EXPERIMENTAL STUDIES

EVALUATION OF THE EFFECTIVENESS OF
5'-NUCLEOTIDASE INHIBITOR AND ALLOPURINOL
IN MYOCARDIAL ISCHEMIA

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The effect of 5'-nucleotidase inhibitor (AMP-C) and xanthine oxidase inhibitor (Allopurinol: ALLO) on myocardial functional recovery and the restoration of myocardial high energy phosphates after 15 min of normothermic global ischemic insult, was studied in the isolated isovolemic Langendorff rat heart model. Fifty nine rats were divided into 4 groups: Group I; saline, Group II; AMP-C plus ALLO, Group III; AMP-C, Group IV; ALLO. Intermittent infusion of drugs was delivered in 3 ml of solution at 5 min intervals during ischemia. Percent recovery of left ventricular systolic function was as follows: Group I; 74.2±3.6%, Group II; 87.7±1.7%, Group III; 83.5±3.1%, Group IV; 86.4±2.6%. Improved recovery was statistically significant only in Group II (p<0.05 vs Group I). Suppression of reactive hyperemia was seen with reperfusion in the groups which had been treated with AMP-C (i.e., Groups II and III). Myocardial adenine nucleotides and purines were measured in 6 hearts in each group using high performance liquid chromatography. Myocardial ATP levels was 0.89±0.16 nmol/mg left ventricular wet weight in Group I, 1.37±0.12 in Group II (p<0.05 vs Group I), 1.42±0.17 in Group III (p<0.05) and 1.17±0.15 in Group IV. This study demonstrates that intermittent infusion of AMP-C plus ALLO during global myocardial ischemia results in improved myocardial functional recovery and improved preservation of high energy phosphates.

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Although many theories concerning the pathophysiology of post-ischemic myocardial dysfunction have been reported, one of the main causes of depressed myocardial function after reperfusion is believed to be “stunned myocardium”1—3. Perioperative hemodynamic impairments associated with stunned myocardium may present some problems during open heart surgery, especially in a patient with a hypertrophied left ventricle. Delayed myocardial recovery after ischemic arrest has been attributed to reperfusion injury secondary to oxygen free radicals and impaired adenosine triphosphate (ATP) regeneration, possibly due to loss of ATP precursors4—10.

Xanthine is a major source of oxygen free radicals. Allopurinol (ALLO) inhibits xanthine oxidase, which catalyzes the production of xanthine from hypoxanthine. Although many studies using allopurinol for the prevention of reperfusion injury have been published, the protective effect of this drug in myocardial ischemia is still controversial11—15. Furthermore, 5'-nucleotidase inhibitor may reduce the loss of ATP

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precursors by inhibiting dephosphorylation of adenosine monophosphate (AMP)\textsuperscript{16,17}.

Therefore, the combined use of ALLO and 5'-nucleotidase inhibitor may reduce ischemic injury by inhibiting the loss of high energy phosphate precursors, and may also reduce reperfusion injury by diminishing the source of injurious oxygen free radicals. It is unclear whether the simultaneous use of these 2 agents will improve functional recovery after ischemia.

In this study, intermittent infusion of allopurinol and 5'-nucleotidase inhibitor *alpha*, beta-methylene ADP: AMP-C) was performed during 15 min of normothermic global ischemic arrest in the isolated isovolemic Langendorff rat heart model.

**MATERIAL AND METHODS**

Fifty nine male Lewis rats weighing 250 to 300 g were used in this study. Each animal was given sodium pentobarbital (60 mg/kg body weight) and heparin (1,000 unit/kg) intraperitoneally. The heart was removed and quickly placed into cold saline (4 °C). Within 3 min, the aortic root was cannulated and the heart was perfused by a modified Langendorff apparatus (Fig. 1). The perfusate was Kumpel solution (NaCl: 109.0 mMol/l, K$_2$HPO$_4$: 1.20 mMol/l, KCl: 4.71 mMol/l, CaCl$_2$: 1.75 mMol/l, Na$_2$EDTA: 0.50 mMol/l, MgSO$_4$: 1.20 mMol/l, NaHCO$_3$: 25.0 mMol/l, Dextrose: 11.0 mMol/l and anhydrous pyruvate: 10.0 mMol/l). The perfusate was bubbled with 95% O$_2$ and 5% CO$_2$. The perfusion pressure was maintained at 100 mmHg by the height of the perfusate reservoir. A saline-filled latex balloon was inserted into the left ventricle through the left atrial appendage and mitral valve, and was connected to a pressure transducer to allow measurement of left ventricular pressure. The volume of the balloon was adjustable and permitted control of left ventricular end-diastolic pressure (LVEDP). Data were recorded on a strip recorder.

After 20 min of stabilization, left ventricular performance was examined by increasing LVEDP in a stepwise manner. Aortic root and coronary sinus effluent samples were drawn for determination of myocardial oxygen consumption (MVO$_2$) at an LVEDP of 5 mmHg. A glass cardiac cradle was filled with coronary sinus effluent and sealed with a latex membrane to avoid oxygen contamination from room air. Perfusion flow was measured with an in-line flow meter and coronary resistance was calculated as:

Coronary Vascular Resistance (CVR) = [Perfusion pressure (100 mmHg)]/[Coronary

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flow (ml/min)/[Heart weight (grams)]

After pre-ischemic evaluation, global ischemia was produced by complete cross clamping of the aortic perfusion line for 15 min. Intermittent infusions of saline (Group I: n=15), AMP-C+ALLO (Group II: n=16), AMP-C (Group III: n=14) and ALLO (Group IV: n=14) were administered at 5 min intervals during ischemia. AMP-C and ALLO were diluted in deoxygenated saline. The AMP-C and ALLO solutions were adjusted to 250 ng and 20 mg/l, respectively. Each intermittent drug injection was carried out in 3 ml of solution over 30 sec. Ischemia was terminated by release of the clamp, and coronary flow measurements were taken at 0, 3, 5, 10, and 20 min. Samples for myocardial oxygen consumption were taken 3 and 20 min after reperfusion.

LVEDP was maintained at 5 mmHg throughout the experiment, except during left ventricular (LV) function analysis. Left ventricular wet weight was measured at the end of the experiment. The experimental protocol is shown in Fig. 2.

Six hearts in each group were prepared for evaluation of adenine nucleotides and purines by high performance liquid chromatography (HPLC: Altex Model 322 Gradient Liquid Chromatography System (Rainin Instrument Co. Inc., Woburn, MA)). The left ventricular apex was biopsied, quickly frozen in liquid nitrogen and stored at -70°C. Frozen samples were homogenized in a Polytron Homogenizer at the temperature of liquid nitrogen and neutralized with 1.0 M KH₂PO₄ buffer (pH: 7.74). For determination of ATP and adenosine diphosphate (ADP), 100 μl of neutralized supernatant was applied to a Radical Pak SAX-10 μ column cartridge. Adenosine monophosphate (AMP), adenosine, hypoxanthine and xanthine levels were determined with 200 μl of neutralized supernatant applied to a C-18 μ Bondapak column cartridge. The adenine nucleotides and purines were quantitated by comparing peak heights with those of standards. All values are expressed in nanomoles per mg of LV wet weight.

To evaluate the preservation of high energy phosphate, energy charge index and phosphorylate intermediates index were calculated by the following formulas\(^{18}\):

Energy charge index = (ATP + 1/2 × (ADP))/(ATP + ADP + AMP)
Phosphorylate intermediates index = ATP + ADP + AMP.

Values are presented as mean ± standard error. Statistical analysis was performed using repeated measures analysis of variance and analysis of variance, as appropriate. A p value, as determined with the F test, of less than 0.05 was regarded as statistically significant.

RESULTS

(I) Left ventricular performance

Fig. 3 shows myocardial systolic functional recovery (developed LVP) in each treatment group. There was no difference between the recoveries in Group III and Group I. Im-
proved systolic functional recovery was obtained in Group II with a pre-load of over 5 mmHg (LVEDP) and in Group IV with a pre-load of over 15 mmHg (LVEDP), as compared to Group I.

(II) Coronary vascular resistance

Fig. 4 shows significant changes in coronary vascular resistance after ischemia in each group. Significant decreases in coronary vascular resistance were seen in each group following reperfusion. However, the coronary vascular resistance soon after the beginning of reperfusion was significantly higher in the groups which had received AMP-C (i.e., Groups II and III) than in the control group. Although there was slight suppression of reactive hyperemia in group IV for the initial 5 min of reperfusion, coronary vascular resistance returned to the same level as that in the control group after 10 min of reperfusion.

(III) Myocardial oxygen consumption

Although MVO₂ increased about 40% following reperfusion, no significant differences were detected between the groups. MVO₂ returned to the pre-arrest value after 20 min of reperfusion in every group (Table I).

(IV) Adenine nucleotides and purines

(1) ATP

Table II shows the myocardial ATP levels after 20 min of reperfusion. The ATP level was 0.89±0.16 nmol/mg LV wet weight in Group I, 1.37±0.12 in Group II (p<0.05 vs Group I), 1.42±0.17 in Group III (P<0.05 vs Group I) and 1.17±0.15 in Group IV (NS vs Group I). Thus, 5'-nucleotidase inhibitor tended to produce better preservation of intracellular ATP. ALLO had no beneficial effect on tissue ATP levels after myocardial

* : P<0.05
** : P<0.01
AMP-C and Allopurinol in Myocardial Ischemia

Fig.4. Changes in coronary vascular resistance
Reactive hyperemia after reperfusion was strongly suppressed in the groups which received 5'-nucleotidase inhibitor.

**TABLE I** MYOCARDIAL OXYGEN CONSUMPTION DURING THE COURSE OF THE EXPERIMENT IN EACH GROUP

<table>
<thead>
<tr>
<th></th>
<th>Pre-arrest</th>
<th>Just after reperfusion</th>
<th>After 20 min of reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>5.45 ± 1.16</td>
<td>7.45 ± 1.71</td>
<td>6.00 ± 1.06</td>
</tr>
<tr>
<td>Group II</td>
<td>4.50 ± 0.52</td>
<td>6.67 ± 1.09</td>
<td>5.80 ± 0.98</td>
</tr>
<tr>
<td>Group III</td>
<td>6.65 ± 0.63</td>
<td>8.64 ± 0.59</td>
<td>7.27 ± 0.68</td>
</tr>
<tr>
<td>Group IV</td>
<td>5.98 ± 0.86</td>
<td>9.12 ± 1.01</td>
<td>6.38 ± 0.43</td>
</tr>
</tbody>
</table>

All values (mmol/min/LV (wt. weight g)) are presented as the mean ± standard error.

ischemia.

(2) Energy charge index
With regard to the energy charge index, no significant differences were observed between any of the groups (Table II).

(3) Phosphoryl intermediates
The preservation of phosphorylate intermediates was 2.76 ± 0.28 nmol/mg LV wet weight in Group I, 3.77 ± 0.21 in Group II (p < 0.05 vs Group I), 3.55 ± 0.24 in Group III (p = 0.058 vs Group I) and 4.21 ± 0.36 in Group IV (p < 0.05 vs Group I).

(4) Purines
Myocardial adenosine, hypoxanthine and xanthine levels after 20 min of reperfusion are presented in Table II. No significant differ-ences were observed between the myocardial levels of purine compounds in any of the groups.

**DISCUSSION**

There is widespread interest in improving functional recovery while minimizing myocardial necrosis and myocardial stunning after ischemic insult during cardiac surgery. Myocardial protection during cardiac surgery is currently achieved by using a cardioplegic solution during ischemia. After the recent introduction of this method, massive myocardial necrosis is now rarely observed. However, the low output syndrome which is related to myocardial stunning still presents many problems perioperatively. Among the many potential causes of myocardial stunning after myocardial ischemia, reperfusion injury by oxygen free radicals and diminished restoration of high energy phosphates are believed to have the greatest effect.

This study has demonstrated improved functional recovery and preservation of ATP following global myocardial ischemia by the use of 5'-nucleotidase inhibitor and allopurinol. Many studies have reported improved myocardial recovery after ischemia using a variety of methods intended to restore the
TABLE II THE EFFECTS OF 5'-NUCLEOTIDASE INHIBITOR AND ALLOPURINOL ON MYOCARDIAL STORAGE OF HIGH ENERGY PHOSPHATES AND PURINES AFTER 15 MIN OF GLOBAL ISCHEMIA AND 20 MIN OF REPERFUSION

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.89±0.16</td>
<td>1.37±0.12*</td>
<td>1.42±0.17*</td>
<td>1.17±0.15</td>
</tr>
<tr>
<td>ADP</td>
<td>1.03±0.09</td>
<td>1.31±0.06</td>
<td>1.17±0.06</td>
<td>1.39±0.04</td>
</tr>
<tr>
<td>AMP</td>
<td>0.85±0.11</td>
<td>1.09±0.14</td>
<td>0.96±0.10</td>
<td>1.65±0.31*</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.06±0.02</td>
<td>0.05±0.02</td>
<td>0.05±0.02</td>
<td>0.02±0.22</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.19±0.03</td>
<td>0.19±0.03</td>
<td>0.18±0.04</td>
<td>0.29±0.06</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.01±0.01</td>
<td>0.03±0.02</td>
<td>0.01±0.01</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td>Energy C.</td>
<td>0.50±0.04</td>
<td>0.54±0.03</td>
<td>0.56±0.03</td>
<td>0.45±0.04</td>
</tr>
<tr>
<td>Phos. Int.</td>
<td>2.76±0.28</td>
<td>3.77±0.21*</td>
<td>3.55±0.24</td>
<td>4.21±0.36*</td>
</tr>
</tbody>
</table>

(All values except those for Energy C. represent nmol/mg LV wet weight)

Energy C.: Energy charge index
Phos. Int.: Phosphorylate intermediates
p<0.05 vs Group I

The effects of exogenous ATP, ATP precursors and adenosine deaminase inhibitor during ischemia and/or reperfusion have been evaluated by many investigators\(^5-8,19-22\). These studies have yielded varying results.

Hearse et al reported that exogenous ATP infusion had a beneficial effect\(^19\). On the other hand, Reibel and Rovetto, and Glower et al showed that exogenous ATP had no myocardial protective effect\(^20,21\). Moreover, Reibel and Rovetto, Foker et al and Humphrey and Seelye reported that adenosine administration had no effect on recovery from myocardial ischemic injury\(^7,8,20\). Foker et al and Humphrey and Seelye added adenosine deaminase inhibitor to adenosine and obtained better preservation of adenosine nucleotides\(^7,8\). The data in the present study indicate that 5'-nucleotidase inhibitor alone improves ATP recovery following global ischemia.

It is well known that dephosphorylation of ATP to ADP and AMP is accelerated by an anoxic insult. Furthermore, AMP is degraded to adenosine by 5'-nucleotidase, and the enzyme activity is increased during periods of ischemia and reperfusion. Adenosine is permeable to myocardial cells. Phosphorylation of adenosine to form AMP, which is mediated by adenosine kinase, is the first step of the primary ATP regeneration salvage pathway\(^22-24\). Therefore, inhibition of AMP catabolization to adenosine may prevent loss of ATP precursors during ischemia. Ward et al did not observe any salutary effect on myocardial protection by administration of 5'-nucleotidase inhibitor alone\(^5\). Our results confirm their data: i.e., 5'-nucleotidase inhibitor did not improve post-ischemic myocardial performance. However, 5'-nucleotidase inhibitor did improve myocardial ATP levels and tended to suppress reactive hyperemia after reperfusion. This is most likely due to inhibition of adenosine (a coronary vasodilator) synthesis during ischemia. We did not detect any differences between the intracellular adenosine concentrations in the 5'-nucleotidase inhibitor group and the control group. This may reflect the sampling time used for the analysis of myocardial adenosine levels. After 20 min of reperfusion, most of the adenosine should have been washed from myocardial cells.

Oxygen free radicals, which have been proposed to be a major factor in reperfusion injury, are produced primarily from xanthine\(^25\). The ubiquitous enzyme xanthine oxidase, which has been demonstrated in rat myocardial cells, converts hypoxanthine to xanthine\(^26\). The controversy regarding the clinical applicability of allopurinol is whether or not the human heart contains xanthine oxidase. Although Krenitsky et al reported that xanthine oxidase showed a tissue activity of 70 mU/g in human heart samples, Eddy et al detected less than 2.0 mU/g of activity in heart samples from surgical patients and organ donors\(^27,28\).

Many investigators have used allopurinol in an attempt to prevent reperfusion injury.
after ischemia. However, the effects of allopurinol during myocardial ischemia have varied with different models and experimental designs. This study has shown that allopurinol induced better myocardial functional recovery only when LVEDP was above 15 mmHg. This was not associated with any changes in intracellular ATP levels. Apparently, a better recovery with a physiological pre-load is obtained when allopurinol and 5'-nucleotidase inhibitor are used together during ischemia. In addition, during the early stages of reperfusion, we noted that reactive hyperemia was slightly suppressed in the group which had been treated with allopurinol. This result may be explained by feedback inhibition during the degradation of adenosine nucleotides.

In the present study, there was no strong correlation between functional recovery and high energy phosphate stores in myocardial cells after 20 min of reperfusion. These data confirm those of Foker et al and Humphrey and Seelye.

In conclusion, intermittent infusion of solutions containing both allopurinol and 5'-nucleotidase inhibitor is effective in maintaining ATP levels, and may also diminish reperfusion injury by suppressing the production of oxygen free radicals. This treatment during global ischemia was effective in producing good myocardial recovery. Our study suggests that combined administration of 5'-nucleotidase inhibitor and xanthine oxidase inhibitor (Allopurinol) during myocardial ischemia may have beneficial metabolic effects resulting in improved myocardial recovery. Moreover, the use of these drugs may have some potential advantages in cardiac surgery involving a hypertrophied left ventricle, because high energy phosphate stores in the subendocardium are frequently diminished under these conditions.

REFERENCES

3. KLOWER RA, PRZYKLENK K, WHITTAKER P: Deleterious effects of oxygen radicals in ischemia/reperfusion. Resolved and unresolved

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10. VARY TC, ANGELAKUS ET, SCHAFFER SW: Relationship between adenosine nucleotide metabolism and irreversible ischemic tissue damage in isolated perfused rat heart. Circ Res 1979; 45: 218—225
12. PARKS DA, BULKLEY GB, GRANGER ON: Role of oxygen free radicals in shock, ischemia and organ preservation. Surgery 1983; 94: 428—432
14. REIMER KA, JENNINGS RB: Failure of the xanthine oxidase inhibitor allopurinol to limit infarct size after ischemia and reperfusion in dogs. Circulation 1985; 71: 1069—1075
17. NAITO Y, LOWENSTEIN JM: 5'-nucleotidase from rat heart. Biochemistry 1981; 20: 5188—5194
19. HEARSE DJ, STEWART DA, BRAIMBRIDGE MV: Cellular protection during myocardial ischemia: The development and characterization of a
27. KRENTISKY TA, TUTTLE JV, CATTAU EL, WANG PA: Comparison of the distribution and electron acceptor specifications of xanthine oxidase and aldehyde oxidase. *Comp Biochem Biophys* 1974; 49: 687－692
29. PEYTON RB, JONES RN, ATTARIAN D, SINK JD, VANTRIGT P, CURRIE WD, WECHSLER AS: Depressed high energy phosphate content in hypertrophied ventricles of animals and man. The biological basis for increased sensitivity to ischemic injury. *Ann Surg* 1982; 196: 278－284