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Cardiac hypoxia and subsequent reoxygenation: sensitivity to L-arginine methylester

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1 The effect of L-arginine methylester (L-Arg-Me) was studied in the isolated heart of the guinea-pig perfused with hypoxic substrate-free medium for 30 min and subsequently reoxygenated with normal saline solution for 30 min.

2 The administration of L-Arg-Me in basal conditions decreases dose-dependently heart rate without any changes in the myocardial structure.

3 On the other hand, the administration of L-Arg-Me (5–10 mM) decreases ventricular arrhythmias, especially during reoxygenation; in fact ventricular fibrillation is abolished.

4 L-Arg-Me treatment increases the recovery of normal electrical and mechanical activity at the end of reoxygenation and reduces the increase in basal tone.

5 Treatment with 10 mM L-Arg-Me decreases lactate dehydrogenase (LDH) release in the effluent and lysosomal fragility in cardiac tissue, while it does not influence calcium gain.

6 L-Arginine (L-Arg) does not mimic any of the effects of L-Arg-Me.

Introduction

Many drugs have been successfully used in experimental hypoxia and subsequent reoxygenation. In this particular situation it has been demonstrated that α-adrenoceptor blocking agents are effective; prazosin treatment in fact reduces lactate dehydrogenase (LDH) release and ventricular arrhythmias (Antonini *et al.*, 1983). The use of β-adrenoceptor blocking agents is more disputable, since some of them are able to reduce ventricular arrhythmias, while others have no beneficial effect (Antonini *et al.*, 1983). The use of calcium entry blockers can reduce the incidence of arrhythmias (Nayler *et al.*, 1976) as can anti-inflammatory drugs (Karmazyn *et al.*, 1981). It has been clearly demonstrated that during reoxygenation there is an increase in calcium uptake (Nakanishi *et al.*, 1982) and calcium overload in cardiac tissue (Franconi *et al.*, 1985).

Calcium-activated neutral proteases have been isolated from cardiac tissue and one of them is activated at millimolar calcium concentrations (Toyo-Oka & Masaki, 1979). Consequently, since this enzyme might be activated in our experimental conditions, it appeared interesting to evaluate the effect of some protease inhibitors. To this end we chose L-arginine methylester (L-Arg-Me) (Troll *et al.*, 1954).

Methods

All experiments were carried out on the male guinea-pig heart. The animals (average weight 250 g) had free access to standard guinea-pig feed and water *ad libitum*.

Heart perfusion

The animals were killed by a sharp blow on the head and their hearts were immediately removed and a polyethylene cannula tied into the cut aorta, for retrograde perfusion of the coronary vessels by the method of Langendorff. A constant rate of perfusion was produced within the range 5–7 ml min^{-1} by a LKB 2115 multiperpex peristaltic pump. The perfusion fluid (composition mM: NaCl 137, $CaCl_2$ 1.8, KCl 2.7, $MgCl_2$ 0.11, $NaHCO_3$ 12, NaH_2PO_4 0.42 and glucose 5; pH 7.4 ± 0.02) was gassed with 3% CO_2 in

O_2 before entering the flow inducer, after which it was passed through a warming coil (37°C) connected with the aortic cannula.

Isometric contractions of the heart were recorded with a force-displacement transducer (MARB 83 F) attached via a pulley to a heart clip on the apex of the ventricles. The transducer, adjusted to apply a resting diastolic tension of 1 g on the heart, was connected to a recorder (Battaglia-Rangoni PFP 2400/A).

Surface electrical activity was recorded by attaching two platinum electrodes to the epicardium of the right atrium and left ventricle connected to a Battaglia-Rangoni electrocardiograph. Ventricular arrhythmias were examined as described (Antonini *et al.*, 1983; Franconi *et al.*, 1985).

The hearts were initially equilibrated for 45 min. In one set of experiments, drugs were administered for 60 min after stabilization. In a second set of experiments, after equilibration the hearts were perfused for 30 min with a glucose-free medium gassed with a 97% N_2:3% CO_2 mixture (hypoxic phase). After the hypoxic phase the hearts were reperfused for 30 min with the original medium gassed with 97% O_2:3% CO_2 (reoxygenation phase). The administration of drugs was begun at the start of the hypoxic phase and maintained throughout the experiment.

Atria perfusion

The hearts were rapidly removed and atria were dissected free and suspended in a 30 ml organ bath containing a saline solution of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10. The temperature was 30°C. The solution in the bath was continuously gassed with a mixture containing 95% O_2 and 5% CO_2. The atria were beating spontaneously and an initial tension of 1 g was applied. After an equilibration period of 70 min the L-Arg-Me was added to the bath for 60 min. Then the tissues were processed as described below for ultrastructural and cytochemical studies.

Biochemical analysis

Measurement of lactate dehydrogenase LDH levels were measured in cardiac effluent during various stages of the experiment according to Wrobleski & Le Due (1955) on a Perkin-Elmer 552 S Spectrophotometer.

Measurement of cathepsin D This was measured in ventricles. The ventricles were minced and gently homogenized in 0.25 M KCl (1:10) to retain lysosomes intact as far as possible. The homogenate was treated as described by Wildenthal (1976). The supernatant was assayed for cathepsin D activity, and measured according to Barret *et al.* (1967) with slight modifications. The pellet was homogenized and incubated with 0.1% Triton X and processed for enzyme determination (sedimentable activity). The index of lysosomal fragility was calculated from the 'non-sedimentable' and total activity (non sedimentable + sedimentable) as follows:

$$\text{Index} = \frac{\text{non-sedimentable} \times 100}{\text{total}}$$

Calcium estimation Dried tissues were digested in concentrated HNO_3 and the calcium level was measured with an atomic absorption spectrophotometer (Perkin-Elmer 303) in the presence of $LaCl_3$ in dried samples as described by Dolara *et al.* (1973).

Proteins were measured according to Lowry *et al.* (1951).

Ultrastructural and cytochemical procedures

Atrial tissue was processed for routine electron microscopy as previously described by Dini *et al.* (1981). Portions of atria were prefixed for cytochemical study in 2.5% (w/v) glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for 90 min on ice and 30–40 μm thick sections were then chopped on a Smith-Farquhart TC-2 tissue chopper (Ivan Sorvall Inc. U.S.A.), (Smith & Farquhart, 1965).

After being rinsed in 0.1 M cacodylate buffer to remove fixative, the sections were incubated at 37°C for 60 min in a modified Gomori medium containing 40 mM TRIS-maleate buffer (pH 5.0), 2.4 mM lead nitrate and 11.5 mM β-glycerophosphate (Sigma Chemical Co., U.S.A.) as substrate (Barka & Anderson, 1962). Control sections were incubated in a medium without substrate. No product reaction was seen in the tissue when incubation for acid phosphatase was carried out in the presence of substrate. After incubation, the specimens were washed in cacodylate buffer, postfixed in 1.33% osmium tetroxide in cacodylate buffer (pH 7.4) at 0°C and stained en bloc with 0.5% uranyl acetate. After dehydratation in a graded series of cold ethanol and toluene, tissue was embedded in Epon mixture. Thin sections were cut with a diamond knife on a Reichert OM_2 ultramicrotome and examined in a Philips 300 electron microscope without further staining.

Drugs

L-Arg-Me and L-arginine (L-Arg) were supplied as hydrochloride by Fluka and L-homoarginine methylester hydrochloride (L-homo-Arg-Me) was a kind gift of Dr G. Sportoletti, Italfarmaco, Italy. All other

reagents were of analytical grade.

Statistics

Statistical analysis of the differences in the incidence of arrhythmias and in the recovery of normal electrical activity was carried out using a Chi-squared test. For change in tension, basal tone, LDH release, calcium content and cathepsin D activity a non-paired Student's t test was used and a level of $P < 0.05$ was considered significant.

Results

The effects of L-Arg, L-Arg-Me and L-homo-Arg-Me were studied in the normal perfused heart. In this set of experiments the administration of L-Arg (10 mM) did not modify any parameter studied, while the administration of 10 mM L-homo-Arg-Me statistically increased the LDH release from 4.7 ± 1.1 to 81.6 ± 15.1 mu ml^{-1} ($n = 7$) ($P < 0.001$). This treatment decreased the contractile force and three of the eight treated hearts ceased to beat (Figure 1).

The administration of L-Arg-Me produced a significant reduction in heart rate (Table 1), leaving biochemical and functional parameters unchanged. L-Arg-Me (10 mM) was also studied in guinea-pig isolated atria, where it did not produce any ultrastructural changes in cardiac tissue. Lysosomes were also normally present and they displayed dense deposits of reaction product for acid phosphatase (Figure 2a and b).

The initiation of perfusion of isolated heart with hypoxic and glucose-free medium followed by reoxygenation was manifested in several distinct changes. Figure 3 depicts the profile of LDH release during control perfusion, hypoxic phase and reoxygenation. It should be noted that reoxygenation produced approximately a 60% increase in LDH release as compared to hypoxic values. The 60 min perfusion of isolated heart with 7.5 and 10 mM L-Arg-Me reduced LDH efflux throughout hypoxic perfusion, maximum reduction being evident with 10 mM. This effect was especially significant during reoxygenation. On the other hand, 5 mM L-Arg-Me did not significantly modify LDH release. During the hypoxic phase there

Table 1 Effect of L-arginine methylester (L-Arg-Me) on heart rate in isolated and perfused guinea-pig heart

Treatment	*Beats min^{-1}*	*Control*	n
L-Arg-Me 1 mM	227 ± 8	235 ± 4	3
L-Arg-Me 5 mM	181 ± 10	223 ± 6*	5
L-Arg-Me 10 mM	190 ± 8	235 ± 8**	7

Values are means ± s.e.: n = number of experiments. *$0.01 > P > 0.001$; **$P < 0.001$.

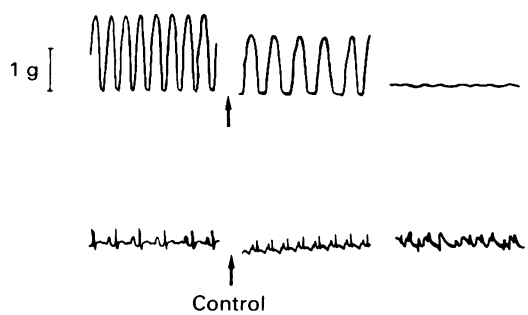

Figure 1 Effect of 10 mM L-homoarginine methylester on mechanogram (upper trace) and on electrocardiogram (lower trace) measured at 30 and 60 min. At arrows perfusion with the compound starts.

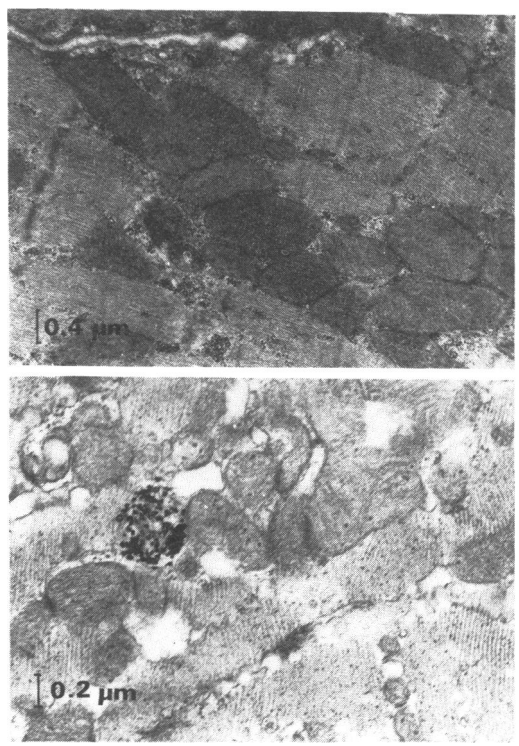

Figure 2 (a) Atria of guinea-pig heart after a 60 min superfusion with L-arginine methylester (L-Arg-Me) 10 mM. Myocytes appear normal (magnification × 25,000). (b) Lysosomes in L-Arg-Me-treated atria show acid phosphatase positive reaction and membrane integrity (magnification × 40,000).

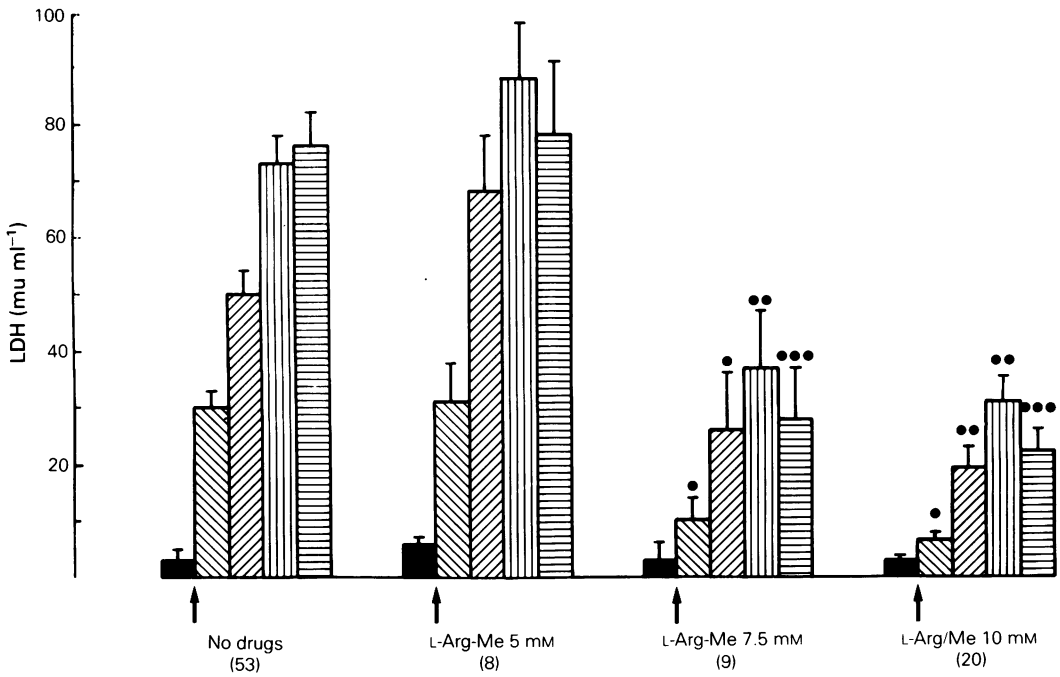

Figure 3 Effect of L-arginine methylester (L-Arg-Me) (10, 7.5 and 5 mM) on lactate dehydrogenase (LDH) release during the last 5 min of stabilization (■), 10–15 (▨) and 25–30 (▨) min of the 'hypoxic phase' and 5–10 (▥) and 15–20 (▤) min of the 'reoxygenation phase'. Results are mean of the number of experiments shown in parentheses. Vertical lines indicate s.e. $*0.05 > P > 0.01$; $**0.01 > P > 0.001$; $*** P < 0.001$.

were ventricular arrhythmias such as ventricular fibrillation (VF), ventricular tachycardia (VT) and ventricular premature beats (VPBs). The incidence of arrhythmias is shown in Table 2. It is known that during the reoxygenation phase, ventricular arrhythmias dramatically increase. The administration of L-Arg-Me (5, 7.5, 10 mM) abolished VF during reoxygenation while only 7.5 and 10 mM abolished VF during the hypoxic phase. VT and VPBs were also reduced by this treatment (Table 2). Moreover, the administration of L-Arg-Me increased the number of hearts that recovered normal electrical activity from about 11% to about 90% and this effect was dose-dependent (Figure 4). Figure 5 summarizes the contractile data. At the end of the hypoxic phase the tension of the control hearts fell to zero and was only partially restored during reoxygenation. On the other hand, in the presence of 7.5 mM L-Arg-Me a smaller decrease in tension was observed and it recovered to about 40% of pre-hypoxic values; 10 mM had effect similar to 7.5 mM. Basal tone was increased during the hypoxic phase and decreased during reoxygenation without reaching the initial values; 10 and 7.5 mM L-Arg-Me decreased the increase in basal tone

Table 2 Percentage of hearts presenting ventricular arrhythmias during hypoxic and reoxygenation phases

		Percentage		
	No drugs	*L-Arg-Me (mM)*		
		5	7.5	10
Hypoxic phase				
VF	31	40	0*	0*
VT	36	20	11*	0*
VPBs	74	60	89	85
Reoxygenation phase				
VF	57	0*	0*	0*
VT	69	20**	11**	15*
VPBs	87	70	89	60*
n	65	8	9	20

The Chi-Square test was used to detect significant differences between control and treated preparations. *n* = number of experiments, VPBs = ventricular premature beats, VF = ventricular fibrillation, VT = ventricular tachycardia.
$*0.01 > P > 0.001$; $**P < 0.001$.

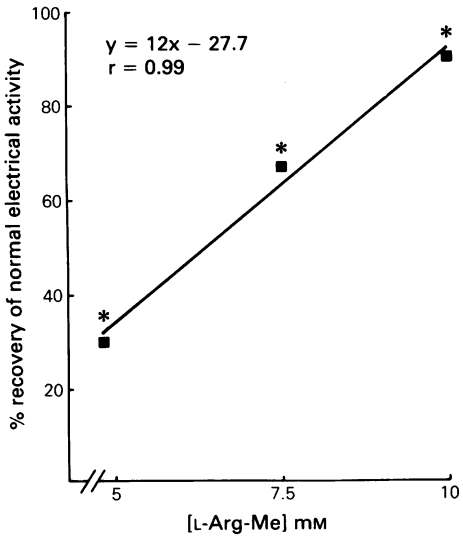

Figure 4 Effect of L-arginine methylester (L-Arg-Me) on electrical recovery after 30 min of hypoxia followed by 30 min of reoxygenation. Recovery of normal electrical activity was considered to exist when ventricular arrhythmias disappeared and there was a simultaneous recovery of sinus rhythm. Each value is the mean of at least 8 experiments.

(Figure 6), while 5 mM was ineffective.

The dosage of cathepsin D activity in ventricles after hypoxia and after hypoxia followed by reoxygenation was an indication of increase in lysosomal fragility. This was more marked at the end of reoxygenation and the treatment with 10 mM L-Arg-Me abolished it (Table 3). After hypoxia followed by reoxygenation, a statistically significant increase in tissue calcium ($ng\,mg^{-1}$ d.w.) was observed. In fact, it rose from 579 ± 50 ($n = 8$) to 985 ± 88 ($n = 11$) ($P < 0.001$), while 10 mM L-Arg-Me did not induce any significant decrease: 838 ± 104 ($n = 7$). In order to verify whether the effect of L-Arg-Me was due to L-Arg, some experiments were performed with L-Arg.

The administration of equimolar concentrations of L-Arg did not reduce LDH release (Figure 7) or ventricular arrhythmias (Table 4), nor did it modify the profile of contractile force.

Discussion

The beneficial effects of L-Arg-Me on the recovery of guinea-pig heart subjected to hypoxia and reoxygenation are clearly shown by functional and biochemical parameters.

The mechanism by which L-Arg-Me exerts its cardioprotective effect is not wholly clear, but the observations in this paper provide some clues. The administration of L-Arg-Me significantly reduces heart rate in guinea-pig isolated hearts perfused with a medium containing glucose and gassed with 97% O_2 and 3% CO_2. A reduction of heart rate, *per se*, cannot however be the sole basis of its cardioprotective effect, though this does not rule out the possibility that L-Arg-Me acts by suppressing the abnormal automacity in the myocardium. The antiarrhythmic activity of this compound has also been revealed in a rat model of coronary ligation and reperfusion, where it does not show any effect on heart rate and exerts an antiarrhythmic action at similar concentrations to the lidocaine antiarrhythmic ones (Pagella *et al.*, 1984).

On the other hand, the effect of L-Arg-Me cannot be attributed to a local anaesthetic-like activity; in fact the administration of 1–4% L-Arg-Me (0.1 ml) in Swiss mice did not produce any anaesthetic activity measured according to Bianchi (1956) (Sportoletti,

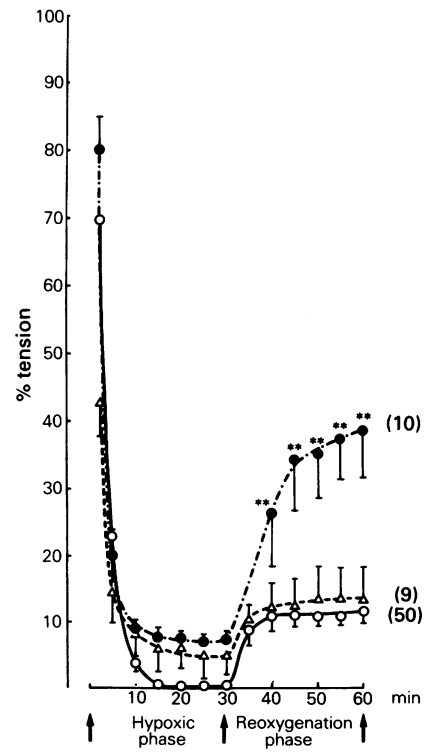

Figure 5 Effect of L-arginine methylester (L-Arg-Me) on changes in contractile force of the heart perfused under hypoxic and under reoxygenation phases: control (○); L-Arg-Me 5 mM (△), 10 mM (●). Each value is the mean of at least 8 experiments; vertical lines show s.e. $^{**}0.01 > P > 0.001$.

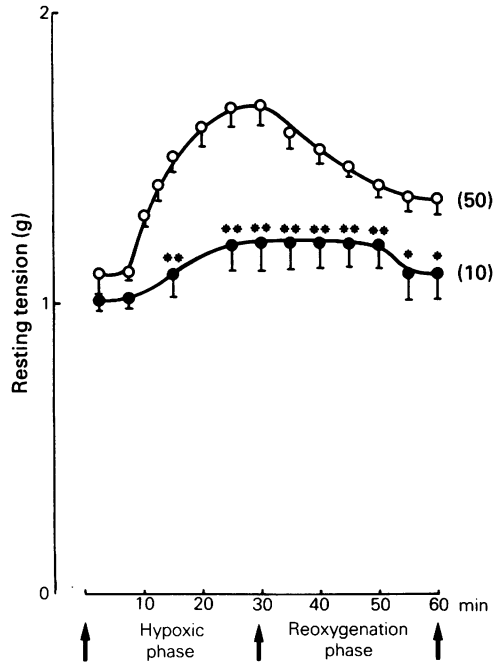

Figure 6 Changes in resting tension developed by guinea-pig isolated hearts. The hearts were perfused with hypoxic substrate-free buffer for 30 min. At arrows perfusion was continued under aerobic conditions. When L-arginine methylester (L-Arg-Me) was present (●) it was infused continuously throughout hypoxic and reoxygenation phases; (○) no drugs. $*0.05 > P > 0.01$; $**0.01 > P > 0.001$. Results are mean with vertical lines showing s.e.

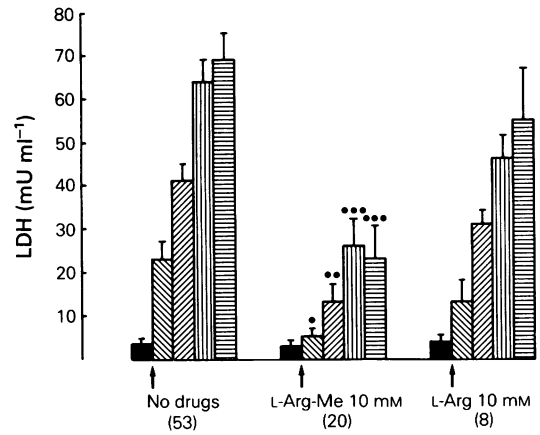

Figure 7 Effect of L-arginine methylester (L-Arg-Me) 10 mM and L-arginine (L-Arg) 10 mM on lactate dehydrogenase (LDH) release during hypoxic and reoxygenation phases; for key, see legend to Figure 3. Values are mean of the number of experiments in parentheses; vertical lines show s.e.

personal communication). In our experimental conditions, an increase in lysosomal fragility was observed at the end of the hypoxic and reoxygenation phases. L-Arg-Me abolishes this increase in lysosomal fragility, suggesting that this compound may stabilize the lysosomal membrane. Cathepsin D activity is not inhibited when L-Arg-Me is tested in the tubes. Our data on cathepsin D distribution in L-Arg-Me-treated hearts are not completely in agreement with those of Decker *et al.* (1980) who found that during reoxygenation, lysosomal fragility decreased in comparison with hypoxia, but unlike us they worked with rats and rabbits, and glucose was present during the hypoxic phase. The effect of L-Arg-Me seems quite specific because the native amino acid is without any activity; thus an osmotic role of L-Arg-Me may be ruled out.

Table 3 Influence of hypoxia and reoxygenation on distribution of cathepsin D activity (mu mg^{-1} protein) in control and during L-arginine methylester (L-Arg-Me) treatment

	Non sedimentable × 100 / total		
Normoxia	*Hypoxic phase* × 30 min	*Hypoxic phase* × 30 min + Reoxygenation phase × 30 min	L-Arg-Me (10 mM) *hypoxic phase* × 30 min + Reoxygenation phase × 30 min
7.4 ± 3.4 (10)	19.5 ± 4.6**[a] (8)	33.6 ± 4.0**[a] (7)	3.5 ± 2.4**[b] (7)

Each value of mean ± s.e.; in parentheses the number of experiments; [a]compared to normoxia, [b]compared to reoxygenation. $0.05 > P > 0.01$; $P < 0.001$.

Table 4 Percentage of hearts showing ventricular arrhythmias during hypoxic and reoxygenation phases

	Percentage	
	No drugs	*L-Arg* (10 mM)
Hypoxic phase		
VF	31	25
VT	36	37
VPBs	74	75
Reoxygenation phase		
VF	57	62
VT	69	87
VPBs	87	100
n	65	9

The Chi-Squared test was used to detect significant differences between control and treated preparations. *n* = number of experiments, VPBs = ventricular premature beats, VF = ventricular fibrillation, VT = ventricular tachycardia.

The fact that L-Arg-Me is a substrate of proteases (Troll *et al.*, 1954) must also be borne in mind, as should the inhibition by L-Arg-Me of ADP-induced platelet aggregation, while L-Arg is ineffective (Salzman & Chambers, 1964).

Recently, calcium-activated proteases have been isolated in cardiac tissue (Toyo-Oka & Masaki, 1979). It is known that [^{3}H]-glutamate binding to hippocampal membranes is a calcium-dependent phenomenon (Baudry & Lynch, 1980) and that it is inhibited by leupeptin, a specific calcium activated protease inhibitor (Vargas *et al.*, 1980).

L-Arg-Me also inhibits calcium activated binding of [^{3}H]-glutamate to hippocampal membranes (Bennardini *et al.*, 1985), so it might be suggested that L-Arg-Me acts as a calcium-activated protease inhibitor. On the other hand while preventing activation of calcium-activated proteases, L-Arg-Me is unable to reduce the calcium overload due to reoxygenation.

Our data suggest the usefulness of investigating calcium-activated protease inhibitors in the context of prevention of ischaemia-anoxia and reperfusion-reoxygenation damage. Studies are in progress to clarify the effect of L-Arg-Me on calcium-activated proteases, in order to assess its specificity of action.

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