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Alteration of glycogen and glucose metabolism in ischaemic and post-ischaemic working rat hearts by adenosine A_1 receptor stimulation

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1 Cardioprotection by adenosine A_1 receptor activation limits infarct size and improves post-ischaemic mechanical function. The mechanisms responsible are unclear but may involve alterations in myocardial glucose metabolism.

2 Since glycogen is an important source of glucose during ischaemia, we examined the effects of N^6-cyclohexyladenosine (CHA), an A_1 receptor agonist, on glycogen and glucose metabolism during ischaemia as well as reperfusion.

3 Isolated working rat hearts were perfused with Krebs-Henseleit solution containing dual-labelled 5-^{3}H and ^{14}C glucose and palmitate as energy substrates. Rates of glycolysis and glucose oxidation were measured directly from the production of ^3H_2O and $^{14}CO_2$. Glycogen turnover was measured from the rate of change of [5-^{3}H and ^{14}C]glucosyl units in total myocardial glycogen.

4 Following low-flow (0.5 ml min^{-1}) ischaemia (60 min) and reperfusion (30 min), left ventricular minute work (LV work) recovered to 22% of pre-ischaemic values. CHA (0.5 μM) improved the recovery of LV work 2 fold.

5 CHA altered glycogen turnover in post-ischaemic hearts by stimulating glycogen synthesis while having no effects on glycogen degradation. CHA also partially inhibited glycolysis. These changes accelerated the recovery of glycogen in CHA-treated hearts and reduced proton production.

6 During ischaemia, CHA had no measurable effect on glycogen turnover or glucose metabolism. Glycogen phosphorylase activity, which was elevated after ischaemia, was inhibited by CHA, possibly in response to CHA-induced inhibition of AMP-activated protein kinase activity.

7 These results indicate that CHA-induced cardioprotection is associated with alterations of glycogen turnover during reperfusion as well as improved metabolic coupling of glycolysis to glucose oxidation.

Keywords: Adenosine A_1 receptor stimulation; cardioprotection; glycogen turnover; glycolysis; proton production; AMP-activated protein kinase; glycogen phosphorylase; glycogen synthase

Abbreviations: AMP, adenosine monophosphate; AMPK, adenosine monophosphate–activated protein kinase; ATP, adenosine triphosphate; CHA, N^6-cyclohexyladenosine; Cr, creatine; CrP, creatine phosphate; CVC, coronary vascular conductance; G_{in}, glycogen synthesis; G_{out}, glycogen degradation; LV work, left ventricular minute work

Introduction

The ability of adenosine to improve recovery of myocardial mechanical function during reperfusion of post-ischaemic hearts is well documented, but its precise mechanism of action remains unclear (Shyrock & Belardinelli, 1997). The role of adenosine A_1 receptors is clearly supported by demonstrations that selective adenosine A_1 receptor agonists such as N^6-cyclohexyladenosine (CHA) mimic the protective actions of adenosine (Lasley & Mentzer Jr., 1993; Finegan *et al*., 1996a; 1996b) while selective adenosine A_1 receptor antagonists inhibit the effects of adenosine and adenosine A_1 receptor agonists (Lasley *et al*., 1990; Finegan *et al*., 1996b).

We have shown previously that the preference of the myocardium for glucose and fatty acid is an important determinant of the extent of recovery of post-ischaemic function (Lopaschuk & Gamble, 1994; Lopaschuk, 1997). In addition, it is well established that drug-induced alterations in glucose and fatty acid metabolism improve recovery of post-ischaemic function (McVeigh & Lopaschuk, 1990; Liu *et al*., 1996; Lopaschuk & Stanley, 1997; Lopaschuk, 1997). Adenosine-induced alterations in myocardial glucose metabolism are demonstrable (Finegan *et al*., 1992; 1993; 1996b) and these effects may contribute to the cardioprotective effects of adenosine A_1 receptor stimulation. In isolated paced working rat hearts perfused under aerobic conditions with both glucose and fatty acid (palmitate), adenosine does not affect mechanical function or rates of glucose or fatty acid oxidation (Finegan *et al*., 1992). However, adenosine partially inhibits glycolysis (the conversion of glucose to pyruvate) thereby reducing the imbalance between rates of glycolysis and glucose oxidation (Finegan *et al*., 1992). This improvement in the metabolic coupling between rates of glycolysis and glucose oxidation reduces the rate of proton production from glycolytically derived ATP. These metabolic effects may explain the adenosine-induced inhibition of intracellular acidosis during ischemia (Fralix *et al*., 1993). The resulting reduction in Na^+/H^+ exchange prevents Na^+ and Ca^{2+} overload, thereby allowing a more rapid and complete recovery of

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mechanical and metabolic function during reperfusion (Tani & Neely, 1989).

Glycogen is also an important energy substrate in the heart, particularly during ischaemia when it is readily degraded (glycogenolysis), following activation of glycogen phosphorylase, to form an endogenous source of glucose phosphate for glycolytic ATP production (anaerobic glucose metabolism). Although the relative activities of the two enzymes, glycogen synthase and glycogen phosphorylase, are tightly coupled, the simultaneous synthesis and degradation of glycogen, termed glycogen turnover, is demonstrable in working rat hearts perfused under aerobic conditions (Goodwin *et al*., 1995; Henning *et al*., 1996; Fraser *et al*., 1998) as well as during conditions of net glycogenolysis (Schonekess *et al*., 1997; Fraser *et al*., 1998) or during post-ischaemic reperfusion (Fraser *et al*., 1998). However, the role of drug-induced modulation of glycogen turnover in cardioprotection has not yet been investigated.

The potential role of changes in glycogen turnover in cardioprotection is strengthened by data that indicate that endogenous glucose, which is released from glycogen under conditions of glycogenolysis, does not undergo the same metabolic fate as exogenous glucose. Endogenous glucose is preferentially oxidized relative to glucose from exogenous sources (Goodwin *et al*., 1996; Henning *et al*., 1996; Schonekess *et al*., 1997). Consequently, rates of glycolysis and oxidation of endogenous glucose are more closely coupled than for exogenous glucose leading to lower rates of proton production (Lopaschuk *et al*., 1993). Thus, cardioprotection due to attenuation of proton production from glucose metabolism may arise in response to a switch to the utilization of endogenous, rather than exogenous, glucose. Although adenosine inhibits the flux of exogenous glucose through glycolysis (Finegan *et al*., 1993; 1996b), thereby reducing proton production, the effects of adenosine on the relative rates of utilization of endogenous and exogenous glucose during ischaemia have not been measured.

Glycogen turnover may be influenced by a number of transduction systems and recent data suggest that a stress kinase, AMP-activated protein kinase (AMPK) may be involved. Activity of this kinase is stimulated by AMP and inhibited by ATP and thus it is well suited to be responsive to the energy status of the cell (e.g., as indicated by the AMP/ATP ratio) (Hardie & Carling, 1997). AMPK influences myocardial energy substrate metabolism in a number of ways including activation of fatty acid oxidation during reperfusion by phosphorylating and inactivating acetyl CoA carboxylase (Kudo *et al*., 1996). Phosphorylase kinase, that activates glycogen phosphorylase, is also stimulated by agents that activate AMPK (Young *et al*., 1996). In this way, AMPK activity may serve as a link between glycogen turnover and myocardial energy status. However, the role of AMPK in glycogen turnover and glucose metabolism during ischaemia and reperfusion and its alteration by adenosine A_1 receptor stimulation have not been defined.

In view of the importance of glycogen metabolism in the heart and its potential contribution to anti-ischaemic mechanisms, this study was designed to assess the effects of the selective adenosine A_1 receptor agonist, CHA, on glycogen turnover and glucose metabolism in ischaemic and post-ischaemic hearts. CHA-induced alterations in the relative rates of metabolism of endogenous and exogenous glucose during low-flow ischaemia were also measured. Studies were performed under appropriate conditions of energy demand and supply in isolated working rat hearts perfused with both glucose and fatty acids.

Methods

Heart perfusions

Male Sprague Dawley rats (300–350 g), that were housed and treated according to the standards set by the Canadian Council of Animal Care, were anaesthetized with pentobarbital (60 mg kg^{-1}, intraperitoneally). Hearts were then rapidly removed, placed in ice-cold Krebs-Henseleit solution and then perfused *via* the aorta in Langendorff mode for a 10 min equilibration period, as described previously (Finegan *et al*., 1996a,b). Langendorff perfusion was then stopped and hearts were switched to working mode and perfused at 37°C under aerobic conditions at an 11.5 mmHg left atrial preload and an 80 mmHg aortic afterload with a modified Krebs-Henseleit solution containing 1.2 mM palmitate pre-bound to 3% bovine serum albumin, 2.5 mM Ca^{2+}, 100 mU l^{-1} insulin and 11 mM glucose. Perfusate was oxygenated with carbogen (95% O_2, 5% CO_2). In order to avoid non-specific effects arising from CHA-induced bradycardia, hearts were paced at 300 beats min^{-1} throughout each phase of the perfusion protocol (voltage adjusted as necessary) with the exception of the initial 5 min of reperfusion when hearts were allowed to beat spontaneously. Aortic systolic and diastolic pressures (mmHg) were measured using a Gould P21 pressure transducer connected to the aortic outflow line. Cardiac output, aortic flow and coronary flow (cardiac output minus aortic flow) were measured (ml min^{-1} using in-line ultrasonic flow probes connected to a Transonic T206 ultrasonic flow meter. Left ventricular minute work (LV work, mmHg × 1 min^{-1}), calculated as cardiac output × left ventricular developed pressure (systolic pressure–preload pressure), was used as a continuous index of mechanical function. Hearts were excluded if LV work decreased more than 20% during the initial 60-min period of aerobic perfusion.

Perfusion protocols

Hearts were perfused under aerobic conditions for 60 min and then subjected to low-flow ischaemia (0.5 ml min^{-1}) for 60 min followed by 30 min of aerobic reperfusion (Figure 1), either in absence (untreated) or presence of CHA at a concentration (0.5 μM) shown previously to possess cardioprotective activity *via* adenosine A_1 receptor activation (Finegan *et al*., 1996a,b). At the end of the perfusion protocol, hearts were rapidly frozen using Wollenberger clamps cooled to the temperature of liquid nitrogen. Additional groups of hearts were frozen at the start of the aerobic perfusion period (time 0-min) as well as immediately before or after the period of low-flow ischaemia. Frozen tissues were pulverized and the resulting powders were stored at −80°C.

Measurement of glycolysis, glucose oxidation and proton production

Rates of glycolysis were measured as previously described (Finegan *et al*., 1996a,b) from the quantitative determination of ^3H_2O liberated from [5-^{3}H]-glucose at the enolase step of glycolysis. Glucose oxidation was determined as previously described (Finegan *et al*., 1996a,b) by measuring $^{14}CO_2$ liberated from [^{14}C]-glucose at the level of pyruvate decarboxylase and throughout the TCA cycle. Perfusate samples were collected after 10, 20, 40, 60, 65, 70, 80, 100, 120, 130, 140 and 150 min of perfusion. Steady state rates were calculated from the averaged rates between 20 and 60 min for aerobic perfusion, between 70 and 120 min for low-flow ischaemia,

and from 130 to 150 min for reperfusion. Rates of glycolysis and glucose oxidation are expressed as μmol glucose metabolized min^{-1} g dry wt^{-1}.

When glucose from exogenous sources is metabolised by glycolysis and is coupled 1 : 1 with glucose oxidation and is associated with the synthesis and hydrolysis of ATP, the net production of protons is zero. However, if glycolysis is uncoupled from glucose oxidation so that its rate exceeds that of oxidation, there is a net production of two protons per molecule of exogenous glucose that passes through glycolysis that is not subsequently oxidised (Dennis *et al*., 1991). Thus, proton production arising from the metabolism of exogenous glucose was calculated as $2 \times$(rate of glycolysis − rate of glucose oxidation).

Proton production from the metabolism of endogenous glucose (glycogen) was calculated in a similar manner. As glycogenolysis yields three ATP per glucosyl unit, there are three protons produced by the hydrolysis of ATP produced from endogenous glucose. However, because one proton is consumed during the release of the glucosyl unit from glycogen, there is a net production of only two protons per molecule of endogenous glucose.

An additional calculation was employed for proton production during low-flow ischaemia that incorporated the rate of metabolism of endogenous and exogenous glucose as well as glycogen turnover. As glycogen synthesis produces one proton when UDP-glucose is added to glycogen (Dennis *et al*., 1991), proton production from endogenous glucose was calculated as $2 \times$(rate of glycolysis − rate of glucose oxidation) $+ 1 \times$(rate of glycogen synthesis). Proton production during aerobic perfusion and reperfusion only approximates true rates because the relative contributions of endogenous and exogenous glucose were not measured.

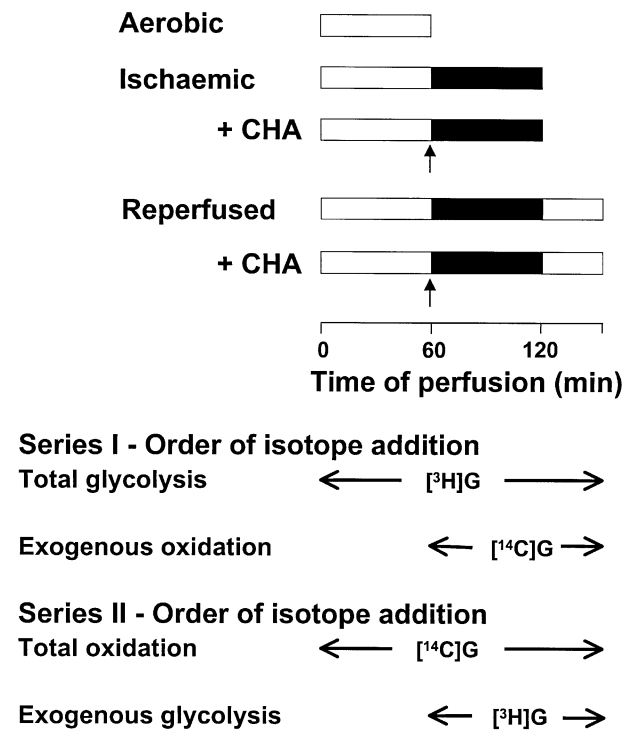

Figure 1 Perfusion protocol for isolated perfused working rat hearts. Hearts were subjected to 60 min of aerobic working perfusion, 60 min of low-flow ischaemia (solid bar) and 30 min of aerobic working reperfusion. Hearts were perfused with modified Krebs-Henseleit solution containing 1.2 mM palmitate, 11 mM glucose, 2.5 mM Ca^{2+}, 100 μU ml^{-1} insulin and 3% BSA. During low-flow ischaemia, coronary flow was reduced to 0.5 ml min^{-1}. During aerobic perfusion and reperfusion, coronary flow was not restricted. Hearts were paced at 5 Hz throughout the protocol except the first 5 min of reperfusion. Hearts were frozen at the end of each perfusion period with Wollenberger clamps cooled to the temperature of liquid nitrogen. Parallel series of hearts were also frozen at time zero, and at the beginning or end of low-flow ischaemia for determination of metabolic parameters. Series I and II depict groups of hearts that were divided into groups based on the order of isotope addition. For further information, see Methods section.

Measurement of the sources and fate of glucose during low-flow ischaemia

A dual label (5-^{3}H-glucose and U-^{14}C-glucose) protocol was designed to label the glycogen pool and then follow separately the fate (rates of glycolysis and glucose oxidation) of glucose arising from either exogenous or endogenous sources. Two series of identical perfusions were performed except the order, and timing, of isotope addition differed (Figure 1). As described previously (Fraser *et al*., 1998), one series of hearts was perfused with [^{3}H]-glucose that was added at the beginning of aerobic perfusion and was present throughout each phase of the perfusion protocol. During aerobic perfusion, a period of net glycogen synthesis, [^{3}H]-glucose became incorporated into glycogen, hereafter referred to as endogenous glucose. During low-flow ischaemia, hearts underwent net glycogenolysis and the fate of endogenous [^{3}H]-glucose liberated from glycogen was followed as described above. As hearts in this series were exposed to both exogenous [^{3}H]-glucose delivered in the perfusate and endogenous [^{3}H]-glucose liberated from glycogen, the rate of ^3H_2O production represents the rate of total glycolysis occurring during low-flow ischaemia. The second isotope, [^{14}C]-glucose, was added at the beginning of low-flow ischaemia. As this isotope was absent during aerobic perfusion and therefore did not become incorporated into glycogen during the period of glycogen synthesis, the rate of production of $^{14}CO_2$ during low-flow ischaemia represents oxidation of only the exogenous source of [^{14}C]-glucose with no contribution from endogenous [^{14}C]-glucose.

In the second series of perfusions, the order of isotope addition was reversed. [^{14}C]-glucose, that was present throughout the perfusion protocol, became incorporated into glycogen during the initial aerobic perfusion, and was subsequently mobilized during low-flow ischaemia. [^{3}H]-Glucose was present only during low-flow ischaemia and reperfusin. Thus, the rate of production of $^{14}CO_2$ during low-flow ischaemia in this series of perfusions represents the rate of oxidation of both exogenous and endogenous sources of [^{14}C]-glucose while the rate of ^3H_2O production represents the rate of glycolysis of only the exogenous source of [^{3}H]-glucose. Rates of glycolysis of endogenous [^{3}H]-glucose and rates of oxidation of endogenous [^{14}C]-glucose were then calculated as the difference between total and exogenous rates (Figure 1).

Metabolite determinations

ATP, AMP, creatine (Cr) and creatine phosphate (CrP) were determined by high performance liquid chromatography of neutralized 6% perchloric acid extractions of frozen heart tissue (Ally & Park, 1992). Myocardial glycogen (μmol glucosyl units g dry wt^{-1}) content was determined by measuring the glucose content in samples of frozen tissue that were subjected to alkaline extraction (30% KOH) to separate glycogen from exogenous glucose. This was followed by ethanol precipitation and acid hydrolysis (2N H_2SO_4) to release endogenous glucose from glycogen, thereby the radiolabelled content of the glycogen pool could be determined

accurately without any contamination from free unincorporated glucose. These samples were also analysed for [^{3}H]-glucose and [^{14}C]-glucose so that the specific activity of [^{3}H/^{14}C]-glycogen and the percentage of glycogen that became labelled with either [^{3}H]-glucose or [^{14}C]-glucose could be determined for hearts frozen at the end of each phase of the perfusion protocol.

Rates of glycogen turnover

Glycogen turnover was assessed by measuring the simultaneous rates (μmol glucose min^{-1} g dry wt^{-1}) of glycogen synthesis (G_{in}) and degradation (G_{out}). Actual rates of G_{in} and G_{out} were calculated as described previously (Fraser *et al*., 1998) by incorporating values for the actual changes in unlabelled and labelled glycogen during each phase of perfusion. During the 60 min period of aerobic perfusion, the rate of change of total glycogen (labelled and unlabelled) between time 0 and the end of the 60 min period of aerobic perfusion is equal to the difference between G_{in} and G_{out}. Similarly, the rate of change of labelled glycogen between time 0 and the end of aerobic perfusion is equal to the difference between glycogen synthesis and the rate of degradation of the labelled component of the glycogen pool. The rate of degradation varied according to the proportion of glycogen that was labelled; this proportion was 0% at time 0 min and was determined experimentally at 60 min. Thus, the average rate of incorporation of radiolabelled glucose into glycogen may be calculated both from the experimentally determined incorporation of radiolabelled glucose, as well as from the average of the rates of incorporation at time 0 and 60 min. Using a similar approach, average rates of G_{in} and G_{out} were calculated for the periods of low-flow ischaemia and reperfusion.

Glucose uptake and % extraction

Rates of glucose uptake during aerobic perfusion and low-flow ischaemia were calculated from the sum of the rates of glycogen synthesis (G_{in}) and glycolysis from exogenous glucose. Glucose extraction, the % of glucose presented to the heart in the coronary perfusate that was metabolised, was calculated from rates of glucose uptake, coronary flow and the perfusate glucose concentration.

Activities of glycogen synthase and glycogen phosphorylase

The activities of glycogen synthase and glycogen phosphorylase were determined in tissue samples frozen at the end of each phase of the perfusion protocol. Glycogen phosphorylase activity, expressed as phosphorylase *a* as a per cent of total, was determined as described previously (Dobson Jr. & Fenton, 1993) from the formation of glucose-6-phosphate in the presence of excess glycogen and in the absence or presence of AMP (3 mM). Glycogen synthase activity, expressed as the active per cent of total activity, was determined as previously described (Passonneau & Rottenberg, 1973) from the consumption of UDP-glucose in the absence and presence of glucose-6-phosphate (5 mM).

Activity of AMP-activated protein kinase

AMPK activity (nmol min^{-1} mg^{-1} protein) was measured in 6% polyethylene glycol (PEG) fractions extracted from 200 mg of frozen LV tissue by determining the incorporation of [γ^{32}P] from [γ^{32}P]-ATP into a synthetic SAMS (HMRSAMSGLHVKRR) peptide, as previously described (Kudo *et al*., 1995; 1996). This peptide is similar to the ser-79 phosphorylation site on acetyl CoA carboxylase-1, except that the serine residue corresponding to the phosphorylation site for cyclic AMP-dependent protein kinase (Ser-77) was replaced by alanine (Ala-77) to prevent phosphorylation by cyclic AMP-dependent protein kinase present in assay extracts. SAMS peptide also contained two additional arginine residues at the C-terminus to facilitate its binding to phosphocellulose paper.

Statistical analysis

Data are expressed as the mean$\pm$s.e.mean. Comparisons between untreated and CHA-treated groups were performed using the unpaired Student's *t*-test. Multiple comparisons were made using analysis of variance followed by Student-Newman-Keuls *post hoc* test. When sample variances were significantly different, a nonparametric test was used (Mann-Whitney *U*-statistic, unpaired, two-tailed test). Differences were judged to be significant when $P<0.05$.

Results

Left ventricular mechanical function

LV work, which was used as an index of mechanical function, was stable throughout the initial 60-min period of aerobic perfusion (Table 1). Coronary flow, aortic flow, cardiac output and coronary vascular conductance were also constant throughout aerobic perfusion. All measurable LV work ceased during low-flow ischaemia (0.5 ml min^{-1}) and recovered to 22$\pm$5% of pre-ischaemic levels by the end of the 30-min period of aerobic reperfusion. During reperfusion, coronary flow, aortic flow, cardiac output and coronary vascular conductance were depressed to 60$\pm$4%, 10$\pm$3%, 27$\pm$5% and 67$\pm$4%, respectively of pre-ischaemic values. CHA (0.5 μM), added 5 min prior to the start of low-flow ischaemia and present throughout reperfusion, improved recovery of LV work 2 fold (to 44$\pm$6% of pre-ischaemic values) (Figure 2). CHA also significantly improved the recovery of coronary flow, aortic flow, cardiac output and coronary vascular conductance compared with untreated hearts to 91$\pm$3%, 30$\pm$4%, 51$\pm$5% and 91$\pm$3%, respectively of pre-ischaemic values (Figure 2).

Table 1 Effects of N^6-cyclohexyladenosine (CHA 0.5 μM) on indices of mechanical function of working rat hearts perfused under aerobic conditions

	Untreated (n = 15)	*CHA* (n = 16)
LV work (mmHg l min^{-1})	7.24$\pm$0.21	7.40$\pm$0.24
Cardiac output (ml min^{-1})	66.6$\pm$1.5	66.4$\pm$1.6
Aortic flow (ml min^{-1})	44.0$\pm$1.3	45.9$\pm$1.5
Coronary flow (ml min^{-1})	22.2$\pm$1.1	22.1$\pm$1.7
CVC (ml min^{-1} mmHg^{-1})	0.28$\pm$0.02	0.28$\pm$0.01

LV work, cardiac output, aortic flow, coronary flow and coronary vascular conductance (CVC) were determined at 10-min intervals prior to the onset of low-flow ischaemia and average values are presented for the 60-min period of aerobic perfusion. CHA (0.5 μM) was added 5 min prior to the onset of low-flow ischaemia. Data are mean$\pm$s.e.mean for *n* observations.

Rates of glycolysis, glucose oxidation and proton production

The steady state rate of glycolysis during aerobic perfusion was greater than that of glucose oxidation (Table 2). This uncoupling of glycolysis from glucose oxidation resulted in substantial rates of proton production from glucose metabolism of 8.1 ± 0.6 μmol min^{-1} g dry wt^{-1}. CHA caused no measurable changes in the relative contributions of endogenous and exogenous glucose to rates of glycolysis and glucose oxidation during low-flow ischaemia. Although rates of glycolysis from exogenous glucose were greater than those from endogenous glucose derived from glycogen, the rates of glucose oxidation arising from exogenous and endogenous sources were similar (Table 2). This indicates that glucose arising from endogenous sources was preferentially oxidized. Thus, the coupling ratio of rates of glycolysis to glucose oxidation was 1.8 fold greater for endogenous glucose (7.6) than for exogenous glucose (13.5). Consequently, proton production arising from the metabolism of endogenous glucose was 53% less than that for exogenous glucose. CHA did not alter any of these rates during low-flow ischaemia.

During reperfusion, CHA inhibited glycolysis but had no effect on glucose oxidation. This improved the metabolic coupling between glycolysis and glucose oxidation in CHA-treated hearts resulting in a significantly lower (64%) rate of proton production during reperfusion compared with untreated hearts (Table 2).

Metabolite contents

After aerobic perfusion, the high energy phosphates ratios AMP to ATP and Cr to CrP were similar to values reported previously for aerobic working rat hearts perfused with fatty acids (Finegan *et al*., 1995). During low-flow ischaemia, ATP and CrP contents declined in both untreated and CHA-treated groups. Thus, the AMP to ATP ratio and the Cr to CrP ratio increased in both untreated and CHA groups. These ratios

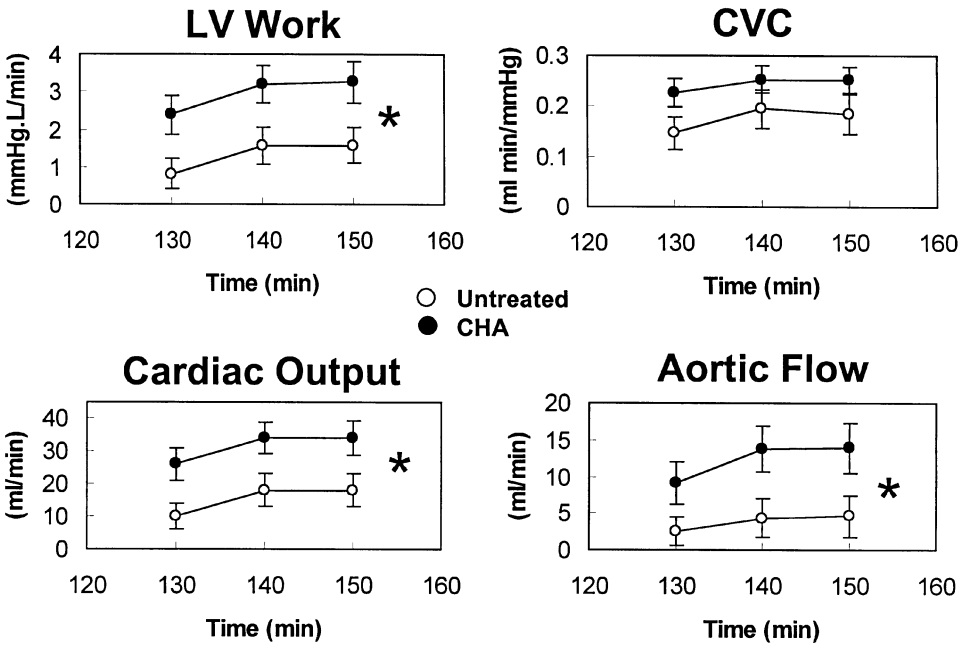

Figure 2 Effect of N^6-cyclohexyladenosine (CHA) on post-ischaemic recovery of left ventricular mechanical function of working rat hearts. Values are means$\pm$s.e.mean for left ventricular minute work (LV work), coronary vascular conductance (CVC), cardiac output and aortic flow for hearts perfused in the absence ($n=15$) or presence of CHA (0.5 μM, $n=16$). *$P<0.05$ compared with untreated group.

Table 2 Effect of N^6-cyclohexyladenosine (CHA) on steady state rates of glucose metabolism and proton production

		Low-flow ischaemia		
	Aerobic	*Exogenous*	*Endogenous*	*Reperfusion*
Glycolysis (μmol min^{-1} g dry wt^{-1})				
Untreated	4.62$\pm$0.28 (7)	2.98$\pm$0.32 (7)	1.37$\pm$0.42$ (7)	3.58$\pm$0.88† (9)
CHA		3.57$\pm$0.52 (7)	1.41$\pm$0.66‡ (7)	1.81$\pm$0.39* (10)
Glucose oxidation (μmol min^{-1}g dry wt^{-1})				
Untreated	0.58$\pm$0.11 (7)	0.22$\pm$0.04 (7)	0.18$\pm$0.06 (7)	0.40$\pm$0.09 (6)
CHA		0.26$\pm$0.04 (7)	0.25$\pm$0.06 (7)	0.68$\pm$0.13 (6)
Calculated proton production (μmol min^{-1} g dry wt^{-1})				
Untreated	8.06$\pm$0.60 (7)	5.53$\pm$0.64 (7)	2.92$\pm$0.86‡ (7)	6.36$\pm$1.77 (6)
CHA		6.62$\pm$1.00 (7)	2.85$\pm$1.35‡ (7)	2.27$\pm$0.80* (6)

Rates of glycolysis, glucose oxidation and proton production (μmol min^{-1} g dry wt^{-1}) were measured throughout the periods of aerobic perfusion, low-flow ischaemia and reperfusion. Contributions of glucose from endogenous and exogenous sources were determined during low-flow ischaemia as described in Methods. Data represent mean$\pm$s.e.mean for n observations; *$P<0.05$ compared with untreated group, †$P<0.05$ compared with aerobic groups, ‡$P<0.05$ compared with exogenous glucose.

decreased to near pre-ischaemic levels after reperfusion (Table 3). During reperfusion, the AMP to ATP ratio and Cr to CrP ratio decreased to a greater degree in the CHA group indicative of an improved energetic state.

Glycogen content (μmol g dry wt^{-1}) of hearts increased substantially during aerobic perfusion from 73.7 ± 8.8 ($n=7$) (at time 0) to 116.2 ± 3.6 ($n=14$) (after 60 min) (Table 4). Low-flow ischaemia elicited marked glycogenolysis and the decrease in glycogen content was similar in untreated and CHA-treated hearts. In untreated hearts, glycogen content remained constant during reperfusion, whereas CHA significantly increased glycogen resynthesis (7 fold) compared with the untreated group (Table 4).

Rates of glycogen turnover (G_{in} and G_{out})

Calculations of glycogen turnover, which incorporate the simultaneous rates of synthesis and degradation of glycogen, indicated that G_{in} was 2.3 fold higher than G_{out} during aerobic perfusion, while during low-flow ischaemia G_{out} was 3.9 fold greater than G_{in} (Figure 3). CHA had no effect on G_{in} or G_{out} during low-flow ischaemia. However, while G_{in} recovered to pre-ischaemic values in untreated hearts during reperfusion, CHA stimulated G_{in} during reperfusion to rates significantly higher than pre-ischaemic values. In contrast, G_{out} remained elevated (3 fold) during reperfusion compared with aerobic values and was not altered by CHA. Although there was no net change in glycogen content during reperfusion in untreated hearts as the simultaneous rates of synthesis and degradation were not significantly different, the high values for G_{in} and G_{out} indicated that there was considerable glycogen turnover during this period (Figure 3).

Glucose uptake and % extraction

Glucose uptake during aerobic perfusion was 5.86 ± 0.28 μmol min^{-1} g dry wt^{-1} and was inhibited by 32% during low-flow ischaemia. In CHA-treated hearts, glucose uptake was not inhibited during low-flow ischaemia. During reperfusion, glucose uptake was similar in CHA-treated and untreated hearts, but both were inhibited compared with pre-ischaemic values (Figure 3).

Glucose extraction during aerobic perfusion was 0.64 ± 0.08%. Extraction significantly increased during low-flow ischaemia to 21 ± 2% and 27 ± 3% in untreated and CHA-treated hearts, respectively. Glucose extraction returned to pre-ischaemic levels during reperfusion in both untreated and CHA-treated hearts.

Activities of glycogen synthase and glycogen phosphorylase

Myocardial glycogen synthase and glycogen phosphorylase activities after aerobic perfusion were similar to those reported previously for rat hearts (McNulty *et al.*, 1995). At the end of low-flow ischaemia, glycogen synthase and glycogen phosphorylase activities were increased significantly compared with aerobic values (Figure 4). CHA inhibited glycogen phosphorylase during low-flow ischaemia, but had no effect on glycogen synthase. During reperfusion, glycogen phosphorylase activity

Table 3 High-energy phosphate ratios in hearts that were frozen at the end of each phase of perfusion

	Aerobic	*Low-flow ischaemia*	*Reperfusion*
AMP/ATP			
Untreated	0.18 ± 0.02 (6)	4.8 ± 1.6† (7)	0.21 ± 0.05 (8)
CHA		5.8 ± 2.2† (7)	0.10 ± 0.02*† (10)
Cr/CrP			
Untreated	4.0 ± 0.7 (6)	11.4 ± 1.6† (7)	2.4 ± 0.4† (8)
CHA		16.5 ± 3.1† (7)	1.5 ± 0.2*† (10)

Data are mean$\pm$s.e.mean for n observations; *$P<0.05$ compared with corresponding untreated group, †$P<0.05$ compared with aerobic group.

Table 4 Glycogen content of hearts frozen at the end of each phase of perfusion

	Glycogen content (μmol g dry wt^{-1}) *Untreated*	*CHA*
End aerobic	116.2 ± 3.6 (14)	
End ischaemia	21.5 ± 3.2† (14)	24.89 ± 2.0† (14)
End reperfusion	24.3 ± 3.2† (15)	45.4 ± 5.2*† (16)

Data are mean$\pm$s.e.mean for n observations; *$P<0.05$ compared with corresponding untreated group, †$P<0.05$ compared with aerobic group.

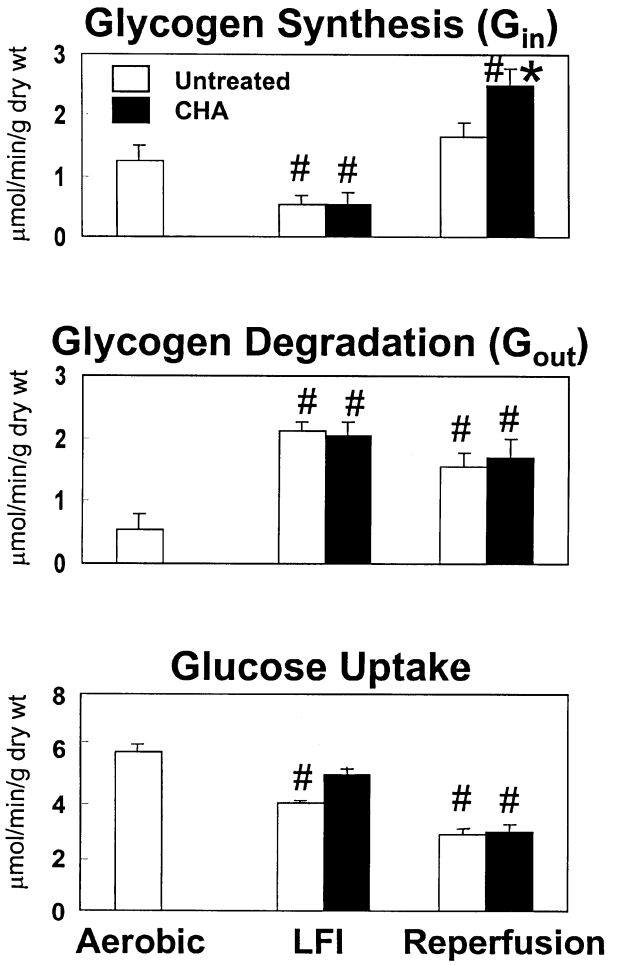

Figure 3 Effect of N^6-cyclohexyladenosine (CHA) on rates of glycogen synthesis (G_{in}), glycogen degradation (G_{out}) and glucose uptake. Values are means$\pm$s.e.mean. Rates (μmol min^{-1} g dry wt^{-1}) were measured during aerobic perfusion ($n=6$) and in hearts subjected to low-flow ischaemia (LFI) (Untreated; $n=5$; CHA, $n=7$) and reperfusion (Untreated, $n=10$; CHA, $n=11$) in the absence or presence of CHA (0.5 μM). *$P<0.05$ compared with untreated group, #$P<0.05$ compared with aerobic value.

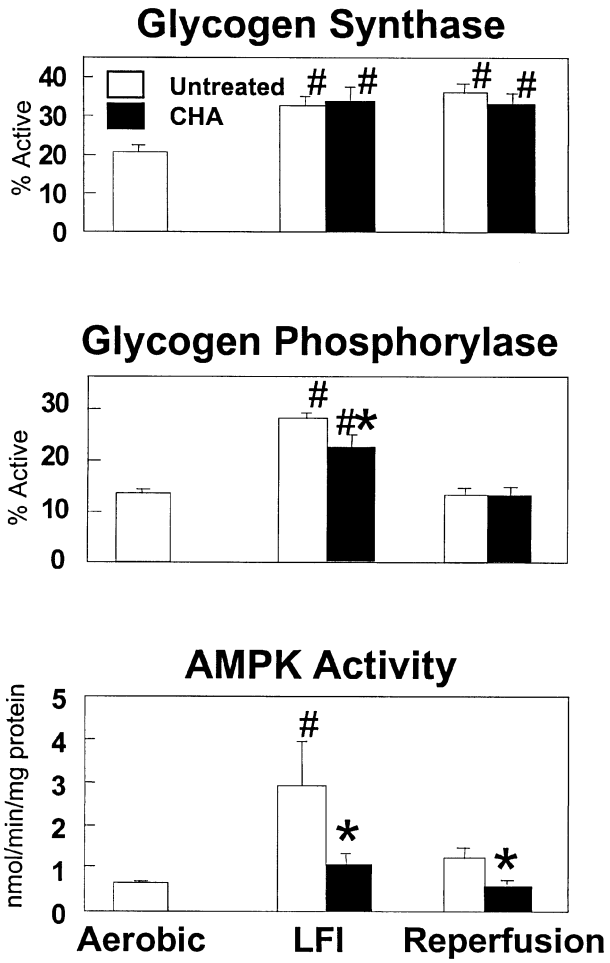

Figure 4 Effect of N^6-cyclohexyladenosine (CHA) on activities of glycogen synthase, glycogen phosphorylase and AMP-dependent protein kinase (AMPK). Values are means ± s.e.mean. Rates (μmol min^{-1} g dry wt^{-1}) were measured in hearts frozen after aerobic perfusion ($n=6$), and in hearts perfused in the absence or presence of CHA (0.5 μM) after low-flow ischaemia (LFI) (Untreated, $n=5$; CHA, $n=7$) and after reperfusion (Untreated, $n=10$; CHA, $n=11$). $^*P<0.05$ compared with untreated group, $\#P<0.05$ compared with aerobic value.

returned to aerobic values in untreated hearts, whereas glycogen synthase activity remained significantly elevated. The activities of glycogen synthase and glycogen phosphorylase at the end of reperfusion were not affected by CHA (Figure 4).

Activity of AMPK

AMPK activity in hearts frozen at the end of aerobic perfusion was similar to that reported previously for fatty acid perfused working rat hearts (Kudo *et al*., 1995). AMPK activity increased significantly during low-flow ischaemia then partially recovered during reperfusion (Figure 4). Relative to untreated hearts, CHA inhibited AMPK activity both during low-flow ischaemia as well as reperfusion.

Discussion

Although the cardioprotective efficacy of adenosine A_1 receptor stimulation is well established, the underlying mechanisms remain unclear. This study investigated the effects of the selective adenosine A_1 receptor agonist, CHA, on glycogen and glucose metabolism both during low-flow ischaemia as well as during reperfusion in isolated working rat hearts. As described previously (Finegan *et al*., 1996a,b), CHA improved the recovery of post-ischaemic mechanical function, decreased glycolysis and inhibited proton production from the hydrolysis of glycolytically derived ATP. CHA also altered glycogen turnover during reperfusion by stimulating G_{in}, an effect that accelerated the recovery of post-ischaemic glycogen content. While CHA inhibited glycogen phosphorylase activity at the end of ischaemia in association with inhibition of AMPK, it had no effect on glycogen or glucose metabolism during low-flow ischaemia. These results indicate that the CHA-induced changes in glucose metabolism are manifest primarily during reperfusion. Moreover, CHA also altered glycogen turnover during reperfusion, possibly in response to the improved functional and energetic state and/or inhibition of glycolysis.

Hearts were perfused under conditions of appropriate energy demand and substrate supply. While fatty acids are important for maintaining appropriate ATP generation, they also significantly influence glucose and glycogen metabolism by maintaining the metabolic uncoupling between rates of glycolysis and glucose oxidation that is observed in human hearts (Wisneski *et al*., 1990), a condition that leads to a significant rate of proton production (Finegan *et al*., 1996a; Liu *et al*., 1996).

The cardioprotective efficacy of adenosine A_1 receptor stimulation (Lasley & Mentzer Jr., 1993; Finegan *et al*., 1996b), as manifest as an improvement of post-ischaemic mechanical function, was associated with an inhibition of glycolysis and proton production arising from glucose metabolism (Finegan *et al*., 1996a,b). Rather than occurring during ischaemia, this beneficial effect of CHA occurs only during reperfusion. As inhibition of glycolysis and proton production is also demonstrable in aerobic hearts (Finegan *et al*., 1993), the CHA-induced inhibition of proton production during reperfusion is not a consequence of improved post-ischaemic function. Instead, these data suggest that the CHA-induced alterations in glucose metabolism contribute to the improved recovery of mechanical function (Finegan *et al*., 1996a,b) by decreasing proton production during the critical early period of reperfusion, limiting the potential for intracellular Na^+ and Ca^{2+} accumulation *via* activation of the Na^+/H^+ and Na^+/Ca^{2+} exchangers, respectively. Moreover, the causal relationship between proton production arising from glycolysis uncoupled from glucose oxidation and the extent of recovery of post-ischaemic function is further supported by our data showing that an increase in proton production from glucose metabolism worsens the recovery of post-ischaemic mechanical function (Finegan *et al*., 1996a).

Previous studies investigating the effects of adenosine A_1 receptor stimulation on glucose metabolism did not examine either the metabolic coupling between glycolysis and glucose oxidation during ischaemia, the potential consequences of alterations in glycogen turnover, or the metabolic fates of endogenous and exogenous glucose. As expected, there was a marked acceleration of glycogenolysis during ischaemia, but G_{in} and thus turnover were still detectable. However, CHA did not affect rates of either G_{in} or G_{out}. This indicates that the cardioprotective effects of CHA do not involve beneficial alterations in glycogen turnover during ischaemia. This study confirms the preferential oxidation of glycogen (Goodwin *et al*., 1996; Henning *et al*., 1996; Schonekess *et al*., 1997; Fraser *et al*., 1998) and indicates that the catabolism of glycogen results in a lower rate of proton production. As CHA had no effect on the metabolic fate of either glycogen or exogenous glucose during ischaemia, the main effects of CHA on glycogen

and glucose metabolism are manifest during reperfusion. The importance of the reperfusion period for the cardioprotective activity of adenosine A_1 agonists is further supported by the equivalent efficacy of CHA when administered prior to ischaemia or only at the onset of reperfusion (Finegan *et al*., 1996b). Several signalling pathways (reviewed in Shyrock & Belardinelli, 1997), including inhibition of adenylyl cyclase, stimulation of PKC, inhibition of Ca^{2+} entry and/or activation of K_{ATP} channels may play a role in the metabolic actions of adenosine A_1 receptor activation. This study did not address transduction mechanisms, but we have recently excluded K^+_{ATP} channels in the metabolic and cardioprotective effects of CHA (Ford *et al*., 1998).

Interestingly, CHA had a small, but significant, inhibitory effect on end-ischaemic glycogen phosphorylase activity. At that time, the AMP/ATP and Cr/Crp ratios, both of which are indicative of cellular energetic state and correlate with AMPK activation (Hardie & Carling, 1997; Ponticos *et al*., 1998), were increased by a similar extent in both untreated and CHA-treated groups. As expected, AMPK activity was increased, but the lower activation of AMPK in CHA-treated hearts, despite similar cellular energetic states, suggests that CHA may directly inhibit AMPK. As AMPK can activate glycogen phosphorylase (Young *et al*., 1996), CHA-mediated inhibition of AMPK may have contributed to the lower activity of glycogen phosphorylase. Nevertheless, the CHA-induced change in glycogen phosphorylase activity did not affect glycogen content at the end of ischaemia or the sources and fates of glucose during ischaemia. Consequently, it is unlikely that inhibition of AMPK is involved in the CHA-mediated alterations in glycogen and glucose metabolism. AMPK plays a role in the regulation of fatty acid metabolism (Hardie & Carling, 1997), thus, CHA, *via* changes in AMPK activity, might have influenced fatty acid oxidation. Fatty acid oxidation was not measured, but we have shown previously these rates are not altered by adenosine (Finegan *et al*., 1992).

By the end of reperfusion, CHA-treated hearts had an improved energetic state as indicated by lower AMP/ATP and Cr/CrP ratios as well as an improved recovery of glycogen content. One possibility for the enhanced glycogen resynthesis is a lower rate of G_{out}. Although AMPK and glycogen phosphorylase activities were lower in CHA-treated hearts at the onset of reperfusion, by the end of reperfusion both AMPK and glycogen phosphorylase activities had recovered to aerobic values in both groups. Consequently, it appears that glycogen phosphorylase activity measured at the onset of reperfusion is not reflective of glycogenolysis during the reperfusion period as the average rate of G_{out} during reperfusion, although higher than in aerobic hearts, was similar in both groups.

Alternatively, facilitation of recovery of glycogen content may have occurred in response to stimulation of G_{in}. CHA did indeed stimulate G_{in} during reperfusion, but this was not in association with altered activities of AMPK or glycogen synthase. Thus, the recovery of glycogen content may have been due to the CHA-mediated inhibition of glycolysis that would have increased the availability of substrate for glycogen synthase.

In summary, CHA-induced inhibition of glycolysis during reperfusion and the associated reduction in proton production from glycolytically derived ATP is an important component of CHA-induced cardioprotection. CHA enhanced the recovery of glycogen content and altered glycogen turnover during reperfusion by facilitating glycogen synthesis. The inability of CHA to alter glycogen and glucose metabolism during ischaemia indicates that the beneficial cardioprotective actions of CHA are due, in part, to alterations in glycogen and glucose metabolism during post-ischaemic reperfusion. Further study of glycogen turnover may help identify new targets for drug-induced cardioprotection.

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