Cardioplegic arrest induces apoptosis signal-pathway in myocardial endothelial cells and cardiac myocytes

Uwe M. Fischer\textsuperscript{a}, Oliver Klass\textsuperscript{a}, Ulrike Stock\textsuperscript{b}, Jerry Easo\textsuperscript{a}, Hans J. Geissler\textsuperscript{a}, Juergen H. Fischer\textsuperscript{c}, Wilhelm Bloch\textsuperscript{b}, Uwe Mehlhorn\textsuperscript{a,*}

\textsuperscript{a}Department of Cardiothoracic Surgery, University of Cologne, Cologne, Germany
\textsuperscript{b}Institute I for Anatomy, University of Cologne, Cologne, Germany
\textsuperscript{c}Institute for Experimental Medicine, University of Cologne, Cologne, Germany

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Abstract

Objective: Myocardial ischemia–reperfusion is associated with free radical-mediated injury and may be involved in cardiac apoptosis. The purpose of our study was to investigate (1) if cardioplegia-induced ischemia–reperfusion initiates cardiac apoptosis signal pathway, and (2) if this is mediated by free radicals.

Methods: We subjected 13 pigs (56 ± 10 kg) to 1 h of cold crystalloid cardioplegic arrest (CA) on cardiopulmonary bypass (CPB), and collected five transmural LV biopsies: prior to CPB (baseline), at 60 min CA, at 15 and 30 min reperfusion on CPB, and at 120 min post CPB. Two additional pigs were subjected to CPB but not CA and two further pigs were neither subjected to CPB nor CA and served as sham-operated time controls. LV specimens were cut at 7 \( \mu \)m and immunocytochemically stained against active caspase-3 and 85 kDa poly(ADP-ribose) polymerase (PARP) as apoptosis signal-pathway key enzymes, nitrotyrosine as indicator for peroxynitrite (ONOO\textsuperscript{−})-mediated tissue injury, and 8-iso-prostaglandin-F\textsubscript{2}a as indicator for oxygen free radical-mediated lipid peroxidation. Specimen were assessed using a scale of 0 (negative) to 3 (highly positive), and cardiomyocytes were quantitatively investigated using TV densitometry.

Results: At 60 min CA, caspase-3 was increased by 9.2 ± 3.7 gray units and remained on this level until 2 h post CPB (\( P = 0.003 \) vs. baseline); nitrotyrosine increased over time to reach a maximum of 8.5 ± 8.1 gray units at 120 min post CPB (\( P = 0.016 \)); and there was a trend for increased 8-iso-prostaglandin-F\textsubscript{2}a at 60 min CA (+3.6 ± 4.7 gray units; \( P = 0.089 \)). At 60 min CA, 92\% of the hearts showed active caspase-3, only 42\% demonstrated nitrotyrosine formation, and 58\% exhibited 8-iso-prostaglandin-F\textsubscript{2}a. At 120 min post CPB, most hearts positive for caspase-3 were also positive for nitrotyrosine (83\%), and 8-iso-prostaglandin-F\textsubscript{2}a (75\%), but no heart showed PARP cleavage. Hearts subjected to CPB but not CA as well as time controls remained negative for all variables. Conclusions: Our data show that CA initiates apoptosis signal-pathway in myocardial endothelium and myocytes; however, this did not result in apoptotic cell death as we did not find PARP cleavage. Further, the data suggest that CA-induced apoptosis signal pathway activation is not mediated by free radicals as caspase-3 activation preceded both nitrotyrosine and 8-iso-prostaglandin-F\textsubscript{2}a formation.

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1. Introduction

Cardiomyocyte apoptosis has been reported in various cardiac pathologies such as myocardial ischemia–reperfusion, heart failure, atherosclerosis, cardiomyopathy, and myocarditis [1–5]. Apoptosis can be triggered through different mechanisms in response to both intracellular and extracellular signals [6]. There is strong evidence that cytokine-release by macrophages and myocytes during reperfusion activates the apoptosis signal cascade [7–9]. Furthermore, the generation of reactive oxygen species (ROS: \( \text{O}_2^\cdot, \text{OH}^\cdot, \text{H}_2\text{O}_2 \)), often called oxidative stress, has been shown to induce apoptosis in cardiomyocytes during ischemia–reperfusion [10,11]. A scheme depicting the most important factors and stimuli involved in the cardiac apoptosis signal-pathway cascade is shown in Fig. 1. In a recent in vitro study apoptosis initiation was reported to occur during global myocardial ischemia [13]. In addition,
we have previously shown in human LV biopsies that cardioplegia-induced ischemia–reperfusion resulted in myocardial injury mediated by both ROS and peroxynitrite (ONOO\textsuperscript{-}) [14]. However, the impact of cardioplegia-induced myocardial ischemia–reperfusion on cardiac apoptosis has not been determined. Therefore, the purpose of our study was to investigate in a clinically relevant animal model[1] if cardioplegia-induced ischemia–reperfusion initiates apoptosis signal pathway in myocardial endothelial cells and myocytes, and [2] if this is mediated by ROS and or ONOO\textsuperscript{-}. As marker for apoptosis we determined activated caspase-3 and 85 kDa poly(ADP-ribose) polymerase (PARP) as apoptosis signal-pathway key enzymes. As marker for ONOO\textsuperscript{-}-mediated tissue injury we determined nitrotyrosine, and as indicator for ROS-mediated lipid peroxidation we used 8-iso-prostaglandin-F\textsubscript{2\alpha} [14].

2. Material and methods

2.1. Experimental protocol

All animal procedures were approved by the Animal Welfare Representative of the University of Cologne and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1985). Seventeen pigs of either sex (56 ± 10 kg) were premedicated with 4 mg/kg intramuscular azaperon (Stresnil, Janssen, Neuss, Germany) and 0.02 mg/kg atropine (Braun, Melsungen, Germany). Anesthesia was started with intravenous administration of 5–10 mg/kg ketamine (Ketanest, Parke-Davis, Berlin, Germany) and maintained with 0.03–0.05 mg per min ketamine. Following tracheal intubation the pigs were mechanically ventilated with 50% oxygen in room air or N\textsubscript{2}O as required using a volume-cycled ventilator (Engstroem 300, Sweden). Pancuronium 0.2 mg/kg was given as required. Fluid-filled catheters were inserted in the right common carotid artery and the right internal jugular vein for arterial pressure monitoring, blood sampling, fluid administration, and central venous pressure determination, respectively.

2.2. CPB and cardioplegic arrest

Following median sternotomy and pericardiotomy, heparin (300 IU/kg) was given intravenously for systemic anticoagulation. Additional doses of 75 IU/kg heparin were administered every 60 min throughout the experiment. In 13 pigs, a 16-F arterial perfusion cannula was introduced into the ascending aorta. A two-stage (34/38-F) venous cannula was placed into the right atrium/inferior vena cava. The LV chamber was vented with a 12-F catheter inserted via the left atrium. Three roller pumps were used for extracorporeal...
circulation, left ventricular drainage, and suction, respectively (HLM-CAPS, Stoecklin Instruments, Munich, Germany). The extracorporeal circuit and the membrane oxygenator (Cobe Cardiovascular Inc., Arvada, CO, USA) were primed with heparinized pig blood. Cardiopulmonary bypass (CPB) was initiated and the body temperature was cooled to 28°C. The aorta was cross-clamped, and 10 ml/kg iced (approx. 4°C) crystalloid cardioplegia (Bretschneider-HTK solution, Custodiol, Dr. F. Koehler Chemie GmbH, Alsbach-Haehnlein, Germany) was infused into the aortic root at 80 mmHg. An additional 3 mg/kg cardioplegia were given at 20 and 40 min of cardioplegic arrest (CA) if mechanical activity of the heart occurred. Whole-body hypothermia at 28°C was maintained for 45 min of aortic cross-clamping, followed by rewarment to 37°C. After 60 min CA, the cross-clamp was removed and the heart was reperfused on normothermic CPB for 30 min. The pigs were then weaned off CPB and all cannulas were removed. At 120 min post CPB, pigs were euthanized with anesthesia overdose and intravenous potassium.

To separate the changes induced by CA from those due to CPB alone, two pigs were subjected to CPB for 45 min at 28°C followed by 45 min at 37°C without CA, weaned off CPB, and euthanized at 120 min post CPB. Two additional pigs were neither subjected to CPB nor CA and served as sham-operated time controls.

2.3. LV biopsies

We collected transmural biopsies from a fat-free area of the LV anterior wall using a 14-gauge biopsy needle (Gallini, Modena, Italy) at the following time points: prior to CPB initiation (baseline), at 60 min CA, at 15 and 30 min reperfusion on CPB, and at 120 min post CPB. All LV biopsies were placed in 4% paraformaldehyde for 4 h and then rinsed in 0.1 M phosphate-buffered saline (PBS) for 24 h, followed by storage for 12 h in PBS solution with 18% sucrose for cryoprotection and frozen at −80°C.

2.4. Immunocytochemistry

Prior to immunohistochemical examination, 7-μm slices from the biopsies were placed in a bathing solution of 3% H2O2 and methanol for 20 min, then cells were lysed with 0.25% Triton X-100 in 0.5 M ammonium chloride. Thereafter, specimens were treated with 5% bovine serum albumin (BSA) solution in 0.05 M TBS. Prior to each step the sections were rinsed three times in 0.05 M TBS buffer. Incubation with primary rabbit anti-active-Caspase-3 antibody (1:500, Pharmigen, San Diego, CA, USA) and rabbit anti-PARP antibody p85 fragment (1:250, Promega, Madison, WI, USA) were performed in a TBS-based solution of 0.8% BSA and 20 mM NaCl for 12 h at 4°C. After rinsing with TBS the sections were incubated with the corresponding secondary biotinylated goat anti-rabbit antibody (1:400, DAKO, Hamburg, Germany) for 1 h at room temperature. For nitrotrotyrine staining we used a monoclonal mouse anti-nitrotyrosine antibody (1:400, Calbiochem) and a secondary goat-anti-mouse antibody (1:400, DAKO); for 8-iso-prostaglandin-F2α detection a polyclonal goat anti-8-Epi-PGF2a antibody (1:1500, Oxford Biomedical Research, MI, USA) and a secondary rabbit-anti-goat antibody (1:400, DAKO) was utilized. A streptavidin–horseradish peroxidase complex was then applied as a detection system (1:150) for 1 h. Finally, staining was developed for 10–20 min with 3,3-diaminobenzidine tetrahydrochloride (DAB) in 0.1 M PBS.

2.5. Semi-quantitative analyses of active caspase-3, nitrotyrosine, and 8-iso-prostaglandin-F2α

For semi-quantitative analyses the alteration of baseline staining against the staining intensity of the various time points for each pig were compared by two independent investigators. All specimens were judged using a semi-quantitative score from 0 (no difference compared with baseline staining) to 3 (highly different to baseline staining) which considered both endothelial and myocyte staining intensities.

2.6. Active caspase-3, nitrotyrosine and 8-iso-prostaglandin-F2α TV densitometry

All LV biopsy slices were incubated and stored under identical conditions. For quantitative intensity analyses of active caspase-3, nitrotyrosine, and 8-iso-prostaglandin-F2α immunostaining in cardiomyocytes we measured the gray values of 30 cardiomyocytes from six randomly selected areas. The intensity of immunostaining was reported as the mean of measured cardiomyocyte gray value minus background gray value. The background gray value was measured at a cell-free area of the slice. For staining intensity detection a Zeiss Axiophot microscope coupled to a 3-chip CCD-camera was used and the analysis was performed using the Optimas 6.01 image analysis program installed on a Pentium PC.

2.7. Identification and comparison of active caspase-3, nitrotyrosine and 8-iso-prostaglandin-F2α

An alteration of staining intensity between baseline and later time points in specimens of individual pigs was ascertained if TV densitometry and semi-quantitative analyses performed by two independent investigators showed differences for cardiomyocytes and/or a clear alteration of endothelial staining. Thereafter, activation of caspase-3 and nitrotyrosine- or 8-iso-prostaglandin-F2α-formation was compared at the different time points for each heart.

2.8. PARP cleavage determination

To determine if CPB and CA (n = 13), CPB only (n = 2)
or sham operation \((n = 2)\) results in apoptotic cell death, LV biopsies collected at the end of the experiments were investigated for 85 kDa PARP-positive cells.

2.9. Statistical analysis

All data presented are mean ± standard deviation (SD). After confirming normal distribution using Shapiro-Wilk’s \(W\)-test, changes vs. baseline data were analyzed for the effect of ‘time’ using analysis of variance (ANOVA) for repeated measures; post hoc comparisons were performed using two-tailed Student’s \(t\)-test for paired samples with Bonferroni correction for multiple comparisons as implemented in the software package SPSS for Windows, Version 10.0. All \(P\)-values reported are Bonferroni-corrected.

3. Results

Changes over time in cardiac myocyte staining intensity for activated caspase-3, nitrotyrosine, and 8-iso-prostaglandin-\(F_{2\alpha}\) are depicted in Fig. 2. ANOVA revealed the effect of ‘time’ to be significant for activated caspase-3 (Fig. 2, upper panel) and nitrotyrosine (Fig. 2, middle panel); there was a trend for the effect of ‘time’ for 8-iso-prostaglandin-\(F_{2\alpha}\) (Fig. 2, lower panel). Compared to baseline, activated caspase-3 was increased by \(9.2 \pm 3.7\) gray units at 60 min CA \((P < 0.001\) vs. baseline), tended to decline at 15 min \((-1.8 \pm 3.5\) gray units; \(P = 0.09\) vs. 60 min CA), and remained on this level until 2 h post CPB \((P \approx 0.003\) vs. baseline). Nitrotyrosine was not increased at 60 min CA \((P = 0.43)\) but increased over time to reach a maximum of \(+8.5 \pm 8.1\) gray units \((P = 0.016)\) at 120 min post CPB. 8-Isoprostaglandin-\(F_{2\alpha}\) increased slightly by \(3.6 \pm 4.7\) gray units \((P = 0.089)\) at 60 min CA and tended to be elevated throughout.

Hearts subjected to CPB but no CA \((n = 2)\) as well as sham-operated time controls with neither CPB nor CA \((n = 2)\) remained negative for activated caspase-3, nitrotyrosine, and 8-iso-prostaglandin-\(F_{2\alpha}\) throughout the experiments (data not shown).

Fig. 3 shows immunocytochemical stainings for baseline, 60 min CA, and 15 min reperfusion. Whereas staining against active caspase-3 at baseline (Fig. 3a) was negative, at 60 min CA (Fig. 3b) all myocytes and endothelial cells were positive and remained positive at 15 min reperfusion (Fig. 3c). In all hearts investigated there was a strong correlation between activation of nitrotyrosine, 8-iso-prostaglandin-\(F_{2\alpha}\), and caspase-3 in both cardiac myocytes and endothelial cells. For technical reasons, TV densitometry is not applicable for endothelial cell staining quantification. This finding was typical for 92% of the hearts investigated (Fig. 4). Compared with baseline (Fig. 3d), staining against 8-iso-prostaglandin-\(F_{2\alpha}\) was slightly increased at 60 min CA (Fig. 3e) and at 15 min reperfusion (Fig. 3f); however, this pattern was only found in 58% of hearts (Fig. 4), 42% did not show 8-iso-prostaglandin-\(F_{2\alpha}\) formation at 60 min CA. Compared to baseline (Fig. 3g), nitrotyrosine was slightly increased at 60 min CA (Fig. 3h; 42% of the hearts) and at 15 min reperfusion (Fig. 3i; 50% of hearts; Fig. 4). In 58% of the hearts investigated, no nitrotyrosine formation at 60 min CA was detected. At 30 min reperfusion and at 120 min post CPB, most hearts positive for caspase-3 were also positive for nitrotyrosine (83%) and 8-iso-prostaglandin-\(F_{2\alpha}\) (75%) (Fig. 4). At 120 min post CPB none of the hearts was positive for staining against 85 kDa PARP cleavage (data not shown).

4. Discussion

Our data show that CA initiates apoptosis signal-pathway in cardiac myocytes and endothelial cells. As we found that at 60 min CA all myocytes and endothelial cells were positive for activated caspase-3 in 92% of the hearts but
only 58% showed 8-isoprostane and 42% showed nitrotyrosine formation at this time, CA-induced apoptosis signal-pathway activation is not exclusively mediated by ROS or peroxynitrite. Thus, ischemia induced by CA probably acts as a stimulus for cardiac apoptosis signal-pathway induction. This is supported by previous work in isolated canine hearts which demonstrated that 90 min of global normothermic ischemia resulted in caspase-3 activation and 85 kDa PARP cleavage [13]. Potential mechanisms for apoptosis signal-pathway induction during ischemia include activation through protooncogenes such as c-fos and c-jun. C-jun and c-fos have been shown to be induced in human right atria during cardioplegic arrest [15] and are involved in both cellular differentiation and apoptosis initiation [16,17].

Following ischemia cessation and myocardial perfusion resumption caspase-3 activity initially tended to decline (−1.8 ± 3.5 gray units from 60 min CA to 15 min reperfusion; P = 0.09; Fig. 2) but remained on that level until 120 min post CPB. This was accompanied with nitrotyrosine formation over time and a trend for increased myocardial 8-iso-prostaglandin-F2α, and thus, it could be that ROS and ONOO− generation may have been involved in caspase-3 activation following CA. Previous studies have demonstrated that ROS generation induced by myocardial ischemia–reperfusion was associated with cardiac apoptosis activation [10,11,13]. This is consistent with our recent findings in human LV myocardium that cardioplegia-induced ischemia–reperfusion resulted in constitutive NO-synthase activation and NO production [18] and was associated with 8-iso-prostaglandin-F2α and nitrotyrosine formation secondary to ROS and ONOO− generation [14]. However, our data do not allow to establish a causal relation between ROS/ONOO− generation and apoptosis signal-pathway activation. Another potential mechanism is cytokine-release by macrophages and myocytes during reperfusion which has been shown to activate the apoptosis signal-cascade [7–9,19,20]. In contrast, cardiopulmonary bypass does not appear to be a source for apoptosis signal-pathway induction because we did neither find caspase-3 activation nor 8-iso-prostaglandin-F2α or nitrotyrosine formation in the two pigs subjected to CPB alone. Similarly, ‘operative stress’ due to thoracotomy and instrumentation in the two sham operated time control pigs resulted neither in apoptosis signal-pathway induction nor in alterations...
mediated by ROS or ONOO\textsuperscript{\textendash}. These data suggest that ischemia may act as a trigger for CA-induced apoptosis signal-pathway activation which is then maintained by several mechanisms including ROS and OONO\textsuperscript{\textendash} generation during reperfusion [12].

Interestingly, at 120 min post CPB none of the 13 hearts subjected to CA showed apoptosis completion as all hearts were negative for 85 kDa PARP cleavage despite active caspase-3 detection throughout the duration of the experiments. Thus, either the apoptotic cascade was interrupted downstream caspase-3 activation prior to PARP cleavage and apoptosis completion or we were not able to detect apoptosis completion within the time frame of our experiments. The latter appears more likely because studies in isolated hearts subjected to 90 min of global ischemia followed by 6 h of reperfusion have shown that cardiac myocyte apoptosis completion as determined by terminal deoxynucleotidyl transferase-dUTP-biotin nick-end labeling (TUNEL) was evident in late reperfusion [13]. In addition, Suzuki et al. [21] showed in adult rat ventricular myocytes that DNA fragmentation detected by TUNEL peaked at 14 h after experimental apoptosis induction. These data suggest that in the hearts of the present study we did not find PARP cleavage or apoptotic myocytes because our experiments were terminated at 2.5 h of reperfusion (30 min reperfusion on CPB followed by 120 min post CPB). Future studies are required to further elucidate apoptosis signal-pathway regulation and the time course of apoptosis completion.

In conclusion, our data indicate that myocardial ischemia induced by CA acts as a trigger for apoptosis signal-pathway activation that is maintained during reperfusion. As nitrotyrosine and 8-iso-prostaglandin-F\textsubscript{2\alpha} were generated post CA, the role of ROS and ONOO\textsuperscript{\textendash} in cardiac apoptosis signal-pathway regulation remains to be elucidated. Despite active caspase-3 determination at the end of our experiments, we did not find PARP cleavage, probably because apoptosis completion requires longer than the 2.5 h of reperfusion according to our experimental protocol. It remains to be investigated if apoptosis activation affects cardiac performance after CPB and CA and if apoptosis inhibition may represent a novel therapeutic strategy to attenuate ischemia\textendash;reperfusion injury as has been suggested by recent experimental work [22].

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References
