6958 ± 419	4492 ± 215	321 ± 8 *‡	23 ± 1 *
Aged transgenic (n = 9)	Adenosine (50 μM)	177 ± 8	7358 ± 296	4893 ± 195	—	35 ± 1 *†

All values represent means ± S.E.M. All parameters except intrinsic hearts rate were measured after 30 min aerobic perfusion in wild-type hearts and hearts overexpressing A_1ARs. Intrinsic heart rate was assessed immediately prior to ventricular pacing (20 min aerobic perfusion). LVDP, left ventricular developed pressure. * $P < 0.05$ *vs.* young wild-type hearts; † $P < 0.05$ *vs.* untreated; ‡ $P < 0.05$ *vs.* wild-type hearts.

endogenous control to correct for minor experimental variations. PCR primers used were:

18S rRNA: forward primer, 5′-CTCAACACGGGAAACCTCAC-3′; reverse primer, 5′-AAATCGCTCCACCAACTAAGAA-3′.

A_1AR: forward primer, 5′-CATTGGGCCACAGACCTACT-3′; reverse primer, 5′-ACCGGAGAGGGATCTTGACT-3′.

Quantitative real-time PCR is based on the measurement of the cycle threshold (C_t) for transcripts of interest. This C_t value is the minimum PCR cycle number at which accumulation of amplicon can be accurately quantified. Hence, higher levels of a transcript in a sample will reach the C_t value earlier. To normalise transcription levels between samples and groups, the C_t for the A_1AR transcript was calculated relative to the C_t for the endogenous house-keeping gene 18S rRNA in each sample – the value ΔC_t represents the difference in C_t for A_1AR transcription *vs.* 18S rRNA transcription. A lower ΔC_t value reflects higher levels of transcription, and vice versa.

For analysis of A_1AR expression, ventricular myocardium was homogenised in 10 volumes of ice-cold buffer (10 mM EDTA, 10 mM Hepes, 0.1 mM benzamidine, pH 7.4), the homogenate was centrifuged at 48 000 *g* for 10 min, and the pellet was resuspended in 3 ml of buffer with EDTA reduced to 1 mM. The pellet was washed an additional two times by resuspension and centrifugation, with the final pellet resuspended in 1 ml of ligand binding buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4). For ligand binding, 50 μl aliquots of membrane suspension, containing 100 μg of protein for wild-types and 10 μg of protein for transgenics, were incubated with 2 U ml^{-1} adenosine deaminase and the A_1AR-selective ligand [^{3}H]DPCPX (Dupont NEN, Boston, MA, USA). After 2 h incubation at 21 °C, 3 ml of ice-cold rinse buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) was added to each sample and membranes were collected onto Whatman GF/C glass-fibre filters. Filters were washed with ice-cold buffer and trapped radioactivity was counted. Non-specific binding was determined by addition of 500 nM N^6-cyclohexyladenosine to incubations. Specific binding was fitted to a single site binding model using non-linear least-squares curve fitting of un-transformed data to calculate receptor density (B_{max}) and the dissociation constant (K_D).

Statistical analysis

Functional responses to ischaemia–reperfusion were assessed via multi-way ANOVA with repeated measures. LDH efflux and receptor expression data were analysed by one-way ANOVA. In all cases, a Tukey *post hoc* test was applied for specific comparisons when significance was detected. Significance was accepted for $P < 0.05$; data shown are means ± S.E.M.

RESULTS

Functional response to ischaemia–reperfusion

Heart mass increased from 110 ± 9 mg in the young group to 159 ± 14 mg in the aged group. Contractile function was similar in the two age groups. Intrinsic heart rate was moderately reduced with age, as was coronary flow rate (Table 1). Global normothermic ischaemia rapidly reduced contractile function with no detectable systolic pressure development after the first 2–3 min. End-diastolic pressure rose gradually throughout the ischaemic episode. The time to ischaemic contracture (rise of 20 mmHg) was similar in young and aged hearts, whereas peak ischaemic contracture achieved was lower in aged *vs.* young hearts (Fig. 1).

During reperfusion there was an initial rapid recovery in contractile function followed by a gradual decline then

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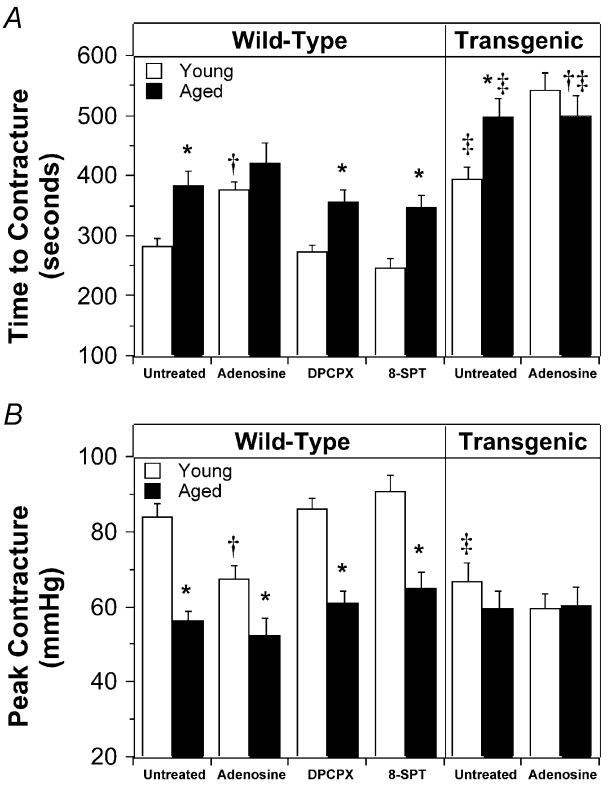

Figure 1. Contracture development during global ischaemia in young and aged hearts

A, time to ischaemic contracture. *B*, peak ischaemic contracture. Wild-type and transgenic hearts overexpressing A_1ARs were untreated or treated with 50 μM adenosine.Wild-type hearts were also treated with 150 nM of the A_1AR antagonist DPCPX or 100 μM of the non-specific antagonist 8-SPT. All values represent means ± S.E.M. * $P < 0.05$ *vs.* young heart; † $P < 0.05$ *vs.* untreated heart; ‡ $P < 0.05$ *vs.* wild-type heart.

slow recovery over the remaining 30 min of reperfusion (Fig. 2). At the end of reperfusion there remained a sustained elevation in diastolic pressure and decline in contractility in both age groups (Fig. 2*A*). Post-ischaemic contractile dysfunction was significantly worsened in aged *vs.* young hearts (Figs 2 and 3). Coronary flow initially recovered to pre-ischaemic levels during early reperfusion, then gradually declined to ~75% of pre-ischaemia (Figs 2*D* and 3*D*). In aged hearts, coronary flow recovery during early reperfusion was similar, although flow tended to be slightly lower in aged *vs.* young hearts. The incidence of reperfusion-induced arrhythmias was similar in the two groups (Fig. 4*A*). Impaired functional recovery with ageing was not associated with differences in the incidence of ectopy during reperfusion (Fig. 4*A*), but was matched by greater loss of myocardial LDH (Fig. 4*B*).

Effects of adenosine and A_1AR overexpression on responses to ischaemia–reperfusion

The data in Fig. 5 demonstrate that adenosine reduces heart rate and coronary resistance in young and aged hearts, and that the 50 μM concentration of adenosine employed in ischaemic studies produced near-maximal and equipotent effects in the two age groups (Fig. 5). Treatment of ischaemic-reperfused hearts with 50 μM adenosine did not significantly alter pre-ischaemic contractile function in

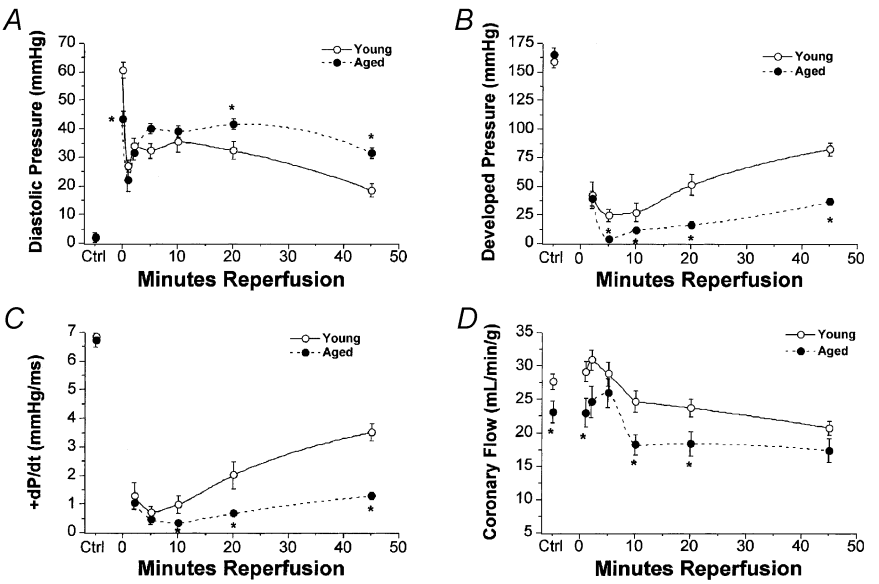

Figure 2. Time course of contractile recoveries following 20 min ischaemia in young and aged wild-type hearts

A, left ventricular diastolic pressure. *B*, left ventricular developed pressure. *C*, +d*P*/d*t*. *D*, coronary flow. All values represent means ± S.E.M. * $P < 0.05$ *vs.* young heart.

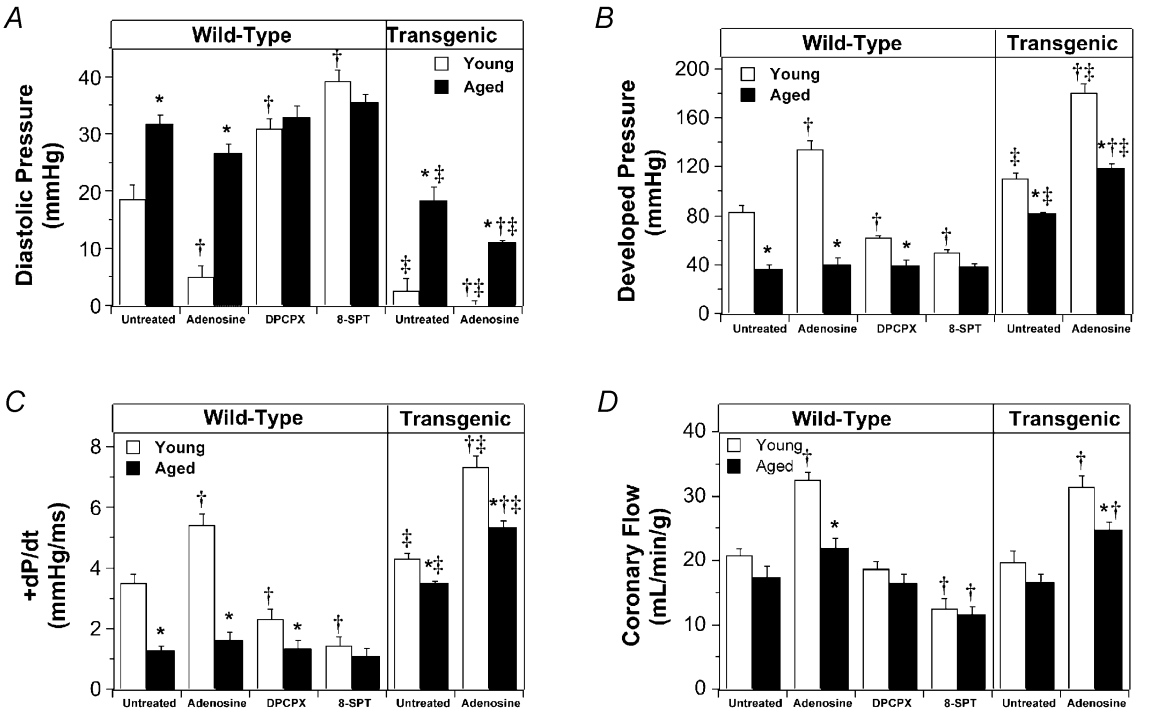

Figure 3. Effects of adenosine, adenosine receptor antagonism and A_1AR overexpression on post-ischaemic functional recoveries

Functional recoveries were assessed after 45 min reperfusion following 20 min ischaemia in young and aged hearts. *A*, left ventricular diastolic pressure. *B*, left ventricular developed pressure. *C*, +d*P*/d*t*. *D*, coronary flow. Wild-type and transgenic hearts overexpressing A_1ARs were untreated or treated with 50 μM adenosine. Wild-type hearts were also treated with 150 nM of the A_1AR antagonist DPCPX or 100 μM of the non-specific antagonist 8-SPT. All values represent means ± S.E.M. * $P < 0.05$ *vs.* young heart; † $P < 0.05$ *vs.* untreated heart; ‡ $P < 0.05$ *vs.* wild-type heart.

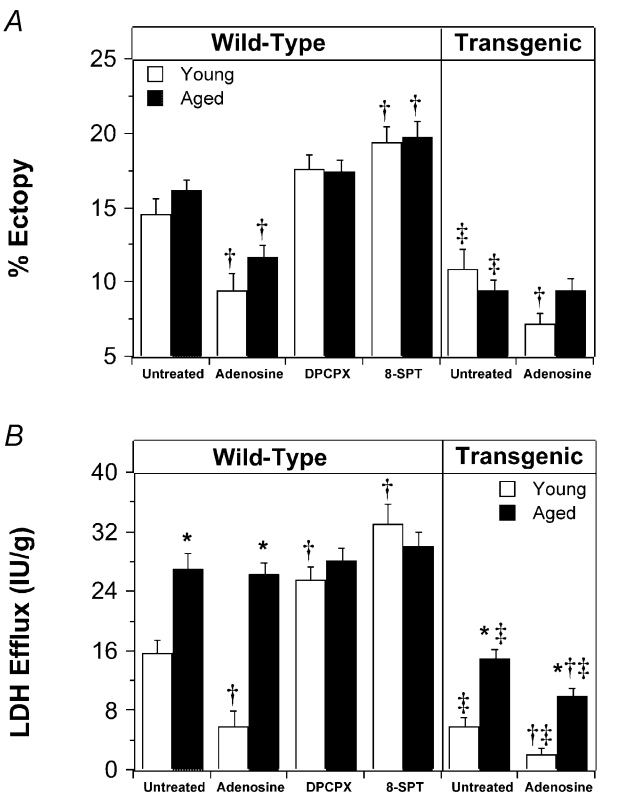

Figure 4. Arrhythmogenesis and LDH efflux in post-ischaemic hearts

A, incidence of tachyarrhythmias was assessed in the initial 10 min of reperfusion following 20 min ischaemia. *B*, myocardial LDH efflux was assessed over the entire 45 min post-ischaemic period. Wild-type and transgenic hearts overexpressing A_1ARs were untreated or treated with 50 μM adenosine. Wild-type hearts were also treated with 150 nM of the A_1AR antagonist DPCPX or 100 μM of the non-specific antagonist 8-SPT. All values represent means ± S.E.M. * $P < 0.05$ *vs.* young heart; † $P < 0.05$ *vs.* untreated heart; ‡ $P < 0.05$ *vs.* wild-type heart.

any experimental groups (Table 1), although coronary flow was elevated. In young hearts adenosine reduced contracture development (Fig. 1), and markedly enhanced post-ischaemic contractile recovery (Fig. 3). Coronary reflow during reperfusion was significantly enhanced by adenosine in young hearts, and was enhanced to a lesser extent in the aged group (Fig. 3*D*). Adenosine also reduced reperfusion arrhythmias and LDH efflux in young hearts (Fig. 4). In aged hearts adenosine failed to alter contractile dysfunction or necrosis, but it did reduce arrhythmogenesis to an extent similar to that for young hearts (Fig. 4*A*). Blockade of A_1ARs with DPCPX did not alter baseline function in young or aged hearts (data not shown), but it impaired ischaemic tolerance in young but not aged hearts (Figs 3 and 4). Similarly, non-selective blockade of adenosine receptors with 8-SPT impaired ischaemic tolerance (Figs 3 and 4). The effects of DPCPX and 8-SPT were similar although DPCPX failed to alter reflow whereas 8-SPT reduced reflow (Fig. 3).

Normoxic contractile function in both young and aged hearts was unaltered by overexpression of A_1ARs, although intrinsic heart rate was reduced in both age groups (Table 1). A_1AR overexpression improved ischaemic tolerance in young and aged hearts, reducing contracture development (Fig. 1), contractile dysfunction (Fig. 3), arrhythmogenesis and LDH efflux (Fig. 4). In contrast to aged wild-type hearts, aged transgenic hearts displayed a significant protective response to 50 μM adenosine. Adenosine treatment in aged transgenic hearts further enhanced contractile recovery, and reduced LDH efflux and arrhythmogenesis (Figs 3 and 4). Nonetheless, post-ischaemic recovery remained much higher in young transgenic hearts treated with adenosine. A_1AR overexpression failed to alter the extent of post-ischaemic coronary reflow in both young and aged hearts untreated or treated with adenosine (Fig. 3*D*).

Effects of diazoxide, 5-HD and chelerythrine on ischaemic tolerance

Treatment with diazoxide enhanced recovery from ischaemia in both young and aged hearts, reducing

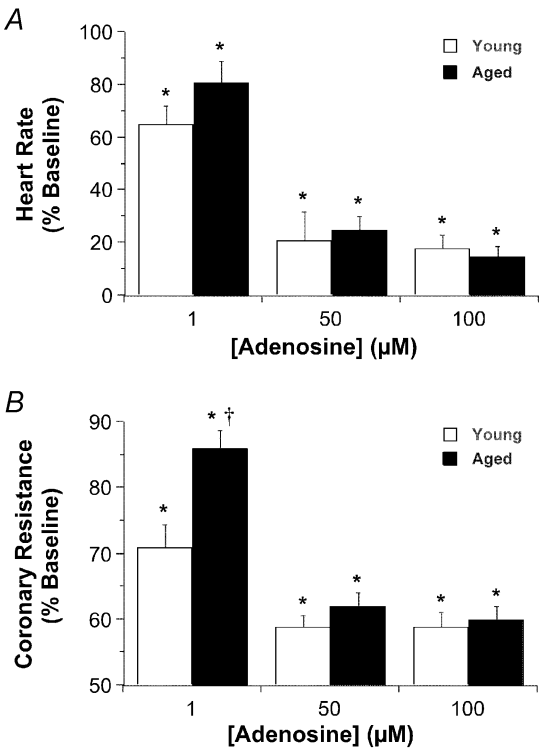

Figure 5. Concentration–response data for A_1 (bradycardia) and A_2 (vasodilatation) receptor-mediated effects of adenosine

Adenosine was infused cumulatively at concentrations of 1, 50 and 100 μM in young and moderately aged hearts. Maximal functional changes were measured at each concentration. All values represent means ± S.E.M. * $P < 0.05$ *vs.* baseline; † $P < 0.05$ *vs.* young hearts.

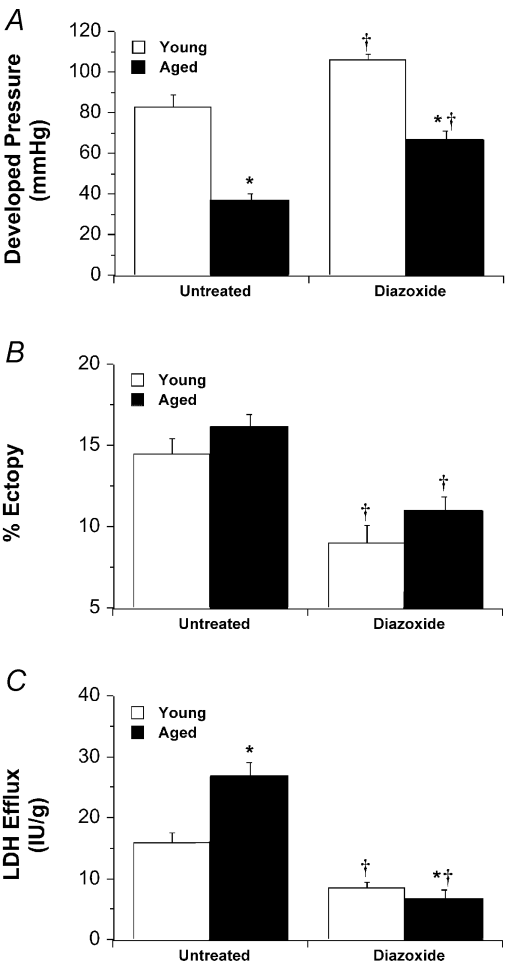

Figure 6. Cardioprotective responses to diazoxide in young and aged hearts

Protective effects of 50 μM diazoxide were assessed in young and aged hearts subjected to 20 min ischaemia and 45 min reperfusion. *A*, final recovery of left ventricular developed pressure. *B*, incidence of tachyarrhythmias in the initial 10 min of reperfusion. *C*, post-ischaemic LDH efflux. All values represent means ± S.E.M. * $P < 0.05$ *vs.* young heart; † $P < 0.05$ *vs.* untreated heart.

contractile dysfunction (Fig. 6*A*), arrhythmogenesis (Fig. 6*B*) and LDH efflux (Fig. 6*C*). The effects of diazoxide on contractile recovery and necrosis were inhibited by the PKC inhibitor chelerythrine and the putative mito K_{ATP} channel inhibitor 5-HD (Fig. 7). However, while the anti-arrhythmic effects of diazoxide were also blocked by 5-HD, they were resistant to chelerythrine (Fig. 7*B*). The cardioprotective effects of adenosine were all abolished by 5-HD (Fig. 7). As with diazoxide, chelerythrine abrogated the effects of adenosine on contractile function and LDH efflux, but failed to modify the effects on reperfusion-induced tachyarrhythmias (Fig. 7*B*).

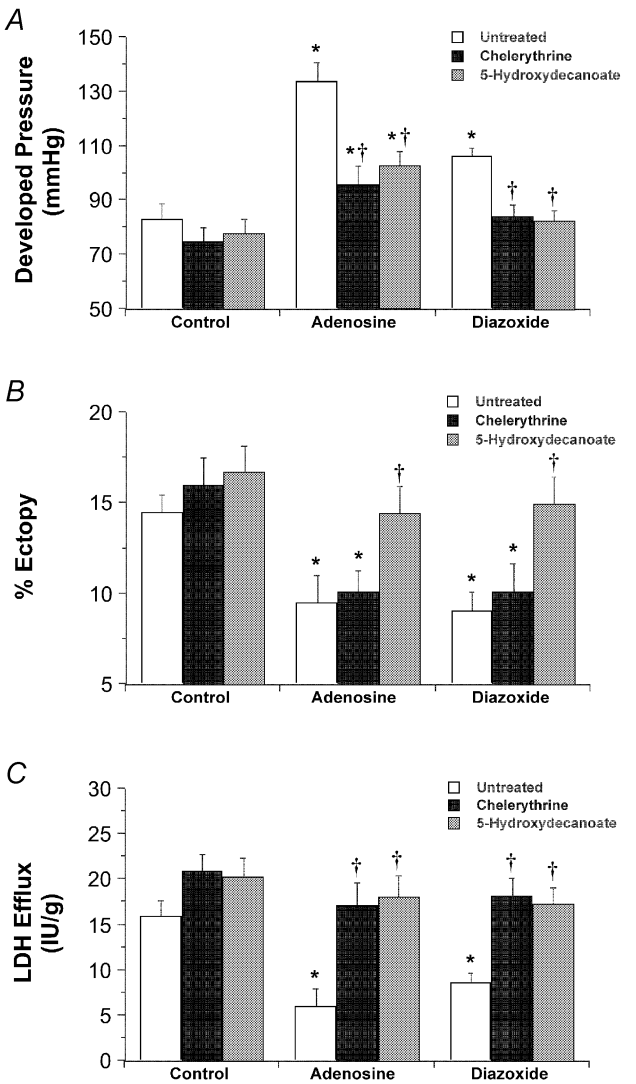

Figure 7. Effects of chelerythrine and 5-HD on cardioprotective effects of adenosine and diazoxide

Ability of 100 μM 5-HD and 3 μM chelerythrine to modify cardioprotection with 50 μM adenosine or 50 μM diazoxide was assessed in young wild-type hearts. *A*, ventricular pressure development. *B*, reperfusion-induced tachyarrhythmias. *C*, LDH efflux. All values represent means ± S.E.M. * $P < 0.05$ *vs.* Control; † $P < 0.05$ *vs.* hearts untreated with chelerythrine or 5-HD.

Effects of age on myocardial A_1AR transcription and expression

Analysis of ventricular myocardium revealed no change in transcription of the A_1AR gene with age in wild-type hearts (Fig. 8*A*). Curiously, we detected a modest age-related increase in A_1AR transcription in transgenic hearts (reflected by reduced ΔC_t relative to 18S rRNA). Radioligand binding analysis failed to detect a difference in A_1AR density in young *vs.* aged wild-type hearts (Fig. 8*B*). However, there was a modest decline in A_1AR density with age in transgenic hearts despite the apparent increase in A_1AR gene transcription. No changes in the dissociation constant for DPCPX were observed. The K_D was 1.2 ± 0.2 and 1.4 ± 0.2 nM in young and aged wild-type hearts, respectively, and was 1.3 ± 0.2 and 1.2 ± 0.2 nM in young and aged transgenic hearts, respectively.

DISCUSSION

The results of this study indicate that ageing substantially limits the tolerance to ischaemic insult, and suggest an abnormality in adenosine-mediated cardioprotection

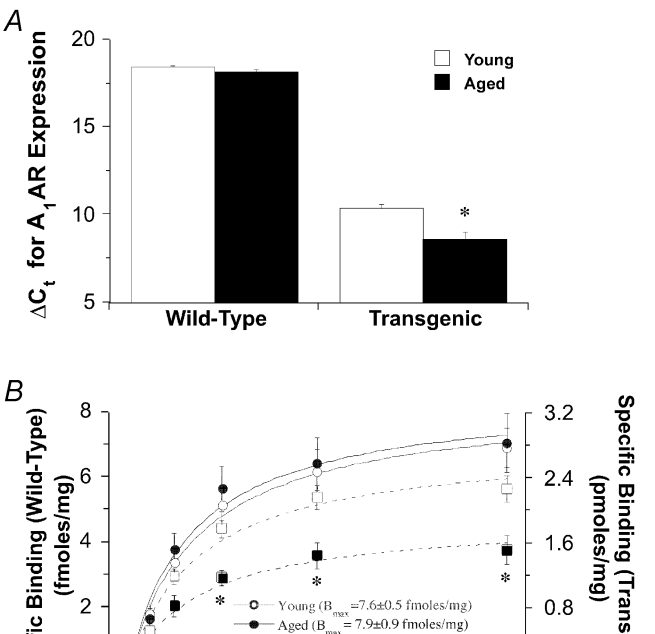

Figure 8. Effects of age on A_1AR gene transcription and protein expression

Relative A_1AR gene transcription and myocardial A_1AR density were assessed in young and aged wild-type hearts. *A*, A_1AR gene transcription determined via quantitative real-time PCR, and presented as the ΔC_t value relative to 18S rRNA transcription (lower ΔC_t value reflects higher transcript levels – see Methods). *B*, [^{3}H]DPCPX binding to myocardial A_1ARs. Density of A_1ARs (B_{max}) is also provided in *B* for all four groups. All values represent means ± S.E.M. * $P < 0.05$ *vs.* young. No differences were detected between B_{max} values in young *vs.* aged hearts in *B*.

plays an important role. The abnormal adenosine response appears to result from impaired activation of mito K_{ATP} channels, and is unrelated to altered transcription or expression of A_1ARs. Enhanced expression of A_1ARs substantially improves tolerance in aged hearts, and restores the protective response to adenosine.

Effects of ageing on functional responses to myocardial ischaemia–reperfusion

An increasing number of studies support reduced ischaemic tolerance with ageing (Pahor *et al.* 1985; Frolkis *et al.* 1991; Misare *et al.* 1992; Lesnefsky *et al.* 1994; Tani *et al.* 1997; Headrick, 1998; Abete *et al.* 1999; Mariani *et al.* 2000; Rosenfeldt *et al.* 2002) and implicate a variety of mechanisms (Boucher *et al.* 1998; Cain *et al.* 1998; Headrick, 1998; Coombes *et al.* 2000; Lesnefsky *et al.* 2001; Bulteau *et al.* 2002). Our data demonstrate substantially worsened post-ischaemic functional recovery and enhanced enzyme leakage in aged *vs*. young hearts (Figs 2 and 4). As opposed to studies in rat (Boucher *et al.* 1998, 2000; Headrick 1998), we observe reduced ischaemic contracture in aged mouse heart (Fig. 1). Nonetheless, post-ischaemic diastolic pressure was elevated in aged hearts (Fig. 2*A*). These observations have parallels in other investigations. In an elegant study, Cross *et al.* (1996) showed that glycogen content and extent of ATP depletion are key determinants of contracture and post-ischaemic diastolic pressure. High pre-ischaemic glycogen limits ischaemic contracture yet worsens recovery due to acidosis and Na^+–H^+ exchange. Elevations in myocardial glycogen with age (Tani *et al.* 1999) may therefore contribute to reduced contracture development (Fig. 1). In addition, since contracture continues to develop until ATP available to myosin-ATPase is consumed, higher pre-ischaemic ATP may enhance contracture (Cross *et al.* 1996). Though the data are equivocal, myocardial ATP may decline with ageing (Ramani *et al.* 1986), limiting ischaemic contracture development.

Whether impaired functional recovery reflects greater stunning *vs*. necrosis is difficult to ascertain. Abete and colleagues observed changes in both end-points (Abete *et al.* 1999). Since we observed elevated LDH loss, enhanced necrosis must contribute to poor functional outcome with age. In terms of reflow, we found no evidence for a substantial role in the decline in tolerance: while reflow was modestly reduced in aged hearts, the difference was small (10–20 %) relative to differences in contractile recovery (50–60 %). Moreover, contractile function and LDH efflux appear independent of reflow. Adenosine increased reflow in aged hearts yet contractile recovery was unaltered (Fig. 3), and A_1AR overexpression enhanced ischaemic tolerance in all groups without altering reflow (Fig. 3*D*). These data agree with earlier findings regarding protection with adenosine and A_1AR activation (Matherne *et al.* 1997; Headrick *et al.* 2000), and the data of Kolocassides *et al.* (1996) regarding the lack of an important role for reflow in the effects of cardioplegia and preconditioning.

Effects of ageing on adenosine-mediated cardioprotection

We and others have acquired evidence implicating adenosine as an endogenous determinant of ischaemic tolerance in immature and mature myocardium (Zhao *et al.* 1993, 1994; Matherne *et al.* 1996; Peart & Headrick, 2000), and a recent study suggests adenosine-mediated protection may decline with age (Gao *et al.* 2000). Unfortunately, the latter study examined an adenosine stimulus selectively modifying heart rate and flow in young but not aged hearts. The lack of protection may therefore have reflected an age-related reduction in activation of effector mechanisms (Cai *et al.* 1997; Gao *et al.* 1997). In the current study we showed that an equipotent and near-maximally effective concentration of adenosine enhanced ischaemic tolerance in young hearts yet failed to modify contractile recovery and enzyme efflux in aged myocardium (Figs 3 and 4). Furthermore, blockade of endogenous adenosine with A_1AR-selective or non-selective antagonists revealed endogenous adenosine also exerts protection in young but not aged hearts (Figs 3 and 4). Thus, the effects of both exogenous and endogenous adenosine are abrogated with ageing. Curiously, the impact of age is selective as adenosine does exert comparable anti-arrhythmic effects in both groups (Fig. 4). This may reflect unique signalling in different forms of protection (see below).

Roles of mito K_{ATP} channels and PKC in adenosine-mediated protection

We have acquired evidence that protection with A_1AR activation depends upon mito K_{ATP} channel activation (Headrick *et al.* 2000), in agreement with other studies (Van Winkle *et al.* 1994; Miura *et al.* 1999). We show here that the mito K_{ATP} channel inhibitor 5-HD and PKC inhibitor chelerythrine both limit protection with adenosine (Fig. 7). Additionally, both agents eliminate protection with the mito K_{ATP} channel opener diazoxide (Fig. 7), which mimicked the effects of adenosine in young hearts (Fig. 6). However, a recent study suggests diazoxide and 5-HD may exert effects on β-oxidation and respiratory chain function (Hanley *et al.* 2002). These authors hypothesise that protection with diazoxide may occur via partial inhibition of respiratory chain complexes. In the light of their observations it might be appropriate to conclude that adenosine-mediated protection occurs via a 5-HD-sensitive process which may involve mito K_{ATP} channels (Garlid *et al.* 1996; Liu *et al.* 1998) and/or a change in respiratory chain function, as proposed by Hanley and colleagues (2002). Furthermore, since the effects of diazoxide and adenosine are sensitive to 5-HD and chelerythrine, and diazoxide protects aged hearts whereas adenosine does not, we conclude that adenosine activates

the same signalling path activated by diazoxide, PKC acts downstream of the site(s) modified by adenosine and diazoxide, and the failure of adenosine-mediated protection with ageing involves ineffective upstream activation of the diazoxide-/5-HD-sensitive process.

That the failure in adenosine- and A_1AR-mediated protection is proximal to the site targeted by diazoxide agrees with recent studies demonstrating impaired protection with preconditioning and PKC activation and preserved protection with diazoxide (Tani *et al.* 2001; Lee *et al.* 2002). Moreover, studies of Korzick *et al.* (2001) and Takayama *et al.* (2001) support an ageing-related disruption of PKC signalling and translocation, potentially explaining the impaired responses to protective stimuli. However, these observations contrast with the data of Schulman *et al.* (2001), who documented a loss of protection with transient A_1 agonism, PKC activation and diazoxide. The reason for this discrepancy is unclear, but may involve the different protective stimulus (i.e. transient activation) assessed by Schulman and colleagues (2001). Our data, and the findings of Tani *et al.* (2001) and Lee *et al.* (2002), support a failure in signalling proximal to molecular processes targeted by diazoxide. It is tempting to speculate that this may involve the PI3 kinase pathway, since myocardial PI3 kinase activity falls with ageing (Martineau *et al.* 1999), the enzyme may activate cardioprotection (Tong *et al.* 2000), and it is located upstream of PKC and mito K_{ATP} channels (Tong *et al.* 2000). However, while adenosine receptors may activate PI3-kinase (Krieg *et al.* 2002), data indicate that the enzyme does not play an essential role in the anti-ischaemic effects of adenosine (Qin *et al.* 2003). Future work might address the precise role of this path in adenosine-mediated cardioprotection.

Despite our evidence for a role for PKC in protective signalling, recent reports question an obligatory function for the enzyme in cardioprotection. These studies support roles for other kinase cascades (Qin *et al.* 2003), lending support to the notion that adenosine and other protective stimuli activate multiple parallel kinase-dependent pathways (Tanno *et al.* 2000; Cohen *et al.* 2001; Li & Sato, 2001; Takeishi *et al.* 2001; Qin *et al.* 2003). As discussed by Cohen *et al.* (2001), this would provide some level of redundancy and explain why blockade of individual paths is not always effective in eliminating protective responses. In this respect, since we show adenosine fails to protect aged hearts, any redundancy provided by multiple signalling paths is clearly ineffective in countering the effects of ageing. This, in turn, implicates an age-related failure at an upstream site in the signalling cascade.

As noted above, the effects of age on arrhythmogenesis *vs.* other end-points differ, suggesting unique signalling. This is verified by the observation that chelerythrine and 5-HD reduce the functional and anti-necrotic effects of adenosine and diazoxide whereas only 5-HD altered anti-arrhythmic responses (Fig. 7). Dissociation of arrhythmogenesis from contractile recovery and necrosis in these groups indicates necrosis and contractile dysfunction are unlikely contributors to arrhythmogenesis. Our findings are consistent with prior studies that showed no role for PKC in post-ischaemic arrhythmogenesis (Du *et al.* 1995) and in anti-arrhythmic effects of preconditioning (Kita *et al.* 1998). Other studies support distinct signalling in the protection against necrosis *vs.* arrhythmogenesis (Wang *et al.* 2001).

Effects of modulating A_1AR expression

We hypothesised that enhanced A_1AR overexpression might restore ischaemic tolerance in aged hearts, based on observations that adenosine receptors are important in determining intrinsic tolerance in younger hearts (Zhao *et al.* 1994; Matherne *et al.* 1996; Peart & Headrick, 2000). Overexpression of A_1ARs does improve ischaemic tolerance (Figs 1, 3 and 4), and recoveries in aged transgenic hearts were equivalent to those for young wild-type hearts. Furthermore, the protective response to adenosine was restored. These findings indicate that mediators and end-effectors of adenosine- and A_1AR-dependent protection are functional in aged myocardium and can be harnessed if the amplitude of the initial stimulus (A_1AR activation or coupling) is sufficiently enhanced. This is consistent with the observations of McCully and colleagues (1998), who found preconditioning and adenosine are individually ineffective in aged hearts while the two together are protective, possibly via additive activation of protective signalling. Our data also indicate that aged myocardium is not necessarily the 'victim' of unavoidable accumulation of cellular damage, and it can possess an apparently normal phenotypic response to ischaemia. Nonetheless, while A_1AR overexpression in aged hearts produces an ischaemia-tolerant phenotype, A_1AR overexpression additionally enhances tolerance in young hearts (Figs 3 and 4). Since the detrimental effects of ageing are not eliminated by A_1AR overexpression, factors other than impaired adenosine-mediated cardioprotection may also play a role in reduced ischaemic tolerance.

The role of A_1AR transcription and expression

As noted above, the data for A_1AR overexpression demonstrate signalling downstream from A_1ARs is intact in aged heart. Thus, a site of 'failure' in aged myocardium may be the A_1AR itself. While prior studies suggest there is no change in cardiac A_1AR expression with ageing (Cai *et al.* 1997), studies in non-cardiac tissue support reduced A_1AR density (Cunha *et al.* 2001). We observed no age-related change in either A_1AR transcription or expression in wild-type mouse hearts (Fig. 8). Thus, the age-related failure in A_1AR-mediated cardioprotection does not occur at the level of A_1AR gene transcription or expression, further implicating impaired activation of downstream effector mechanisms. Interestingly, we did observe a

modest decline in A_1AR expression in aged transgenic hearts, despite an apparent increase in A_1AR transcription. The cause of this apparent dissociation between transcription and expression in transgenic hearts is unclear, but may reflect age-related changes in expression amplification via the α-MHC promoter.

Conclusions

This study documents a substantial decline in myocardial ischaemic tolerance with moderate ageing, which involves a failure in cardioprotection mediated by endogenous and exogenous adenosine. The data indicate that the effects of ageing are selective for protection against contractile dysfunction and necrosis *vs.* arrhythmogenesis and support differing signalling in these responses. The inability of adenosine to protect against contractile dysfunction and necrosis is unrelated to changes in A_1AR transcription or translation, and appears to involve a failure in one or more signalling elements upstream of mito K_{ATP} channels. Overexpression of A_1ARs improves ischaemic tolerance in aged hearts, and restores protection with exogenous adenosine. However, since an age-related difference in ischaemic tolerance persists in transgenic hearts, it is probable that factors additional to impaired adenosine-mediated cardioprotection play some role in the ischaemia-intolerant aged phenotype.

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