

# Isoflurane Alters Energy Substrate Metabolism to Preserve Mechanical Function in Isolated Rat Hearts following Prolonged No-Flow Hypothermic Storage

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**Background:** Isoflurane enhances mechanical function in hearts subject to normothermic global or regional ischemia. The authors examined the effectiveness of isoflurane in preserving mechanical function in hearts subjected to cardioplegic arrest and prolonged hypothermic no-flow storage. The role of isoflurane in altering myocardial glucose metabolism during storage and reperfusion during these conditions and the contribution of adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channel activation in mediating the functional and metabolic effects of isoflurane preconditioning was determined.

**Methods:** Isolated working rat hearts were subjected to cardioplegic arrest with St. Thomas' II solution, hypothermic no-flow storage for 8 h, and subsequent aerobic reperfusion. The consequences of isoflurane treatment were assessed during the following conditions: (1) isoflurane exposure before and during storage; (2) brief isoflurane exposure during early non-working poststorage reperfusion; and (3) isoflurane preconditioning before storage. The selective mitochondrial and sarcolemmal  $K_{ATP}$  channel antagonists, 5-hydroxydecanoate and HMR 1098, respectively, were used to assess the role of  $K_{ATP}$  channel activation on glycogen consumption during storage in isoflurane-preconditioned hearts.

**Results:** Isoflurane enhanced recovery of mechanical function if present before and during storage. Isoflurane preconditioning was also protective. Isoflurane reduced glycogen consumption during storage under the aforementioned circumstances. Storage of isoflurane-preconditioned hearts in the presence of 5-hydroxydecanoate prevented the reduction in glycogen consumption during storage and abolished the beneficial effect of isoflurane preconditioning on recovery of mechanical function.

**Conclusions:** Isoflurane provides additive protection of hearts subject to cardioplegic arrest and prolonged hypothermic no-flow storage and favorably alters energy substrate metabolism by modulating glycolysis during ischemia. The effects of isoflurane preconditioning on glycolysis during hypothermic no-flow storage appears to be associated with activation of mitochondrial  $K_{ATP}$  channels.

CARDIAC transplantation remains the only viable treatment for patients with end-stage cardiac failure.<sup>1</sup> Donor hearts are protected before reimplantation by cardioplegic arrest and no-flow hypothermic storage. This form of

protected ischemia is not optimal, is associated with a variable degree of myocyte and endothelial injury,<sup>2</sup> and, if prolonged, impairs contractile activity on reperfusion.<sup>3</sup> Preconditioning, a phenomenon whereby an intervention occurring before an episode of prolonged ischemia mitigates the adverse functional and cellular effects of such ischemia, can be initiated by (among many other factors) volatile anesthetic agents, in particular isoflurane.<sup>4</sup> Although it is clear that isoflurane-induced preconditioning is effective in reducing infarct size in models of regional ischemia<sup>5</sup> and confers additional protection in isolated hearts subjected to prolonged low-flow hypothermic perfusion,<sup>6</sup> the role of isoflurane in preserving mechanical functional recovery on reperfusion in hearts subject to arrest and prolonged no-flow hypothermic storage has not been clearly established.

Energy substrate metabolism in the heart, in particular the role of glucose during low and no-flow ischemia, has recently been the subject of intense investigation.<sup>7</sup> The heart is capable of metabolizing multiple substrates to meet its energy requirements.<sup>8</sup> During aerobic conditions, myocardial adenosine triphosphate (ATP) is produced mainly by the mitochondrial oxidation of acetyl CoA derived from fatty acids and glucose.<sup>7</sup> During severe ischemia, glucose oxidation ceases and energy is produced by the conversion of stored glycogen into lactate.<sup>9</sup> No-flow ischemia is accompanied by the accumulation of the products of glycolysis, including protons,  $NADH_2$ , and lactate, leading to impaired contractile activity on reperfusion.<sup>10</sup> Modulation of glycolysis during ischemia attenuates glycolytic product accumulation and is associated with improved functional recovery of the heart. Preconditioning with ischemia modifies the metabolism of exogenous supplied glucose and stored glycogen by decreasing the rate of glycolytic activity and improving the coupling of glycolysis to glucose oxidation, thereby reducing proton production.<sup>11</sup> This effect of ischemic preconditioning is seen in hearts that undergo aerobic perfusion after the preconditioning process and in hearts subsequently subjected to prolonged ischemia and reperfusion. The effect of isoflurane on glycogen consumption during prolonged global no-flow hypothermic storage and on exogenous glucose metabolism during subsequent aerobic reperfusion has not been examined. There is convincing evidence that preconditioning evoked by exposure to isoflurane involves activation of cardiac ATP-dependent potassium ( $K_{ATP}$ ) channels.<sup>12</sup> We

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### Perfusion Protocol

	10 min	8 hours	10 min	60 min
PHASES	L	S	LR	WR

**Fig. 1. Working heart perfusion protocol.** Hearts underwent an initial 10-min Langendorff perfusion (L) phase, a storage (S) phase, a Langendorff reperfusion (LR) phase, and a working mode reperfusion (WR) phase.

therefore assessed the consequences of sarcolemmal and mitochondrial  $K_{ATP}$  channel antagonism on glucose use during the period of prolonged no-flow hypothermic storage and, in the case of the mitochondrial  $K_{ATP}$  antagonist, on mechanical functional recovery following isoflurane-induced preconditioning.

## Materials and Methods

The study was performed in accordance with guidelines of the Canadian Council on Animal Care and the University of Alberta Health Sciences Animal Welfare Committee.

### Study Design

Male Sprague-Dawley rats aged 7–8 weeks, weighing 300–350 g, and that had been fed *ad libitum*, were killed after induction of anesthesia with sodium pentobarbital, and their hearts were excised. The aortae were cannulated and a retrograde Langendorff perfusion using Krebs-Henseleit solution (37°C, pH 7.4, gassed with a 95%  $O_2$ –5%  $CO_2$  mixture), containing 11 mM glucose and 2.5 mM  $Ca^{2+}$ , was initiated for 10 min at a constant perfusion pressure of 60 mmHg. Thereafter, hearts were perfused with 25 ml of ice-cold St. Thomas' II cardioplegia solution (pH 7.8, 110 mM NaCl, 10 mM  $NaHCO_3$ , 16 mM KCl, 6 mM  $MgCl_2$ , 16 mM  $H_2O$ , 1.2 mM  $CaCl_2$ ) administered at constant perfusion pressure of 60 mmHg for 2 min to induce arrest. Hearts were immediately removed from the perfusion apparatus and immersed in St. Thomas' II solution at  $3 \pm 1^\circ C$  for 8 h. Following storage, hearts were perfused at 37°C in Langendorff mode for 10 min, before undergoing working mode reperfusion at 300 beats/min (Grass SD9 stimulator; Astro-Med Inc., West Warwick, RI) for 60 min. During working reperfusion, hearts were perfused with modified Krebs-Henseleit solution at 37°C, containing 2.5 mM  $Ca^{2+}$ , 11 mM glucose, 100  $\mu U/ml$  insulin, and 1.2 mM palmitate prebound to 3% bovine serum albumin (fraction V).

A group of hearts, serving as a time control group ( $n = 13$ ), was not subjected to cardioplegic arrest and hypothermic no-flow storage, but underwent excision, aerobic Langendorff perfusion for 10 min, and then 60 min of aerobic perfusion in the working mode during normal aerobic conditions. The remaining groups of hearts were subject to the four phases of the perfusion protocol (fig. 1). Hearts were randomly assigned to an untreated group ( $n = 20$ )

and isoflurane-treated groups. The consequence of isoflurane on mechanical function was assessed in four isoflurane-treated groups. One group of hearts were perfused with Krebs-Henseleit solution equilibrated with 1.5% isoflurane during the initial and reperfusion Langendorff phases and stored in St. Thomas' II solution equilibrated with 1.5% isoflurane ( $n = 15$ ). In an isoflurane reperfusion group, hearts were perfused with Krebs-Henseleit equilibrated with 1.5% isoflurane only during the Langendorff reperfusion phase ( $n = 6$ ). Finally, two groups of hearts were subject to isoflurane-induced preconditioning, where hearts were exposed to 1.5% isoflurane only during the initial Langendorff perfusion and were subsequently stored in St. Thomas' II solution in the absence ( $n = 7$ ) or presence of 5-hydroxydecanoate (5-HD; 500  $\mu M$ ;  $n = 6$ ), a selective mitochondrial  $K_{ATP}$  channel antagonist. Isoflurane was purchased from Abbott Laboratories Ltd. (Saint-Laurent, Québec, Canada) and 5-HD from Sigma-Aldrich Co. (St. Louis, MO).

We measured rates of glycolysis and glucose oxidation during the working reperfusion phase in time control hearts ( $n = 13$ ), untreated stored hearts ( $n = 7$ ), and hearts treated with isoflurane during both Langendorff phases and during storage ( $n = 9$ ). Exogenous glycolysis was measured by determining the amount of  $^3H_2O$  produced (which is liberated from [5- $^3H$ ]glucose at the enolase step of glycolysis). Separation of  $^3H_2O$  from [ $^3H$ ]/[ $^{14}C$ ]glucose was achieved as described previously.<sup>13</sup> Measurement of exogenous glucose oxidation was achieved by quantitatively collecting  $^{14}CO_2$  produced (liberated from [U- $^{14}C$ ]glucose at the level of pyruvate dehydrogenase complex and in the trichloroacetic acid cycle). Briefly, the  $^{14}CO_2$  produced as a gas was collected from the sealed perfusion system through a 1-M methylbenzethonium hydroxide solution that acted as a  $^{14}CO_2$  trap. Perfusate samples containing [ $^{14}C$ ]bicarbonate were collected and stored under mineral oil to prevent the liberation of  $^{14}CO_2$  and subsequently injected into closed metabolic reaction flasks containing 9N  $H_2SO_4$ . The  $^{14}CO_2$  released from the perfusion buffer was trapped in center wells containing filter paper saturated with 1 M methylbenzethonium hydroxide. Afterward, the papers were removed and counted in aqueous counting scintillant using standard counting procedures. Therefore, total  $^{14}CO_2$  production was determined by analyzing samples of the methylbenzethonium hydroxide used as a  $^{14}CO_2$  gas trap and perfusate samples that contained [ $^{14}C$ ]bicarbonate. Steady state rates of glycolysis and glucose oxidation from exogenous glucose were expressed as micromoles of [5- $^3H$ ]glucose and [U- $^{14}C$ ]glucose metabolized per minute per gram dry weight, respectively. The rate of proton production attributable to the hydrolysis of ATP arising from exogenous glucose metabolism was calculated as  $2 \times$  (rate of glycolysis – rate of glucose oxidation).

### *Delivery and Measurement of Isoflurane*

Isoflurane was administered *via* a calibrated agent-specific vaporizer (Datex-Ohmeda Tec 5; Datex-Ohmeda, Madison, WI) and the delivered concentration (1.5%) continuously monitored by an infrared analyzer (Datex Capnomac Ultima; Datex Instrumentarium Corporation, Helsinki, Finland) at a constant fresh gas flow (2.5 l/min). Isoflurane-treated groups were perfused with Krebs-Henseleit solution equilibrated with isoflurane at 37°C for 10 min before the initial Langendorff perfusion and continued throughout the Langendorff perfusion period. Samples of the perfusion fluid were collected immediately before and at the end of the Langendorff phase for later analysis of isoflurane content. St. Thomas' II solution was equilibrated with isoflurane at 1.5°C for 20 min and stored in gas-tight containers at 3°C until required for experimental purposes. Samples were collected for later analysis of isoflurane content. Fresh anesthetic-containing solutions were prepared on the day experiments were performed.

Isoflurane was assayed quantitatively by a Perkin-Elmer Autosystem XL gas chromatograph equipped with a Perkin-Elmer headspace auto sampler (Perkin-Elmer Corp., Norwalk, CT), using an Rtx<sup>®</sup> capillary column (Restek Corp., Bellefonte, PA). The internal standard was n-propanol. Intraassay and interassay imprecision over a concentration range of 100–700 mg/l were 3.3% and 6.2%, respectively. Samples with an initial concentration in excess of the calibration range were reanalyzed on a 1:5 dilution in the appropriate matrix. The concentrations of isoflurane achieved in Krebs-Henseleit at 37°C were within the clinically relevant range, being 0.89 mM and 0.92 mM at 10 and 20 min, respectively (1.8 vol%, equivalent to ~1.3 minimum alveolar concentration in the rat). The concentration of isoflurane measured in St. Thomas' II solution at 1.5°C was 5.4 mM, being, as expected, in excess of that achieved clinically.

### *Measurements of Mechanical Function and Coronary Perfusion*

In the working mode, heart rate and systolic and diastolic aortic pressures (millimeters of mercury) were measured using a Gould P21 pressure transducer (Gulton-Statham Transducers Inc., Amherst, NY) attached to the aortic outflow line. Cardiac output (milliliters per minute) and aortic flow (milliliters per minute) were measured using ultrasonic flow probes (Transonic Systems Inc., Ithaca, NY) placed in the left atrial inflow line and the aortic outflow line, respectively. Left ventricular minute work (LV work; liters per minute × millimeters of mercury), calculated as cardiac output × (systolic aortic pressure – left atrial preload pressure), was measured and served as an index of mechanical function. Measurements of coronary flow were made during the periods of Langendorff perfusion.

### *Biochemical Analysis*

Further groups of hearts were studied to allow assessment of the consequences of the treatment strategies on the contents of glycogen, high-energy phosphates, and intermediate metabolites. Tissue was obtained from hearts frozen at the end of the initial Langendorff phase in the time control group (n = 6). Similarly, groups of untreated hearts were frozen at the end of storage (n = 6) and at the end of Langendorff reperfusion (n = 6). Hearts treated with isoflurane (1.5%) were also frozen at the end of storage (n = 6) and at the end of Langendorff reperfusion (n = 6). To evaluate the potential role of sarcolemmal and mitochondrial K<sub>ATP</sub> channel activation in the mechanism of isoflurane-induced preconditioning, tissue was obtained from groups of hearts frozen at similar time points but treated with isoflurane only during the initial Langendorff phase. Groups of hearts were treated with 5-HD (500 μM) coadministered with isoflurane (n = 6) or administered only during the storage phase (n = 6). Similarly, HMR 1098 (30 μM; Aventis Pharma, Laval, Quebec, Canada), a sarcolemmal K<sub>ATP</sub> channel-selective blocker, was coadministered with isoflurane (n = 6) or administered only during the storage phase (n = 6). Concentrations of 5-HD and HMR 1098 used in these experiments were chosen based on previous studies.<sup>14,15</sup> Hearts in the above groups were cooled to the temperature of liquid N<sub>2</sub> and stored at –70°C for subsequent metabolic analysis.

Frozen ventricular tissue was powdered using a mortar and pestle maintained at the temperature of liquid N<sub>2</sub>. Adenine nucleotides were extracted from approximately 100 mg of frozen ventricular tissue into 1 ml 6% ice-cold perchloric acid by homogenization with a pestle in a cold mortar. The tissue perchloric acid mixture was centrifuged at 4°C, and the supernatant was neutralized with K<sub>2</sub>CO<sub>3</sub>. High-performance liquid chromatography analysis (with the operator blinded to the origin of each sample) was used to measure nucleotide concentrations in the neutralized extracts.<sup>16</sup> Glycogen in approximately 200 mg of frozen ventricular tissue was hydrolyzed to glucose by reacting with 2N H<sub>2</sub>SO<sub>4</sub>; the amounts were measured using a glucose kit (Sigma-Aldrich, St. Louis, MO) and expressed as glucose units.

Glycogen and ATP contents measured at the end of the initial Langendorff phase and the storage phase allowing the calculation of the amount of glycogen (Δglycogen) and ATP (ΔATP) being consumed during the period of no-flow hypothermic storage. Proton production during ischemia was calculated as (Δglycogen) × 3 + ΔATP.<sup>11</sup> The reliability of this calculation as an index of pH changes during ischemia in isolated hearts has recently been validated using nuclear magnetic resonance spectroscopy.<sup>17</sup>

### *Statistical Analysis*

Data are expressed as mean ± SEM and have been subject to two-way analysis of variance. If significant,

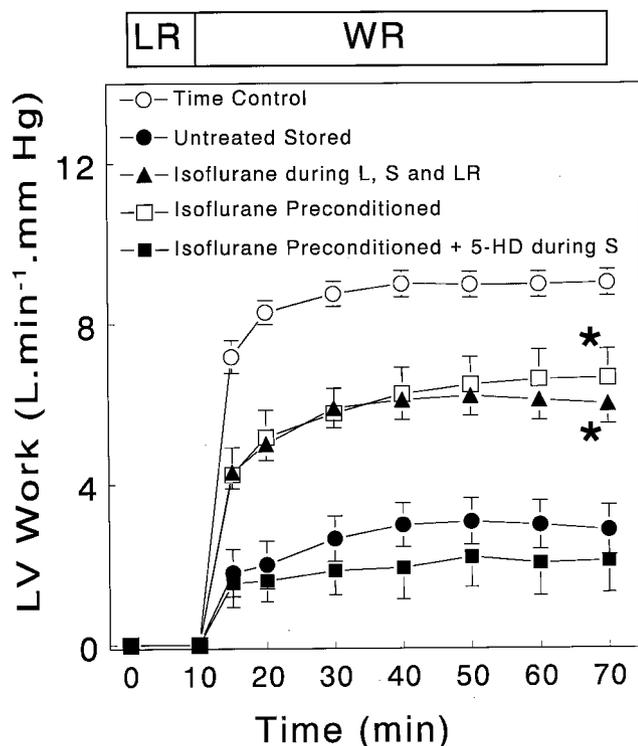


Fig. 2. Effect of isoflurane on the time course for recovery of mechanical work during Langendorff (LR) and working (WR) reperfusion. Left ventricular minute work (LV work) values are shown for time control hearts ( $n = 13$ ); untreated stored hearts ( $n = 20$ ); hearts treated with isoflurane during the initial 10-min Langendorff perfusion (L) phase, the storage (S) phase, and Langendorff reperfusion (LR) phase ( $n = 15$ ); isoflurane preconditioned hearts ( $n = 7$ ); and isoflurane-preconditioned hearts treated with 5-hydroxydecanoate (5-HD) during storage ( $n = 6$ ). \* $P < 0.05$  versus untreated stored hearts and isoflurane-preconditioned hearts treated with 5-HD during storage. Data are mean  $\pm$  SEM.

selected data sets were compared by Bonferroni multiple comparison test. Test differences were considered significant at  $P < 0.05$  (two-tailed).

## Results

### Effect of Isoflurane on Mechanical Functional Recovery and Hemodynamic Indices

Cardioplegic arrest and no-flow hypothermic storage for 8 h was associated with a marked reduction in me-

chanical function after 60 min of reperfusion, with LV work recovering to only 20% of the values measured in the time control group (fig. 2). Isoflurane, present before and during storage and early reperfusion, was associated with enhanced recovery of mechanical function, with LV work recovering to 68% of that observed in the time control group. Remarkably, preconditioning with isoflurane resulted in an equivalent recovery in LV work. The presence of 5-HD during the storage period, which had no effects *per se*, abolished the protection afforded by isoflurane preconditioning, suggesting that mitochondrial  $K_{ATP}$  activation mediated the observed protection. Developed pressures, cardiac output, and aortic flow values in the groups taken through working reperfusion are shown in table 1. Again, significant improvements in these parameters were seen in isoflurane-treated hearts relative to the untreated hearts. Treatment with isoflurane only on reperfusion was not a useful cardioprotective strategy, as LV work recovered to a similar extent to that measured in the untreated group (data not shown).

Coronary flow measured during the initial Langendorff perfusion in time control hearts was 12.5 ml/min. Isoflurane increased coronary flow by approximately 20% when present during the this phase of the perfusion.

### Effect of Isoflurane on Exogenous Glucose Metabolism during Working Reperfusion

Subjecting hearts to cardioplegic arrest and hypothermic storage reduced the rates of exogenous glucose oxidation on reperfusion below those measured in the time control group (fig. 3). Interestingly, if isoflurane was present before and during storage and during Langendorff reperfusion, glucose oxidation was similar to that observed in the time control group. Glycolytic rates were similar in the three groups studied. Calculated proton production during working reperfusion was greater in the untreated hearts, whereas the rate of proton production in the group of hearts treated with isoflurane before and during storage and during early reperfusion was comparable with that of the time control group.

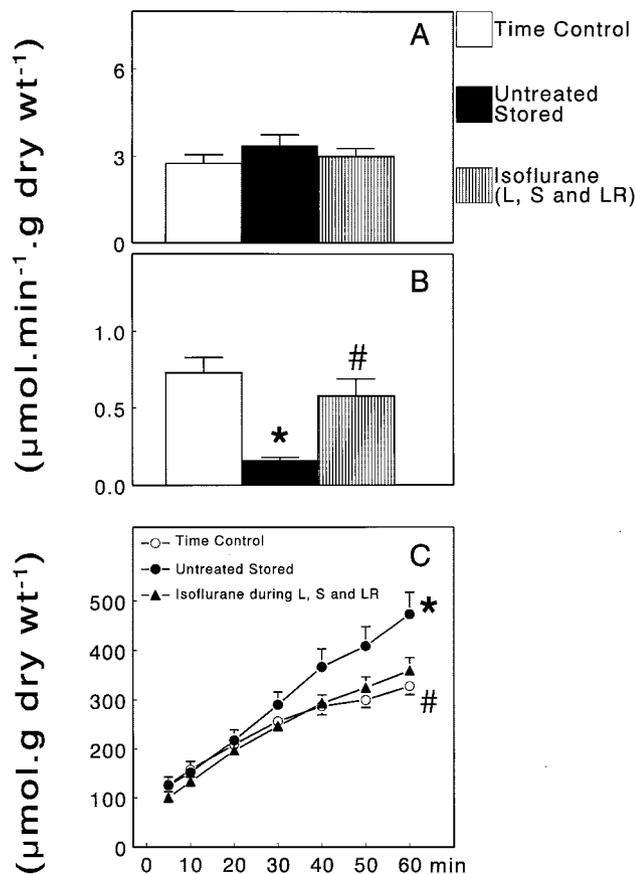
Table 1. Mechanical Function at 60 min of Working Mode Reperfusion

Hearts	DP, mmHg	CO, ml/min	AF, ml/min
Time control ( $n = 13$ )	$70 \pm 2$	$70 \pm 2$	$50 \pm 3$
Untreated stored ( $n = 20$ )	$28 \pm 4^*$	$29 \pm 4^*$	$14 \pm 3^*$
Isoflurane during L, S, and LR phases ( $n = 15$ )	$56 \pm 4^\dagger$	$50 \pm 4^\dagger$	$33 \pm 4^\dagger$
Isoflurane during LR phase ( $n = 6$ )	$41 \pm 5$	$37 \pm 8$	$24 \pm 7$
Isoflurane preconditioned ( $n = 7$ )	$47 \pm 5^\dagger$	$61 \pm 6^\dagger$	$44 \pm 5^\dagger$
Isoflurane preconditioned + 5-hydroxydecanoate ( $n = 6$ )	$20 \pm 7^\ddagger$	$23 \pm 8^\ddagger$	$11 \pm 4^\ddagger$

Data are mean  $\pm$  SEM.

\*  $P < 0.05$  versus time-control hearts.  $^\dagger P < 0.05$  versus untreated stored hearts.  $^\ddagger P < 0.05$  versus isoflurane-preconditioned hearts.

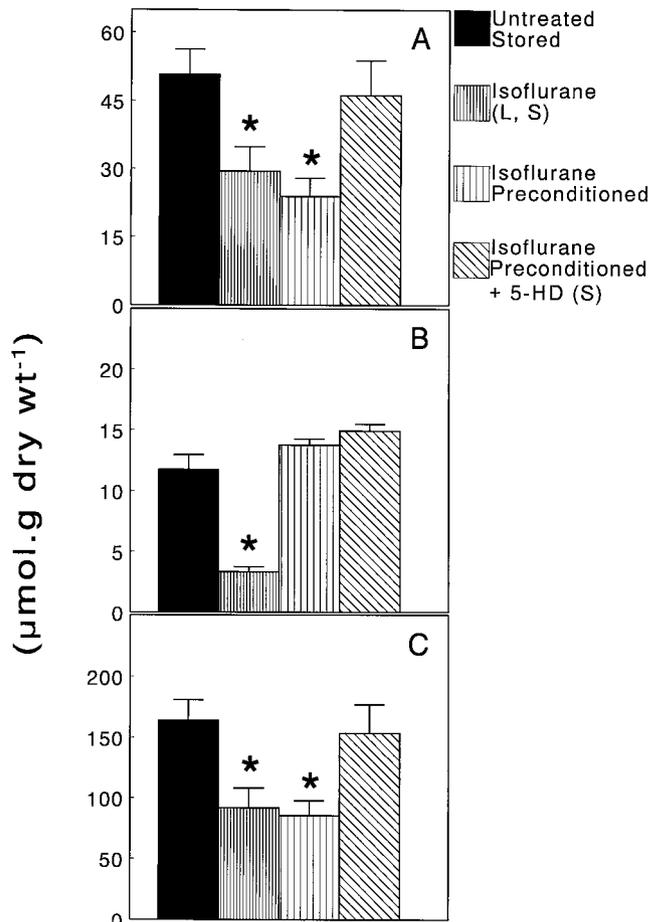
AF = aortic flow; CO = cardiac output; DP = developed aortic pressure; L = initial 10-min Langendorff perfusion phase; LR = Langendorff reperfusion phase; S = storage phase.



**Fig. 3.** Isoflurane effect on glucose metabolism averaged over 60 min of working reperfusion. Rates for glycolysis (A), glucose oxidation (B), and calculated proton production (C) are shown for time control hearts (n = 13), untreated stored hearts (n = 7), and hearts treated with isoflurane during the initial 10-min Langendorff perfusion (L) phase, the storage (S) phase, and Langendorff reperfusion (LR) phase (n = 9). \*P < 0.05 versus time control hearts. #P < 0.05 versus untreated stored. Data are mean  $\pm$  SEM.

*Effect of Isoflurane on Glycogen Use during No-Flow Hypothermic Storage*

In the time control group, glycogen content was  $113.5 \pm 7.4 \mu\text{mol/g dry weight}$  at the end of the initial Langendorff perfusion. In untreated hearts, glycogen content at the end of the storage phase was  $40.4 \pm 5.4$  and  $38.9 \pm 5.1 \mu\text{mol/g dry weight}$  at the end of the Langendorff reperfusion. In hearts where isoflurane was present, either during the initial Langendorff and storage phases or present before or during storage and Langendorff reperfusion, glycogen content was significantly greater than in untreated hearts ( $63.0 \pm 4.1$  and  $59.6 \pm 2.9 \mu\text{mol/g dry weight}$ , respectively). In the group of hearts preconditioned with isoflurane, glycogen content was equivalently preserved at the end of the storage phase ( $67.0 \pm 4.0 \mu\text{mol/g dry weight}$ ). In hearts preconditioned with isoflurane but treated with 5-HD during preconditioning, glycogen content was not significantly altered at the end of the storage phase ( $56.9 \pm 3.2 \mu\text{mol/g dry weight}$ ). However, if 5-HD was present during the storage period, glycogen was not preserved and



**Fig. 4.** Change in glycogen (A) and adenosine triphosphate (ATP; B) content and calculated proton production (C) during 8 h of hypothermic storage. Values are shown for untreated stored hearts (n = 6), hearts treated with isoflurane during the initial 10-min Langendorff perfusion (L) and storage (S) phases (n = 6), isoflurane-preconditioned hearts (n = 6), and isoflurane-preconditioned hearts treated with 5-hydroxydecanoate (5-HD) during storage (n = 6). \*P < 0.05 versus untreated stored hearts. Data are mean  $\pm$  SEM.

was reduced to  $33.9 \pm 5.5 \mu\text{mol/g dry weight}$ . The coadministration of HMR 1098 during preconditioning with isoflurane or the storage of isoflurane-preconditioned hearts in the presence of HMR 1098 did not alter isoflurane-induced glycogen preservation during storage, with end-storage glycogen contents of  $68.2 \pm 8.6$  and  $59.1 \pm 8.1 \mu\text{mol/g dry weight}$ , respectively. Calculated proton production during the storage phase was reduced by isoflurane preconditioning and if isoflurane was present before and during storage. However, if 5-HD was present during storage, the beneficial effect of isoflurane preconditioning on this calculated parameter was abolished (fig. 4).

*Effect of Isoflurane on Adenosine Triphosphate Content and Energy Charge*

Adenosine triphosphate content at the end of storage was reduced from  $30.9 \pm 0.6 \mu\text{mol/g dry weight}$  in the time control group to  $19.2 \pm 1.2 \mu\text{mol/g dry weight}$  in

the untreated group. In contrast, when isoflurane was present during both the initial Langendorff and storage phases, ATP content at the end of storage was  $27.65 \pm 0.4 \mu\text{mol/g}$  dry weight, equivalent to that measured in the time control group. Repletion of ATP occurred rapidly in untreated hearts during the Langendorff reperfusion phase, although ATP content remained greater in the group of hearts where isoflurane was present before and during storage and during early reperfusion ( $26.5 \pm 1.0$  and  $35.7 \pm 2.4 \mu\text{mol/g}$  dry weight, respectively). Hearts preconditioned with isoflurane did not show ATP conservation following storage (ATP content of  $17.2 \pm 0.5 \mu\text{mol/g}$  dry weight). The presence of 5-HD either during the initial Langendorff or storage phases in isoflurane treated hearts did not influence ATP content at the end of storage. Energy charge, a measure of the metabolic energy stored in the adenine nucleotide system, was significantly improved in isoflurane-treated hearts at the end of storage, relative to untreated hearts. The presence of 5-HD significantly attenuated energy charge values in isoflurane-treated hearts (data not shown).

## Discussion

Hypothermia in cold-sensitive mammals forcibly suppresses metabolism and induces a mismatch between ATP supply and demand pathways.<sup>18</sup> In the current study, a significant reduction in LV work on warm reperfusion occurred following cardioplegic arrest and no-flow hypothermic storage of hearts for a period of 8 h at 4°C with St. Thomas' II cardioplegia solution. Recovery of LV work was improved if the volatile anesthetic isoflurane was present, either before arrest and no-flow hypothermic storage or during arrest, storage, and early reperfusion. Of particular interest was the finding that LV work recovered to an equivalent extent in hearts preconditioned by isoflurane as in those exposed to isoflurane before ischemia, throughout ischemia and early reperfusion.

There are few studies assessing ischemic or drug-induced preconditioning in the clinically relevant model of prolonged cardioplegic arrest and no-flow hypothermic storage. Ogino *et al.*<sup>19</sup> demonstrated in nonworking rat hearts that ischemic preconditioning improved diastolic but not systolic ventricular function in hearts subject to 6 h of hypothermic storage. Hachida *et al.*,<sup>20</sup> also in a nonworking heart model, found that ischemic preconditioning improved both systolic and diastolic indices of mechanical function and reduced  $\text{Ca}^{2+}$  accumulation. Preconditioning with diazoxide, a  $\text{K}_{\text{ATP}}$  channel agonist, before storage in a similar model improved diastolic parameters of ventricular function only.<sup>21</sup> Our novel observation that isoflurane improves LV work over and above the protection afforded by hypothermia is intriguing and suggests that isoflurane-induced preconditioning

may be an effective and simple cardioprotective strategy to extend the viability of hearts subject to arrest and hypothermic storage.

Isoflurane has been shown to be protective in prolonged low-flow hypothermic perfusion if continuously present in the perfusate.<sup>6</sup> Our data extend these observations to the no-flow hypothermic setting. We did not find that isoflurane present only during early reperfusion enhanced mechanical functional recovery. This finding appears to conflict with that of Preckel *et al.*,<sup>22</sup> who found 1.5 minimum alveolar concentration isoflurane to be protective when administered immediately before reperfusion following 30 min of cardioplegic arrest in a nonworking heart model. Close inspection of their data indicates that isoflurane-induced protection, as measured by LV developed pressure, was not consistently apparent during reperfusion. Other measures of myocyte protection, including LVEDP and  $\text{dP/dt}_{\text{max}}$ , appear to have been improved only transiently in isoflurane-treated hearts relative to the cardioplegic control group, with no significant differences noted between the two groups in the aforementioned parameters after 5 min of reperfusion. Furthermore, in the study by Preckel *et al.*,<sup>22</sup> cumulative creatine kinase release on reperfusion did not appear to have been significantly different between hearts subject to cardioplegic arrest reperfused in the absence or presence of isoflurane, suggesting minimal isoflurane-induced protection during the reperfusion period. Our study was performed in hearts subject to a prolonged period of cardioplegic arrest, but the period of exposure to isoflurane on reperfusion was brief, occurring only during the 10-min nonworking reperfusion phase. We did not study the consequences of prolonged exposure to isoflurane on reperfusion, which is beneficial in reducing infarct size after regional ischemia,<sup>23</sup> but may not, at least in the short term, improve contractility.<sup>24</sup>

Cardiac surgery involving cardiopulmonary bypass profoundly alters the composition of energy substrate availability for cardiac metabolic activity. Free fatty acid concentrations are increased threefold.<sup>25</sup> The experimental groups in this study were perfused with a 1.2-mM concentration of fatty acids to simulate these conditions. Free fatty acids depress glucose use by inhibiting glucose transport and the activation of hexokinase, phosphofructokinase, and pyruvate dehydrogenase kinase. The inhibitory effect of fatty acids on the rate of exogenous glucose oxidation during reperfusion was evident in our study, with steady state rates lower than those previously measured in working hearts perfused with 0.4 mM palmitate, a concentration of fatty acids found during normal aerobic conditions.<sup>26</sup> Hydrolysis of ATP derived from glycolysis results in a net production of two  $\text{H}^+$  per molecule of glucose that passes through glycolysis but is not oxidized. Consequently, during no-flow ischemia,  $\text{H}^+$  ions accumulate and on reperfusion are exchanged

for  $\text{Na}^+$ , which in turn is exchanged for  $\text{Ca}^{2+}$  via the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger.<sup>10</sup> A mismatch between glucose oxidation and glycolysis on reperfusion add to the  $\text{H}^+$  load at a critical time and is detrimental to mechanical function. Significantly, during reperfusion in our study, the coupling of glycolysis to glucose oxidation was improved in hearts treated with isoflurane, and mechanical functional recovery was better preserved.

Consumption of glycogen during global ischemia is an important determinant of reperfusion recovery, with either glycogen depletion before ischemia, by ischemic preconditioning<sup>27</sup> or by pharmacologically inhibiting glycogenolysis using glucosidase inhibitors,<sup>28</sup> being remarkably effective in reducing myocardial injury in hearts exposed to prolonged no-flow ischemia. We observed a reduction in glycogen use during the period of prolonged no-flow hypothermic storage in hearts preconditioned by isoflurane and when isoflurane was present before and during storage and during early reperfusion, indicating that the effect of isoflurane on glycogenolysis is intact in hearts subject to hypothermic storage. Ventricular myocytes subject to deep hypothermia exhibit marked intracellular  $\text{Ca}^{2+}$  accumulation.<sup>29</sup> Glycogen phosphorylase, the key regulator of glycogenolysis, is activated by cyclic adenosine monophosphate-dependent protein kinase or by  $\text{Ca}^{2+}$ -activated phosphorylase kinase. The mechanism of reduced glycogenolysis in hearts exposed to isoflurane during ischemia may involve an isoflurane-induced decrease in myocyte  $\text{Ca}^{2+}$  availability<sup>30</sup> and consequent modulation of phosphorylase kinase activity. We did not assess this possibility. An alternative explanation is that isoflurane activated adenosine  $\text{A}_1$  receptors during the ischemic period. Kersten *et al.*<sup>31</sup> have shown, in a canine model of regional ischemia, that the protective effects of isoflurane against stunning were decreased if hearts were pretreated with 8-cyclopentyl-1,3-dipropylxanthine, a selective adenosine  $\text{A}_1$  receptor antagonist, suggesting that the cardioprotective effects of isoflurane may be mediated *via* a process initiated by activation of the adenosine  $\text{A}_1$  receptor. In human tissue, 8-cyclopentyl-1,3-dipropylxanthine pretreatment markedly attenuates isoflurane-induced protection from anoxia.<sup>32</sup> Interestingly, activation of adenosine  $\text{A}_1$  receptors alters glucose use in the heart by decreasing glycolysis and improving the coupling between glycolysis and glucose oxidation.<sup>13,33</sup> Our findings of better ATP preservation and calculated energy charge in isoflurane-preconditioned hearts and in the group of hearts treated with isoflurane before and during storage and during early reperfusion confirm those of Boutros *et al.*<sup>34</sup> and suggest that efficiency of energy use during the period of no-flow hypothermic storage was enhanced by isoflurane treatment. Glycolytically derived ATP is preferentially used by the myocyte to maintain  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity<sup>35</sup>; in consequence, modulating glycolysis and ATP use throughout the ischemic interval may be an important component in isoflurane-induced protection.

There is a significant body of evidence that isoflurane exerts protective effects by  $\text{K}_{\text{ATP}}$  channel activation,<sup>36,37</sup> a process that also occurs as a consequence of adenosine  $\text{A}_1$  receptor activation.<sup>38</sup> We observed that, while sarcolemmal  $\text{K}_{\text{ATP}}$  channels did not appear to be involved in the metabolic changes observed in isoflurane-preconditioned hearts, the presence of the selective mitochondrial  $\text{K}_{\text{ATP}}$  channel antagonist 5-HD, during no-flow hypothermic storage but not during the preconditioning period, effectively blocked the favorable effects of isoflurane preconditioning both on metabolism and subsequent mechanical functional recovery on reperfusion. These data suggest the isoflurane triggers a mechanism that is mediated during ischemia by mitochondrial  $\text{K}_{\text{ATP}}$  channels. Supporting this concept, Kohro *et al.*<sup>39</sup> demonstrated that mitochondrial  $\text{K}_{\text{ATP}}$  currents, indirectly measured in isolated cardiac myocytes by assessment of flavoprotein fluorescence, are increased by isoflurane, and that these currents are inhibited in the presence of 5-HD. Opening of mitochondrial  $\text{K}_{\text{ATP}}$  channels is associated with preservation of mitochondrial membrane integrity following ischemia and improved mitochondrial bioenergetics, in particular preservation of mitochondrial oxidative phosphorylation on reperfusion following ischemia.<sup>40</sup> Improved oxidative capacity may be particularly important following hypothermia given the multiple effects of such a stress on cellular homeostatic mechanisms.<sup>18</sup> Our studies were performed in hearts subjected to no-flow ischemia and hypothermia, and it is during these conditions that  $\text{K}_{\text{ATP}}$  channel activation appears to be relevant as a mechanism of isoflurane-induced cardioprotection. Interestingly, isoflurane also provides additive protection during prolonged nonischemic hypothermia, as elegantly investigated by Stowe *et al.*,<sup>6</sup> by a  $\text{K}_{\text{ATP}}$  channel-independent mechanism.

In conclusion, our findings indicate that isoflurane, administered briefly before, or if present before and throughout the period of hypothermic storage and early reperfusion, improve LV work on reperfusion. The mechanism of protection involves attenuation in glycogen consumption and proton production and activation of mitochondrial  $\text{K}_{\text{ATP}}$  channels during the period of prolonged no-flow hypothermic storage. Our studies were conducted in isolated rat hearts perfused with crystalloid subsequent to hypothermic no-flow storage and arrested with and stored in St. Thomas' II cardioplegic solution. It remains to be proven whether isoflurane administered in the manner described in this study is effective in the human blood-reperfused, intact working heart.

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