

Reduction of myocardial infarction by postischemic administration of the calpain inhibitor A-705253 in comparison to the Na⁺/H⁺ exchange inhibitor Cariporide[®] in isolated perfused rabbit hearts*

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Abstract

The calpain inhibitor A-705253 and the Na⁺/H⁺-exchange inhibitor Cariporide[®] were studied in isolated perfused rabbit hearts subjected to 60 min occlusion of the ramus interventricularis of the left coronary artery (below the origin of the first diagonal branch), followed by 120 min of reperfusion. The inhibitors were added to the perfusion fluid solely or in combination at the beginning of reperfusion. Hemodynamic monitoring and biochemical analysis of perfusion fluid from the coronary outflow were performed. Myocardial infarct size and area at risk (transiently not perfused myocardium) were determined from left ventricular slices after a special staining procedure with Evans blue and 2,3,5-triphenyltetrazolium chloride. The infarcted area (dead myocardium) was 72.7±4.0% of the area at risk in untreated controls, but was significantly smaller in the presence of the inhibitors. The largest effect was observed with 10⁻⁶ M A-705253, which reduced the infarcted area to 49.2±4.1% of the area at risk, corresponding to a reduction of 33.6%. Cariporide[®] at 10⁻⁶ M reduced the infarct size to the same extent. The combination of both inhibitors, however, did not further improve cardioprotection. No significant difference was observed between the experimental groups in coronary perfusion, left ventricular pressure, heart rate, or in the release of lactate dehydrogenase and creatine kinase from heart muscle.

Keywords: coronary occlusion; coronary perfusion; enzyme release; infarct size; left ventricular pressure.

Introduction

In addition to their physiological functions in cytoskeletal remodeling and signal transduction, calpains (calcium-dependent cytosolic cysteine proteases) are also implicated in pathophysiological processes, especially with disturbed calcium homeostasis (Goll et al., 2003). Thus, calpains were found to be involved in myocardial tissue damage resulting from ischemia and reperfusion, such as sarcomere disorder, desmin degradation and proteolysis of caldesmon and fodrin (Yoshida et al., 1995; Papp et al., 2000; Fukunaga et al., 2006). Calpain activity increases during myocardial hypoxia (Iizuka et al., 1991) and mechanical unloading of the heart (Razeghi et al., 2007), and activated calpains were localized by specific antibodies in infarcted regions of the human heart (Kunimatsu et al., 1999). Calpain inhibition, on the other hand, attenuates or prevents myocardial tissue damage and mitochondrial dysfunction by ischemia and reperfusion (Neuhof et al., 2003). Increased calpain activity during hypoxia (Iizuka et al., 1991) can be reduced with peptidyl aldehyde calpain inhibitor-I. Calpain inhibitors are also found to be protective in prolonged hypothermic cardiac preservation (Saito et al., 1999), in virus-induced apoptotic myocardial injury (DeBiasi et al., 2001) and against structural and contractile dysfunction in atrial fibrillation (Xue et al., 2007). So far, only a few studies are available regarding the effect of calpain inhibition in experimental models of myocardial infarction, in which the infarct size could be reduced by pretreatment with the calcium-activated neutral protease inhibitor E-64c in dogs (Toda et al., 1989) or with calpain inhibitor-I in isolated perfused rat hearts (Iwamoto et al., 1999; Perrin et al., 2003). Recently, we could show that myocardial infarct size is reduced approximately 36.7% by preischemic administration of calpain inhibitor A-705253 (Lubisch et al., 2003) in isolated perfused rabbit hearts (Neuhof et al., 2004) and in pigs following temporary coronary occlusion (Khalil et al., 2005). In the present study, we evaluated the cardioprotective effect of this calpain inhibitor after postischemic administration, which better reflects the clinical situation of coronary intervention in acute myocardial infarction.

Since inhibition of the Na⁺/H⁺-exchange inhibitor Cariporide[®] is known to attenuate intracellular calcium overload (Cun et al., 2007) and calpain activation (Chen et al., 2002) and to reduce myocardial infarct size after preischemic administration in a pig model (Klein et al., 1995), we also studied the effect of Cariporide[®] without and

*Dedicated to the memory of Professor Darrel Goll (Tucson, USA), an extremely successful scientist in the calpain field with optimism, sense of humor and warmth.

in combination with A-705253 after postischemic administration.

Results

Exclusion of experiments from statistical analysis

Out of 10 control experiments without inhibitor, 2 had to be ended untimely because of severe arrhythmias and ventricular fibrillation. Likewise, 2 out of 10 experiments with an A-705253 10^{-6} M inhibitor concentration, and 1 heart out of 9 experiments each with inhibitor concentrations of 10^{-7} M or 10^{-8} M had to be ended also because of the severe arrhythmias and ventricular fibrillation. Furthermore, 1 experiment out of 9 with Cariporide[®] (10^{-6} M) and 3 experiments out of 11 treated with A-705253 (10^{-6} M)+Cariporide[®] (10^{-6} M) also had to be ended untimely because of ventricular fibrillation. Due to imperfect staining 1 heart out of the A-705253 (10^{-6} M) and Cariporide[®] (10^{-6} M) treatment group each had to be excluded from histological and statistical analysis.

The high exclusion rate due to severe arrhythmias and ventricular fibrillation, as usually observed in animal models of myocardial infarction (Ravn et al., 1999; Waagstein et al., 1999), however, did not differ significantly between the experimental groups, and thus did not influence the results.

Coronary perfusion, systolic left ventricular pressure, and heart rate

Coronary occlusion was immediately followed by a significant ($p < 0.01$) reduction of the coronary perfusion by approximately 16.9% from its baseline at 29.7 ± 0.6 to 24.7 ± 0.5 ml/min in all perfused hearts ($n=48$). With reperfusion, coronary flow increased initially about 6.7% to 26.2 ± 0.7 ml/min. After 10 min of reperfusion, the flow rate continuously decreased again to a range between

18.3 and 21.1 ml/min up to the end of the experiment. Untreated and treated hearts showed the same reaction (Figure 1).

Similarly, systolic left ventricular pressure dropped significantly ($p < 0.01$) in all perfused hearts ($n=48$) immediately after coronary occlusion as a mean to approximately 26.3% from its baseline of 81.4 ± 2.1 to 69.0 ± 2.0 mm Hg and did not recover any more during reperfusion (Figure 2).

Heart rate was in the range between 185 and 154 min^{-1} during the steady state period and only lightly dropped to a range between 156 and 123 beats min^{-1} up to the end of reperfusion.

There was no significant difference in the reaction of coronary perfusion, systolic left ventricular pressure, and heart rate between the experimental groups.

Enzyme release from heart muscle and potassium concentration

The concentrations of lactate dehydrogenase and creatine kinase in the perfusate increased significantly ($p < 0.001$) during reperfusion in all experiments (Figure 3). Lactate dehydrogenase increased approximately 28.8 up to 57.3 U/l and creatine kinase approximately 65.0 up to 109.8 U/l. No significant difference, however, was found between the different experimental groups. Potassium level in the perfusion fluid remained constant between 4.587 ± 0.078 and 4.812 ± 0.044 mM during all experiments.

Area at risk and infarct size

The area of myocardium, the so-called area at risk below the level of coronary occlusion, which was temporarily excluded for 60 min from perfusion, was $42.4 \pm 1.3\%$ of the total left ventricle wall in all evaluated hearts ($n=46$) without a significant difference between the experimental groups. An infarct size (dead tissue/necrosis) of

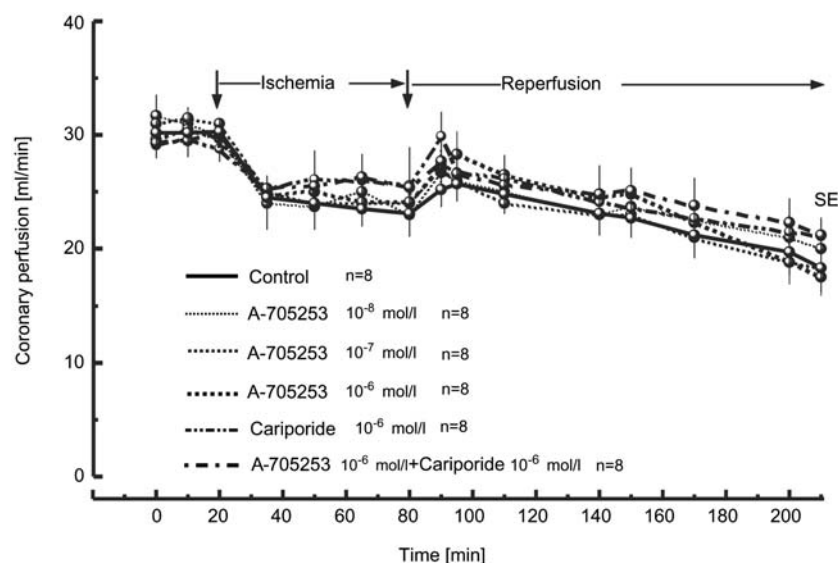


Figure 1 Time course of coronary perfusion of isolated perfused rabbit hearts subjected to transient occlusion of the ramus interventricularis of the left coronary artery for 60 min, followed by 120 min of reperfusion. Control experiments without inhibitor are represented by a solid line and inhibitor treated hearts by dotted lines. Data are expressed as means \pm SEM.

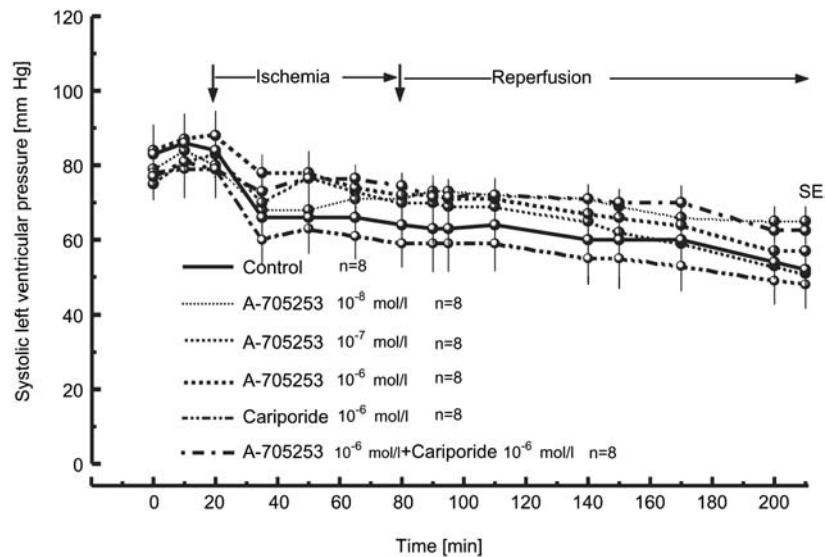


Figure 2 Time course of systolic left ventricular pressure of isolated perfused rabbit hearts subjected to transient occlusion of the ramus interventricularis of the left coronary artery for 60 min, followed by 120 min of reperfusion. Control experiments without inhibitor are represented by a solid line and inhibitor treated hearts by dotted lines. Data are expressed as means \pm SEM.

72.7 \pm 4.0% of the area at risk was observed in untreated control hearts after 120 min of reperfusion (Figure 4). In the presence of calpain inhibitor A-705253 using a concentration of 10⁻⁸ M in the perfusion fluid, the infarcted area was not significantly reduced to 63.9 \pm 3.9% of the area at risk. With increasing inhibitor concentrations, however, the infarct size decreased significantly ($p < 0.01$) to 49.2 \pm 4.1% with 10⁻⁷ M and to 48.3 \pm 2.3% with 10⁻⁶ M, corresponding to an infarct reduction of 33.6%. Sole addition of Cariporide[®] (10⁻⁶ M) or addition of Cariporide[®] (10⁻⁶ M) in combination with A-705253 (10⁻⁶ M) resulted in a significant ($p < 0.01$) infarct size reduction to 50.2 \pm 3.4% and 52.1 \pm 3.7%, respectively, of the area at risk.

Discussion

This study demonstrates for the first time a postischemic cardioprotective effect of calpain inhibition by A-705253 or inhibition of the Na⁺/H⁺-exchanger by Cariporide[®] in a model of myocardial infarction in isolated perfused rabbit hearts. Both inhibitors significantly reduced the infarct size, when administered with the beginning of reperfusion after coronary occlusion. Even with postischemic administration of these inhibitors infarct size could be reduced around an almost equal degree (33.6%) as with preischemic treatment (36.7%; Neuhof et al., 2004) in an identical model. These findings suggest that the final infarct size is determined by an ongoing myocardial damage during reperfusion.

Increased attention has been focused over the past two decades on calpains, besides cardiomyocyte contracture, reactive oxygen species and lysosomal proteases, as leading players in lethal myocardial cell injury during ischemia and reperfusion. Cytosolic Ca²⁺ overload during ischemia and reperfusion (Kihara et al., 1989; Marban et al., 1990) is considered to be mainly responsible for the activation of calcium-dependent calpains, local-

ized primarily in the Z-disk/I-band region (Yoshimura et al., 1986; Kumamoto et al., 1992), and the following breakdown of myocardial proteins. Thus, the calpain-dependent degradation of desmin, calpectrin (Yoshida et al., 1995; Papp et al., 2000), of troponin I and T, and sarcoplasmic reticulum proteins (Singh et al., 2004), corresponding to the myofilament disintegration established by electron microscopy (Gao et al., 1997; Goette et al., 2002), has been reported in ischemia-reperfusion models. Further, calpain activation in ischemia and reperfusion is accompanied by the downregulation of calpastatin, its natural inhibitor (Sorimachi et al., 1997; Enns et al., 2002). Besides direct myofilament disintegration, mitochondrial dysfunction is also considered to contribute essentially to cardiac dysfunction and myocyte injury during ischemia and reperfusion (Lesnefsky et al., 2001). This assumption is supported by observations that calpain inhibition (Neuhof et al., 2003) or the prevention of calpain activation by Na⁺/H⁺ exchange inhibition (Chen et al., 2002) reduces mitochondrial dysfunction during myocardial ischemia and reperfusion. Meanwhile, preischemic calpain inhibition has proved to decrease infarct size in experimental models in rats (Iwamoto et al., 1999; Perrin et al., 2003), in rabbits (Neuhof et al., 2004), and in dogs (Toda et al., 1989; Khalil et al., 2005).

Plasma components and blood cells are not used for perfusion in our experiments. Thus, a reduced expression of cell adhesion molecules and neutrophil-mediated organ injury by calpain inhibition (Ikeda et al., 2002) or other immunologic reactions are not involved in our experimental model.

A cardioprotective effect has also been documented of the Na⁺/H⁺-exchange inhibitor Cariporide[®] in some studies. Klein et al. (1995) found a reduction of the infarcted area in reperused pig hearts following local ischemia by pretreatment with Cariporide[®], and Otani et al. (2000) observed an improved left ventricular function and a diminished enzyme release upon global ischemia in rat

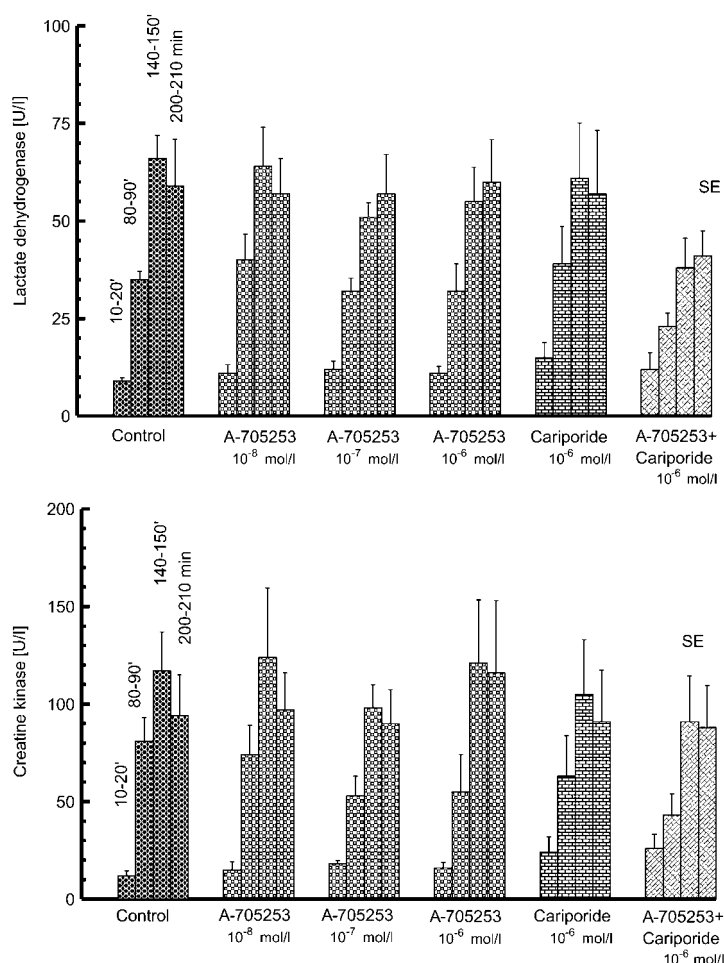


Figure 3 Release of lactate dehydrogenase and creatine kinase into the perfusion fluid of isolated perfused rabbit hearts subjected to transient occlusion of the ramus interventricularis of the left coronary artery for 60 min, followed by 120 min of reperfusion. Control experiments without inhibitor are represented by a dark-colored column and inhibitor treated hearts by light-colored columns. Data are presented as means \pm SD.

hearts. Even in patients undergoing coronary bypass surgery, pretreatment with Cariporide[®] reduced mortality and the risk of myocardial infarction (Boyce et al., 2003); however, cerebrovascular events increased (Mentzer et al., 2008). Cardioprotective effects are also reported in animal experiments with other Na⁺/H⁺-exchange inhibitors (Knight et al., 2001; Lee et al., 2005) or Na⁺/H⁺-channel blockers, which limit the upregulation of the cardiac calpain system after myocardial infarction (Sandmann et al., 2002). The Na⁺/H⁺-exchange inhibitor Cariporide[®] reduced the infarct size in our experiments in the same degree as the calpain inhibitor A-705253.

This protective effect can be attributed in our model exclusively to an influence on the calpain system, since the combination of both inhibitors did not augment the protective effect of sole calpain inhibition. The calpain inhibitor A-705253 is known to directly block the catalytic center of activated calpains, whereas the Na⁺/H⁺-exchange inhibitor Cariporide[®] prevents or reduces the ischemic intracellular Ca²⁺-overload and thus prevents or reduces the following calpain activation. This assumption is supported by Chen et al. (2002) who observed a reduced calpain activation in postischemic perfused rat hearts, and by Cun et al. (2007) who found a reduced

calcium overload in global ischemic rabbit hearts preconditioned with Cariporide[®].

No reduction in the release of lactate dehydrogenase and creatine kinase by inhibition of calpain or the Na⁺/H⁺-exchanger, as observed after global myocardial ischemia (Chen et al., 2002; Neuhof et al., 2003; Liu and Schellmann, 2003) was found in our study with transient local ischemia. This may be due to an enzyme loss from small affected tissue volumes with insufficient concentration for detection in the open non-recirculating perfusion system. Differences in coronary flow between treated and untreated hearts are not expected, because infarcted areas are reperfused as well. The slowly decreasing coronary flow up to the end of experiment can be related to an increasing vascular resistance (by endothelial swelling), also observed in normal perfused hearts without ischemia. Inhibitor treated hearts with significantly reduced infarct size did not show a better myocardial function (ventricular pressure performance) than untreated controls during 120 min of reperfusion. This behavior becomes clear by observations that salvage of myocardium from necrosis after local ischemia by reperfusion has little instant effect on systolic myocardial performance, as its functional recovery takes up to 4 weeks (The-

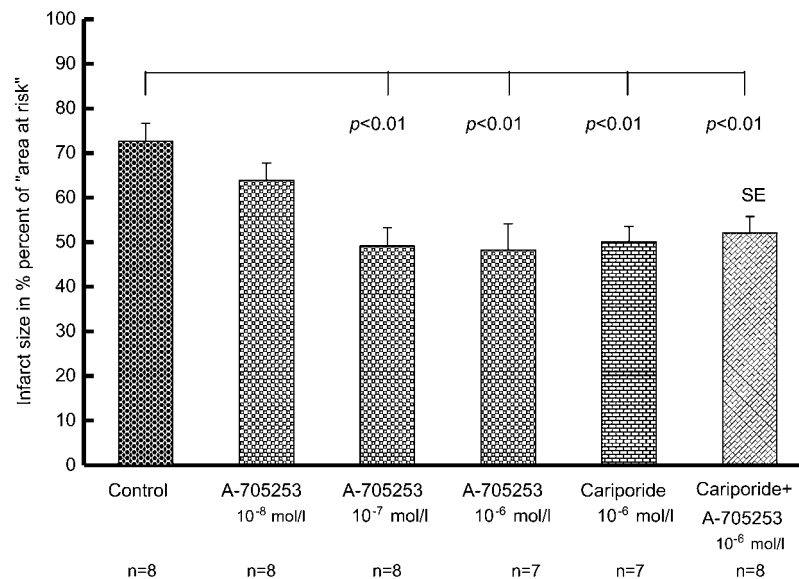


Figure 4 Development of myocardial infarction in isolated perfused rabbit hearts after occlusion of the ramus interventricularis of the left coronary artery for 60 min, followed by 120 min of reperfusion.

Infarct size is expressed in percentage of the area at risk (the transiently not perfused myocardium). Control experiments without inhibitor are represented by a dark-colored column and inhibitor treated hearts by light-colored columns. Data are presented as means \pm SD. Infarct size is significantly reduced by calpain inhibition in all treated hearts compared to untreated controls.

roux et al., 1976; Lavalley et al., 1983; Ellis et al., 1985). To evaluate not only the salvage from necrosis but also the return of function, long-term experiments have to be performed in intact animals. In contrast to the infarct model in which the time of transient local ischemia is chosen long enough to develop necrosis, in models of global ischemia the duration of perfusion stop is restricted to enable at least a speedy recovery with reperfusion.

As the calpain inhibitor used in our study is also inhibiting the lysosomal cysteine proteases cathepsin B and L, it has to be discussed whether inhibition of both proteases could account for the cardioprotective effect. So far, only poor information is available for both cathepsins in the heart. Thus, activation of cathepsin L was found in the intralysosomal compartment of cardiomyocytes during coronary-aortal bypass (Turski and Zaslanka, 2000). The increase of functional recovery and the decrease of protein degradation of total activity of cathepsin B and L and of cathepsin B leakage by cysteine proteinase inhibitors in experimental models of myocardial ischemia and infarction (Tsuchida et al., 1986; Shibata et al., 1992), however, cannot be related to both proteinases, as these inhibitors could also inhibit calpains. Since calpain inhibitor A-705253 and the Na⁺/H⁺-exchange inhibitor Cariporide[®] reduce infarct size in the same degree without an additional effect by their combination, these findings suggest that cardioprotection in our study is due to the inhibition of calpain and not of cathepsin B and L. These cathepsins are not calcium-activated proteases and their activity would not be inhibited by a Na⁺/H⁺ exchange inhibitor.

Both the calpain inhibitor A-705253 and the Na⁺/H⁺ exchange inhibitor Cariporide[®] showed excellent protection against myocardial infarction after transient local coronary occlusion when administered with the onset of reperfusion. These findings may suggest a prophylactic

calpain inhibition in clinical situations of coronary surgery and coronary angioplasty, which are often threatened by acute myocardial failure and infarction.

Materials and methods

This study was approved by the Institutional Animal Care and Use Committee of the Justus Liebig University of Giessen and was performed in accordance with the guidelines of the National Institute of Health (USA).

Reagents and materials

Calpain inhibitor A-705253 (Abbott GmbH & Co. KG, Ludwigshafen, Germany) is a representative of a novel class of non-peptidic calpain inhibitors, as described by Lubisch et al. (2003). These inhibitors are benzoylalanine-derived ketoamides carrying vinylbenzyl amino residues in the P²/P³ region and inhibit calpain in nanomolar concentrations. A-705253 inhibits calpain-I with a K_i of 27 \pm 2.5 nM for the enzyme inhibitor complex. Cathepsins B and L are inhibited by A-705253 with a K_i of 62 \pm 9 and 149 \pm 15 nM, respectively. No inhibition is detected for yeast proteasome, papain, and human ICE/caspase1 at concentrations up to 10 μ M. Negative side effects of this inhibitor are not reported up to now and were not observed in our experiments previously performed in pigs and isolated rabbit hearts. A-705253, however, is not yet approved for use in humans.

Cariporide[®] (4-isopropyl-3-methylsulfonyl-benzoylguanidinemethansulfonate), a Na⁺/H⁺-exchange inhibitor (Scholz et al., 1999), was developed and obtained from Hoechst-Marion-Roussel AG (Frankfurt/Main, Germany). Poly(0-2-hydroxyethyl)-starch (HAES-steril[®] 10%) was obtained from Fresenius (Bad Homburg, Germany), and Custadiol[®] from Dr. F. Köhler Chemie GmbH (Alsbach-Hänlein, Germany). Na-pyruvate and 2,3,5-triphenyltetrazolium chloride were purchased from Sigma-Aldrich (Deisenhofen, Germany), and Evans blue from Merck (Darmstadt, Germany).

Isolated heart preparation

Half-breed rabbits of either sex weighing between 2.5 and 3.2 kg were anesthetized with pentobarbital sodium (60–80 mg/kg) and anticoagulated with heparin sodium (1000 IU/kg) injected into an ear vein. A tracheotomy was performed, and the rabbits were mechanically ventilated with room air by means of a Starling pump (B. Braun, Melsungen, Germany). The thorax was opened via the diaphragm followed by a median sternotomy. The aorta was cannulated retrogradely and cardioplegia was induced by injection of 25–30 ml of ice-cold (4°C) Bretschneider solution (Custadiol®). The hearts were excised, mounted onto a modified Langendorff perfusion system and excess tissue was removed. A latex balloon (1.3 ml capacity) for pressure monitoring was inserted into the left ventricle and connected to a second extracorporeal circuit according to the 'working' heart model, as described by Bardenheuer and Schrader (1983). Left end-diastolic influx pressure (preload) was set hydrostatically to 7 mm Hg and 'aortic' pressure (afterload) to 50 mm Hg by means of a water filled reservoir and the height of the outflow tube, respectively. After a cardioplegic arrest of 3–4 min, the hearts were perfused retrogradely with a constant hydrostatic pressure of 70 mm Hg with oxygenated Krebs-Henseleit-HAES buffer at 37°C, which was filtered by a 0.2- μ m Pall filter (Pall Biomedical Inc., Fajardo, PR, USA). The buffer was bubbled with a mixture of 95% O₂ and 5% CO₂ resulting in a PO₂ of >400 mm Hg in the perfusate and >200 mm Hg in the coronary effluent, and the pH was maintained between 7.35 and 7.45 by increasing or decreasing the CO₂ admixture. During the experiments, the spontaneously beating hearts were submerged into a container (25 ml capacity) at 37°C filled with the coronary effluent to prevent exciccation. Coronary perfusion pressure and coronary flow were measured continuously by a pressure transducer (Combi-trans®, B. Braun) and electromagnetic flow probe (Empo/Gould, Oxnard, CA, USA), respectively, in the perfusion line. Left ventricular pressure and end-diastolic pressures were measured by another pressure transducer connected to the balloon inserted into the cavity of the left ventricle. Isovolumetric left ventricular pressure was measured intermittently by clamping off the connections to the inflow reservoir and the outflow tube. A bipolar electrocardiogram was conducted from apex versus heart base. All these parameters were monitored continuously and recorded on a multichannel recorder.

Perfusate

For perfusion, a modified Krebs-Henseleit buffer solution (KHHB) with Na-pyruvate (0.5 g/l) was prepared, and hydroxyethyl starch (HAES-steril® 10%) was added to keep colloid oncotic pressure between 23 and 25 mm Hg, yielding the following final concentrations: starch 50 g/l; Na⁺ 138 mM; K⁺ 4.5 mM; Mg²⁺ 1.33 mM; Cl⁻ 135 mM; Ca²⁺ 2.38 mM; glucose 12 mM; Na-pyruvate 4.5 mM, and HCO₃⁻ 12 mM. Osmolality was approximately 330 mosmol/kg. The pH of the perfusate was adjusted to 7.4 with 1 M NaHCO₃ before usage. Oncotic pressure and osmolality were controlled by an Onkometer (BMT 921, Dr. Karl Thomae, Biberach, Germany) and a Mikro-Osmometer (Roebbling Messtechnik, Berlin, Germany).

Experimental protocol

Perfused hearts were stabilized for 20 min. After this steady state period, the ramus interventricularis of the left coronary artery was blocked by means of a Tourniquet just below the origin of the first diagonal branch for 60 min. Hearts selected for the study were those that developed a constant systolic left ventricular pressure of more than 60 mm Hg during the steady state period. After 60 min of occlusion the Tourniquet was reopened and the coronary arteries reperfused for 120 min.

In 8 perfused hearts each, calpain inhibitor A-705253 was added to the perfusate from the beginning of reperfusion in final concentrations of 10⁻⁸, 10⁻⁷ and 10⁻⁶ M, respectively. In 8 experiments the Na⁺ / H⁺-exchange inhibitor Cariporide® was administered in a final concentration of 10⁻⁶ M, and in a further 8 hearts a combination of A-705253 (10⁻⁶ M)+Cariporide® (10⁻⁶ M) was used. Eight hearts were reperfused without inhibitors as control.

Measurements

Isovolumetric left ventricular pressure amplitude and heart rate were determined by pressure records and electrocardiogram, respectively. Coronary flow was monitored continuously by an electromagnetic flow transducer. Samples of coronary effluent were collected intermittently for control of pH, PO₂, and PCO₂, and for determination of creatine kinase and lactate dehydrogenase activity using kits from Boehringer (Mannheim, Germany).

Determination of area at risk and infarct size

At the end of each experiment, perfusion was stopped and the ramus interventricularis of the left coronary artery was closed again by the Tourniquet. A total of 5 ml of Evans blue (5%) was injected via the perfusion catheter into the coronary circulation to stain the non-ischemic zone blue and to demarcate the area at risk. The atria, right ventricle, and great vessels were removed and the left ventricle was cut in 7–8 slices of approximately 3 mm thickness, which then were incubated in 2,3,5-triphenyltetrazolium chloride (1%) for 15 min at a temperature of 37°C. By this procedure, normal perfused myocardium was stained blue and the viable tissue within the area at risk was stained red, whereas the infarcted dead myocardium remained unstained. The stained slices were photographed on both sides by means of a digital camera (Coolpix 950, Nikon Corp., Tokyo, Japan). The computer programs Paint Shop Pro6 (Jasc Software Inc., Eden Prairie, MN, USA) and Image J (NIH, Bethesda, MD, USA) were used for color discrimination and planimetric evaluation of normal perfused myocardium, area at risk, and infarct size. Both sides of each left ventricle slice were evaluated and the areas added up, corresponding to their different color. Infarct size (dead myocardium) was expressed in percentage of area at risk (the temporarily non-perfused myocardium due to the 60 min of coronary occlusion).

Statistics

The data were analyzed by means of the Statgraphics-Plus® statistical analysis package from Statistical Graphics Corporation (Rockville, MD, USA). Intergroup analysis was performed by analysis of variance. Fisher's LSD test was used for multiple group comparison. Data are presented as means±standard error of the mean. A *p*-value <0.05 was considered as statistically significant.

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