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Precursor Trapping: A "Neonatal" Mechanism of Myocardial Protection

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During induced ischemia for cardiac surgery, 5′-nucleotidase (5NT) catalyzes nucleotide breakdown by dephosphorylating AMP and IMP to diffusible precursors—adenosine and inosine. These precursors become unavailable upon reperfusion washout limiting nucleotide resynthesis, resulting in poor postischemic function. Neonatal hearts, which are more resistant to ischemia than adults, have low 5NT activity, trapping available precursors. Adult rabbit hearts given cardioplegia with a 5NT inhibitor, pentoxifylline, demonstrated improved postischemic contractility, compliance, and myocardial oxygen consumption after 120 min of 34°C ischemia. To determine if this improved function was a result of enhanced nucleotide precursor availability during or following ischemia, total nondiffusible nucleotides, ATP, ADP, AMP, and IMP, and total diffusible nucleotides, adenosine, inosine, hypoxanthine, and xanthine, were measured by HPLC at end ischemia, 1 and 15 min after reperfusion. While all preischemic values were equivalent, pentoxifylline-treated hearts had significantly greater total nondiffusible nucleotides at end ischemia, 1 and 15 min after reperfusion. Additionally, pentoxifylline-treated hearts had significantly greater total diffusible nucleosides at end ischemia and 1 min after reperfusion, but were equal to control at 15 min after reperfusion. Furthermore, coronary sinus effluent had a significantly higher release of total diffusible nucleosides in control vs pentoxifylline-treated hearts. The data indicate that precursor trapping with pentoxifylline prevented nucleotide catabolism to diffusible precursors and enhanced postischemic nucleotide availability. We postulate the increased precursor availability augmented myocardial nucleotide resynthesis and correlated with the improved functional recovery noted. This strategy may have application in adult cardiac surgery.

INTRODUCTION

The breakdown of ATP into diffusible precursors during ischemia is a key factor limiting the functional recovery of the myocardium following reperfusion. Postischemic myocytes have the metabolic ability to utilize precursors [1]. Adenosine, when given during ischemia, has been shown to enhance the recovery of myocardium following ischemia and reperfusion by providing the needed precursor pool for ATP resynthesis utilizing the salvage pathway [1]. The cardiac enzyme 5′-nucleotidase (5NT) is responsible for final nucleotide breakdown by dephosphorylating AMP and IMP to adenosine and inosine, which then diffuse out of the cell, where they can no longer act effectively as precursors for ATP resynthesis. Because neonatal hearts exhibit low 5′-nucleotidase activity, they maintain a greater ATP precursor pool by trapping the precursors in the cell and are thus generally more resistant to ischemia.

Consequently, a "neonatal" mechanism of myocardial preservation was proposed. In previous studies adult hearts given the 5′-nucleotidase inhibitor pentoxifylline during ischemia showed enhanced myocardial preservation [2]. Accordingly, the present study was undertaken to confirm this beneficial effect and elucidate the mechanism of myocardial preservation. We tested the hypothesis that the myocardium is preserved through increasing the ATP precursor pool by trapping precursors in the cell, thereby leading to improved functional recovery upon reperfusion.

MATERIAL AND METHODS

Studies were conducted using isolated, perfused male New Zealand White rabbit hearts. Rabbit hearts were excised through a median sternotomy following sodium pentobarbitol anesthesia (10 mg/kg) and immediately immersed in cold (4°C) Krebs–Ringer bicarbonate solution. The aorta was cannulated and the heart suspended on a perfusion column within 30 sec from excision. Perfusion was initiated with an oxygenated (450–550 mm Hg O2) Krebs–Ringer bicarbonate solution maintained

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at 37°C in a heat-exchange bath. The perfusate (pH 7.44 to 7.48, 300 to 310 mOsm/liter) was filtered through a Millipore 8.0-μm filter and was not recirculated.

After initiation of perfusion, the pulmonary veins as well as the superior and inferior vena cavae were ligated to eliminate escape of perfusate through these vessels. An incision was made in the left atria and the function of the mitral valve was destroyed using blunt forceps, after which a latex balloon connected to saline-filled tubing was placed in the left ventricle. The balloon was sutured in to allow passive venting of the left ventricular cavity. Inside the tubing and extending into the balloon was a Millar pressure transducer to record left ventricular (LV) pressure. Output from the pressure transducer was electronically differentiated to record maximum dP/dt. The pulmonary artery was cannulated with a small piece of tubing so that coronary effluent could be collected.

Hearts were allowed to stabilize for a period of 30 min at normothermia. During this period normal sinus rhythm was regained and all hearts had a rate of 160 beats/min. A volume of saline was introduced into the balloon in the LV to produce an end-diastolic pressure (EDP) of 10 mm Hg. Ventricular systolic function was evaluated isovolumically by introducing the same volume into the LV balloon during reperfusion. Diastolic stiffness was estimated at different time points by generating EDP vs end-diastolic volume curves. Coronary flow was measured volumetrically. Oxygen consumption was measured by passing coronary effluent through a chamber with an oxygen electrode and calculating myocardial oxygen consumption (MVO_2). MVO_2 was calculated as $MVO_2 = CF \times [(P_aO_2 - P_vO_2) \times (c/760)]$, where CF is coronary flow (ml/min/g), $(P_aO_2 - P_vO_2)$ is the difference in the partial pressure of oxygen (PO_2, mm Hg) between perfusate and coronary effluent flow, c is the Bunsen solubility coefficient of O_2 in perfusate at 37°C (22.7 μl $O_2 \cdot atm^{-1} \cdot ml^{-1}$ perfusate), and the PO_2 of the perfusate is 663 mm Hg in our laboratory. Coronary flow was measured by performing timed collections of the pulmonary effluent flow with a graduated cylinder.

After the 30-min stabilization period, control measurements of developed pressure (DP; peak systolic − EDP), EDP, peak positive dP/dt, coronary flow, oxygen consumption, and diastolic stiffness were made. Perfusion was then stopped, and the heart rendered globally ischemic with 60 ml of modified St. Thomas's cardioplegia. The intraventricular balloon was deflated and the heart maintained at 34°C in a circulating water jacket. Ischemia lasted for 120 min during which all hearts received 15 ml cardioplegia every 30 min. Control hearts received St. Thomas's cardioplegia while experimental hearts received 500 mg/liter pentoxifylline (Trental; Hoescht–Roussel, Somerville, NJ) in the cardioplegia.

Reperfusion was initiated at 80 mm Hg with 37°C perfusate. The balloon remained deflated for the first 15 min of reperfusion to simulate the beating, nonworking condition. If needed, defibrillation was performed within the first few minutes of reperfusion. After 15 min the balloon was reinflated to control volume, DP, EDP, dP/dt, coronary flow, oxygen consumption, and diastolic stiffness measurements were made. These measurements were also made at 30 and 45 min of reperfusion. Following 45 min of reperfusion, the hearts were removed from the perfusion column and water content determinations were made. The myocardium was weighed, dessicated for 48 hr at 80°C, and reweighed. Water content was determined using the formula [(1 − dry wt/wet wt) × 100 − %water].

A parallel simultaneous series of experiments was performed using the same protocol in order to obtain nucleotide and nucleoside levels in the left ventricular myocardium. Nucleotide and nucleoside levels were also determined in the reperfusion coronary effluent. To assay nucleotide and nucleoside levels, left ventricular myocardial tissue was biopsied and snap frozen in liquid nitrogen. Tissue was lyophilized for 24–48 hr at −40°C and under a 200-torr vacuum to remove water and prevent the degradation of nucleotides and nucleosides. Tissue aliquot duplicates were weighed (10–20 mg) and placed in conical tubes after which 2.5 ml of 0.73 M trichloroacetic acid was added. Samples were then homogenized with a Brinkman homogenizer (Model PT 10/35) for 15 sec, vortexed, and centrifuged (2900 rpm at 4°C, GPR $\approx$ 1700 g) for 2 min to separate the pellet. Supernatant (1.8 ml) was removed, equal volumes tri-n-octylamine and freon were added, and the sample was subsequently vortexed for 30 sec then centrifuged for 5 min as above. The aqueous phase was pipetted into limited-volume inserts and stored at −70°C for later automatic sampling injection using Waters WISP 712 and column peak separation with a μBondpac C^{18} column (3.9 × 300 mm).

Mobile phase was prepared in the following manner. Buffer A consisted of 10% acetonitrile (acn) in distilled, deionized water, 1.47 mM tetrabutylammonium phosphate (TBAP) as a pairing ion, and 73.5 M potassium dihydrogen phosphate (pdp). Buffer B consisted of 10% acn/water, 1.33 mM TBAP, and 66 M pdp. The final concentration of acn/water was adjusted by a two-pump control method for achieving optimum peak resolution and separation of nucleotides. A 2.5% acn/water final concentration and a 1.6 ml/min flow rate separated ATP at a retention time of 60 min. Standards used were ATP, ADP, AMP, and IMP for nucleotides and adenosine, hypoxanthine, xanthine, and inosine for nucleosides. All were purchased from Sigma Chemical (St. Louis, MO). Standard curves were generated from serial dilutions of standards at 10, 25, 50, 100, and 500 μM/liter. Peak areas from standards were integrated and least square curves plotted. A Waters 484 uv absorbance detector was set at 254 nm λ_{max} (AUFS 0.005) for all nucleotide and nucleoside determinations. Statistical analysis of all functional and HPLC data was performed using a Student t test and analysis of variance with a P value less

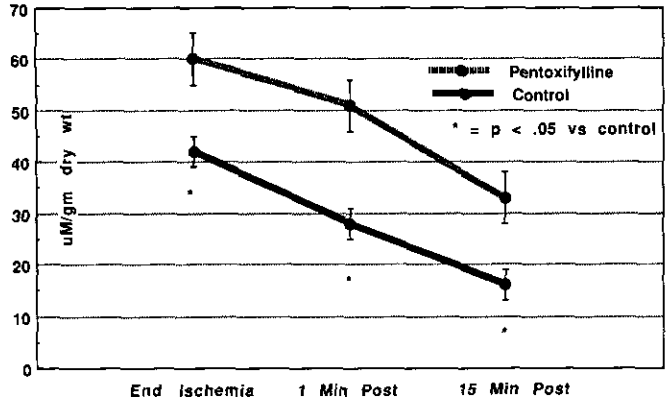

FIG. 1. Total nondiffusible nucleotide levels (ATP, ADP, AMP, and IMP) at end ischemia, 1 and 15 min following reperfusion, comparing control hearts to pentoxifylline-treated hearts. Nucleotide levels are presented as μM nucleotide/g dry wt left ventricular tissue. * $P < 0.05$ vs controls.

than 0.05 considered significant. Results are expressed as means ± standard deviation. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Pub. No. 80-23, revised 1978).

RESULTS

Functional Results

No significant differences existed in preischemic baseline values for DP, EDP, dP/dt, coronary flow, oxygen consumption, or diastolic stiffness between any of the groups. In terms of systolic function following 120 min of hypothermic ischemia and 45 min of reperfusion, control hearts recovered 37 ± 8% of developed pressure and 43 ± 10% of $+dP/dt$. Pentoxifylline (PEN)-treated hearts exhibited significantly better left ventricular function with a 59 ± 8% recovery of developed pressure and a 69 ± 10% recovery of $+dP/dt$.

In terms of diastolic function following 120 min of hypothermic ischemia and 45 min of reperfusion, control hearts recovered 38 ± 10% of $-dP/dt$. PEN-treated hearts exhibited significantly better recovery of $-dP/dt$ at 66 ± 5%. Left ventricular end-diastolic pressure was significantly lower during reperfusion in PEN-treated hearts than in control hearts. Control hearts exhibited an elevated end-diastolic pressure following 45 min of reperfusion with a 19 ± 11 mm Hg increase in pressure. PEN-treated hearts displayed only a 7 ± 5 mm Hg increase in pressure following 45 min of reperfusion. The slope of linearized end-diastolic pressure vs end-diastolic volume curves was determined for each heart in the prearrest period as well as during reperfusion. Diastolic stiffness in the PEN-treated hearts was significantly lower at all times during reperfusion compared to untreated controls. After 45 min of reperfusion the control hearts were characterized by a diastolic stiffness slope value of 80 ± 10 (an 8.0 ± 1.0 mm Hg increase in end-diastolic pressure for each 0.1 ml increase in end-diastolic volume) vs a diastolic stiffness value of 37 ± 6 in the PEN-treated hearts.

Recovery of myocardial oxygen consumption as a percentage of preischemic baseline in PEN-treated hearts was also significantly better during reperfusion than in control hearts. After 45 min of reperfusion, PEN-treated hearts had a myocardial oxygen consumption of 91 ± 17% of preischemic baseline versus 51 ± 19% in control hearts. There were no significant differences in coronary flow at any time after reperfusion between control and PEN-treated hearts. There were no significant differences in myocardial water content between control and PEN-treated hearts.

Nucleotide/Nucleoside Results

To assess nucleotide and nucleoside levels in the myocardium, total nondiffusible nucleotides (TNN = ATP, ADP, AMP, and IMP) as well as total diffusible nucleosides (TDN = adenosine, inosine, hypoxanthine, and xanthine) were measured using HPLC. These levels were assayed in control hearts (CTL) and pentoxifylline-treated hearts (PEN) at baseline, at the end of the ischemic period, at 1 min of reperfusion, and at 15 min of reperfusion. (see Figs. 1–3) All results are shown as micromolar nucleotide/nucleoside per gram dry weight of left ventricular cardiac tissue and expressed as means ± standard deviation.

During the baseline period, TNN (Fig. 1) for all hearts was 53 ± 7, while TDN (Fig. 2) was 5 ± 2. At the end of the ischemic period, TNN for CTL was 42 ± 2, while

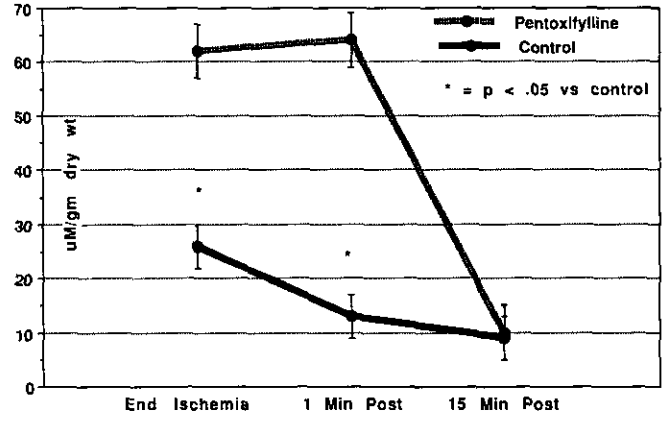

FIG. 2. Total diffusible nucleoside levels (adenosine, inosine, hypoxanthine, and xanthine) at end ischemia, 1 and 15 min following reperfusion, comparing control hearts to pentoxifylline-treated hearts. Nucleoside levels are presented as μM nucleotide/g dry wt left ventricular tissue. * $P < 0.05$ vs controls.

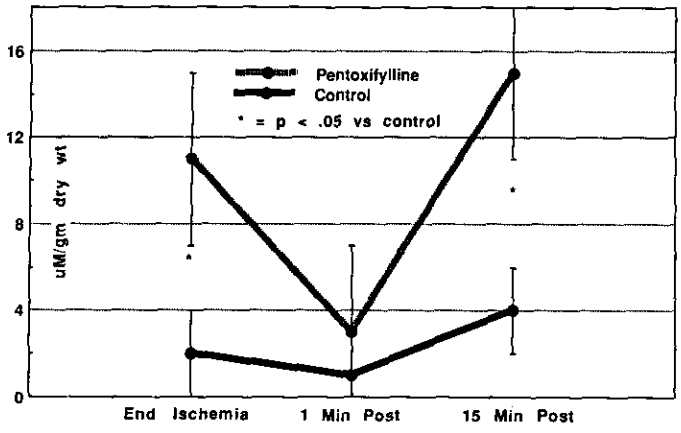

FIG. 3. Adenosine triphosphate levels at end ischemia, 1 and 15 min following reperfusion, comparing control hearts to pentoxifylline-treated hearts. ATP levels are presented as μM nucleotide/g dry wt left ventricular tissue. * $P < 0.05$ vs controls.

TDN was 26 ± 5. At the end of the ischemic period, PEN exhibited significantly ($P < 0.05$) higher TNN and TDN with values of 60 ± 4 and 62 ± 7, respectively. As expected, pentoxifylline appears to have trapped nucleotides in the cells by rendering them nondiffusible.

After 1 min of reperfusion, TNN and TDN remained significantly higher in PEN than in CTL. TNN was 51 ± 7 in PEN vs 28 ± 5 in CTL, while TDN was 64 ± 4 in PEN vs 13 ± 4 in CTL (both $P < 0.05$). After 15 min of reperfusion TDN in PEN at 10 ± 4 was not significantly higher than TDN in CTL at 9 ± 4. However, after 15 min of reperfusion TNN still remained significantly higher in PEN than in CTL with TNN values of 33 ± 5 vs 16 ± 3, respectively.

ATP levels at baseline were 22 ± 8. At the end of the ischemic period, ATP for CTL was 2 ± 2 while ATP in the PEN group was 11 ± 9, which was significantly ($P < 0.05$) higher (Fig. 3). Furthermore, ATP levels were assessed following reperfusion to determine whether resynthesis had been accelerated in PEN-treated hearts. The ATP levels at 1 and 15 min of reperfusion in PEN were 3 ± 2 and 13 ± 8, respectively, significantly higher than the ATP levels of 1 ± 1 and 4 ± 1, respectively, at 1 and 15 min of reperfusion in CTL. Finally, the rate of increase of ATP was higher in the PEN-treated hearts, suggesting that ATP was resynthesized more rapidly in PEN.

In addition to myocardial tissue nucleoside levels, nucleosides were also measured in coronary sinus effluent immediately upon reperfusion. TDN as measured in control hearts was 341 ± 206 μM while TDN in PEN hearts was significantly lower at 147 ± 69 μM, indicating that PEN trapped the nucleotide precursors more effectively.

DISCUSSION

Poor cardiac function after ischemia is thought to arise in part from the depletion of high-energy phosphates [1, 3–7]. The high-energy phosphates are broken down into diffusible precursors which diffuse out of and leave the myocyte. The enzyme 5NT is responsible for this breakdown by dephosphorylating AMP to adenosine and IMP to inosine [8]. Once the precursors have diffused out of the myocyte, they are no longer available for resynthesis of high-energy phosphates upon reperfusion of the ischemic cardiac tissue. Furthermore, isolated myocyte studies have shown that salvage and resynthesis of ATP from intracellular precursors is eight times that of myocytes dependent on extracellular adenosine [9]. This scenario severely impairs cardiac recovery following ischemia, as many studies have demonstrated that postischemic cardiac function can be roughly correlated with the availability of precursors at end of ischemia [1, 5, 7, 10].

Neonatal hearts, which are more resistant to ischemia, have low 5NT activity, which has been well documented. One study [11] correlated 5NT activity with functional recovery by comparing adult and neonatal rabbit hearts. Adult hearts had greater baseline 5NT activity (13,275 ± 2060 nmole/mg vs neonatal hearts 3900 ± 300 nmole/mg). When subjected to ischemia followed by reperfusion, neonatal hearts recovered function better than adult hearts. Also, when adult hearts were reperfused with theophylline, they recovered 72% of baseline function compared to only 48% recovery in untreated adult hearts. Additionally one study showed that embryonic myocardial ATP and AMP levels were maintained higher than those in adult myocardium when measured after normothermic ischemia [12]. Finally, a human study compared the adenine nucleotide levels of patients 2 months to 8 years of age undergoing cardiac operations. [13] In patients younger than 18 months of age, ATP loss was lower and AMP and inosine accumulated. These findings suggest that a 5NT deficiency exists up to 18 months in humans. Therefore, using a "neonatal" strategy of trapping nondiffusible precursors in the myocyte by decreasing 5NT activity may be a beneficial strategy for improving postischemic cardiac function.

Pentoxifylline, a theophylline-derived compound, is an inhibitor of 5NT. A study in rat kidneys showed that pentoxifylline treatment enhanced the recovery of renal energy metabolism through improved recovery of nucleotide precursor profiles [14, 15]. ATP was also higher in pentoxifylline-treated kidneys than in control kidneys. Previous studies from this laboratory showed enhanced myocardial protection and functional recovery during global ischemia with the 5NT inhibitor pentoxifylline [2]. The present study tried to elucidate the mechanism of this protection by assaying nucleotide levels of pentoxifylline-treated hearts. The results clearly indicate that treatment with pentoxifylline resulted in preservation of precursors, presumably by inhibition of 5NT activity. Both total nondiffusible nucleotides and, interestingly, total diffusible nucleosides were significantly

elevated. The elevation of total diffusible nucleosides, which was only noted up until 1 min following reperfusion, is probably due to the interruption in the kinetics of nucleotide breakdown by pentoxifylline, forcing a higher state of equilibrium. Additionally, total resynthesis and rate of resynthesis of ATP appeared augmented due to the increased precursor pool. Thus, pentoxifylline seems to exert its beneficial effect in adult hearts using a "neonatal" strategy by inhibiting 5NT activity.

Although pentoxifylline does exhibit 5NT inhibitory effects, other postulated actions of pentoxifylline could effect outcome following cardiac ischemia. Recently, pentoxifylline was shown to have beneficial effects on hepatocellular function after trauma-induced hemorrhage and subsequent resuscitation [16]. The beneficial effect was felt to be due to downregulation of the inflammatory cytokines TNF and IL-6. In other investigations [17], pretreatment of animals with pentoxifylline inhibited lipopolysaccharide-induced tumor necrosis factor in a dose-dependent fashion. Tumor necrosis factor, a mononuclear-derived peptide produced in response to lipopolysaccharide mediates certain aspects of septic shock. Pentoxifylline prevented sequestration of neutrophils in animals given intravenous lipopolysaccharide and protected animals from the lethal effects of a challenge with lipopolysaccharide, by inhibiting lipopolysaccharide-induced tumor necrosis factor and cytokine effects.

Pentoxifylline has also been used clinically as a treatment for patients with claudication. The benefit to these patient may be due to altered nucleotide profiles in red cells, leading to increased red cell deformability [18]. Furthermore, pentoxifylline has been shown to decrease the pool of circulating activated neutrophils and to reduce neutrophil adhesion to endothelium [19]. All of these reported benefits of pentoxifylline apply to *in vivo* blood-perfused models. Since our model is an isolated, crytalloid-perfused model, these mechanisms of pentoxifylline are not likely to be responsible for the results demonstrated in the present study.

When nucleotide precursors accumulate to adenosine concentrations higher than 0.1 to 0.3 m*M*/liter which can occur with 5NT inhibition, there can be pronounced vasodilation [20]. However, in a crystalloid-perfused isolated heart, maximal vasodilation occurs at all times, probably because of low total oxygen delivery. Therefore, any favorable action of pentoxifylline can not be attributed to an increase in coronary flow, as was confirmed by our coronary flow data, which did not demonstrate any difference in coronary flow between groups.

Other investigators [21] showed that pentoxifylline improved tissue oxygenation after hemorrhagic shock. This improved oxygenation effect was hypothesized to result from enhanced microcirculatory blood flow due to alterations in red cells themselves or blood viscosity changes even though the mechanism remains unclear and has not been elucidated. While the present study did show improved oxygen extraction and MVO_2 following reperfusion in pentoxifylline-treated hearts, this improved extraction is consistent with previous studies, which showed improved oxygen extraction and MVO_2 with augmented intracellular adenosine alone [1]. The increased oxygen extraction in the present study is thought to arise from the increased intracellular ATP precursor pools enhancing postischemic metabolic function, not from a direct effect of pentoxifylline on oxygen extraction.

In conclusion, this study demonstrated that pentoxifylline significantly improved functional recovery of the myocardium following an ischemic insult and subsequent reperfusion. Intracellular ATP precursor levels remained elevated at end ischemia due to inhibition of their breakdown and washout from the myocyte. ATP resynthesis was therefore augmented because of the greater availability of precursors. Presently, adults undergoing coronary bypass are being treated with nucleotide precursor-preserving tactics and inhibition of 5NT may prove a useful addition in cardiac surgery, especially in those adult patients at risk for myocardial injury during ischemia.

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