Induction of cardioplegic arrest immediately activates the myocardial apoptosis signal pathway

Uwe M. Fischer,1,4 Charles S. Cox, Jr.,1 Glen A. Laine,2 Uwe Mehlhorn,3 Wilhelm Bloch, and Steven J. Allen1

1Center for Microvascular and Lymphatic Studies, Department of Surgery and Anesthesiology, the University of Texas-Houston Medical School, Houston, Texas; 2Michael E. DeBakey Institute for Comparative Cardiovascular Science, Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas; 3Department of Molecular Medicine, German Sports University, Cologne, Germany; and 4Department of Thoracic and Cardiovascular Surgery, University of Cologne, Cologne, Germany

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Fischer UM, Cox CS Jr., Laine GA, Mehlhorn U, Bloch W, Allen SJ. Induction of cardioplegic arrest immediately activates the myocardial apoptosis signal pathway. Am J Physiol Heart Circ Physiol 292: H1630–H1633, 2007. Published by the American Physiological Society. doi: 10.1152/ajpheart.00006.2005.—Myocardial ischemia–reperfusion, including cardioplegic arrest (CA), has been associated with cardiomyocyte apoptosis induction. However, the time course of apoptosis activation and the trigger mechanisms are still unclear. Because apoptosis inhibition may represent a novel therapeutic strategy for long-term myocardial preservation, we sought to investigate the time course of apoptosis signal-pathway induction during CA. As to method, Sprague-Dawley rats (300–350 g) were anesthetized, intubated, and mechanically ventilated. CA was initiated by infusion of ice-cold crystalloid solution (Custodiol, 10 ml/kg) into the aortic root, and hearts were rapidly excised and stored for 0, 30, 60, and 120 min in 0.9% sodium chloride solution (28°C). In controls, no CA was initiated before removal and storage at 28°C. In another group, calcium-rich cardioplegia was used, and an additional group received a caspase-8 inhibitor before CA induction. Left ventricular cytosolic extracts were isolated and investigated for the activity of caspase-3 and -6 (effector caspses) and caspase-8 and -9 (involved in extrinsic and intrinsic pathways of apoptosis induction). Fluorometric activity assays were performed by using specific substrates. As a result, activities of all tested caspses were significantly increased immediately after CA induction compared with controls. Administration of the caspase-8 inhibitor significantly reduced activities of all caspses. With calcium-rich cardioplegia, caspase activities were significantly lower compared with low-calcium CA. Control hearts also showed an increase of caspase activities during cold-storage ischemia without CA but had significantly different time courses compared with hearts with CA. In conclusion, our data show rapid apoptosis signal-pathway induction immediately following CA exposure. Thus apoptosis signal-pathway inhibition as a potential strategy for improved myocardial preservation would have the greatest effect when applied before CA exposure.

AMONG SEVERAL CARDIAC INSULTS myocardial ischemia–reperfusion (I/R) has been associated with cardiomyocyte apoptosis (20). Cardiomyocytes that undergo apoptosis contribute to cell loss due to I/R (17, 20). Induction of the myocardial apoptotic signal pathway has also been shown in hearts subjected to cardiopulmonary bypass and cardioplegic arrest (CA) (1, 5). However, the time course and mechanisms of apoptosis induction during I/R are still unclear. Whereas some studies attribute apoptosis induction mainly to oxygen radicals during reperfusion (8), recent evidence suggests initiation of the apoptosis signal pathway during CA (5–7, 17, 18). Furthermore, in recent experiments performed in pigs with cardiopulmonary bypass/CA, we found that caspase-3, one of the terminal apoptosis effector enzymes, was already activated in myocytes and myocardial endothelium at the end of 60 min of ischemia (5).

Several stimuli have been reported to be involved in the initiation of the caspase cascade, including activation of caspase-8 by cell surface death receptors and the mitochondrial pathway with subsequent caspase-9 activation (3). Because myocardial apoptosis contributes to cell damage and cell loss after I/R (11), it is a potential target for new strategies in myocardial protection. Effective inhibition of myocardial apoptosis, however, requires knowledge of the inducers, pathways, and enzymes involved in the apoptosis process.

Therefore, we performed the present study to determine the time course and pathways of apoptosis induction with CA under moderate hypothermic conditions (28°C) in a rat model mimicking clinical conditions in cardiac surgical procedures in terms of induction of CA and associated global myocardial ischemia. We analyzed myocardium for extrinsic (mediated through caspase-8) and intrinsic (mitochondrial, mediated through caspase-9) apoptosis induction and for further activation of the apoptosis signal-pathway effector enzymes caspase-3 and -6.

METHODS

Experimental protocol. The University of Texas Animal Welfare Committee approved all procedures. The investigation conforms with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

Male Sprague-Dawley rats (300–350 g) were anesthetized with ketamine (intraperitoneal), intubated, and mechanically ventilated with 1.2% isoflurane. The chest was opened through a median sternotomy, the heart was exposed, and a 2-0 silk thread was placed around the ascending aorta for later occlusion. The inferior vena cava was incised, and a 22-gauge feeding needle was inserted in the ascending aorta. After occlusion of the aorta, 10 ml/kg ice-cold crystalloid low sodium, low calcium cardioplegic solution [Custodiol histidine-tryptophan-ketoglutarate (HTK) solution, Koehler Chemie]

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Address for reprint requests and other correspondence: U. M. Fischer, Dept. of Cardiothoracic Surgery, Univ. of Cologne, Joseph-Stelzmann-Str. 9, 50924 Cologne, Germany.
was infused into the aortic root (CA group). After cardiac arrest, the hearts were rapidly excised and stored in 0.9% sodium chloride solution at 28°C for 0 (baseline, n = 10), 30, 60, and 120 min, respectively (n = 5, for each group) and snap frozen in liquid nitrogen. Another 25 animals in which cardioplegia was not infused served as ischemic controls. In an additional five animals (caspase-8 inhibition), the cell permeable caspase-8 inhibitor Z-IETD-FMK (Calbiochem) was administered (0.1 μmol/l iv) 10 min before induction of CA and also added to the cardioplegia. We also supplemented the cardioplegic solution with calcium (Custodiol HTK, supplemented with calcium to a concentration of 2.4 mmol/l) and induced CA in another group of five animals (calcium group).

**Cytosolic extracts.** Frozen left ventricles were homogenized (Ultra-Turrax, Jahnke & Kunkel) in a buffer containing (in mM) 150 sucrose, 10 KCl, 1.5 MgCl₂, 1.0 EDTA, 1.0 EGTA, and 20 HEPES, supplemented with a protease inhibitor cocktail (Complete, Roche) and centrifuged at 400 g for 10 min to pellet cell debris and nuclei. The supernatant was centrifuged at 10,000 g for 30 min and stored at −80°C until further usage. Protein concentration was determined by using a BCA Protein Assay Kit (Pierce).

**Caspase activities.** Caspase activities were measured in the cytosolic fraction, as described previously (9). Caspase 3-, 6-, 8-, and 9-like activities were measured by following the cleavage of the fluorescent substrate analogs Ac-DEVD-AMC, Ac-VEID-AMC, Z-IETD-AFC, and Ac-LEHD-AFC, respectively (Calbiochem), in a fluorescent plate reader (PE Biosystems). The rate of fluorescent change, an average of three replicate measurements, was normalized to the protein content.

**Statistical analysis.** The results are expressed as means ± SE. Data analysis was performed by using ANOVA with Tukey’s test for post hoc comparison and Student’s t-test. A P value <0.05 was considered significant.

**RESULTS**

Activities of all measured caspases were significantly increased immediately after induction of CA (Fig. 1) compared with those in control hearts. Caspase-8 activity decreased progressively over 120 min of 28°C cold storage after CA induction. Caspase-3 and -9 activities also decreased throughout the first 60 min but went up again after 120 min of CA. Activity of caspase-6 further increased after induction of CA, peaked at 30 min, and decreased there after.

All caspase activities in ischemic control hearts showed a significantly different development during the 120-min time course of ischemia with cold storage at 28°C without CA. Caspase-3 and -8 activities increased after baseline until 60 min of ischemia, decreased thereafter, but remained significantly elevated. Caspase-6 activity increased gradually throughout 120 min of ischemia. Activity of caspase-9, in contrast, remained at baseline levels during the first 60 min but increased significantly during the last hour of ischemia.

Figure 2 depicts the relative caspase activities immediately after induction of CA in the hearts with and without caspase-8 inhibition and in hearts subjected to calcium-supplemented cardioplegia. As shown in Fig. 2, caspase-8 activity was significantly inhibited. Caspase-3, -6, and -9 activities were also significantly reduced in the hearts with caspase-8 inhibition compared with noninhibited hearts. Caspase activities in hearts subjected to CA with calcium-supplemented cardioplegic solution were significantly lower for caspase-3, -6, and -8. Caspase-9 activity, however, was significantly higher compared with non-calcium-supplemented cardioplegia and caspase-8 inhibition.

**DISCUSSION**

Our data show that CA results in myocardial caspase activation, which is significantly different from non-CA ischemic hearts. We also show that, in the early phase of CA, caspase-8 plays a crucial role in activation of caspase-3, -6, and -9. Furthermore, our data demonstrate that an increased cardioplegic...
caspase-8 inhibition. 0.05, caspase-8 inhibition vs. noninhibition; /H11001

Custodiol HTK (15). However, although rapid caspase-8 activation has been shown to be possible, the mechanism by which, in our study, this rapid caspase activation was involved in rapid apoptosis induction is unclear. These findings are in contrast to a study from Scarabelli et al. (18) in which caspase-8 cleavage was only found in myocardium during reperfusion after 35 min of global ischemia. Even considering different experimental conditions compared with previous studies, our results demonstrate that ischemia alone is a sufficient trigger for apoptosis induction and involves both the death receptor-mediated activation by caspase-8 and activation of caspase-9 representing mitochondrial pathway. Activation of these initiator caspases occurred at different time points and depended on global ischemia duration.

Caspase activities in the CA group not only had a significantly different time course compared with ischemic controls but also showed a surprisingly fast increase within seconds after CA induction (baseline values in Fig. 1). Furthermore, with inhibition of caspase-8, this rapid caspase activation was significantly reduced, suggesting a key role for caspase-8 in this process.

Rapid caspase-8 activation in certain TNF/Fas-treated cells occurred within 5 s of receptor triggering and led to simultaneous activation of the apoptotic effector caspase-3 and -7 (15). However, although rapid caspase-8 activation has been shown to be possible, the mechanism by which, in our study, the induction of CA activated caspase-8 is unclear.

We used a low sodium, low calcium crystalloid solution (Custodiol HTK = Bretschneider’s solution) for CA induction. This cardioplegic solution induces cardiac arrest by lowering the transsarcolemmal gradient for sodium and thus reducing the excitability of the myocytes (2). Furthermore, the low calcium concentration reduces cellular energy consumption and ameliorates myocardial protection during ischemia (2). Because perturbations in cellular Ca$^{2+}$ homeostasis have been reported to induce apoptosis in both normal and malignant cells (13), alterations of intracellular Ca$^{2+}$ concentrations due to the cardioplegic solution might be involved in rapid apoptosis induction. Therefore, we increased the cardioplegic calcium concentration to 2.4 mmol/l in one group of animals. Although caspase activities immediately following CA induction were still higher than control levels, increased calcium concentration resulted in significant activity reduction for caspase-3, -6, and -8. Interestingly, higher calcium concentration led to a significant caspase-9 activity increase compared with controls, CA with low-calcium, and caspase-8 inhibition. The caspase-9 activation due to higher calcium concentrations is consistent with studies that report apoptosis induction with increased intracellular calcium influx (16, 19). However, in these studies, apoptosis activation occurred over several hours of increased intracellular calcium concentrations and not within seconds. Furthermore, although increased calcium concentration of the cardioplegic solution reduced activation of caspase-3, -6, and -8, these activities were still significantly higher than control levels. Therefore, factors other than cardioplegia calcium concentration also impact apoptosis signal-pathway induction after induction of CA.

In summary, global ischemia without prior CA induces apoptosis via two pathways, both initiated by cell surface death receptors and mitochondria. Global ischemia induced by CA induction also activates caspases but much faster and with a significantly different time course. Caspase-8 seems to play a major role in this rapid apoptosis induction as well as the cardioplegia calcium concentration. Myocyte apoptosis contributes to cell loss after I/R (11). In addition, caspase inhibition has been shown to reduce myocyte cell death and to improve posts ischemic myocardial recovery in different species (10, 12). Furthermore, as an energy-dependent process, activation of the caspase cascade increases ATP consumption (4, 14). Thus myocyte apoptosis further diminishes cellular resources that are necessary to maintain myocyte integrity during ischemia and to achieve functional recovery during reperfusion. Because reduction of cellular energy consumption and avoidance of cell loss are major principles of myocardial protection during I/R, inhibition of apoptosis induction is a promising new strategy. With a consideration of our results in combination with previous evidence on beneficial effects of caspase inhibition during ischemia on left ventricular function, inhibition of apoptosis induction would have the greatest effect when applied before CA induction. Despite the experimental limitation that hearts were excised from the animals, we trust that our data give relevant insights into myocardial ischemia pathophysiology with potential for new strategies in myocardial protection. However, future studies are required to investigate apoptosis signal-pathway induction in human myocardium subjected to cardioplegic arrest.

GRANTS

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