Effect of ACE Inhibitor Captopril and L-Arginine on the Metabolism and on Ischemia-Reperfusion Injury of the Isolated Rat Heart

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Summary
We investigated the effects of in vivo treatment with the angiotensin-converting enzyme inhibitor (ACE-I) captopril and/or of in vitro administration of L-arginine on the metabolism and ischemia-reperfusion injury of the isolated perfused rat myocardium. Captopril (50 mg/l in drinking water, 4 weeks) raised the myocardial content of glycogen. After 25-min global ischemia, captopril treatment, compared with the controls, resulted in lower rates of lactate dehydrogenase release during reperfusion (8.58±1.12 vs. 13.39±1.88 U/heart/30 min, p<0.05), lower myocardial lactate contents (11.34±0.93 vs. 21.22±4.28 µmol/g d.w., p<0.05) and higher coronary flow recovery (by 25 %), and prevented the decrease of NO release into the perfusate during reperfusion. In control hearts L-arginine added to the perfusate (1 mmol/l) 10 min before ischemia had no effect on the parameters evaluated under our experimental conditions, presumably because of sufficient saturation of the myocardium with L-arginine. In the hearts of captopril-treated rats, L-arginine further increased NO production during reperfusion and the cGMP content before ischemia. Our results have shown that long-term captopril treatment increases the energy potential and has a beneficial effect on tolerance of the isolated heart to ischemia. L-arginine added into the perfusate potentiates the effect of captopril on the NO signaling pathway.

Key words
ACE inhibitors • L-arginine • Nitric oxide • Cyclic GMP • Isolated rat heart • Ischemia-reperfusion injury

Introduction
Angiotensin-converting enzyme inhibitors (ACE-I) are well-known as antihypertensive agents. In addition, they exert several cardioprotective effects such as prevention and regression of left ventricular hypertrophy or reduction of infarct size in both clinical and experimental studies, and may represent an important therapeutic approach in the treatment of congestive heart failure (Awan et al. 1981, Lund-Johansen and Omvik 1993, Pecháňová et al. 1997, Šimko and Šimko 1999). However, the mechanism of the beneficial effect of ACE-I is not fully understood.
ACE-I have been shown to interfere not only with the renin-angiotensin system through inhibition of angiotensin II production, but also with the kallikrein-kinin system through attenuation of endogenous bradykinin degradation by inhibiting kininase II, an enzyme identical with angiotensin-converting enzyme (Baumgarten et al. 1993, Hartman 1995). Bradykinin binding to B\textsubscript{2} kinin receptors has been demonstrated to activate several intracellular messenger cascades (Linz et al. 1995), one of them being the nitric oxide synthase (NOS) system, which produces the short-lived signaling compound nitric oxide (NO) (Wiener et al. 1991). By activating soluble guanylate cyclase NO raises the levels of cyclic guanosine 3’,5’-monophosphate (cGMP) in target cells (Wiener et al. 1991).

Most studies dealing with the effects of ACE-I on the isolated myocardium have focused on functional parameters (for review see Heusch et al. 1997), but only a few on metabolism, in particular on energy reserves. Moreover, ACE-I are often used in non-physiological doses in vitro, thereby eliminating the possibility of the evaluation of long-term effects. Therefore, we have chosen chronic treatment with low captopril doses and studied its effects on myocardial metabolism, namely ATP and glycogen content, under basal conditions and during ischemia in normotensive rats. As NO and cGMP is a part of the mode of captopril action that may participate in the myocardial metabolic status, we sought to enhance NO production directly by administering L-arginine, the substrate for NO synthesis, into the perfusate.

Controversial data regarding the effect of L-arginine on the isolated myocardium have been reported (Wang et al. 1997, Takeuchi et al. 1995). However, no information is available on the effect of L-arginine on energy metabolism. We therefore assessed myocardial metabolic parameters under basal conditions and also in the heart with ischemic injury after administration of L-arginine in vitro without previous ACE-I treatment.

The aims of the present study were (1) to examine the effects of long-term captopril treatment on myocardial metabolism and ischemic tolerance in normal rats, (2) to test whether the effects of captopril can be further enhanced by stimulation of NO production induced by concomitant in vitro L-arginine administration, and (3) to clarify the controversial effect of L-arginine alone on myocardial metabolism and ischemia-reperfusion injury.

**Methods**

**Animals**

The study was carried out on adult male Wistar rats weighing 330-420 g. The animals were fed standard chow and water ad libitum. Half of them were administered the ACE-I captopril (Sigma, St. Louis, USA) in a dose of about 2.5 mg/kg body weight per day (in drinking water, 50 mg/l, free access) for 4 weeks prior to decapitation. At the beginning of the experiments and prior to decapitation, blood pressure was measured by tail-cuff plethysmography in control animals and captopril-treated groups.

**Perfusion of isolated hearts**

After decapitation under ether anesthesia, the hearts were removed and rapidly placed into ice-cool saline. Once the heart had stopped beating, the aorta was connected to a perfusion system. Each heart was perfused retrogradely under constant pressure (60 mm Hg) with oxygenated (equilibrated with 95 % O\textsubscript{2} + 5 % CO\textsubscript{2}) non-recirculating Krebs-Henseleit bicarbonate solution (KRB), at pH 7.4 and 37 °C according to Langendorff (1895). The perfusate contained (in mmol/l): 118.0 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 25.0 NaHCO\textsubscript{3}, 0.5 Na-EDTA, 5.5 glucose. The stabilization period lasted 20 min and was followed by a 10-min period during which the hearts were perfused with either KRB or with KRB containing L-arginine (Sigma, St. Louis, USA) at a final concentration of 1 mmol/l. This was followed by 25-min global (no-flow) ischemia and 30-min retrograde reperfusion. The hearts were maintained at 37 °C in a heated-water jacketed chamber throughout the perfusion period. Coronary flow was measured by timed coronary effluent collection. Coronary effluent was used for further analysis. Lactate dehydrogenase was assessed as total amount of enzyme released into the perfusate during 30 min of reperfusion, lactate release was determined at minutes 1 and 2 of reperfusion and NO production in the perfusate was evaluated 1 min before ischemia and at 5 min of reperfusion. The whole hearts prior to ischemia, at the end of ischemia, and after 30-min reperfusion were frozen in liquid nitrogen using Wollenberger forceps,
then weighed and the ventricles were used for further analysis.

**Chemical analysis of coronary effluent**

Lactate dehydrogenase (L-lactate: NAD⁺-oxidoreductase EC 1.1.1.27) in the medium was determined by a standard enzymatic procedure based on the reduction of NAD to NADH (Mosinger et al. 1978) as a test of myocardial injury. Lactate was determined by spectrophotometric assay using lactate dehydrogenase (Hohorst 1963). NO production in the perfusate was evaluated as the concentrations of stable NO metabolites $\text{NO}_2^-/\text{NO}_3^-$ measured using the Griess reagent (Green et al. 1982) with minor modifications. Briefly, samples were incubated for 1 hour at room temperature with nitrate reductase (0.1 U/100 µl, *Aspergillus* species, Boehringer Mannheim GmbH, Germany) in the substrate buffer (pH 7.8) containing imidazole, NADPH, and FAD to convert all $\text{NO}_3^-$ to $\text{NO}_2^-$. Total $\text{NO}_2^- + \text{NO}_3^-$ was then analyzed by means of Griess reagent and absorbances were measured at 543 nm using a UV/VIS spectrophotometer (Secomam, France). Concentrations of $\text{NO}_2^-$ in the samples were determined from the standard curve of NaNO₂. Nitrate recovery was 97 %.

**Heart tissue biochemistry**

Frozen heart ventricles were powdered in liquid nitrogen and ATP was determined in a neutralized perchloric acid extract by a spectrophotometric assay using hexokinase and glucose-6-phosphate-dehydrogenase (Lamprecht and Trautschold 1963). In the same extract, lactate was determined by a spectrophotometric assay using lactate dehydrogenase (Hohorst 1963) and cGMP was assessed using a radioimmunoassay kit (Immunotech, France). Glycogen was estimated enzymatically in the chloroform-methanol sediment after acid hydrolysis as glucose using the glucose oxidase method (Huggett and Nixon 1957).

**Statistical analysis**

Results are given as means ± S.E.M. Differences between the groups were evaluated by ANOVA and Tukey-Kramer multiple comparisons test. Differences were considered statistically significant at the level of $p<0.05$.

**Results**

The dose of captopril used resulted in a decrease in blood pressure (Captopril: 123±1 (n=20) vs. Controls: 148±2 mm Hg (n=16), $p<0.0001$), but did not affect the absolute heart weight (Captopril: 1.589±0.032 (n=11) vs. Controls: 1.552±0.059 g (n=12), N.S.) or the heart weight/body weight (HW/BW) ratio (Captopril: 0.0039±0.0001 vs. Controls: 0.0041±0.0001, N.S.).

**Parameters of ischemia-reperfusion injury**

Ischemia-reperfusion injury of the heart was evaluated both by measuring the release of lactate dehydrogenase into the coronary effluent during reperfusion, by determining the myocardial lactate content before ischemia, at the end of ischemia and at the end of reperfusion and by measuring coronary flow recovery. The total lactate dehydrogenase released into the perfusate during reperfusion is shown in Figure 1. Four-week captopril administration decreased lactate dehydrogenase release to almost 64% of the control group level (Captopril: 8.58±1.12 vs. Controls: 13.39±1.88 U/heart/30 min, $p<0.05$). Addition of L-arginine into the perfusate had no effect on the above parameter (14.77±1.05 U/heart/30 min), even when
combined with long-term captopril administration (8.01±1.61 U/heart/30 min).

After global ischemia, the myocardial lactate content rose (Fig. 2) to the same level in all experimental groups (Controls: 148.6±9.5, L-arginine: 132.0±4.9, Captopril: 147.0±5.9, Captopril + L-arginine: 169.0±3.0 µmol/g d.w., N.S.). At the end of reperfusion, the lactate content was significantly lower in captopril-treated groups than in those non-treated (Captopril: 11.3±0.9 vs. Controls: 21.5±3.0, Captopril + L-arginine: 9.3±1.0 vs. L-arginine: 23.3±4.7 µmol/g d.w., p<0.05). Lactate is supposed to have been washed out more effectively in the captopril-treated groups. This explanation is supported by the measurement of lactate released from the heart into the coronary effluent in the first two minutes of reperfusion (not shown) that was amplified in captopril-treated rats (Captopril: 34.9±0.9 vs. Controls: 29.7±1.2 µmol/2 min, p<0.01). Moreover, the beneficial effect of captopril is manifested by improved coronary flow recovery, which could also contribute to the enhanced lactate washout. At minute 5, 15 and 30 of reperfusion, coronary flow recovered to 121.0±7.7, 110.0±6.0 and 107.5±5.7% in the captopril-treated group vs. 94.2±8.5, 86.4±8.5 and 81.9±7.8% in the controls (p<0.05). Preischemic infusion of L-arginine had no effect on postischemic coronary flow recovery, even when combined with captopril. Baseline values of coronary flow (not shown) did not differ significantly either after captopril treatment or after addition of L-arginine into the perfusate (Controls: 14.4±0.6, L-arginine: 13.8±0.5, Captopril: 12.5±0.5, Captopril + L-arginine: 13.4±0.8 ml/min, N.S.).

**Fig. 2.** Effect of captopril in vivo and/or L-arginine in vitro (1 mM) on myocardial lactate content before ischemia and after 25-min ischemia (isch) and after 30-min reperfusion (reperf). Statistical significance: *p<0.05 vs. non-treated groups; ♦ p<0.0001 vs. preischemic levels; Data are means ± S.E.M., n = 3-8 hearts per group.

**Table 1.** Effect of captopril in vivo and/or L-arginine in vitro (1 mM) on myocardial ATP and glycogen contents.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>L-arginine</th>
<th>Captopril</th>
<th>Captopril + L-arginine</th>
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</thead>
<tbody>
<tr>
<td><strong>Preischemia</strong></td>
<td></td>
<td></td>
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<tr>
<td>ATP</td>
<td>19.0±0.5 (10)</td>
<td>21.4±0.8 (10)</td>
<td>21.0±0.7 (7)</td>
<td>21.3±1.0 (6)</td>
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<tr>
<td>Glycogen</td>
<td>91.7±4.1 (7)</td>
<td>107.6±2.3 (6)</td>
<td>119.0±6.1 (6)**</td>
<td>112.0±5.9 (6)*</td>
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<tr>
<td><strong>After 30-min reperfusion</strong></td>
<td></td>
<td></td>
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<tr>
<td>ATP</td>
<td>8.0±1.0 (5)</td>
<td>8.7±1.6 (5)</td>
<td>7.7±0.8 (7)</td>
<td>7.0±0.6 (8)</td>
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<tr>
<td>Glycogen</td>
<td>31.2±3.5 (5)</td>
<td>27.0±.9 (5)</td>
<td>25.6±1.4 (7)</td>
<td>25.5±2.7 (6)</td>
</tr>
</tbody>
</table>

Data (in µmol/g d.w.) are given as means ± S.E.M., number of hearts per group is given in parentheses. Significantly different from the control group: *p<0.05, **p<0.01.

**ATP and glycogen contents**

As is shown in Table 1, long-term captopril administration increased the myocardial glycogen content and also tended to increase the myocardial ATP content (significant only when the captopril group was compared with controls using the t-test, p = 0.034). L-arginine...
administered into the perfusate had no significant effects. The differences observed in the heart before ischemia were no longer present at the end of reperfusion.

Release of nitric oxide and myocardial cGMP content

The amount of NO released into the coronary effluent in the stabilized state before ischemia did not differ significantly between the groups (Fig. 3). At minute 5 of postischemic reperfusion, there was a decrease of NO release in the hearts of rats not receiving captopril; L-arginine likewise did not lead to improved NO release. By contrast, NO release from the hearts of captopril-treated rats was not attenuated after ischemia, and the addition of L-arginine into the perfusate still enhanced NO production (Captopril: 184.2±23.0 nmol/min vs. Captopril + L-arginine: 304.0±28.1 nmol/min, p<0.01).

Fig. 3. Effect of captopril in vivo and/or L-arginine in vitro (1 mM) on NO release into coronary effluent before ischemia and at minute 5 of postischemic reperfusion. *** p<0.001 vs. non-treated groups; ♦ p<0.02, ♦♦ p<0.01 vs. preischemic levels; ♦♦♦ p<0.01 vs. Captopril group without L-arginine. Data are means ± S.E.M., n = 4-7 hearts per group.

As is shown in Figure 4, the cGMP content in the equilibrated heart before ischemia was slightly increased in the group of rats receiving captopril for 4 weeks (61.3±8.5 pmol/g d.w.), but it was significantly higher only when captopril treatment was combined with the addition of L-arginine into the perfusate (73.5±18.5 vs. Controls: 43.1±3.3 pmol/g d.w., p<0.05). The addition of L-arginine into the perfusate of the controls did not result in a significant increase. The 25-min ischemia was associated with a reduction in cGMP content in all the studied groups. It is evident that the effect of captopril promoting cGMP production was no longer present at the end of 30-min reperfusion (45.8±4.3 pmol/g d.w.), whereas it persisted when captopril was combined with L-arginine (70.2±9.7 pmol/g d.w.). However, this point cannot be statistically evaluated because of an insufficient number of samples.

Discussion

In the present study, we investigated the effects of either long-term in vivo treatment with the ACE-I captopril or acute in vitro administration of L-arginine on metabolic parameters and ischemia-reperfusion injury in isolated hearts of normotensive rats. As the common denominator of both ACE-I treatment and L-arginine administration is the activation of the NO pathway in the heart, we also evaluated the effects of a combination of
both stimuli. While numerous studies have been designed to monitor the effects of ACE-I on the myocardium (Schölkens et al. 1988, Massoudy et al. 1994), only a few have assessed the action of long-term ACE-I administration in vivo on the metabolism and function of the isolated heart (Werrmann and Cohen 1994). We have chosen a model of the heart perfused according to Langendorff under constant pressure because it eliminated changes in NOS activity due to shear stress (Curtis and Ellwood 1998, Vavříková et al. 1999).

The four-week treatment with captopril improved myocardial resistance to ischemia as was confirmed by the decreased lactate dehydrogenase release into the coronary effluent during postischemic reperfusion. Captopril treatment lowered the myocardial lactate content at the end of reperfusion. It is supposed that the reduced accumulation of lactate in the captopril-treated groups was a consequence of its facilitated washout due to improved coronary flow recovery. It was not possible to measure other functional parameters under our experimental conditions, but there is also convincing evidence of a beneficial effect of ACE-I on the functional parameters of ischemia-reperfusion injury (Werrmann and Cohen 1994, Massoudy et al. 1994). The beneficial effect of ACE-I on ischemia-reperfusion injury of the rat heart was also demonstrated after the addition of another ACE-I (ramipril) into the perfusate or after a single oral dose one hour prior to decapitation (Schölkens et al. 1988). The results of our study have demonstrated that the changes induced in vivo by captopril also persisted in the isolated myocardium. This finding could possibly be explained by protracted binding of captopril to the angiotensin-converting enzyme which is persisting even in the isolated heart (Brown et al. 1998). Apart from inhibiting angiotensin II production, this binding results in a reduced degradation of bradykinin which is released in the heart by the local kallikrein-kinin system (Nolly et al. 1994). Inhibition by ACE-I leads to increased NO formation through bradykinin-mediated NOS activation (Wiemer et al. 1991), as we demonstrated during postischemic myocardial reperfusion, where captopril prevented the decrease in NO production. Enhanced production of vasorelaxant NO may have contributed to improved coronary flow recovery during reperfusion and may also have been associated with increased supply of O₂ into the tissue and with the elimination of anaerobic metabolic products. A protective role may also be played by reduced O₂ consumption, demonstrated after ACE-I administration in the isolated heart (Zhang et al. 1997). A certain role in the improved myocardial ischemic tolerance may also have been played by the increased cGMP level prior to ischemia, that can reduce Ca²⁺ entry into cells (Méry et al. 1991) associated with ischemic injury.

In our experiments, captopril administration raised the glycogen content and tended to increase the ATP content in perfused hearts under basal conditions. This is consistent with the results published by Gohlke et al. (1994) who reported increased ATP and glycogen levels after chronic administration of the ACE-I perindopril in “stroke-prone” hypertensive rats. The increased glycogen content observed after captopril administration could be a consequence of enhanced glucose uptake. A number of studies have shown that ACE-I or bradykinin enhance tissue glucose uptake (Henriksen and Jacob 1995). Another mechanism by which the ACE-I could have raised the content of ATP and glycogen is their cleavage controlled by catecholamines. It is just the release of neurotransmitters from the nerve endings and their myocardial uptake which are affected by the ACE-I (Kawai et al. 1999). This is under the inhibitory control of endogenous NO (Schwarz et al. 1995).

NO formation may be enhanced by ACE-I as a result of increased bradykinin levels (Wiemer et al. 1991). In our study, long-term captopril administration prevented the postischemic decrease of NO release into the perfusate, which was noted in the control group. A similar reduction in NO release in the same phase of reperfusion was reported by Maulik et al. (1996). It has been suggested that this was due to a major decrease in NOS activity during ischemia (70-90% reduction) (Giraldez et al. 1997, Wang et al. 1997).

An important role in the NO signaling pathway is played by cGMP. Compared with the controls, the cGMP content in the heart before ischemia was elevated after the treatment with captopril, especially when potentiated by L-arginine administration. A rise in the cGMP content after long-term administration of ramiprilat was also detected in the aortas of SHR rats (Gohlke et al. 1993). In our experiments, the cGMP content declined at the end of 25-min ischemia. Similar results were reported by Maulik et al. (1996) and Mizuno et al. (1998). On the contrary, Dépré and Hue (1994)
noted an increase in the cGMP content in isolated hearts after 10-min ischemia. The cGMP content in the heart, which is varying during ischemia, depends on the duration of ischemia and on the status of the myocardium. This might be related to the fact that cGMP can be affected by bradykinin activation, which occurs at the onset of ischemia, but declines as early as in minute 15 of ischemia (Baumgarten et al. 1993).

In our experiments, attention was also paid to the effects of L-arginine, the substrate for NO synthesis. It had no significant effect either on ATP and glycogen contents or on myocardial tolerance to ischemia. This finding is consistent with that reported by Masini et al. (1999). Neither did it increase NO formation or myocardial cGMP content before ischemia. Similarly, Takeuchi et al. (1995) did not find increased NO release following the addition of L-arginine into the perfusate. In our experiments, L-arginine did not enhance NO release and myocardial cGMP content even during reperfusion. By contrast, Maulik et al. (1996) described increased NO release and Mizuno et al. (1998) reported an elevated myocardial cGMP content during reperfusion in the presence of L-arginine. These controversial findings can presumably be explained by differences in the model used. Maulik et al. (1996) and Mizuno et al. (1998) used the working heart, whereby the coronary arteries are affected by shear stress, contrary to our model of non-working perfusion (Pinsky et al. 1997). The changes in NOS activity produced by shear stress (Curtis and Ellwood 1998, Vavříková et al. 1999) were only negligible in our experiments, hence the consumption of L-arginine may have been lower than in the working models. The low consumption of L-arginine and its high cellular concentrations (200-800 µM) (Baydoun et al. 1990), which are several times above the K_m for NOS (35 µM; Mayer et al. 1991), explain why a further supply of L-arginine did not result in any additional changes.

Our results indicate that the situation is different in the hearts of captopril-treated rats. In this case, the addition of L-arginine to the perfusate increased NO release into the perfusate at minute 5 of reperfusion. In a similar situation, Vavříková et al. (2001) found an increased NO content directly in the myocardium using electron-spin resonance. It can reasonably be assumed that NOS activity and hence also the consumption of L-arginine were higher in the hearts of rats treated by captopril. Consequently, there may have occurred a local deficiency of L-arginine, which may also take place at the site of NO synthesis in vivo (Huk et al. 1997). Some results indicated a beneficial effect of exogenous supply of L-arginine that can only be observed in a situation of its local deficiency (Gold et al. 1990). Another possible contribution of L-arginine to NO production enhancement is the fact that the NOS enzyme, bound to substrate in excess, is more resistant to its deactivation during ischemia (Huk et al. 1997). Last but not least, it should also be mentioned that at suboptimal concentrations of L-arginine the complex NOS enzyme catalyzes the generation of superoxide radicals instead of NO formation (Pou et al. 1992).

It can thus be summarized that the effect of long-term administration of the angiotensin-converting enzyme inhibitor captopril to normotensive rats persists in vitro and improves some metabolic parameters in the isolated rat heart, enhances its energy potential and increases its tolerance to ischemia. Addition of L-arginine to the perfusate had no effect on the parameters of myocardial ischemia-reperfusion injury except for potentiating the effect of captopril on NO release during reperfusion and the cGMP content before ischemia. Under basal conditions, the myocardium in our non-working heart model was presumably sufficiently saturated with L-arginine and its increased availability was not effective except when captopril had activated the NO pathway.

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References


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