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Influence of β-adrenoceptor tone on the cardioprotective efficacy of adenosine A_1 receptor activation in isolated working rat hearts

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1 This study investigated the role of β-adrenoceptors in the cardioprotective and metabolic actions of adenosine A_1 receptor stimulation.

2 Isolated paced (300 beats min^{-1}) working rat hearts were perfused with Krebs-Henseleit solution containing 1.2 mM palmitate. Left ventricular minute work (LV work), O_2 consumption and rates of glycolysis and glucose oxidation were measured during reperfusion (30 min) following global ischaemia (30 min) as well as during aerobic conditions.

3 Relative to untreated hearts, N^6-cyclohexyladenosine (CHA, 0.5 μM) improved post-ischaemic LV work (158%) and reduced glycolysis and proton production (53 and 42%, respectively). CHA + propranolol (1 μM) had similar beneficial effects, while propranolol alone did not affect post-ischaemic LV work or glucose metabolism. Isoprenaline (10 nM) impaired post-ischaemic function and after 25 min ischaemia recovery was comparable with 30 min ischaemia in untreated hearts (41 and 53%, respectively). Relative to isoprenaline alone, CHA + isoprenaline improved recovery of LV work (181%) and reduced glycolysis and proton production (64 and 60%, respectively).

4 In aerobic hearts, CHA, propranolol or CHA + propranolol had no effect on LV work or glucose oxidation. Glycolysis was inhibited by CHA, propranolol and CHA + propranolol (50, 53 and 52%, respectively). Isoprenaline-induced increases in heart rate, glycolysis and proton production were attenuated by CHA (85, 57 and 53%, respectively).

5 The cardioprotective efficacy of CHA was unaffected by antagonism or activation of β-adrenoceptors. Thus, the mechanism of protection by adenosine A_1 receptor activation does not involve functional antagonism of β-adrenoceptors.

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Abbreviations: BSA, bovine serum albumin; CF, coronary flow; CHA, N^6-cyclohexyladenosine; CO, cardiac output; CVC, coronary vascular conductance; HR, heart rate; LV work, left ventricular minute work; MVO_2, myocardial oxygen consumption; PDC, pyruvate dehydrogenase complex; pH_i, intracellular pH, PSP, peak systolic pressure

Introduction

Myocardial energy substrate preference is an important determinant of the extent of recovery of mechanical function following ischaemia. The oxidation of free fatty acids, levels of which are elevated in human plasma after myocardial infarction (Lopaschuk *et al*., 1994), is the major source of energy for ATP generation in aerobic hearts (Saddik & Lopaschuk, 1991). However, fatty acids impair the recovery of post-ischaemic mechanical function, in part by altering glucose metabolism (Lopaschuk *et al*., 1990). This is due to an inhibition of pyruvate dehydrogenase activity, the rate limiting enzyme of glucose oxidation, by acetyl CoA arising from the β-oxidation of fatty acids (Lopaschuk, 1997; Lopaschuk & Stanley, 1997). Consequently, in the presence of fatty acids, rates of glucose oxidation are reduced and the uncoupling between rates of glycolysis and glucose oxidation is intensified. Under these conditions, the hydrolysis of ATP derived from glucose metabolism becomes a significant source of proton production (Lopaschuk *et al*., 1993). During reperfusion, the accumulation of protons from ischaemia, together with a continued uncoupling of glycolysis from glucose oxidation, delays the recovery of intracellular pH (pH_i) and accelerates Na^+/H^+ exchange. This increases Na^+ accumulation that ultimately induces Ca^{2+} overload (Lazdunski *et al*., 1985; Tani & Neely, 1989), both of which are important determinants of ischaemia-reperfusion injury and impaired recovery of mechanical function during reperfusion (Bolli & Marban, 1999; Murphy *et al*., 1999). Alteration of energy substrate metabolism, either by increasing glucose oxidation (McVeigh & Lopaschuk, 1990; Lopaschuk & Stanley, 1997) or by inhibiting glycolysis (Finegan *et al*., 1993), reduces proton production and enhances the recovery of post-ischaemic mechanical function.

Adenosine is an endogenous nucleoside that can exert a variety of effects throughout the body by interacting with several types of cell-surface receptors. Recent interest in the cardiovascular effects of adenosine has focused on its cardioprotective efficacy (Ely & Berne, 1992) and on its role as a trigger or mediator of ischaemic preconditioning (Mullane, 1992). Marked reductions in infarct size, (Toombs *et al*., 1992; Zhao *et al*., 1993; 1994), attenuation of dysrhythmias (Pantely & Bristow, 1990) and improved recovery of mechanical function during reperfusion have been noted following administration of adenosine (Lasley *et al*., 1990; Lasley & Mentzer, 1992; Finegan *et al*., 1993; Janier *et al*., 1993; Nomura *et al*., 1993). The precise mechanism underlying adenosine-induced cardioprotection has not been established, but adenosine can influence cardiac function either

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directly *via* stimulation of adenosine A_1 receptors located on cardiac myocytes (Martens *et al*., 1987) or indirectly by inhibition of noradrenaline release (Richardt *et al*., 1987) as well as *via* stimulation of adenosine A_2 receptors located on the coronary and systemic vasculature (Collis, 1989). Cardioprotection arising from adenosine A_1 receptor stimulation (Lasley *et al*., 1990; Thornton *et al*., 1992) may also be due, in part, to beneficial alterations in glucose (Finegan *et al*., 1996b) and glycogen (Fraser *et al*., 1999) metabolism. However, the detailed mechanisms underlying these cardioprotective effects of adenosine have not been elucidated and may occur by actions either directly on the myocyte or indirectly by an anti-adrenergic action (Richardt *et al*., 1987).

Sympathetic activation normally accompanies myocardial ischaemia *in vivo* and may also occur *in vitro* in response to ischaemia-induced release of noradrenaline (Schomig *et al*., 1992; Kurz *et al*., 1995). While it is well established that adrenoceptor activation dramatically increases cardiac workload and energy demand, it also influences energy substrate preference. The increased ATP demand is met mainly from increases in glucose metabolism (Collins-Nakai *et al*., 1994) rather than from increases in fatty acid oxidation (Goodwin *et al*., 1998). The relative rates of myocardial glycolysis and glucose oxidation are also affected by adrenoceptor activation (Collins-Nakai *et al*., 1994) and under normal aerobic conditions, glycolysis is markedly accelerated while glucose oxidation is increased to a lesser extent. These changes cause a further uncoupling of glycolysis from glucose oxidation that enhances proton production. Although the detailed effects of adrenoceptor activation on energy substrate utilization during ischaemia and reperfusion are unknown, the exaggerated uncoupling of glycolysis from glucose oxidation, in combination with the increased energy demand during reperfusion, suggest a high potential for acidosis, Na^+ and Ca^{2+} overload and impaired recovery of mechanical function.

Adenosine exerts an anti-adrenergic action by both pre-synaptic (inhibition of noradrenaline release) and post-synaptic mechanisms (inhibition of adenylyl cyclase) that serves to decrease energy demand (Dobson *et al*., 1986; Romano *et al*., 1991; Romano & Dobson, 1990). In addition, adenosine increases left ventricular (LV) efficiency in catecholamine-stimulated non-paced hearts (Headrick & Willis, 1989). The metabolic and cardioprotective actions of adenosine in the presence of adrenoceptor activation and the role of these potential anti-adrenergic mechanisms in the effects of adenosine have not been established.

This study was designed to assess the therapeutic potential and mechanisms of adenosine-induced inhibition of glycolysis and cardioprotection. We hypothesized that, if these actions of adenosine A_1 receptor activation were exerted directly on the myocyte, they would also persist during β-adrenoceptor blockade. Moreover, as sympathetic activation likely occurs during myocardial ischaemia *in vivo*, we determined whether the metabolic actions and cardioprotective efficacy of adenosine A_1 receptor activation would be altered in the presence of β-adrenoceptor activation.

Methods

Heart perfusions

Male Sprague-Dawley rats (250–400 g), which had been fed *ad libitum* were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}). The heart was rapidly excised and placed in ice-cooled Krebs-Henseleit solution. The aorta was cannulated and a Langendorff perfusion was initiated using Krebs-Henseleit solution (pH 7.4, gassed with a 95% O_2 / 5% CO_2 mixture) at a hydrostatic pressure of 60 mmHg. Each heart was then trimmed of excess tissue and the pulmonary artery and left atrium was cannulated. After 10 min, Langendorff perfusion was stopped and hearts were switched to working mode by clamping the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow line. Working hearts were electrically paced at 300 beats min^{-1} (Grass S88 stimulator) during periods of aerobic perfusion. Working hearts were perfused in a closed recirculating system at 37°C using an oxygenator with a large surface area in constant contact with the 95% O_2 / 5% CO_2 mixture. The perfusate (volume 100 ml) was a modified Krebs-Henseleit solution (mM: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, Ca^{2+} 2.5, glucose 11, 100 mU l^{-1} insulin and palmitate 1.2 pre-bound to 3% bovine serum albumin (BSA, Fraction V, hsp). Perfusions were performed at a constant left atrial preload of 11.5 mmHg with the afterload hydrostatic pressure set to a column height equivalent to 80 mmHg.

Measurement of mechanical function

Heart rate and systolic and diastolic aortic pressures (mmHg) were measured using a Gould P23 pressure transducer connected to the aortic outflow line. Cardiac output (CO) and aortic flow (ml min^{-1}) were measured using ultrasonic flow probes (Transonic T206) placed in the left atrial inflow and aortic outflow lines, respectively. Coronary flow (ml min^{-1}) was calculated as the difference between CO and aortic flow. Aortic developed pressure (mmHg) was calculated as the difference between systolic and diastolic pressure. O_2 content of the coronary effluent was measured using an oxygen probe (YSI 5331) that was placed in the pulmonary artery outflow line and connected to a YSI oxygen meter (Model number 5300). Coronary effluent was then returned to the perfusate reservoir. Myocardial oxygen consumption (MVO_2, μmol min^{-1}) was calculated as the product of coronary flow and the difference in oxygen content between the left atrial inflow and the coronary effluent. Left ventricular (LV) developed pressure was calculated as the difference between aortic systolic pressure and preload pressure. LV minute work (LV work, Joules) was calculated as (LV developed pressure) $\times$ CO $\times$ 0.133 and myocardial efficiency was calculated as LV work (Joules) expressed as a percentage of the total potential work (Joules) based on MVO_2 where total potential work was calculated as MVO_2 (μmol min^{-1}) $\times$ 0.447). Coronary vascular conductance (CVC) was calculated as coronary flow (ml min^{-1}) / mean aortic pressure (mmHg).

Measurement of rates of glycolysis and glucose oxidation during aerobic perfusion and during reperfusion following ischaemia

Glycolysis and glucose oxidation were measured directly by the quantitative collection of ^3H_2O (liberated at the enolase step of glycolysis) and $^{14}CO_2$ (liberated at the level of pyruvate dehydrogenase complex (PDC) and in the citric acid cycle) from hearts perfused with solution containing tracer amounts of [5-^{3}H]-glucose and [U-^{14}C]-glucose. Samples of perfusate were removed at 10 min intervals during aerobic perfusion, stored under liquid paraffin oil, and used to determine metabolic rates.

To measure glycolysis, the ^3H_2O in perfusate samples was separated from [^{3}H]-glucose and [^{14}C]-glucose using columns containing Dowex 1-X4 anion exchange resin, as described

previously (Finegan *et al*., 1996b). Glycolysis rates are expressed as μmol glucose metabolized min^{-1} g^{-1} dry weight. The closed perfusion system allowed the collection of gaseous $^{14}CO_2$ by means of a hyamine trap (volume 40 ml). Samples of hyamine, containing trapped $^{14}CO_2$ were taken at the same times as the perfusate samples. $^{14}CO_2$ trapped as bicarbonate in the perfusate together with $^{14}CO_2$ trapped in the hyamine were measured and glucose oxidation determined as previously described (Finegan *et al*., 1996b). Rates of glucose oxidation rates are expressed as μmol glucose metabolized min^{-1} g^{-1} dry weight.

Calculation of the rate of proton production from glucose metabolism

When the rates of glycolysis and glucose oxidation are identical, the net production of protons from glucose metabolism is zero. However, if the rate of glycolysis exceeds that of glucose oxidation, there is a net production of two protons per molecule of glucose that passes through glycolysis that is not subsequently oxidised (Dennis *et al*., 1991). Consequently, the rate of proton production attributable to the hydrolysis of ATP arising from glucose metabolism can be calculated as $2\times$(rate of glycolysis − rate of glucose oxidation).

Experimental protocols

Hearts were randomized to untreated or drug-treated groups. Drug treatments consisted of N^6-cyclohexyladenosine (CHA, 0.5 μM), propranolol (1 μM) alone or in combination with CHA (0.5 μM) and isoprenaline (10 nM) alone or in combination with CHA (0.5 μM). CHA at this concentration (0.5 μM) has been shown previously to exert marked cardioprotective efficacy in working rat hearts (Finegan *et al*. 1996b). Isoprenaline was used at 10 nM as this concentration elicited a sustained increase in mechanical function while propranolol at 1 μM antagonized the effects of isoprenaline (10 nM). Hearts from each group were assigned to either an aerobic or ischaemia-reperfusion protocol.

Ischaemia-reperfusion protocol All hearts were perfused aerobically in working mode for 50 min (Pre-Ischaemia) and were then subjected to global, no-flow ischaemia that was followed by 30 min of aerobic working mode reperfusion (Reperfusion). In the absence of isoprenaline, the duration of no-flow ischaemia was 30 min. However, when isoprenaline was present, the duration of no-flow ischaemia was reduced to 25 min. When isoprenaline was included in the drug treatment regimen, it was added after 15 min of aerobic perfusion and was present throughout the remainder of the perfusion protocol. Drug treatments, other than isoprenaline, were administered 5 min before the onset of ischaemia and were also present for the remainder of the perfusion protocol. Mechanical function, rates of glycolysis and glucose oxidation were measured at 10 min intervals throughout periods of aerobic perfusion (Pre-Ischaemia and Reperfusion). Comparisons of mechanical function and glucose metabolism were made between values obtained at the end of Pre-Ischaemia and Reperfusion periods within each drug treatment group, as well as by time-matched comparisons of values obtained at the end of Reperfusion for the different drug-treated groups.

Aerobic protocol Mechanical function, rates of glycolysis and glucose oxidation were measured at 10 min intervals throughout an 80 min period of aerobic perfusion (equivalent to the combined lengths of the baseline and reperfusion periods in the ischaemia-reperfusion protocol). Isoprenaline was introduced after 15 min of aerobic perfusion and other drugs were added after a further 30 min period of baseline aerobic perfusion (Baseline) and were present throughout the remaining 35 min (treatment). The effects of drug treatments on glucose metabolism and mechanical function were assessed by comparing values obtained at the end of baseline and treatment periods within each drug treatment group. Also time-matched comparisons were performed for values obtained at the end of Baseline and Treatment periods among the different drug-treated groups.

At the end of the Aerobic as well as the Ischaemia-Reperfusion protocols, heart ventricles were rapidly frozen with Wollenberger clamps cooled to the temperature of liquid N_2 for the later determination of their total dry weight.

Drugs and reagents

D-[5-^{3}H]-glucose and D-[U-^{14}C]-glucose (5 mCi $mmol^{-1}$) were purchased from Dupont Canada Inc, Ontario. Hyamine hydroxide was purchased from ICN Biomedicals Inc, OH, U.S.A. Bovine serum albumin (BSA fraction V, hsp) was obtained from Boehringer Mannheim, IN, U.S.A. Dowex 1-X4 anion exchange resin (200–400 mesh, chloride form) was obtained from Bio-Rad Laboratories, CA, U.S.A. Drugs (and their sources) were as follows: insulin (Connaught Novo, Ontario, Canada), N^6-cyclohexyladenosine (Research Biochemicals International, MA, U.S.A.), isoprenaline (Sigma Chemical Co., MI, U.S.A.) and propranolol (Sigma Chemical Co., MI, U.S.A.).

Statistical analysis

Data are expressed as mean$\pm$s.e.mean. Groups of data compared to a single control group were analysed using a one-way analysis of variance supported by Dunnett's test for multiple comparisons against a single control. For multiple comparisons, a one-way analysis of variance supported by the Bonferroni Multiple Comparison Test for inter-group differences was used. Where only two groups of data were compared, a Student's *t*-test was used. For paired comparisons, a paired Student's *t*-test was used. Differences were considered significant when $P<0.05$.

Results

Ischaemia-reperfusion in absence of isoprenaline

Prior to the onset of ischaemia and addition of drugs, mechanical function, MVO_2, myocardial efficiency, and rates of glucose metabolism and proton production were similar in the untreated, CHA, propranolol and propranolol+CHA groups (Figures 1 and 2, and Table 1). Rates of glycolysis exceeded those of glucose oxidation in each group and this uncoupling of glycolysis from glucose oxidation leads to a rate of proton production of 4.74 ± 0.66 μmol min^{-1} g^{-1} dry weight. After 30 min reperfusion, LV work in untreated hearts recovered to 53 ± 10% of pre-ischaemia values (Figure 1 and Table 1). PSP and cardiac output were also depressed ($P<0.05$) to 75 ± 9% and 60 ± 10%, respectively, of pre-ischaemia values. Coronary flow and CVC did not differ significantly between pre-ischaemia and reperfusion; values at the end of reperfusion recovered to 91 ± 12% and 97 ± 11% respectively. MVO_2 after 30 min of reperfusion was also not

significantly different from pre-ischaemia (79±13%). However, as LV work was depressed, myocardial efficiency was significantly impaired and at the end of reperfusion was 56±12% ($P<0.01$) of pre-ischaemia values (Table 1). While rates of glucose oxidation during reperfusion were similar to those obtained during pre-ischaemia (Figure 2), rates of glycolysis and proton production were significantly elevated ($P<0.05$).

CHA significantly enhanced the recovery of LV work and myocardial efficiency to 83±4% ($P<0.05$) and 86±6% ($P<0.05$), respectively, of pre-ischaemia values (Figure 1 and Table 1). CHA also partially inhibited rates of glycolysis and proton production during reperfusion whereas glucose oxidation rates were not significantly different from the time-matched untreated group (Figure 2). Propranolol did not significantly affect the recovery of mechanical function, MVO_2 myocardial efficiency and after 30 min of reperfusion values were similar to those in the time-matched untreated group (Figure 1 and Table 1). Relative to the time-matched untreated group, rates of glycolysis, proton production and glucose oxidation during reperfusion were also not affected by propranolol (Figure 2).

CHA, in combination with propranolol, retained its cardioprotective efficacy and, relative to propranolol alone, enhanced the recovery of LV work and myocardial efficiency (to 75±6% and 79±7%, respectively, $P<0.05$, Figure 1). Values for coronary flow, CVC and MVO_2 after 30 min of reperfusion were similar to those obtained with propranolol alone (Table 1). Relative to the time-matched Untreated group, CHA in combination with propranolol reduced rates of glycolysis and proton production during reperfusion but the rate of glucose oxidation was unaffected (Figure 2).

Ischaemia-reperfusion in presence of isoprenaline

Compared with values in the Untreated group, isoprenaline increased heart rate, rate-pressure product, LV work and MVO_2 whereas myocardial efficiency was depressed (Table 2). The recovery of mechanical function following ischaemia was impaired by isoprenaline such that the extent of recovery of LV work after 25 min of global ischaemia (41±11% of pre-ischaemia values) was similar to that observed in untreated hearts subjected to 30 min of ischaemia (53±10%) (Figure 3). After 30 min of reperfusion, heart rate and CVC were similar to pre-ischaemia values for isoprenaline-treated hearts but rate-pressure product (61±11%), cardiac output (47±12%), PSP (62±11%) and coronary flow (64±15%) were each depressed ($P<0.05$) at the end of reperfusion (Table 2). The recovery of myocardial efficiency was also reduced to 41±11% ($P<0.05$). Isoprenaline increased ($P<0.05$) rates of glycolysis and proton production during pre-ischaemia compared with the untreated group whereas the rate of glucose oxidation was not significantly affected (Figure 2). In contrast to hearts perfused in the absence of isoprenaline, rates of glycolysis, glucose oxidation and proton production were not different between pre-ischaemia and reperfusion (Figure 2).

CHA, when given in combination with isoprenaline enhanced ($P<0.05$) the recovery of LV work (to 79±5% of pre-ischaemia values) when compared with hearts treated with isoprenaline alone (Figure 3). While CHA did not affect the recovery of rate-pressure product and heart rate, it enhanced ($P<0.05$) the recovery of PSP (98±2%), cardiac output (81±4%), coronary flow (103±9%) and CVC (109±10%) (Table 2). CHA, when given in combination with isoprenaline, significantly reduced rates of glycolysis and proton production

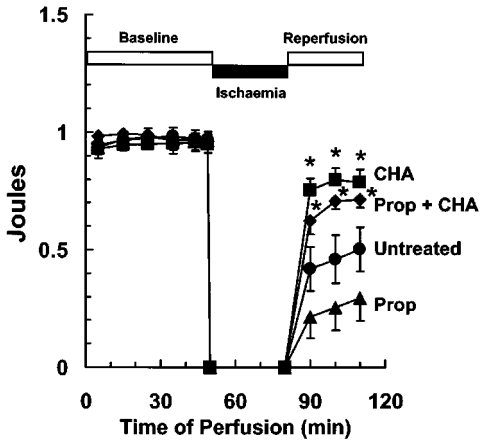

Figure 1 LV Work in working rat hearts during pre-ischaemic baseline perfusion, 30 min global ischaemia and 30 min reperfusion. Values are means±s.e.mean for n observations and are shown for hearts that were either untreated ($n=12$) or perfused in the presence of CHA (0.5 μM, $n=9$), propranolol (Prop, 1 μM, $n=11$), propranolol in combination with CHA (Prop+CHA, $n=9$). *Indicates a significant difference value during reperfusion in each of the drug-treated groups and the untreated group (ANOVA+Dunnetts).

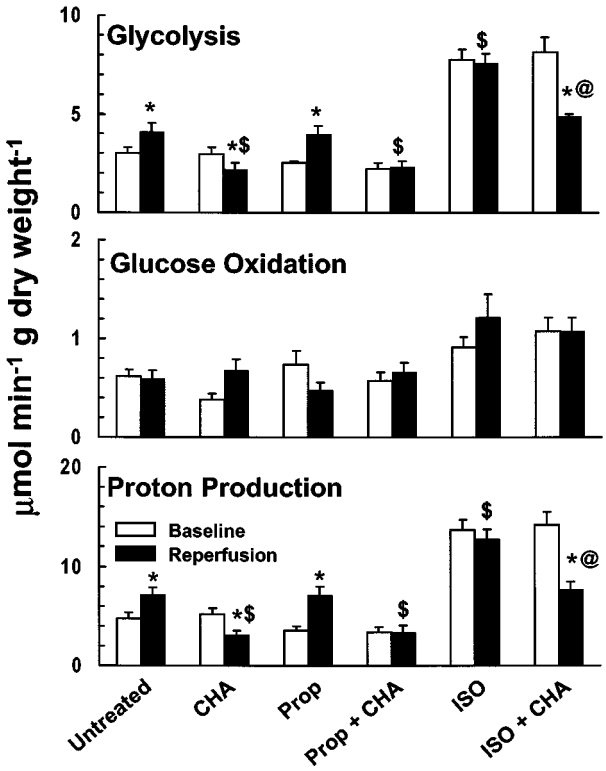

Figure 2 Rates of glycolysis, glucose oxidation and proton production in working rat hearts during pre-ischaemic baseline perfusion and during reperfusion following global, no-flow ischaemia. Values are means±s.e.mean for n observations and are shown hearts that were either untreated ($n=6$) or subjected to ischaemia and reperfusion in the presence of CHA (0.5 μM, $n=6$), propranolol (Prop, 1 μM, $n=6$), propranolol in combination with CHA (Prop+CHA, $n=7$), isoprenaline (ISO, 10 nM, $n=7$) or isoprenaline in combination with CHA (ISO+CHA, $n=7$). *Indicates a significant difference between reperfusion and corresponding pre-ischaemia value (by Student's paired t-test), \$ indicates a significant difference between values during reperfusion in each of the drug-treated groups and the untreated group (ANOVA+Dunnetts) and @ indicates a significant difference between Reperfusion values in the isoprenaline and isoprenaline+CHA groups (Student's unpaired t-test).

Table 1 Effects of CHA, propranolol and CHA in combination with propranolol on mechanical function, MVO_2 and myocardial efficiency following 30 min of global, no-flow ischaemia in working rat hearts

	HR	*PSP*	*CO*	*CF*	*CVC*	*PSP*HR*	*LV Work*	*MVO_2*	*Efficiency*
Untreated hearts ($n=12$)									
Pre-ischaemia	300	119±2	67±2	22±1	0.28±0.01	35.8±0.5	0.97±0.03	52±4	17±1
Reperfusion	300	89±10*	40±7*	19±3	0.26±0.03	26.7±3.1*	0.50±0.09*	38±7	10±2*
Hearts treated with CHA (0.5 μM, $n=9$)									
Pre-ischaemia	300	124±2	64±2	23±1	0.27±0.01	37.1±0.5	0.95±0.04	58±5	14±1
Reperfusion	300	117±2*	56±3*	26±1*	0.30±0.01*	35.0±0.6*	0.79±0.05*,$	55±4	12±1
Hearts treated with propranolol (1 μM, $n=11$)									
Pre-ischaemia	300	121±2	66±2	22±2	0.26±0.02	36.3±0.7	0.97±0.04	48±3	17±1
Reperfusion	300	64±14*	26±6*	16±3	0.4±0.13	19.2±4.3*	0.30±0.10*,$	28±7	5±2*
Hearts treated with CHA in combination with propranolol ($n=9$)									
Pre-ischaemia	300	122±3	66±3	20±1	0.23±0.01	36.6±0.8	0.97±0.05	45±2	18±1
Reperfusion	300	111±2*,@	53±2*,@	23±1	0.29±0.02	33.3±0.7*,@	0.72±0.03*,@	44±4	14±1@

Values (mean±s.e.mean for n observations) are presented for heart rate (HR, beat min^{-1}), peak systolic pressure (PSP, mmHg), cardiac output (CO, ml min^{-1}), coronary flow (CF, ml min^{-1}), coronary vascular conductance (CVC, ml min^{-1} $mmHg^{-1}$), rate-pressure product (PSP*HR, beats min^{-1} mmHg 10^{-3}), LV work (Joules), myocardial oxygen consumption (MVO_2 μmol min^{-1} g dry $weight^{-1}$) and myocardial efficiency (%). *Indicates a significant difference between reperfusion and corresponding pre-ischaemia values (by Student's paired t-test), $ indicates a significant difference between values in each of the drug-treated groups and the Untreated group during reperfusion (ANOVA + Bonferroni), @indicates a significant difference between values in the propranolol and propranolol + CHA groups during reperfusion (ANOVA + Bonferroni).

Table 2 Effects of CHA on mechanical function, MVO_2 and myocardial efficiency following 25 min of global, no-flow ischaemia in working rat hearts perfused in the presence of isoprenaline

	HR	*PSP*	*CO*	*CF*	*CVC*	*PSP*HR*	*LV Work*	*MVO_2*	*Efficiency*
Isoprenaline (10 nM) ($n=11$)									
Pre-ischaemia	349±14	126±3	74±2	26±2	0.29±0.02	43.7±1.1	1.13±0.04	78±5	13±1
Reperfusion	339±18	77±13*	37±10*	17±4*	0.22±0.04	27.2±5.1*	0.48±0.14*	44±10	6±2*
Isoprenaline (10 nM) + *CHA* (0.5 μM) ($n=12$)									
Pre-ischaemia	368±13	121±3	76±2	28±1	0.31±0.01	44.2±1.2	1.10±0.04	76±4	12±1
Reperfusion	300*	118±3@	61±3*,@	28±2@	0.33±0.02@	35.5±1.0*	0.87±0.06*,@	66±4	11±1

Values are mean±s.e.mean for n observations. For abbreviations and units, see legend to Table 1. *Indicates a significant difference between reperfusion and corresponding pre-ischaemia value (by Student's paired t-test), @indicates a significant difference between values in the isoprenaline and isoprenaline + CHA groups during reperfusion (Student's unpaired t-test).

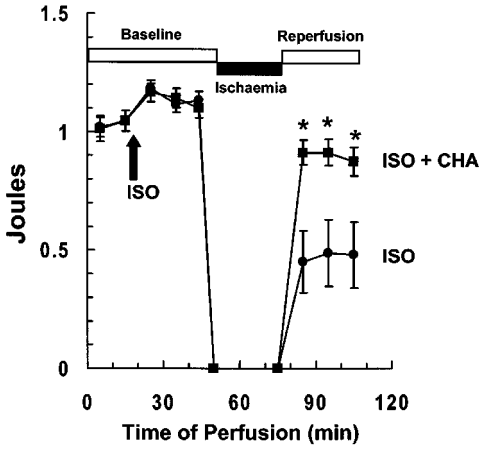

Figure 3 LV Work in working rat hearts during pre-ischaemic baseline perfusion, 25 min global ischaemia and 30 min reperfusion. Values are means±s.e.mean for n observations and are shown for isoprenaline-treated hearts that were perfused in the absence (ISO, 10 nM, $n=11$) or presence of 0.5 μM CHA (ISO + CHA, $n=12$). *Indicates a significant difference between reperfusion values in the isoprenaline and isoprenaline + CHA groups (Student's unpaired t-test).

during reperfusion, whereas the rate of glucose oxidation was unaffected (Figure 2).

Aerobic perfusion in absence of isoprenaline

During aerobic perfusion prior to drug treatment (baseline), mechanical function, MVO_2, myocardial efficiency (Table 3), and rates of glucose metabolism and proton production were similar in the untreated, CHA, propranolol and propranolol + CHA groups (Figure 4). In the untreated group, during the subsequent period of aerobic perfusion (treatment), PSP, cardiac output, rate-pressure product and LV work were slightly, but significantly, lower than during baseline whereas coronary flow, CVC, MVO_2 and myocardial efficiency were not significantly different from baseline values (Table 3). Rates of glycolysis and proton production during treatment were similar to rates observed during baseline. Glucose oxidation increased gradually throughout aerobic perfusion and the average rate during treatment was significantly higher than during baseline (Figure 4).

The presence of CHA during treatment had no significant effect on mechanical function, MVO_2 or myocardial efficiency compared with the time-matched untreated group (Table 3). However, CHA significantly inhibited rates of glycolysis and proton production compared with rates measured in the time-

Table 3 Effects of CHA, propranolol, CHA in combination with propranolol, isoprenaline and CHA in combination with isoprenaline on mechanical function, MVO_2 and myocardial efficiency in working rat hearts during aerobic reperfusion

	HR	*PSP*	*CO*	*CF*	*CVC*	*PSP*HR*	*LV Work*	*MVO_2*	*Efficiency*
Untreated ($n=11$)									
Baseline	300	119±2	70±2	23±1	0.28±0.02	35.6±0.5	1.00±0.02	57±4	15±1
Treatment	300	115±2*	66±2*	22±2	0.27±0.02	34.5±0.5*	0.91±0.03*	53±4	16±2
CHA (0.5 μM) ($n=10$)									
Baseline	300	124±2	71±2	22±1	0.26±0.02	37.1±0.7	1.06±0.04	56±3	16±1
Treatment	300	121±3	66±3*	24±1*	0.29±0.01*	36.2±1.2	0.97±0.06	57±2	14±1
Propranolol (1 μM) ($n=5$)									
Baseline	300	121±1	67±1	24±3	0.29±0.03	36.2±0.4	0.98±0.02	57±8	16±1
Treatment	300	115±3	65±1	22±4	0.27±0.05	34.6±1.0	0.89±0.02*	54±10	19±6
Propranolol+CHA ($n=8$)									
Baseline	300	118±1	64±2	23±2	0.27±0.02	35.3±0.4	0.89±0.04	56±4	15±1
Treatment	300	114±2	59±3	24±2	0.29±0.02	34.2±0.6	0.81±0.05	57±4	13±1
Isoprenaline (10 nM) ($n=6$)									
Baseline	345±16$^{\$}$	117±4	71±5	26±2	0.31±0.02	40.1±1.3$^{\$}$	1.00±0.08	67±5	12±1
Treatment	353±8	109±5	63±7	26±3	0.31±0.03	38.4±1.2	0.84±0.10	66±6	10±1
Isoprenaline+CHA ($n=6$)									
Baseline	328±11$^{\$}$	125±2	73±3	27±2	0.29±0.03	41.0±1.5$^{\$}$	1.10±0.05	70±8	12±1
Treatment	300$^{@}$	120±3	62±5	27±2	0.31±0.02	36.0±1.0$^{@}$	0.91±0.10	54±5$^{@}$	13±1

Values are mean±s.e.mean for n observations. For abbreviations and units, see legend for Table 1. *Indicates a significant difference between treatment and corresponding baseline value for each group (by Student's t-test), $ indicates a significant difference between baseline values in each of the drug-treated groups and the untreated group (ANOVA + Bonferroni), #indicates a significant difference in treatment values between each of the drug-treated groups and the untreated group (ANOVA + Bonferroni). @indicates a significant difference in treatment values between isoprenaline and isoprenaline + CHA groups (by Student's unpaired t-test).

matched untreated group, whereas the rate of glucose oxidation was not significantly affected (Figure 4). Propranolol had no significant effect on mechanical function, MVO_2 or myocardial efficiency compared with the time-matched untreated group (Table 3). Propranolol also inhibited the rate of glycolysis ($P<0.05$) and proton production whereas the rate of glucose oxidation was similar to the rate obtained in the time-matched untreated group (Figure 4).

CHA in combination with propranolol had no significant effect on mechanical function, MVO_2 or myocardial efficiency in aerobic hearts compared with propranolol alone. Rates of glycolysis, glucose oxidation and proton production during treatment were also similar to those obtained with propranolol alone.

Aerobic perfusion in presence of isoprenaline

Isoprenaline increased ($P<0.05$) baseline heart rate and rate-pressure product whereas other indices of mechanical function, MVO_2 and myocardial efficiency were not affected when compared with values in the time-matched untreated group (Table 3). There was a trend for isoprenaline to increase LV work and reduce myocardial efficiency but the differences did not attain statistical significance. Isoprenaline increased rates of glycolysis and proton production during baseline compared with values in the time-matched untreated group, whereas glucose oxidation was unaffected (Figure 4). Mechanical function, MVO_2 and myocardial efficiency were stable throughout isoprenaline administration as values measured during treatment were similar to those during baseline. While rates of glycolysis and proton production were also stable throughout the entire period of aerobic perfusion, the rate of glucose oxidation increased in a time-dependent manner and was significantly higher during treatment than during baseline (Figure 4).

CHA, when given to isoprenaline-treated hearts, reduced heart rate, rate-pressure product and MVO_2 but had no effect on other indices of mechanical function or on myocardial efficiency. CHA also reduced ($P<0.05$) rates of glycolysis and proton production, but had no effect on the rate of glucose oxidation when compared with rates measured in hearts treated only with isoprenaline (Table 3 and Figure 4).

Discussion

This study has determined that pharmacological inhibition or elevation of β-adrenoceptor tone does not alter the cardioprotective efficacy of adenosine A_1 receptor stimulation in isolated working rat hearts. This finding indicates that the mechanism by which adenosine A_1 receptor stimulation improves the recovery of post-ischaemic mechanical function does not involve functional antagonism of β-adrenoceptor-mediated responses. Moreover, the cardioprotective efficacy of adenosine A_1 receptor agonists are unlikely to be altered in the presence of elevated sympathetic tone that would be an expected deleterious component of myocardial ischaemia *in vivo*.

Mechanism of cardioprotection by adenosine A_1 receptor stimulation

The efficacy of adenosine A_1 receptor agonists as cardioprotective agents has been extensively documented (Lasley *et al*., 1990; Lasley & Mentzer, 1992; Thornton *et al*., 1992; Finegan *et al*., 1996b) but the underlying mechanism and the associated transduction systems are still unclear. As has been documented previously (Finegan *et al*., 1996b) in fatty acid perfused working rat hearts, adenosine A_1 receptor stimulation improves the recovery of post-ischaemic LV work. This is due

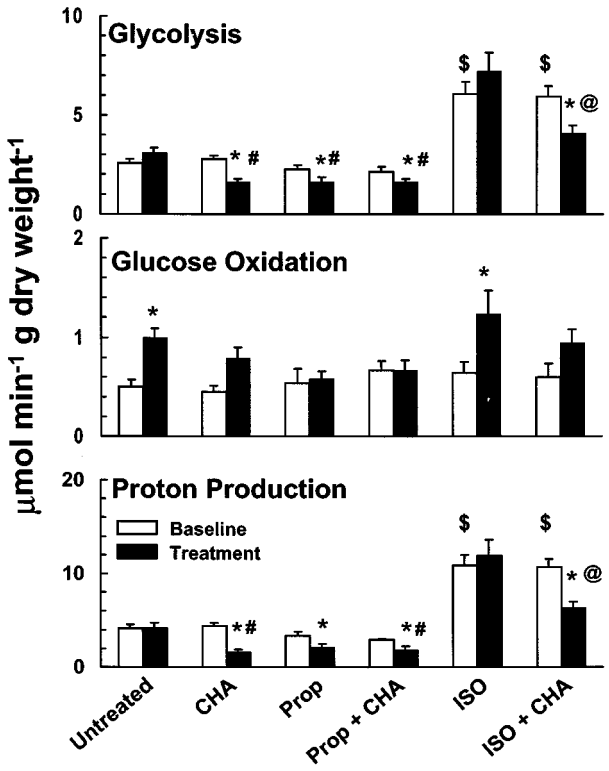

Figure 4 Rates of glycolysis, glucose oxidation and proton production in working rat hearts during aerobic baseline perfusion and during a subsequent aerobic treatment period. Values are means ± s.e.mean for n observations and are shown hearts that were either untreated ($n=11$) or perfused in the presence of CHA (0.5 μM, $n=8$), propranolol (Prop, 1 μM, $n=5$), propranolol in combination with CHA (Prop + CHA, $n=5$), isoprenaline (ISO, 10 nM, $n=7$) or isoprenaline in combination with CHA (ISO + CHA, $n=7$). *Indicates a significant difference between treatment and corresponding baseline value (by Student's paired t-test). $ Indicates a significant difference between baseline values in each of the drug-treated groups and the untreated group (ANOVA + Bonferroni). # Indicates a significant difference in treatment values between each of the drug-treated groups and the untreated group and @ indicates a significant difference between treatment values in the isoprenaline and isoprenaline + CHA groups (Student's unpaired t-test).

mainly to an effect mediated during reperfusion that reduces stunning rather than to a reduction of irreversible cell damage. Indeed, there is little evidence that infarction (irreversible cell damage) influences the extent of post-ischaemic recovery after 30 min global ischaemia in this model. Adenosine, when given immediately prior to the onset of reperfusion also improves post-ischaemic recovery.

In this study, the improved recovery of post-ischaemic mechanical function by CHA was accompanied by a partial inhibition of glycolysis. This effect improves the coupling between glycolysis and glucose oxidation and thereby reduces proton production. Inhibition of the source of protons during reperfusion accelerates the recovery of pH_i and reduces the concomitant accumulation of Na^+ by activation of Na^+ / H^+ exchange. Thus, recovery of post-ischaemic mechanical function and efficiency of coupling between MVO_2 and contractile work are both improved. While it is not possible to confirm cause and effect relationships between improved coupling of glucose metabolism and post-ischaemic mechanical function, additional studies performed in working hearts perfused under aerobic conditions confirm that the alterations in glucose metabolism are not simply a consequence of changes in mechanical function or ischaemic injury. Under aerobic conditions, the selective adenosine A_1 receptor agonist, CHA, also reduced rates of glycolysis and proton production in the absence of any changes in mechanical function. These data provide clear support for a causal relationship between adenosine A_1 receptor stimulation, improved coupling of glycolysis and glucose oxidation and improved post-ischaemic function. Moreover, recent data have shown that under conditions where adenosine stimulates glycolysis and proton production, the recovery of post-ischaemic function is impaired (Finegan *et al.*, 1996a).

Effects of β-adrenoceptor stimulation

Stimulation of β-adrenoceptor tone was achieved with the addition of the β-adrenoceptor agonist, isoprenaline (10 nM) at a concentration that was chosen to elicit a submaximal acceleration of heart rate while exerting no significant effect on LV work. Despite the modest increase in energy demand, isoprenaline markedly accelerated the rate of glycolysis whereas the rate of glucose oxidation was unaltered. This preferential stimulation of glycolysis exacerbated the uncoupling between glycolysis and glucose oxidation, leading to a 3 fold increase in the rate of proton production. Under aerobic conditions, this increase in proton production did not influence mechanical function or myocardial efficiency, indicating that adequate energy was available to correct the associated acidosis and ionic imbalances resulting from Na^+/H^+ exchange. However, isoprenaline had a marked deleterious effect on the recovery of post-ischaemic mechanical function such that recovery of LV work after 30 min of ischaemia was zero. In order to evaluate the cardioprotective efficacy of CHA in the presence of elevated β-adrenoceptor tone, it was necessary to reduce the ischaemic interval in isoprenaline-treated hearts to 25 min. While in the absence of isoprenaline, recovery of mechanical function after 25 min of ischaemia was 100%, in the presence of isoprenaline recovery after 25 min was equivalent to that of untreated hearts subjected to reperfusion after 30 min of ischaemia. These results are in general agreement with previous studies where β-adrenoceptor stimulation has been shown to affect adversely the outcome following ischaemia-reperfusion injury by (1) augmenting metabolic demand; (2) stimulating glycolysis (Collins-Nakai *et al.*, 1994); (3) exacerbating acidosis (Sakai & Abiko, 1985); (4) decreasing myocardial efficiency (Opie, 1988) and (5) increasing the incidence of ventricular fibrillation (Lubbe *et al.*, 1981).

Despite these adverse effects of isoprenaline, the cardioprotective efficacy of CHA was unchanged. The ability of CHA to enhance the recovery of post-ischaemic mechanical function in the presence of the exaggerated uncoupling of glycolysis and glucose oxidation was similar to that in untreated hearts. If the adenosine A_1 receptor-mediated improvements in post-ischaemic contractile function had been mediated by an anti-β adrenoceptor mechanism, cardioprotection afforded by CHA would have been attenuated in the presence of isoprenaline due to functional antagonism at the level of adenylyl cyclase. However, the degree of post-ischaemic recovery was similar in both untreated and isoprenaline-treated hearts and so an anti-β adrenoceptor mechanism is not likely to mediate cardioprotection in response to adenosine A_1 receptor stimulation.

In the presence of isoprenaline, CHA-induced protection was again related to an inhibitory effect on glycolysis and proton production during reperfusion. Similarly, under aerobic conditions, CHA inhibited the isoprenaline-induced acceleration of heart rate, glycolysis and proton production.

Thus, these data confirm that the CHA-induced alterations of glycolysis and proton production during reperfusion are not a consequence of alterations in mechanical function. Rather, they indicate that the cardioprotective efficacy of adenosine A_1 receptor stimulation, that is due in part to the effects on glucose metabolism, is maintained in the presence of elevated β-adrenoceptor tone. This suggests that under clinical conditions of ischaemia, the associated release of endogenous catecholamines and the resulting acceleration of glycolysis and proton production will not limit the beneficial actions of adenosine A_1 receptor stimulation.

Effects of β-adrenoceptor antagonism

Inhibition of β-adrenoceptor tone was achieved by the addition of the β-adrenoceptor antagonist, propranolol (1 μM) at a concentration that was confirmed to cause a right-ward shift in the concentration response curve to isoprenaline (data not shown) while having no significant effect *per se* on mechanical function, coronary flow, MVO_2 or myocardial efficiency in hearts perfused under aerobic conditions. Propranolol partially inhibited rates of both glycolysis and proton production in aerobic hearts but the rate of glucose oxidation was not significantly altered. Data from this and previous studies (Collins-Nakai *et al*., 1994) have demonstrated that β-adrenoceptor stimulation preferentially stimulates glycolysis. Thus, a component of the propranolol-induced changes in glucose metabolism might be due to antagonism of endogenous β-adrenoceptor tone. It is also possible that inhibition of myocardial glucose metabolism was related to a general depressant action of propranolol on myocardial energy demand, rather than to antagonism of β-adrenoceptor-mediated activation of glycolysis.

In hearts subjected to ischaemia and reperfusion, propranolol *per se* did not affect the recovery of post-ischaemic mechanical function, MVO_2, myocardial efficiency or glucose metabolism. The lack of cardioprotective efficacy of propranolol in this study is unlikely to be due to ineffective β-adrenoceptor antagonism as the responses to isoprenaline were effectively inhibited. Previous studies have identified bradycardia and the associated decrease in metabolic demand, as critical components of propranolol-induced cardioprotection (Hillis *et al*., 1979). This mechanism is not operative in the hearts in this study as heart rate was maintained by electrical pacing. However, the lack of cardioprotective efficacy of propranolol is consistent with its failure to alter the coupling of glycolysis and glucose oxidation and the associated proton production and ionic imbalances.

In the presence of propranolol, CHA exerted no significant effects on mechanical function of hearts perfused under aerobic conditions and did not inhibit rates of glycolysis and proton production further than were observed with propranolol alone. This contrasts with results obtained in the absence of propranolol and may be related the effects of propranolol *per se* that inhibited glycolysis by such an extent that no further CHA-induced inhibition was possible. Importantly, in the presence of propranolol, CHA maintained its ability to improve the coupling of glycolysis and glucose oxidation during reperfusion and to enhance the recovery of post-ischaemic mechanical function. If cardioprotection induced by adenosine A_1 receptors was mediated by an anti-β adrenoceptor mechanism then propranolol would have blocked this beneficial response as the relative contribution of β-adrenoceptor activation to post-ischaemic dysfunction would have been reduced. Thus, the cardioprotection afforded by adenosine A_1 receptor stimulation apparently does not involve an anti-adrenergic mechanism mediated by functional antagonism of β-adrenoceptor stimulation in response to ischaemia-induced release of endogenous noradrenaline.

Summary

The cardioprotective efficacy of the adenosine A_1 receptor agonist, CHA, is unaffected by either stimulation (with isoprenaline) or antagonism (with propranolol) of β-adrenoceptor tone. These results indicate that the cardioprotective mechanism of adenosine A_1 receptor activation does not involve a functional antagonism of the consequences of β-adrenoceptor activation. Although β-adrenoceptor stimulation exacerbates ischaemia-reperfusion injury, the cardioprotective efficacy of CHA and its modulation of glucose metabolism were maintained when β-adrenoceptors were stimulated by isoprenaline. Thus, the cardioprotective efficacy of adenosine A_1 receptor activation should not be limited by the marked sympathetic activation that occurs during ischaemia-reperfusion *in vivo*.

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