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Mechanisms of apoptosis after ischemia and reperfusion: Role of the renin-angiotensin system

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Background: **Apoptosis plays a key role in the pathogenesis of cardiac diseases. We examined the influence of the renin-angiotensin system (RAS) on different regulators of apoptosis using an isolated hemoperfused working porcine heart model of acute ischemia (2 h), followed by reperfusion (4 h).** ***Methods and Results:*** **23 porcine hearts were randomized to 5 groups: hemoperfused non-infarcted hearts (C), infarcted hearts (MI: R. circumflexus), infarcted hearts treated with quinaprilat (Q), infarcted hearts treated with angiotensin-I (Ang I), and infarcted hearts treated with angiotensin-I and quinaprilat (QA). Fas, Bax, bcl-2 and p53 proteins were increased in MI hearts and further elevated by Ang I. Quinaprilat reduced Bax and p53. Bcl-2 was elevated in Q and reduced in QA. An early upregulation of caspase-3 gene and protein expression was detected in MI and Ang I hearts compared to C. Q reduced caspase-3 gene expression, but had no effect on caspase-3 and Fas protein.** ***Conclusions:*** **These data suggest that the RAS plays a pivotal role in cardiac apoptosis which is the early and predominant form of death in myocardial infarction. Ischemia/reperfusion induces programmed cell death via extrinsic and intrinsic pathways. Early treatment with quinaprilat attenuated cardiomyocyte apoptosis.**

Keywords: ACE-inhibition; apoptosis; fas; myocardial infarction; P53

Abbreviations: Ang: angiotensin; LV: left ventricle; Q: quinaprilat; MI: infarct group.

Introduction

Cardiomyocyte apoptosis is a rare event in healthy myocardium. However, hypertrophied,[1] infarcted,[2] senescent,[3] and failing hearts,[4] as well as hibernating myocardium[5] contain apoptotic cardiomyocytes. Apoptosis is the early and predominant form of cell death in infarcted myocardium. Programmed cell death in the heart has been linked to ischemia and reperfusion injury.[6] Apoptotic cardiomyocytes can already be detected 2 h after coronary artery occlusion, whereas necrosis peaks at day 1.[7] They may be found in fibrotic areas as well as in non-fibrotic regions in ischemic porcine hearts. Their number usually increased during the period of reperfusion. Several therapeutic strategies to prevent apoptosis of cardiomyocytes have been evaluated during the last years. Caspase inhibition reduced myocyte cell death induced by myocardial ischemia and reperfusion *in vivo*.[8] The actual concentrations of bcl-2 and Bax play an important role in the protection or acceleration of cardiomyocyte apoptosis after ischemia and/or reperfusion.[9] The ratio of bcl-2 to Bax plays a major role in determining whether or not a cell dies after it has received an apoptotic signal.[9] The regulation of bcl-2 and Bax may be affected by myocardial infarction as well as heart failure by increased mechanical load.[10] The ratio may also be influenced by p53 signaling.[11] p53 is a direct transcriptional activator of the human Bax gene.[12] It enhances the cardiomyocyte renin-angiotensin system and decreases the bcl-2/Bax ratio in the cells.[11] Changing the bcl-2 /Bax ratio is not enough to induce apoptosis. For example, if Fas, an important promotor of apoptosis, is overexpressed, bcl-2 is unable to inhibit apoptosis.[13]

Apoptosis of cardiomyocytes may further be influenced by Ang II, which binds to the AT_1 receptor.[14] Thereby, it enhances intracellular calcium and stimulates calcium dependent endogenic endonucleases, which cause DNA laddering, cell shrinking and formation of apoptotic bodies. Mechanical stretching of cardiomyocytes *in vitro* causes Ang II release, increased Bax expression, and apoptosis.[14] Diastolic stress induces programmed cell death *in vivo* and *in vitro*. This is accompanied by an increase in Bax and Ang II and a simultaneous decrease of bcl-2 and may be inhibited by the AT_1-blocker losartan.[14,15]

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Little is known concerning the mechanisms modulating apoptotic cell death in ischemia and reperfusion. The implication of p53 and p53-dependent genes in the onset of apoptosis has been excluded *in vivo*.[16] This finding contrasts *in vitro* investigations which have documented the involvement of p53.[17]

The principal aim of this study was *first* to investigate the effects of the Renin-Angiotensin System (RAS) on different regulators of apoptosis in infarcted porcine hearts using an ischemia/reperfusion model, *and second* to answer the question, whether local ACE plays a role in the induction of apoptosis and whether p53 is involved in the induction of programmed cell death. For this purpose, we perfused the hearts with Ang I and investigated the influence of Ang II on apoptosis following the ischemia-reperfusion injury. Our third objective was to test the effects of ACE-inhibition with quinaprilat on regulators of apoptosis.

Materials and methods

Organ selection, transport

Twenty eight porcine hearts were obtained from a slaughterhouse. Health conditions of animals were controlled and verified by a veterinarian. Only female German landrace pigs with a total body weight range of 60–80 kg were chosen and killed according to the rules established by the Veterinary Council of the European Community. Immediately after bleeding, sternotomy was performed and the heart was carefully excised and placed in cardioplegic solution as published earlier.[18,19] The organs were transported to the laboratory within 1 h at 4°C.

Organ perfusion

Twenty-three hearts were prepared and connected to the perfusion apparatus developed in collaboration with Mediport Biotechnik GmbH (Berlin, Germany) exactly as described earlier.[18,19] Five additional non-perfused hearts served as controls. When connected, the hearts were warmed up to 37°C and stimulated to beat by starting the perfusion apparatus. Autologous blood which had been collected from the respective donor at the slaughterhouse was diluted 2:1 with a modified Krebs Henseleit solution,[18] warmed up to 37°C by a heat exchanger connected to a water bath and oxygenated by a flow meter (Krohne DK800R Duisburg, Germany) with a mixture of oxygen and carbon dioxide (95% O_2 and 5% CO_2) was pumped through the organ by the machine.

Infarction

When the hearts beat regularly after one initial hour of perfusion, 5 of the 23 working hearts were perfused for another 6 h without further intervention (group: control, C). The remaining 18 hearts were infarcted by occluding the Ramus circumflexus of the left coronary artery for 2 h before reperfusion was continued for another 4 hours (group: infarct, MI, $n = 5$). The Ramus circumflexus was closed by a three-way valve (stop-cock). The perfusion was stopped but it was possible to apply drugs such as Ang I and quinaprilat via one way. After 2 h of occlusion, the three-way valve was opened again and the heart was reperfused for 4 hours (reperfusion time). Ten hearts were treated with Ang I (Sigma Deisenhofen, Germany). 100 μl 10^{-7} M Ang I were injected in the R. circumflexus. The dose was chosen according to Farquharson *et al.* 2002.[20] Moreover, the cardioprotective effects of the ACE-inhibitor quinaprilat (5 mg) were tested (group: QA: $n = 5$ and group Q: $n = 3$).[21,22] Ang I and Quinaprilat were applied for 30 min 10 min before start of the reperfusion.

Tissue samples

Left ventricular (LV) endomyocardial biopsies were obtained using a disposable bioptome (Cordis, Miami, FL, USA). Before coronary occlusion and at the end of the occlusion (2 h) biopsies were taken for morphological investigation. At the end of the reperfusion (4 h), the whole left ventricles were cut serially from apex to base. Sections were investigated from each myocardial sample. Samples for angiotensin measurements, histochemistry, electron microscopy, immunohistochemistry, western blot analysis, and PCR were frozen immediately in liquid nitrogen and stored at –80°C. For electron microscopy, freshly isolated myocardium was cut into 2 mm squares and submerged in 3% gluteraldehyde/0.1 M phosphate buffer, [pH 7.2–7.4].

Measurements of angiotensins in cardiac tissue

Tissue angiotensin I and II levels were measured in pooled samples from the various groups (2–8 samples per group), after SepPak extraction and high-performance liquid chromatography (HPLC) separation, by radioimmunoassay.[23] A known amount of ^{125}I-Ang I was added as an internal standard before the extraction procedure, and the recovery of ^{125}I-Ang I after HPLC separation was used to correct for losses (maximally 20-30%) that occurred during extraction and separation.[24]

Histological analysis

Cardiac tissue was fixed in formalin, embedded in paraffin and sectioned with a microtome. Hematoxylin and eosin, was used to stain paraffin sections.

Table 1. Angiotensin I and II levels in pooled LV myocardial tissue samples

	Treatment	Area	Ang I fmol/g	Ang II fmol/g	Ang II/Ang I ratio
Control	None		1.0	32.0	32.0
MI	None	Non-infarcted	1.9	11.0	5.8
		Infarcted	2.0	8.0	4.0
MI	Q	Non-infarcted	3.2	5.0	1.6
		Infarcted	5.3	10	1.9
MI	Ang I perfusion	Non-infarcted	2.8	37.0	13.2
		Infarcted	12.9	34.0	2.6
MI	Ang I perfusion + Q	Non-infarcted	11.9	20.0	1.7
		Infarcted	21.6	15.0	0.7

Immunohistochemistry

The monoclonal antibodies used for this study were against caspase-3, Fas, Bax, bcl-2 (all Chemicon, Hofheim, Germany), and p53 (Cymbus Biotechnology, Chandlers Ford, UK). Antigen-antibody complexes were visualized with the labeled streptavidine-biotin method.

Automatic image analysis

Automatic image analysis was performed as published earlier.[19,25,26]

RNA isolation

To isolate RNA from myocardium, we used the TRIzol® Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.[19]

Reverse transcription

1 μg of total RNA was reverse transcribed in a total volume of 20 μl using the First Strand cDNA Synthesis Kit™ (MBI Fermentas) according to the manufacturer's recommendations.

TaqMan-PCR

To quantify expression levels of genes of interest we employed the real-time quantitative RT ("TaqMan") PCR. Appropriate primers and fluorogenic probes were designed with the Primer Express® software. The ABI PRISM® 7000 SDS instrument in conjunction with the ABI TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany) was used to perform the assays. The reaction volume was 25 μl with a final concentration of 400 nM for the primers and 200 nM for the probes. PCR conditions were used as recommended by the manufacturer (2 min 50°C, 10 min 95°C, 45 cycles of 15 s 95°C and 1 min 60°C). Fluorogenic probes were synthesized by TIB Molbiol (Berlin, Germany) and the primers (caspase-3, 18S) were obtained from Proligo (Evry Cedex, France).

Relative quantitation was done using the standard curve method. For each gene, a PCR fragment ("standard") containing the sequence of the TaqMan-system was generated (Table 1) and purified with the QIAquick® PCR purification kit (Qiagen, Hilden, Germany). Seven serial 1:10 dilutions of this fragment served as a standard curve that was assayed together with the corresponding unknown samples on each plate. Every sample was measured in triplicate. To normalize our expression data, we used 18S-rRNA as the housekeeping gene.

Western blot analysis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and immuno-blotting were carried out following routine protocols.[25,26] The following antibodies were used for this study: Bax, bcl-2, Fas and PARP (Transduction Laboratories, NJ, USA), p53 and caspase-3 (R & D Systems, Wiesbaden, Germany).

Transmission electron microscopy (TEM)

A detailed description of the technique used for the following experiments was published by Shakibaei *et al.* [27]

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

In order to identify apoptotic cells containing DNA fragments, the tissue samples were labeled by immunohistochemical staining with the Apopdetect Peroxidase Kit (Qbiogene, Heidelberg, Germany) according to

Figure 1. Electron micrographs of normal porcine heart without signs of pathological changes at the ultrastructural levels (**A**) with intact cardiomyocytes (arrows) embedded in well-developed numerous cardiomyofibers (∗). Figure 1**B** shows porcine heart of the infarct area which represents myopathic fibers and apoptotic cardiomyocytes. Most myofibers of this area are clusters of degenerated myofibers (∗), and the most cardiomyocytes (arrows) manifest features typical of apoptosis, e.g., membrane blebbing, nuclear changes (with peripheral segregation and aggregation of chromatin into dense areas) and the presence of apoptotic bodies. The inset (**C**) shows a higher magnification view of the extensive expression of extracellular matrix sheath (M) of cardiomyocytes. A: × 2.000; bar = 2 μm, B: × 2.000; bar = 2 μm, C: × 5.000; bar = 2 μm.

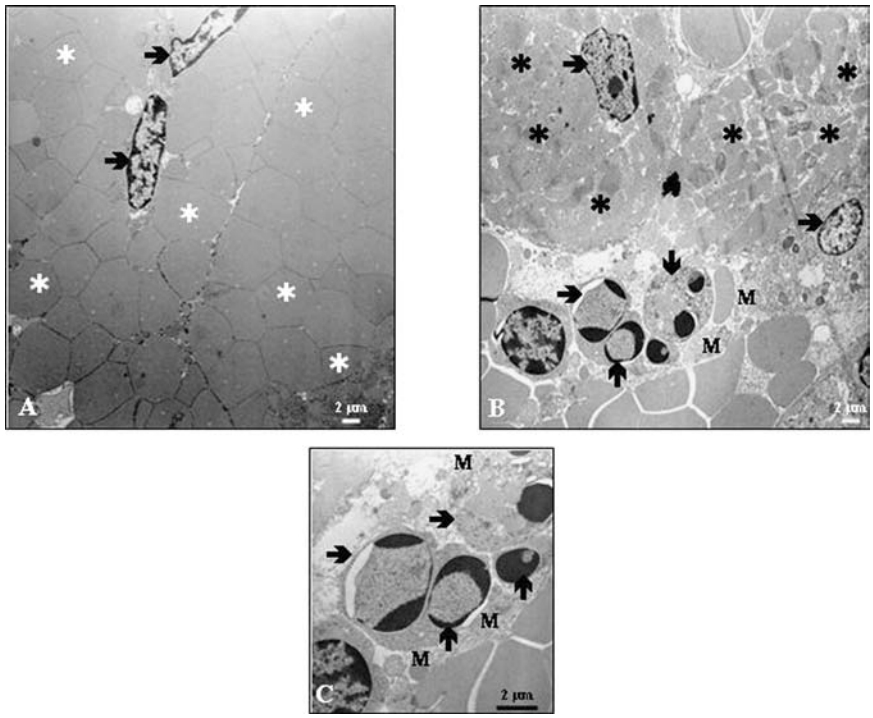


Figure 2. Hematoxylin-eosin staining: **A, B:** Control myocardium. No inflammatory cells were detectable in post-capillary vessels and the interstitium. **C–E**: Infarct area: Neutrophils adhered to capillary endothelium and were found in the interstitial space in ischemia/reperfusion.

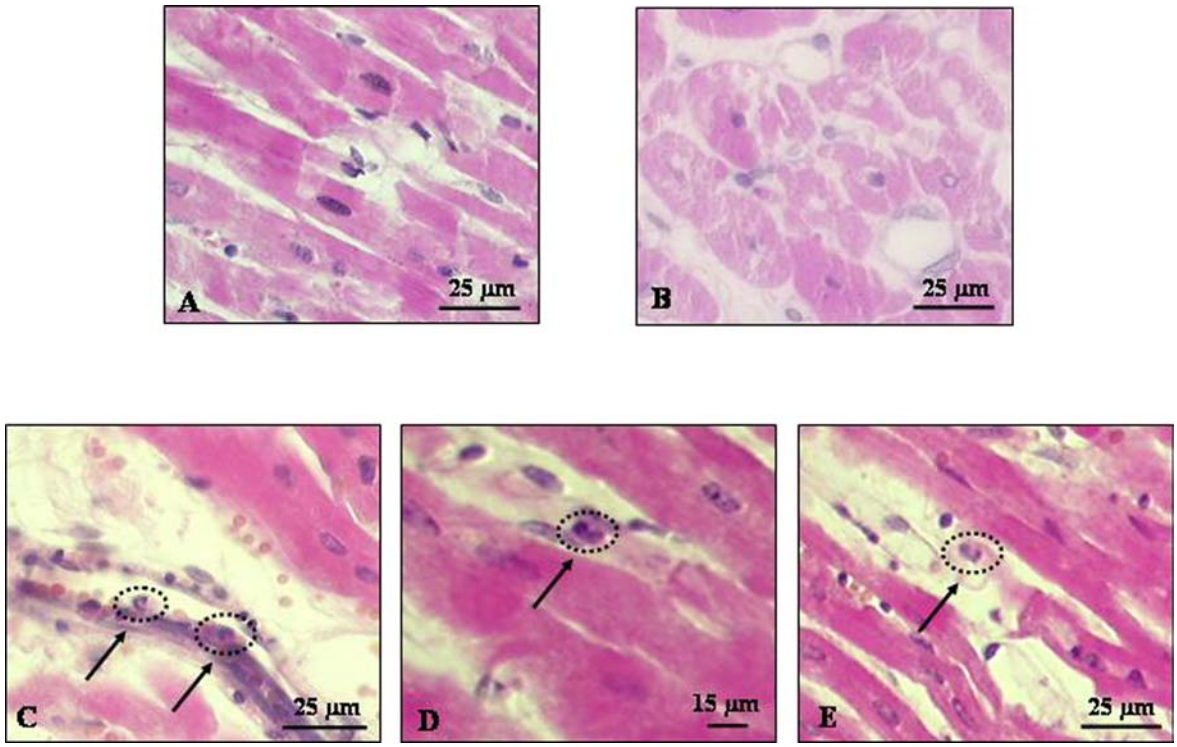
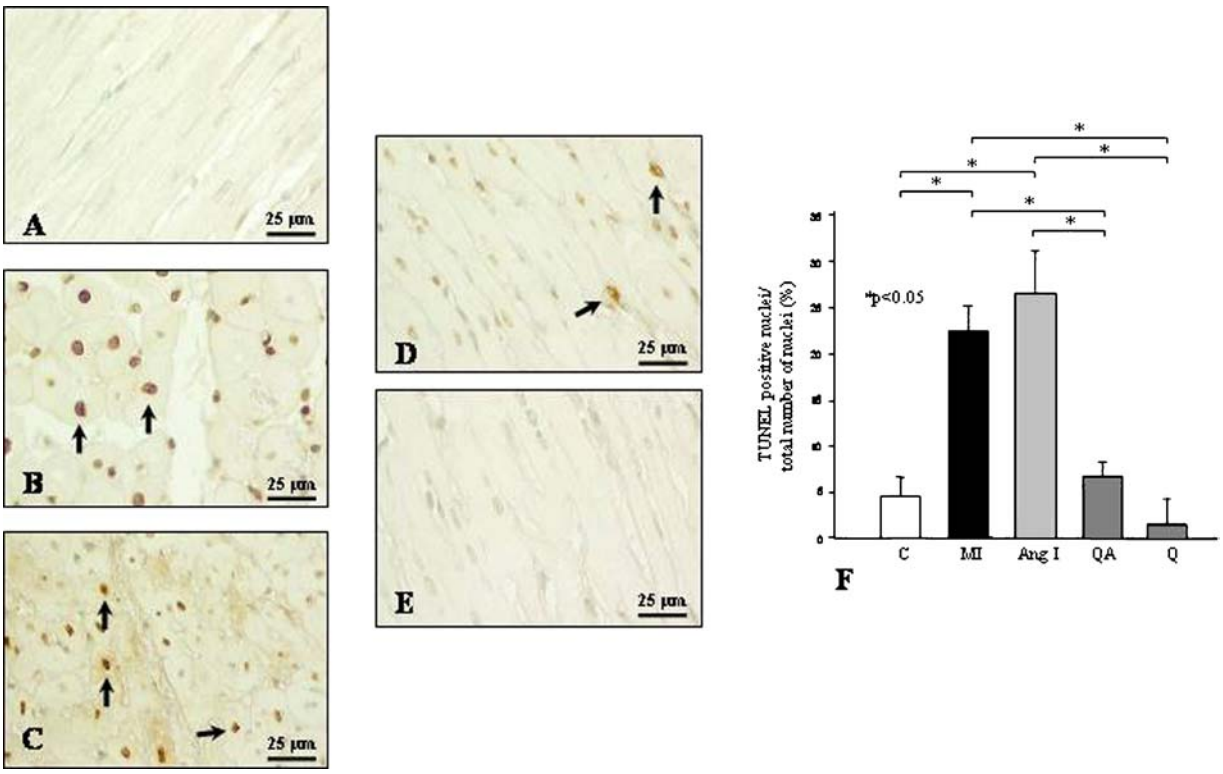


Figure 3. TUNEL staining: **A:** No positive cells were detectable in C hearts. **B:** Numerous TUNEL positive nuclei (arrows) were found in the infarct area of MI hearts. **C:** Similar results were obtained in Ang I hearts. **D:** Quinaprilat applied together with Ang I significantly reduced the number of apoptotic cardiomyocytes. **E:** Quinaprilat given alone after MI normalized the amount of programmed cell death in cardiomyocytes. **F:** Quantitative image analysis. Apoptotic cardiomyocytes were significantly increased in all hearts with ischemia and reperfusion. Ang I application further elevated the amount of TUNEL positive nuclei compared to MI hearts. This increase was attenuated by Quinaprilat application.



the manufacturer's protocol. The method was described earlier.[28]

Statistical evaluation

Statistical analysis was performed using SPSS 11.5. All data are expressed as means ± standard deviation (SD). We tested all parameters for deviations from Gaussian distribution by Kolmogorov-Smirnov test and compared the cases by the use of independent-samples *t*-test or Mann-Whitney test (dependent of the results from the normality test). Differences were considered significant at the level of $P < .05$.

Results

Morphological features of apoptosis

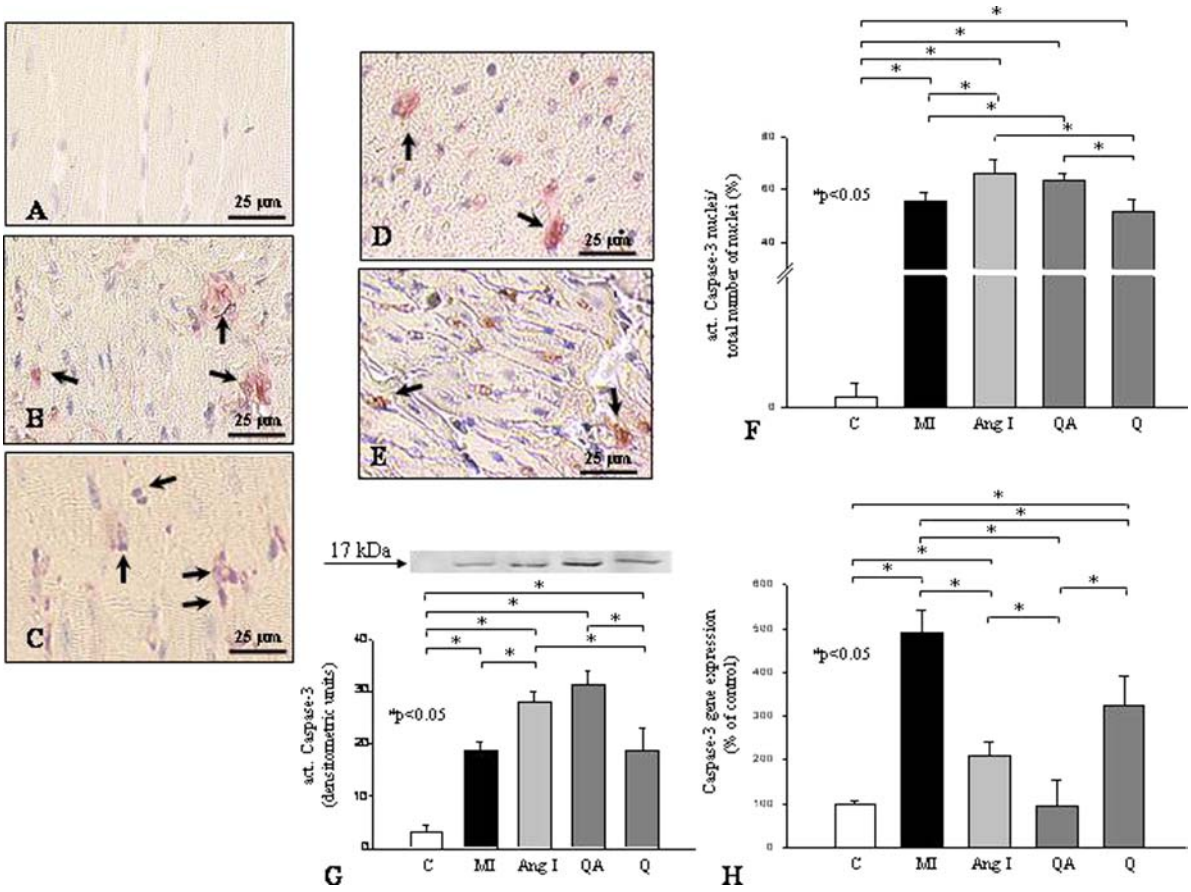
Electron microscopy of the infarct area revealed nuclei with chromatin condensation, membrane blebbing as well as cellular fragmentation and formation of apoptotic bodies (Fig. 1B, C) which are characteristic signs of programmed cell death. Hematoxylin and eosin staining revealed a slight interstitial infiltration of inflammatory cells (neutrophils) after 2 h of ischemia followed by 4 h of reperfusion (Fig. 2D, E). Neutrophils adhered to capillary endothelium (post-capillary venules) as sign for an early stage of acute inflammation (Fig. 2C).

A clear increase in apoptotic cardiomyocytes in the infarct area and remote area of MI (6.08-fold/3.5-fold; $P < 0.009$ vs. C) and Ang I (6.01-fold/3.42-fold) left ventricles was detected by TUNEL staining. Quinaprilat reduced this amount in the infarct area by 73.3% and in the remote area by 66% in the QA group and normalized it in the Q group (Fig. 3A–F). There was no statistical difference between perfused and non-perfused control hearts.

Cardiac levels of Angiotensin I and II

MI increased Ang I two-fold (Table 1), and Ang I perfusion of MI hearts with or without Q further increased Ang I. MI reduced Ang II 3–4-fold. Ang I perfusion of MI hearts increased cardiac Ang II to control levels. Q abolished this

Figure 4. **A:** Immunohistochemical investigation of activated caspase-3. No caspase-3 positive cells were detectable in C hearts. **B:** Numerous caspase-3 positive nuclei (arrows) were detectable in the infarct area of MI hearts. **C:** Similar results were found in Ang I hearts. **D:** Quinaprilat (QA group and **E:** Q group) had no effect on caspase-3 protein. **F:** Quantitative image analysis of acitivated caspase-3: Activated caspase-3 was significantly increased in all hearts with ischemia and reperfusion. Ang I application significantly enhanced the amount of capase-3 compared to MI hearts. **G:** Western blot analysis and densitometry confirmed the data obtained by immunohistochemistry. **H:** Gene expression of caspase-3 measured by TaqMan PCR revealed a clear elevation of the gene in MI hearts compared to C hearts. Ang I, Q and QA hearts exerted a significantly lowered gene expression of activated caspase-3.



increase. The 5-10-fold reduction of the Ang II/I ratio during Q demonstrates that ACE inhibtion was effective (Table 1).

Detection of activated caspase-3 protein

Detection of activated caspase-3 in cardiomyocyte nuclei indicates programmed cell death. A significant elevation of caspase-3 positive nuclei was detectable in MI hearts compared to control hearts ($P < 0.009$). The amount of activated caspase-3 in perfused and non-perfused control hearts was not significantly different from each other. Ang I significantly increased the amount of activated caspase-3 positive nuclei (Fig. 4A–E). Quinaprilat had no effect on activated caspase-3. The 17 kDa-protein is not detectable in control myocardium and clearly elevated in all infarcted groups (Fig. 4F, G).

Gene expression of caspase-3

Caspase-3 gene expression as measured by TaqMan PCR is clearly enhanced in MI hearts, only slightly elevated in Ang I myocardium, and reduced by quinaprilat (Q and QA group) ($P < 0.05$) (Fig. 4H).

Poly(ADP-ribose)polymerase activity (PARP) protein

PARP 116-kDa protein is 2.35-fold increased in MI hearts, 3.2-fold in Ang I treated hearts, and 2.6-fold in quinaprilat treated hearts as compared to control hearts (Fig. 5A). In parallel, the 85-kDa apoptosis-related cleavage fragment, which results from enhanced PARP activity and apoptosis, was significantly elevated in MI, Ang I, QA and Q hearts (Fig. 5B). Quinaprilat or Ang I treatment had no further effect on the amount of PARP protein.

Figure 5. Western blot analysis and densitometry of Poly (ADP) ribose polymerase. **A:** 116- kDa PARP was clearly enhanced in all infarcted groups. **B:** In parallel, the 85-kDa cleavage fragment of PARP was elevated in the ischemic and reperfused hearts. Quinaprilat had no effect on PARP protein.

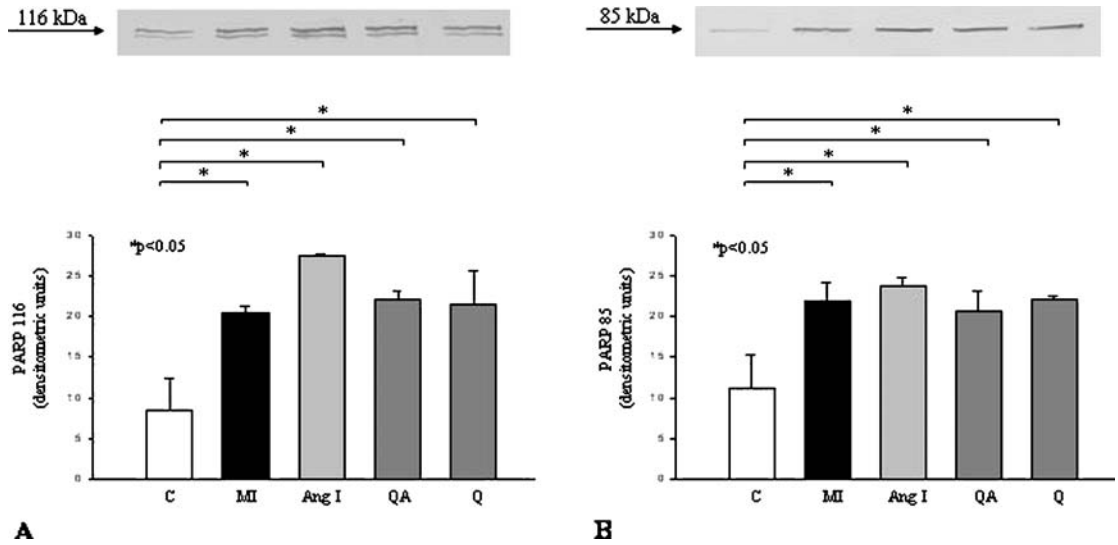
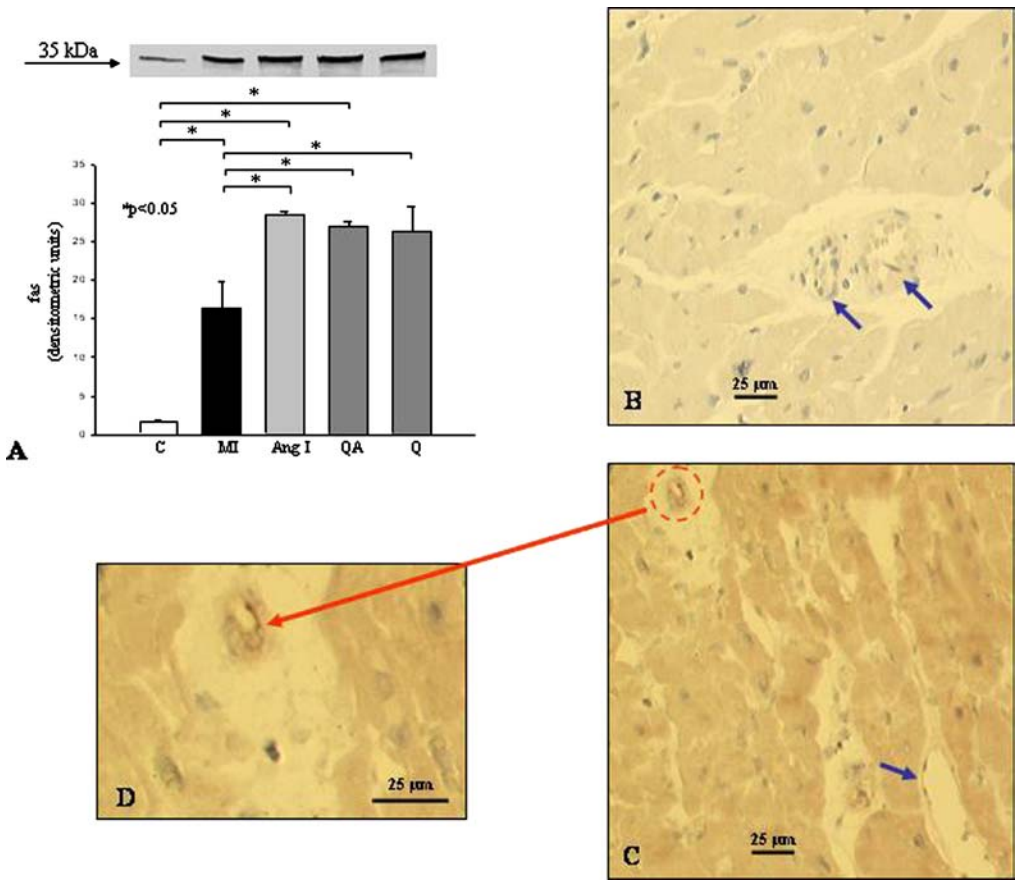


Figure 6. Western blotting and densitometry of **A:** Fas protein. Fas was increased in MI hearts in the infarct area and further enhanced by Ang I. Quinaprilat did not change the content of Fas protein in the infarct area. **B:** Fas was negative in control hearts. **C, D:** Fas was detectable in cardiomyocytes and endothelial cells in the infarct area. Also Fas-negative endothelial cells were found (arrow). **E:** Bax was clearly enhanced in all infarcted groups. Quinaprilat slightly decreased the content of Bax protein in the infarct area. **F:** Bax staining of control hearts and **G:** of infarcted hearts. Cardiomyocytes were Bax-positive, endothelial cells were negative. **H:** Bcl-2 was elevated in MI hearts and further enhanced by Ang 1 and Q, but decreased in QA hearts. **I:** Normal myocardium was Bcl-2 negative. **J:** Bcl-2-positive cardiomyocytes in the infarct area. Endothelial cells in post-capillary vessels were Bcl-2- negative.



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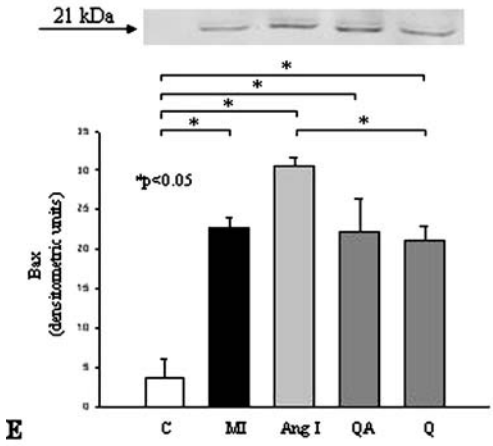
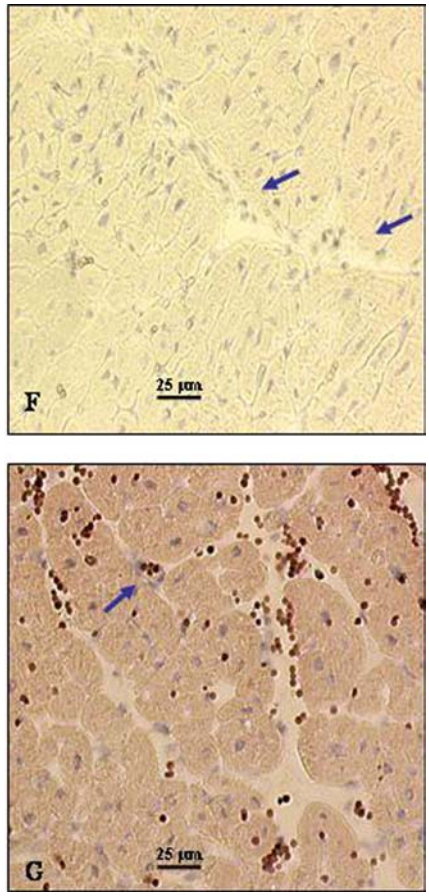




Detection of Fas protein

Fas protein is significantly increased in LV myocardium of MI hearts. Ang I application significantly enhanced Fas protein in the infarct area. Quinaprilat had no effect on the amount of Fas protein in the infarct area of the left ventricle (Fig. 6A). Fas-positive cardiomyocytes and endothelial cells were detectable by immunohistochemistry (Fig. 6C, D).

Bax and bcl-2 proteins

Bax protein is significantly increased in MI hearts, and further elevated in Ang I myocardium. Quinaprilat application reduced Bax protein (Fig. 6E). In parallel, bcl-2 protein is elevated in the infarct area of ischemic hearts. Quinaprilat elevated the amount of bcl-2 in infarcted hearts (Q group). Ang I significantly induced bcl-2 protein and quinaprilat reduced it (QA group; Fig. 6H). Ischemia/reperfusion elevates the amount of Bax and bcl-2 positive cardiomyocytes. Endothelial cells were negative in the infarct area (Fig. 6G, J).

P53 protein

Immunohistochemistry demonstrated that p53 is significantly elevated in MI hearts (4.37-fold) and further elevated in Ang I treated hearts (5.83-fold). Quinaprilat significantly reduced the amount of p53-positive cells in the infarct area (Fig. 7D–F).

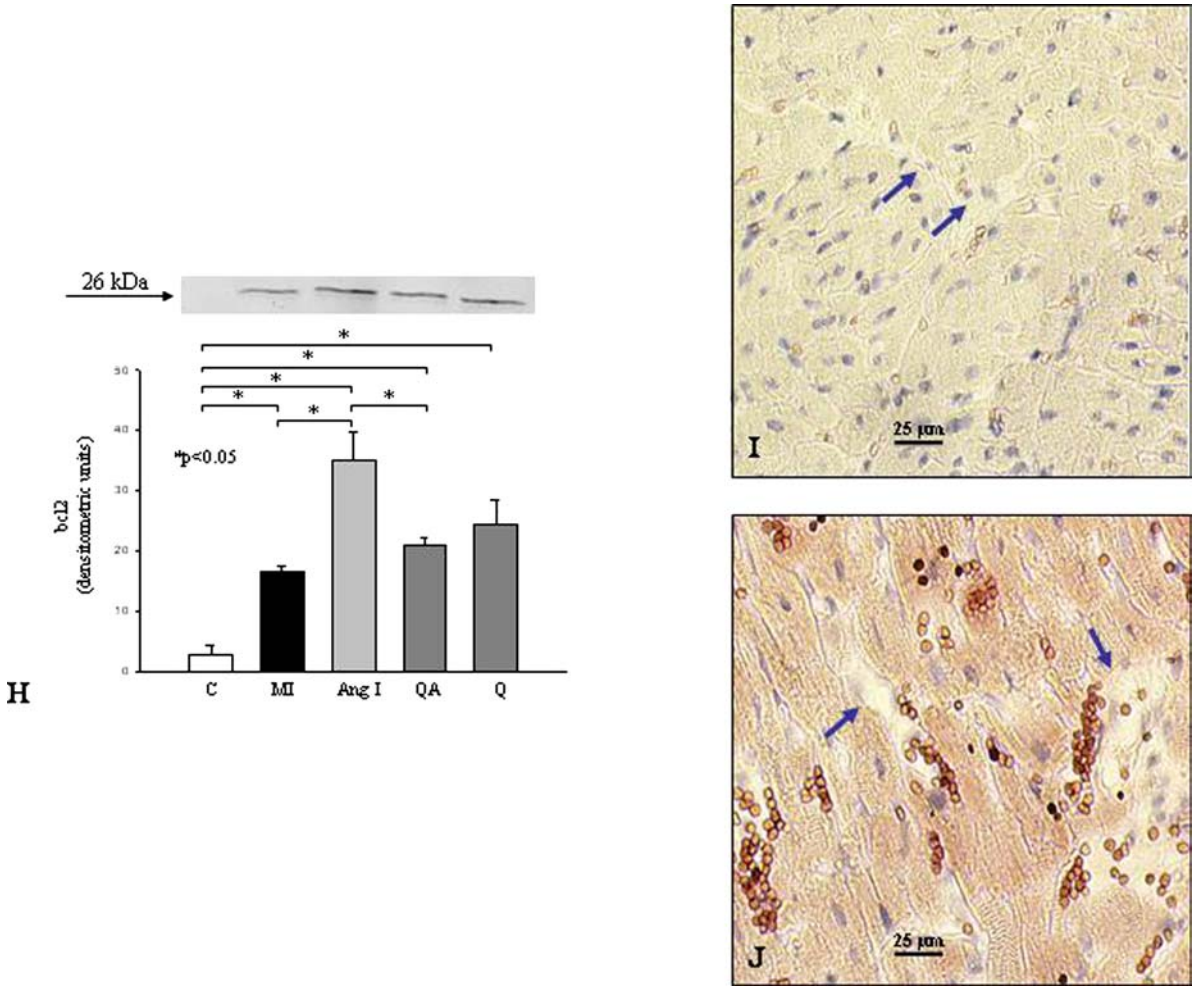
Western blot analysis confirmed the data obtained by immunohistochemistry and revealed a 4.9-fold increase in p53 protein in MI hearts and a 5.4-fold elevation in Ang I hearts. Quinaprilat significantly attenuated the amount of p53 in the infarct area (Q group; Fig. 7G).

Discussion

The present study demonstrates that ischemia (2 h) and reperfusion (4 h) increased Bax, bcl-2, Fas and p53. Ang I application further elevated these parameters in the infarct area. Ischemia/reperfusion led to caspase-3 activation, PARP cleavage, and subsequent DNA fragmentation. In addition, quinaprilat application reduced cardiomyocyte apoptosis.

To evaluate the impact of the RAS, the levels of Ang I and Ang II were measured. Interestingly, the lowest Ang II levels were observed in MI hearts. Since no reduction was observed in cardiac Ang I, this cannot be attributed to a reduced renin uptake from the perfusion medium due to the reduced blood flow in MI hearts. A more likely explanation,

Figure 6 Continued

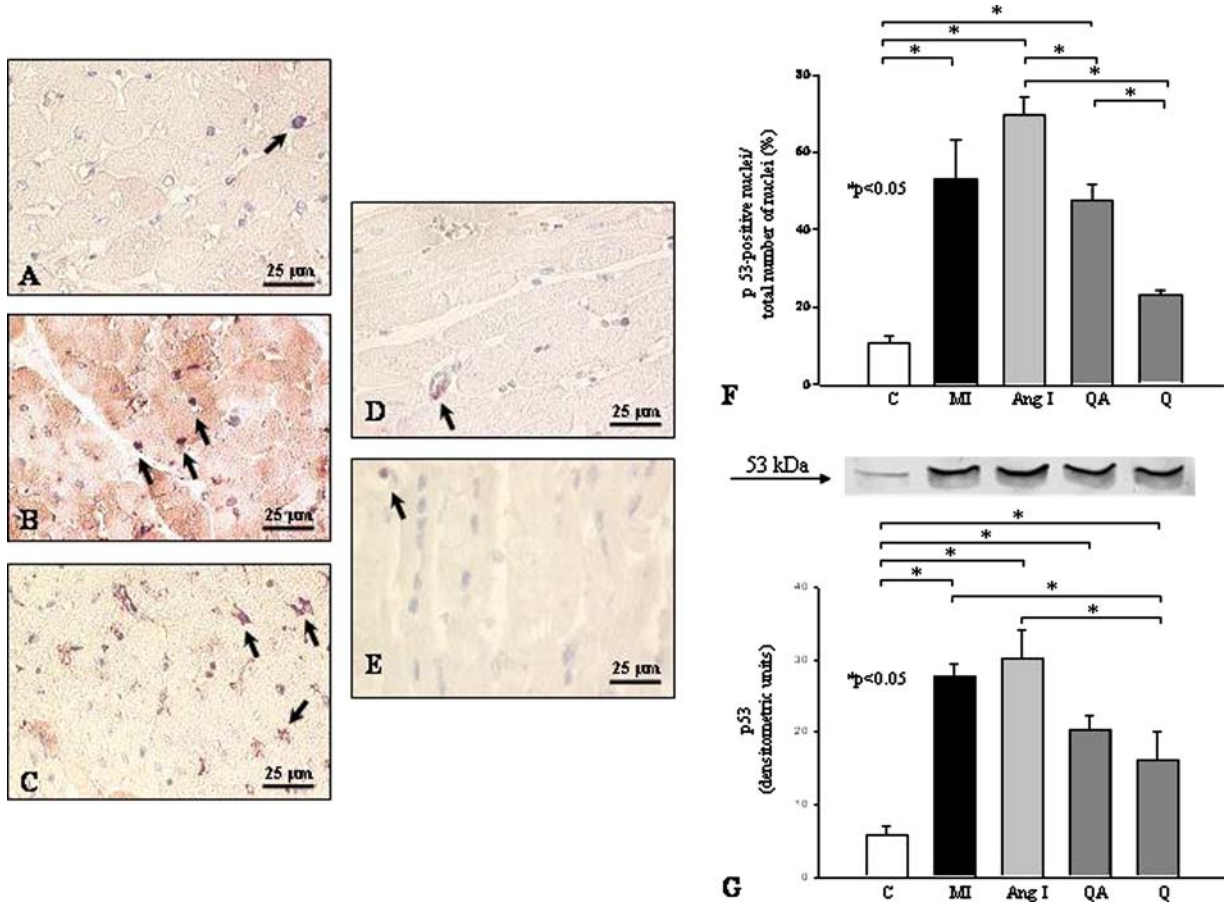


in agreement with a previous study in porcine hearts *in vivo*,[29] is that these reduced levels are the consequence of increased local Ang II degradation. As expected, Ang I perfusion elevated cardiac Ang II, and Quinaprilat prevented this rise. Although Quinaprilat reduced the cardiac Ang II/I ratio 5–10-fold, it did not suppress cardiac Ang II to zero. This indicates that either not all cardiac ACE was blocked by Quinaprilat or that Ang I–II conversion by non-ACE enzymes has occurred.

Cardiomyocyte death in the infarct area of MI and Ang I hearts was demonstrated by electron microscopy. This finding was confirmed by others.[6,30,31] Fliss and Gattinger[32] detected apoptosis in rat hearts after a 2 h ischemic period without reperfusion. Cleutjens *et al.*[33] showed that within 6-8 h after the initial insult neutrophil granulocytes migrated in the infarct area. Secondary necrosis was visible starting at 12 h and up to 4 days post infarct. Several studies have demonstrated the loss of cardiomyocytes due to apoptosis in infarcted hearts.[30,34] These results based on the detection of specific nuclear features of cardiomyocytes were visualized by TUNEL staining. Anversa *et al.*[7] demonstrated 6–11% TUNEL-positive nuclei after ischemia of 30 min and following reperfusion of 2 h or 4 h. We also detected apoptosis in the remote myocardium. This was also measured by others.[10,35] They showed that apoptosis also affects the viable myocardium after infarction in rats. The mechanical load produced by myocardial infarction and ventricular failure may trigger apoptosis and remodeling of the non-infarcted left ventricle. Olivetti *et al.*[36] measured a percentage of 11.6% apoptotic nuclei in the border zone of the infarct. Nurala *et al.*[37] demonstrated values ranging from 5 to 35% in dilated human myocardium. These differences can be explained by the use of different animal models and different metabolism characteristics of different species. Our data obtained from an isolated working porcine heart are comparable to the results of Okamura *et al.*[38] who used a rat model.

In addition, we addressed the impact of Ang I. Ang I-perfused MI hearts exerted a higher amount of apoptotic nuclei in the infarct area and the remote region compared to controls. Compared to untreated MI hearts however, this rate was not significantly higher. This finding indicates

Figure 7. Immunohistochemical investigation of p53. **A:** A few positive cells were detectable in C hearts. **B:** Numerous p53 positive nuclei (arrows) were shown in the infarct area of MI hearts. **C:** Similar results were found in Ang I hearts. **D:** In QA hearts and **E:** In Q hearts p53 was significantly reduced. **F:** Quantitative image analysis of p53. p53 positive nuclei were increased in MI hearts and further elevated by Ang I. This increase was attenuated by Quinaprilat. **G:** Western blotting and densitometry confirmed these data.



that the increase in cardiac Ang II during Ang I perfusion did not exert additional effects on apoptosis. Quinaprilat reduced apoptosis in infarcted and remote left ventricles. Moreover, Wang and coworkers[39] showed that application of temacapril reduced apoptosis by more than 50%. Inhibition of apoptosis might be one important mechanism by which ACE inhibitors improve cardiac function in heart failure patients.

Ischemia/reperfusion induced a significant increase of caspase-3 activity in infarcted regions of MI and Ang I hearts. This was also confirmed by others.[8,40] Quinaprilat reduced the gene expression of caspase-3, but not the amount of caspase-3 protein. Odaka and Mizuochi[41] have demonstrated that ACE-inhibitors like captopril and others are able to inhibit caspase-3-like activity independently from their effects on the RAS. Additionally, inactivation of caspase-3 leads to a stabilization of the now functionless p12–p17 complex.[42] These effects might explain the contradiction between our gene expression and protein data and also account for the fact, that caspase-3-positive tissue shows little or no apoptosis, as demonstrated by TUNEL staining.

The cleavage of PARP 116-kDa to PARP 85-kDa was found in all groups in the infarct area. An increase of both proteins was detectable in all infarcted groups. Quinaprilat or Ang I did not change the cleavage of PARP in infarcted hearts.

Fas was markedly upregulated in neonatal cardiomyocytes during ischemia/reperfusion.[43] Fas protein was markedly increased in infarcted hearts (cardiomyocytes, endothelial cells) compared to controls, whereas quinaprilat application had no effect. Hypoxia has been shown to induce CD 95 mRNA in neonatal cardiomyocytes.[44] Kajstura *et al.*[13] published a 131-fold increase of Fas after infarction and reperfusion. An enormous up-regulation of Fas was detectable in a rabbit model of ischemia/reperfusion injury. Carvedilol reduced the amount of apoptotic cardiomyocytes by 77%.[31] Isolated hearts of lpr-mice without functioning Fas receptor exerted a clearly reduced rate of programmed cell death after ischemia and reperfusion.[45] Until today the relation between Fas and the RAS remains unclear. Investigations on cell cultures of lung alveolar epithelial cells showed that lisinopril significantly inhibited Fas-induced apoptosis.[46]

Furthermore, it is known that the ratio of bcl-2 to Bax determines survival or death after an apoptotic stimulus. Bcl-2 was induced in salvaged myocytes at the acute stage of MI. In control hearts both protein levels of bcl-2 and Bax were very low. This was also confirmed by others.[47] A marked increase of bcl-2 and Bax proteins was detectable in cardiomyocytes of the infarct area. *In vivo* experiments on ischemic (60 min) and reperfused (6 h) dog hearts resulted in a decrease of bcl-2 and increase in Bax in the infarct area. After a permanent ischemia of 7 h no change of bcl-2 or Bax was detected.[34] Ischemia/reperfusion clearly enhanced Bax protein, and Ang I application led to a further elevation of Bax. Quinaprilat displayed a reduction of Bax in the infarct area. In parallel, bcl-2 was significantly increased by ischemia and reperfusion, further significantly elevated by Ang I and reduced again by quinaprilat application. These data indicate that cardiac Ang II formation induces directly Bax and bcl-2 proteins. Moreover, we could demonstrate that the RAS has direct impact on the intrinsic pathway of apoptosis.

Comparable to caspases and the bcl-2 family, p53 modulates myocardial apoptosis. Miyashita *et al.* demonstrated that p53 induced apoptosis via activation of caspases and Bax and reduction of bcl-2.[12,48,49] Here, we showed that p53 is elevated early after coronary occlusion and reperfusion. Ang I significantly induced p53 in the infarct area compared to MI hearts. Quinaprilat application significantly reduced p53. These data support the conclusion that the RAS directly influences the expression of p53 in myocardial infarction. Another study by Bialik[16] demonstrated the existence of a p53-independent pathway that mediated cardiomyocyte apoptosis. Therefore, we can assume that several signal pathways exist in the heart that are used by p53 to induce apoptosis and that the local RAS has an important impact on p53.

The external death stimulus ischemia/reperfusion induced release of Ang II, activated p53, and upregulated Bax and Fas. Ang I application further elevated pro-apoptotic proteins. Thus, the hypothesis is hypoxia-mediated enhanced RAS and p53 may be involved in the induction of apoptosis. Ischemia/reperfusion resulted in caspase-3 activation, PARP cleavage and subsequent DNA fragmentation. Application of quinaprilat could reduce the amount of apoptotic cardiomyocytes in the early phase of myocardial infarction using an *ex vivo* hemoperfused porcine working heart model. Our data suggest that ACE inhibition is sufficient to block ischemia/reperfusion induced apoptosis. We have demonstrated that the RAS influences different regulators of apoptosis and has a clear effect on p53.

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