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Comparison of Protection by Propranolol, Bepridil, Verapamil, and Captopril on Depleting ATP, ADP, and AMP in Heart, Brain, and Liver by Anoxia Plus Isoprenaline

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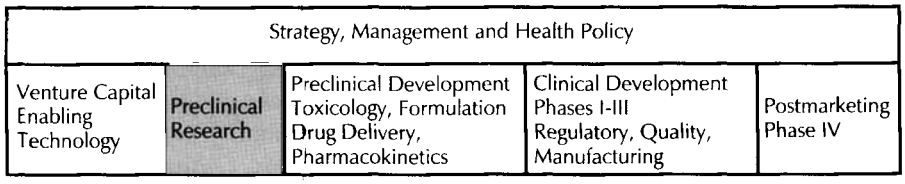

ABSTRACT Mice were injected with 5 mg/kg isoprenaline 45 min prior to being subject to 12 min global anoxia and ATP, ADP, and AMP were assayed in the heart, brain, and liver by reverse phase HPLC with UV detection at 254 nm. In myocardium the depletion of ATP by global anoxia was 72.5% of the total and only 7.5% due to the addition of isoprenaline. In the brain and liver, globally anoxic depletion of ATP was much less; a large fraction of ATP depletion was caused by isoprenaline. The depletion of ATP, ADP, and AMP in the heart by anoxia plus isoprenaline was partially protected by propranolol, and the protection by bepridil, verapamil, and captopril was weak. In the cerebrum bepridil, propranolol, and verapamil were potent protectors of ATP and total high energy bonds. Bepridil was the most effective, and its protection against cerebral ischemia was reproduced by limiting the infarcted zone and relieving the abnormal behavior after occlusion of the middle cerebral artery in rats. In liver all four drugs exerted a mild protection. In summary, isoprenaline is more toxic to the brain and liver in depleting ATP in the presence of global ischemia and the protection by bepridil against cerebral ischemia is attributed to its dual blocking effect on sodium and calcium channels. Drug Dev. Res. 39:125–130. © 1997 Wiley-Liss, Inc.

Key words: adenosine triphosphate; ischemia; cerebral anoxia; propranolol; verapamil; bepridil; captopril

INTRODUCTION

Beta-blockers, calcium antagonists, and angiotensin converting enzyme inhibitors are effective in protection of the heart against ischemic injury [Rong et al., 1990a,b]. Bepridil, a calcium antagonist which also blocks sodium channels, is used in the treatment of myocardial ischemia and arrhythmias [Cosnier et al., 1977; Vogel et al., 1979; Kane and Winslow, 1980; Harder and Sperelakis, 1981; Hasegawa, 1988; Fuchs et al., 1988; Winslow et al., 1983; Sharma et al., 1988]. It is well known that an excess of isoprenaline (Isop) can produce an infarct-like lesion in the heart. Catecholamines, which may be harmful to the cerebrum, are released under stress and may precipitate stroke in the presence of cerebral ischemia. A stress state may also cause a further impairment in a compromised liver.

The mechanisms by which an altered Ca^{2+} homeostasis induces cell injury are poorly understood in detail [Borgerds et al., 1991]. However, it is generally assumed that ATP depletion during ischemia triggers an impairment of ion homeostasis. A cellular over-load of calcium through L-type Ca^{2+} channels may not be the first event, since before the appearance of an excess of calcium ion, an increment of sodium ion in the cytosol is apparent [Silverman and Stern, 1994]. Blockade of sodium channels is considered to be a target for cerebral protection [Taylor and Meldrum, 1995]. The sodium channel blocking effect possessed by bepridil may afford it neuroprotective properties. Angiotensin converting enzyme

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inhibitors may also be beneficial to protect cerebral neurons from ischemic injury.

Stress is related to an over-activity of the sympathetic system which causes an excess of endogenous catecholamines to be released. The incidence of cerebral vascular insufficiency is increased in the aged population and in this case an excess of catecholamines might be harmful to the existing ischemic lesion. Accordingly, we have developed a dual ischemic model by combining global ischema with the addition of isoprenaline to determine whether this might cause a further reduction in ATP levels in the cardiac, cerebral, and hepatic tissues.

Protection of the brain, heart, and liver was compared among propranolol (Pro, beta-blockade combined with membrane stabilizing effect), bepridil (Bep, calcium antagonist with sodium channel blockade), verapamil (Ver, calcium antagonist), and captopril (Cap, angiotensin converting enzyme inhibitor) in terms of ATP preservation against the dual ischemic injury.

METHODS

Duplicate Ischemic Injury

Mice, ddy strain (18–23 g), were subjected to a dual anoxic procedure: mice were left in an asphyxiator for 12 min to develop global ischemia [Rong et al., 1990a] referred to as simple anoxia and dual anoxia was created by addition of isoprenaline (5 mg/kg sc) 45 min prior to asphyxia. After cervical dislocation the heart, brain, and liver were removed quickly and frozen immediately in liquid nitrogen.

HPLC Assay of ATP, ADP, and AMP

Tissue samples (100 mg) were homogenized in 0.4 M $HClO_4$ and centrifuged at 3,000 rpm for 10 min. The supernatant was neutralized to pH 7.5 with solid $KHCO_3$, and then centrifuged again at 3,000 rpm for 10 min. An aliquot of 20 µl was injected into an HPLC system for nucleotide determination.

The assays were based on previous work [Gu et al., 1989; Zhu et al., 1994] and were briefly as follows: the composition of the mobile phase was (M): KH_2PO_4, 0.0667, KH_2PO_4, 0.0997, prepared in redistilled water, mixed 1 to 9 in volume to produce a pH of 5.92. The speed of the mobile phase was 1.5 ml/min. The wavelength used was 254.0 nm. ATP was a product of Boehringer Mannheim Gbh., Mannheim, Germany, Na_2-5-ADP from Fluka Chemical AG, Buchs, Switzerland, and AMP from Sigma (St. Louis, MO).

Occlusion of Middle Cerebral Artery in Rats

Rats of the Sprague-Dawley Strain of either sex and of body weight 200–260 g were used. The occlusion of the middle cerebral artery (MCA) was performed [Garcia, 1984; Karpiak et al., 1989] under urethane anesthesia (1 5 g/kg, ip). Observations were made 24 h after the operation and a scoring system of assessing changed behavior compared with the sham-operated group in which the skull was opened without occlusion of the MCA. The score system for assessment of the abnormal behavior was set as follows: 0, no abnormal behavior; 1, slight imbalance in walking and inclined to the opposite side on which the weakness of muscular tone was noticed; 2, imbalance and decline and walking in large circles toward the opposite side; 3, imbalance and decline and walking in small circles; 4, standing silently, inclined markedly to one side and not walking in 3 min.

After recording the changes in behavior, the brain was removed and six slices were made and the infarcted size was determined by the TTC technique [Yu et al., 1991] and expressed as the percentage of the infarcted mass over the whole cerebrum.

Calculation and Statistics

A calibration curve was prepared by injecting six concentrations of ATP, ADP, and AMP (n = 3), yielding a linear regression equation for: ATP, $Y = 0.08206x + 0.06364$, $r = 0.9981$; ADP, $Y = 0.03886x - 0.03362$, $r = 0.9994$; AMP, $Y = 0.068148x - 0.227$, $r = 0.9998$. The within-day variation was assayed with four doses, each being an average of three measurements (CV 2.2 –6.5%). The retention times of ATP, ADP, and AMP were 4.4, 5.0, and 11.26 min, respectively. The height of the peak was used to calculate the amount detected and the units are presented as µmol/g of tissue. Data are presented as means ± s.e. The total high energy bond (HEB) of nucleotide phosphates is the sum of 3ATP + 2ADP + AMP. An energy index (EI) was defined as the ration of 3ATP/HEB.

The Student's *t*-test was used for the statistical significance set at $P < 0.05$ based on one-way analysis of variance between groups.

RESULTS

Dual Depletion of ATP, ADP, and AMP

During global anoxia for 12 min the depletion of ATP in the heart, brain, and liver was 13, 31, and 35% ($P < 0.001$), respectively. The extent of ATP depletion was twice as much as in the myocardium as in cerebral and hepatic tissues. The depletion of ADP and AMP varied between organs, but changes in HEB and EI during simple anoxia were consistent ($P < 0.001$) (Table 1).

Further depletion of ATP was evident in tissues following isoprenaline treatment. The decline in ATP was greatest in the brain (47%, $P < 0.001$), and liver (35%, $P < 0.001$) and much less in the heart (7%). The remain-

TABLE 1. Depletion of Nucleotide Phosphates in Cardial, Cerebral and Hepatic Tissue in Mice by Anoxia and Its Combination With Isoprenaline†

	μmol/g wet weight				
	ATP	ADP	AMP	HEB	EI
Heart					
Normal	3.32 ± 0.15	0.98 ± 0.02	2.69 ± 0.06	14.6 ± 0.52	0.68 ± 0.01
Anoxia	0.91 ± 0.12*	1.22 ± 0.16	2.23 ± 0.16	7.41 ± 0.48*	0.36 ± 0.03*
Anoxia and Isop	0.66 ± 0.06*	0.18 ± 0.02*	1.97 ± 0.09	4.30 ± 0.27*	0.45 ± 0.01*
Cerebrum					
Normal	2.19 ± 0.15	0.53 ± 0.04	1.57 ± 0.08	9.21 ± 0.57	0.71 ± 0.01
Anoxia	1.52 ± 0.02*	0.38 ± 0.02*	1.52 ± 0.07	6.83 ± 0.38*	0.66 ± 0.02
Anoxia and Isop	0.53 ± 0.05*,**	0.24 ± 0.03*,**	1.19 ± 0.09*,**	3.28 ± 0.24*,**	0.49 ± 0.2*,**
Liver					
Normal	2.03 ± 0.06	0.48 ± 0.02	1.79 ± 0.03	8.86 ± 0.24	0.69 ± 0.01
Anoxia	1.33 ± 0.07*	0.61 ± 0.01*	1.48 ± 0.05*	6.68 ± 0.25*	0.69 ± 0.59*
Anoxia and Isop	0.62 ± 0.04*,**	0.27 ± 0.01*,**	1.56 ± 0.01	3.96 ± 0.28*,**	0.47 ± 0.01*,**

†N = 10. x ± se. HEB: high energy bonds (3ATP + 2ADP + AMP); EI: energy index (3ATP/HEB); Isop: isoprenaline.
*$P < 0.001$, vs. normal.
**$P < 0.001$, vs. anoxia.

ing parameters were reduced significantly against simple anoxia ($P < 0.01$, $P < 0.001$) save for AMP in the heart and liver.

Protection Against Ischemic Depletion in the Heart

Propranolol was effective in protecting the heart from depletion of ATP and other parameters except the EI. The increment in ATP preserved by propranolol was 39% over untreated, reaching the level depleted by simple anoxia. The effects of bepridil, verapamil, and captopril were weak, but did raise the levels of ADP, AMP, and the HEB values, but not ATP (Table 2).

Protection Against ATP Depletion in Brain

In the brain significant preservation of ATP was afforded by propranolol (123%), bepridil (326%), and verapamil (96%) ($P < 0.001$) relative to control. The ATP protected, including the EI by bepridil, was at the control levels despite dual anoxia. Captopril exerted no effect at all on any measurement of decline (Table 3).

Protection Against ATP Depletion in Liver

All four drugs possessed a modest protective effect on ATP and HEB depletion ($P < 0.05$ to $P < 0.001$), but their effect on ADP and AMP was varied (Table 4).

Bepridil Limitation of Cerebral Infarction

After recovery for 24 h after MCAO, an infarcted zone occupying 14.6% of cerebral mass, including a part of the striatum, was defined and a reduction in spontaneous activities and the appearance of abnormal behavior were noticed. The latter are listed in Table 5. Bepridil given ip prior to MCAO was effective in limiting the infarcted size by 29% ($P < 0.05$) and improved the changes in behavior. A benefit in cerebral ischemic damage was also found with 41% reduction in the infarcted size and a beneficial effect to behavioral changes with nimodipine (Table 5).

DISCUSSION

The dual ischemic event in the heart, cerebrum, and liver from anoxia plus isoproterenol was shown by

TABLE 2. Comparison of Protective Effect of Propranolol, Bepridil, Verapamil, and Captopril on Myocardial ATP, ADP, AMP, and HEB and EI by Combining Anoxia With Isoprenaline†

	μmol/g wet weight				
Groups	ATP	ADP	AMP	HEB	EI
Untreated	0.66 ± 0.06	0.18 ± 0.02	1.97 ± 0.01	4.30 ± 0.27	0.45 ± 0.01
Propranolol	0.91 ± 0.04**	0.39 ± 0.02***	3.22 ± 0.11***	6.73 ± 0.28***	0.41 ± 0.01
Bepridil	0.60 ± 0.02	0.24 ± 0.01*	2.70 ± 0.09	4.99 ± 0.07*	0.36 ± 0.01
Verapamil	0.78 ± 0.03	0.30 ± 0.02***	3.23 ± 0.01***	6.19 ± 0.18***	0.38 ± 0.01
Captopril	0.67 ± 0.03	0.19 ± 0.01	2.46 ± 0.10***	4.85 ± 0.14	0.42 ± 0.01

†N = 10. x ± se. HEB: high energy bonds (3ATP + 2ADP + AMP); EI: energy index (3ATP/HEB).
*$P < 0.05$, vs. untreated.
**$P < 0.01$, vs. untreated.
***$P < 0.001$, from untreated.

TABLE 3. Protective Effect of Propranolol, Bepridil, Verapamil, and Captopril on Depletion of ATP, ADP, and AMP in Brain by Anoxia and Isoprenaline†

	μmol/g wet weight				
	ATP	ADP	AMP	HRB	EI
Untreated	0.53 ± 0.05	0.24 ± 0.03	1.19 ± 0.09	3.28 ± 0.24	0.49 ± 0.03
Propranolol	1.18 ± 0.08**	0.33 ± 0.02	1.96 ± 0.08**	3.28 ± 0.24**	0.57 ± 0.02*
Bepridil	2.6 ± 0.14**	0.44 ± 1.6**	2.11 ± 0.04	9.78 ± 0.40**	0.69 ± 0.02**
Verapamil	1.04 ± 0.14**	0.29 ± 0.02	1.60 ± 0.13*	5.30 ± 0.34**	0.57 ± 0.04
Captopril	0.64 ± 0.03	0.21 ± 0.01	1.30 ± 0.04	3.66 ± 0.07	0.53 ± 0.02

†N = 10. x ± se. HEB: high energy bonds (3ATP + 2ADP + AMP); EI: energy index (3ATP/HEB).
*$P < 0.05$ vs. untreated.
**$P < 0.001$ vs. untreated.

the two steps of depletion of ATP and its derivatives. Depletion of ATP in the myocardium after global or local ischemia has been widely studied [Reimer et al., 1981; Keith, 1981; Gu et al., 1989; Rong et al., 1990a,b; Zhu et al., 1994]. In our work isoproterenol is sufficient to cause further ATP depletion in tissues compared to simple global anoxia. Depletion of HEB was more uniform among organs. The EI is assumed to reflect the ration of ATP vs. HEB.

The extent of depletion of ATP and HEB by simple anoxia was greater in the heart (73%) than in the liver (34%) or cerebrum (31%). The order of ATP depletion by dual anoxia, however, in the three organs was different: cerebrum (65%), liver (53%), and heart (27%). The difference in depletion of HEB among the three organs was not large (Table 6).

ATP depletion in the heart is very sensitive to simple anoxia, while the cerebrum and liver are relatively tolerant, but more sensitive to isoproterenol. A delayed response of myocardial infarction and stroke is likely to follow stress in mice, which is related to the consequence of an excessive release of endogenous catecholamines. In the presence of ischemia, a harmful effect of catecholamines is expected, possibly more toxic to the cerebrum than to the heart.

In this global ischemia model the possibility of relief by increasing collateral blood flow is totally absent. Therefore, the protective effect of bepridil, verapamil, and captopril on the ischemic myocardium was greatly reduced and considered to be direct cellular protection [Boddeekje et al., 1989]. The results are compatible with our previous findings [Rong et al., 1990a].

In the brain further depletion of ATP by isoproterenol, which is protected by propranolol, may not be the result of beta-blockade, but rather from the membrane-stabilizing (Na^+ channel blockade) effect of propranolol. The effect of bepridil, which improves ATP preservation against ischemia, was greater in the cerebrum than in the heart and this may be mediated by a blockade of Na^+ channels and/or Na^+:Ca^2 reverse exchange [Silverman and Stern, 1994; Taylor and Meldrum, 1995]. The primary action of bepridil is likely to correct an increase in sodium ion in the cytosol and, thereafter, a reduction in cytosol Ca^{2+} and ATP depletion. A blockade of Na^+ channels may be important for neuroprotection [Taylor and Meldrum, 1995].

The neuroprotection by bepridil was confirmed by its limiting the infarcted size after MCAO. The observation period of the abnormal behavior was set at 24 h after the operation based on this suggestion. The extent of injury is likely to be more severe at this time from the com-

TABLE 4. Comparison of Beneficial Effect of Propranolol, Bepridil, Verapamil, and Captopril on the Depleted ATP, ADP, and AMP in Liver by Anoxia and Isoprenaline†

	μmol/g wet weight				
	ATP	ADP	AMP	HEB	EI
Untreated	0.62 ± 0.04	0.27 ± 0.01	1.56 ± 0.13	3.96 ± 0.28	0.47 ± 0.01
Propranolol	0.95 ± 0.04***	0.39 ± 0.01**	2.21 ± 0.07***	5.75 ± 0.17***	0.49 ± 0.01
Bepridil	0.79 ± 0.13*	0.32 ± 0.10	2.34 ± 0.46	5.33 ± 0.32**	0.44 ± 0.01
Verapamil	0.81 ± 0.05**	0.28 ± 0.02	2.56 ± 0.13**	5.54 ± 0.29**	0.43 ± 0.01
Captopril	0.91 ± 0.07	0.35 ± 0.03**	2.33 ± 0.22**	1.28 ± 0.04**	0.48 ± 0.02

†N = 10. x ± se. HEB: high energy bonds (3ATP + 2ADP + AMP); EI: energy index (3ATP/HEB).
*$P < 0.05$ vs. untreated.
**$P < 0.01$ vs. untreated.
***$P < 0.001$ vs. untreated.

TABLE 5. Cerebral Infarct Size and Behavior Changes Assessed 24 H After Middle Cerebral Artery Occlusion in Rats†

Groups (mg/kg ip)	N	Infarcted size (%)	Neurological scores	
			Spontaneous activity	Abnormal behavior
Sham-operation	6		0.5 ± 0.2	0.5 ± 0.2
Untreated	8	14.6 ± 1.3	2.2 ± 0.2*	3.7 ± 0.2*
Nimodipine 8	6	8.7 ± 0.85*	1.0 ± 0.2*,**	1.8 ± 0.12*,**
Bepridil 8	6	10.4 ± 0.78*	1.6 ± 0.2*,**	1.6 ± 0.2*,**

†x ± se.
*$P < 0.001$ vs. sham-operation.
**$P < 0.01$ vs. untreated.

bination of local cerebral edema and ischemic damage. The dysfunction of the right side of the striatum was noticed as the weakness of the opposite muscle tension and the circling movement toward the opposite side. The improvement in abnormal behavior was correlated with a reduction in cerebral infarction.

Verapamil was weak in raising cerebral and myocardial ATP levels, although it was more active in the brain. This accords with an increase in tolerance to severe hypoxia of the brain [Cartheuser, 1988]. The neuroprotection by nimodipine is significant in the MCAO model, but its effect is likely not mediated by vasodilatation [Welsch et al., 1990].

Dopamine exerts neurotoxic effects in cerebral neurons [Globus et al., 1987]. Ischemic stroke in Parkinson's patients was reported to be much less than in the normal population: the dopamine deficiency in the central nervous system of these patients possibly confers a protective mechanism [Struck et al., 1990]. The neurotoxic effect of NO may be mediated by release of dopamine [Zhu and Luo, 1992] and a blockade of release of dopamine is achieved by angiotensin receptor blockade [Ge and Barnes, 1995]. In this study the ACE inhibitor captopril showed no effect on the brain.

Catecholamines are toxic to cerebral neurons [Rosenberg, 1988] and we have shown the neurotoxic effect of isoprenaline in depleting ATP in the presence of simple anoxia. The toxicity of isoprenaline in the central nervous system is likely mediated by the c-AMP-dependent phosphorylation of ion channels, including L-type Ca^{2+} channels, and possibly involving a direct action mediated by G proteins. Catecholamines released endogenously in stress may render the cerebral neurons more sensitive to the effects of a compromised energy source.

The protective action in the liver by the four drugs was very similar. This suggests that a blockade of beta-receptors, calcium and sodium channels, and the angiotensin converting enzyme are beneficial to exert a non-specific protection on the impaired hepatic function and preserving the energy supply. However, the effectiveness of these interventions are mild and any clinical significance unclear.

In conclusion, the brain responds dramatically to isoprenaline imposed on simple anoxia and more sensitively than the heart to deplete ATP. The ability of bepridil to offer protection is proposed to be due to its dual blockade of sodium and calcium channels.

TABLE 6. Sensitivity to ATP Depletion by Simple and Dual Anoxia*

Deletion of ATP (%)		Depletion of HEB (%)	
Depleted from the normal			
Simple anoxia			
Heart	73	Heart	49
Liver	34	Cerebrum	26
Cerebrum	31	Liver	25
Dual anoxia (Isoprenaline sc prior to Simple Anoxia)			
Heart	80	Heart	70
Cerebrum	76	Cerebrum	64
Liver	69	Liver	55
Depleted from the simple Anoxia			
After adding isoprenaline			
Cerebrum	65	Cerebrum	52
Liver	53	Heart	42
Heart	27	Liver	41

*HEB: high energy bonds.

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