Warm blood cardioplegic arrest induces mitochondrial-mediated cardiomyocyte apoptosis associated with increased urocortin expression in viable cells

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Objectives: This study assesses the mechanisms of apoptosis in patients after on-pump coronary artery bypass graft surgery and the potential involvement of the endogenous cardiac peptide urocortin as a cardiomyocyte salvage mechanism. We have previously described the mechanisms of apoptosis after ischemia-reperfusion injury in the rat heart and shown that endogenous urocortin is cardioprotective. Here we extend these findings to the human heart exposed to ischemic-reperfusion injury.

Methods: Two sequential biopsy specimens were obtained from the right atriums of 24 patients undergoing coronary artery bypass grafting at the start of grafting and 10 minutes after release of the aortic clamp. Apoptosis was identified by means of immunocytochemical colocalization between terminal deoxynucleotidyl transferase-mediated nick end-labeling positivity and active caspase-3. Immunostaining for active caspase-9 and caspase-8 was performed to identify the pathways of apoptosis induction. Urocortin and adenosine triphosphate-dependent potassium channel expression was also assessed by means of immunocytochemistry.

Results: Myocyte apoptosis ($<0.1\%$ before coronary artery bypass grafting) was increased after coronary artery bypass grafting and reperfusion and was greater in patients with longer periods of cardioplegic arrest ($3.3\% \pm 0.5\%$ with <55 minutes and $5.1\% \pm 0.9\%$ with 85-100 minutes, $P < .001$). Processing of caspase-9 was always more pronounced than that of caspase-8 ($P < .05$). Cardioplegic arrest was also associated with increased urocortin expression (up to $29\% \pm 3.5\%$ vs $<3\%$ in samples obtained before coronary artery bypass grafting, $P < .001$) but only in nonapoptotic myocytes. These and surrounding viable myocytes also showed increased Kir6.1 adenosine triphosphate-dependent potassium channel expression.

Conclusions: Cardioplegic arrest and subsequent reperfusion result in cardiomyocyte apoptosis, largely through mitochondrial injury, as well as exclusive urocortin expression in viable cells. This finding might suggest a cardioprotective role for endogenous urocortin in human subjects.

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During cardiopulmonary bypass, cardioplegic arrest and subsequent reperfusion inevitably expose the heart to an iatrogenic ischemia-reperfusion injury (IRI). Although different cardioplegic techniques (crystalloid, cold blood, and warm blood cardioplegia) have been developed to prevent this injury,¹ the protection given to the heart by cardioplegia is often inadequate, especially for surgical procedures requiring prolonged cardiac arrest. Previous reports have shown, for instance, that both crystalloid^{2,3} and cold blood cardioplegia^{4,5} are still associated with functional and ultrastructural changes in cardiac myocytes and the coronary circulation, although no corresponding data are yet available concerning warm blood cardioplegia.⁶

Apoptotic cell death has been implicated in the pathogenesis of several cardiovascular diseases, including IRI.⁷ However, although apoptosis has been frequently proposed as one mechanism sustaining the loss of ischemic-reperfused myocytes, no definitive evidence has been provided thus far for the contribution of apoptosis to the iatrogenic IRI associated with on-pump cardiac surgery.

The apoptotic process is mediated by activation of a cascade of proteases (caspases) that are normally present as inactive zymogens and that cleave substrates essential for the maintenance of cellular integrity. This proteolytic cascade can be initiated in 2 ways. Mitochondrial damage results in activation of the apical caspase-9, whereas binding of a specific ligand, such as Fas ligand, to its cognate death receptor, Fas, leads to activation of caspase-8. These 2 upstream caspases subsequently activate downstream effector caspases, such as caspase-3 and caspase-7. We have previously shown that in the isolated perfused rat heart exposed to ischemia and reperfusion, apoptosis of endothelial cells, which precedes that of myocytes,⁸ is initiated solely by caspase-9 activation, whereas myocyte apoptosis involves activation of both signaling pathways.⁹ Although processing of both caspase-9 and caspase-8 has also been described in the failing human myocardium,¹⁰ the relative contribution of the aforementioned signaling pathways to any apoptosis associated with cardioplegic arrest in the human subjects is currently unknown.

Several molecules have also been shown to inhibit cardiac apoptosis. One inhibitor, urocortin, is a 40-amino-acid member of the corticotrophin-releasing factor family¹¹ and is endogenously expressed in the human heart.¹² The coexpression of urocortin and its cognate receptor in cardiac myocytes suggests that endogenous urocortin might exert autocrine-paracrine effects on the normal and diseased human heart. Biologically active urocortin, for instance, is released from isolated myocytes exposed to simulated ischemia and confers on the culture medium cardioprotective properties that are abrogated by corticotrophin-releasing factor receptor antagonists.¹³

This background encouraged us to evaluate the occurrence of apoptosis and the relative contribution of its signaling pathways in human myocytes from patients exposed to cardiopulmonary bypass, warm blood cardioplegia, and subsequent reperfusion. Furthermore, we investigated whether the above surgical IRI modifies the cardiac expression of urocortin, as well as its potential involvement as a salvage mechanism.

Materials and Methods

Patient Population

The study was approved by the Institutional Ethics Committee of S. Rocco Hospital (Ome, Italy), and all patients selected provided informed consent before enrollment. Twenty-four patients (11

TABLE 1. Clinical characteristics of the enrolled patients

Variables	Group A	Group B
No. of patients	12	12
Female sex	7	5
Male sex	5	7
Age (y)	62 ± 8	64 ± 6
Aortic crossclamp time (min; mean ± SD)	48 ± 6	91 ± 8
Range (min)	40-55	85-100
Grafts	3.8 ± 0.6	4.0 ± 0.8
LV ejection fraction (% before CABG)	53 ± 6	55 ± 8
LV ejection fraction (% after CABG)	51 ± 11	52 ± 13

Data are expressed as means ± SD.

LV, Left ventricular; CABG, coronary artery bypass grafting.

female patients and 13 male patients) admitted for elective on-pump coronary artery bypass graft (CABG) surgery were classified into one of 2 groups (groups A and B) on the basis of the duration of cardioplegic arrest (Table 1). Aortic crossclamping time ranged from 40 to 55 minutes (48 ± 6 minutes [mean ± SD]) for group A (12 patients) and from 85 to 100 minutes (91 ± 8 minutes) for group B (12 patients) because of dissimilarities in coronary artery anatomy (ie, intramyocardial, unusually small, or calcified coronary arteries), although the mean number of grafts between the 2 groups was not significantly different (2.2 and 2.35 in groups A and B, respectively; $P > .05$). The patients in both groups had multiple-vessel coronary disease with symptoms of class II/III according to the Canadian Cardiovascular Society Angina Classification. Exclusion criteria were as follows: (1) symptoms of angina pectoris, heart failure, or both during the week preceding the operation; (2) reduced left ventricular ejection fraction ($<40\%$); (3) enlargement or hypertrophy of ventricular cardiac chambers, atrial cardiac chambers, or both; (4) concomitant valvular disease; (5) arrhythmias; (6) diabetes and other metabolic disorders; and (7) concomitant liver, pulmonary, or kidney diseases. The intentional enrollment of subjects who were otherwise healthy, unlike the patient population typically undergoing CABG surgery, although representing a limitation of our study, also has the merit of restricting the degree of interference that comorbidities might have exerted. All the patients were receiving calcium channel blockers and β -blockers, which were discontinued together with all other medications 2 days before the operation. The enrolled patients underwent multiple aortocoronary bypass grafts with internal thoracic arteries and saphenous veins (Table 1).

Surgical Procedure

Anesthesia and muscle relaxation were induced with propofol (2.0-2.5 mg/kg) and atracurium (0.08 mg/kg), respectively. Propofol was continuously infused ($4-6 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) to maintain anesthesia. Ventilation was controlled with 50% O₂ in air. After median sternotomy, the aorta and the inferior vena cava were cannulated, and heparin (3 $\mu\text{g/kg}$) was administered. Cardiopulmonary bypass was conducted with nonpulsatile flow at a rate of $2.4 \text{ L} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$. After aortic crossclamping, intermittent antegrade and retrograde warm blood cardioplegia was initiated through the infusion of whole blood plus 20 mEq/L of potassium.

Figure 1. A, Control precardioplegic heart (group A) exhibiting no TUNEL-positive staining or processing of caspase-3. Orange nuclei are stained with propidium iodide. B, Control precardioplegic heart (group B) showing no processing of caspase-8 or caspase-9. Cells are counterstained with propidium iodide. C, Postcardioplegic heart (group A): yellow TUNEL-positive nuclei colocalize with cytoplasmic anti-active caspase-3-positive labeling (bright red). D, Postcardioplegic heart (group B): cardiac myocytes showing colocalization between TUNEL-positive (yellow/green nuclei) and cytoplasmic anti-active caspase-3-positive staining (bright red). (Original magnifications: A, B, and C, 400 $\times$; D, 650 $\times$.)

TABLE 2. Percentages of TUNEL-, caspase-, urocortin-, and Kir6.1-positive labeling in cardiac myocytes in precardioplegia and postcardioplegia biopsy specimens

Groups	TUNEL	Caspase-3	Caspase-8	Caspase-9	Urocortin	Kir6.1
Pre-CP, group A	0%	0%	0%	0%	2.3% $\pm$ 0.5%	0.9% $\pm$ 0.3%
Pre-CP, group B	0%	0%	0%	0%	1.9% $\pm$ 0.4%	0.6% $\pm$ 0.2%
Post-CP, group A	3.3% $\pm$ 0.5%*	4.5% $\pm$ 0.8%*	1.5% $\pm$ 0.4%*	4.3% $\pm$ 1.2%*	17% $\pm$ 2.3%***	2.3% $\pm$ 0.6%*
Post-CP, group B	5.1% $\pm$ 0.9%**	6.9% $\pm$ 1.1%**	3.2% $\pm$ 0.6%*	7.3% $\pm$ 1.5%**	29% $\pm$ 3.5%***	6.7% $\pm$ 1.3%**

Data, in percentages $\pm$ SD, are expressed as the means of 12 to 15 high-power fields.

TUNEL, Terminal deoxynucleotidyl transferase-mediated nick end labeling; CP, cardioplegia.

* $P < .05$, ** $P < .01$, and *** $P < .001$ versus corresponding precardioplegia group.

The cardioplegic solution was injected at 37°C for 2 minutes into the aortic root (flow rate, 300 mL/min) and then for an additional 2 minutes into the coronary sinus at approximately 150 mL/min (approximately 40 mm Hg). This dose of cardioplegic solution was injected every 15 to 20 minutes, partially antegrade followed by retrograde. Cardiopulmonary bypass was maintained with moderate hemodilution (hematocrit, 24%-28%). After completion of the distal anastomoses, the aortic crossclamp was removed, and the construction of the proximal anastomoses was begun. At the end of the grafting procedure, protamine (3 μ g/kg) was injected to reverse the effect of heparin. Inotropic drugs were not used.

Tissue Sampling

Two sequential biopsy specimens were obtained from a virgin site in the right atrium by the same experienced cardiac surgeon (M.F.). The first biopsy specimen was taken as a control sample at the start of cardiopulmonary bypass but before cardioplegia. The second specimen was obtained as near as possible to the site of the previous sampling about 10 minutes after the release of the aortic crossclamp. There were no clinical complications related to the sampling procedure. Immediately after collection, a section of the biopsy material was fixed in 4% formaldehyde and stored at 4°C for a maximum of 48 hours. The remainder was promptly

Figure 2. Processing of caspase-3 (C3), caspase-8 (C8), and caspase-9 (C9) in precardioplegic (pre-CP) and postcardioplegic (post-CP) samples from groups A and B analyzed by means of Western blotting. The housekeeping gene actin was used as a protein-loading control.

collected in cryovials, snap-frozen in liquid nitrogen, and stored at -80°C .

Immunocytochemistry Assessment

Serial 5- μm sections were cut from paraffin blocks, and after dewaxing and heat-mediated antigen retrieval, they were stained with terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) reagents, propidium iodide, and an antibody against the cleaved active form of caspase-3 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), as previously described.⁸ Colocalization of cleaved caspase-3 and TUNEL-positive staining was used as marker of myocyte cell apoptosis. Other myocardial sections were stained with an antibody recognizing the cleaved form of caspase-8 or caspase-9 (BioVision, Mountain View, Calif) and subsequently counterstained with propidium iodide. In another staining reaction, after TUNEL staining, serial myocardial sections were incubated with an antibody against urocortin or the Kir6.1 adenosine triphosphate-dependent potassium (K_{ATP}) channel subunit (Santa Cruz Biotechnology, Inc) and, where appropriate, counterstained with propidium iodide. After washing and incubation with suitable secondary antibodies (DakoCytomation, Glostrup, Denmark), sections were analyzed by a confocal micros-

Figure 3. Serial myocardial sections from a postcardioplegic heart (group B) showing activation of caspase-9 (B, bright red) and caspase-8 (C, bright green) in cardiac myocytes, as identified by an anti-desmin red banding running perpendicularly to the cell bodies (A). (Original magnification 400 $\times$.)

Figure 4. Basal expression of urocortin in cardiac myocytes from a control heart (group A). No TUNEL-positive cells are detected (B). Postcardioplegic heart (group B): cardiac myocytes exhibiting cytosolic urocortin-positive staining (bright red) are consistently TUNEL negative. TUNEL-positive nuclei appear yellow (D). Cardiac myocytes are recognizable by the anti-desmin red banding running perpendicularly to their cellular body (A and C). (Original magnification 400 $\times$.)

copist who was blinded to the origin and sequence of the specimens (T.M.S.). Data were expressed as the means of 12 to 15 high-power fields $\pm$ SD.

Western Blotting

Cardiac specimens before and after cardioplegia from both groups of patients were homogenized in lysis buffer, electrophoresed on 8% sodium dodecylsulfate–polyacrylamide gels, and Western blotted with anti-active caspase-3, caspase-8, and caspase-9 antibodies, as previously described.⁹

Statistical Analysis

Significance was evaluated by using the 2-tailed analysis of variance test.

Results

As shown in Figure 1, A and B, in the control precardioplegic specimens from patients assigned to both groups, there was no TUNEL staining or processing of any tested caspases. The absence of cleavage of caspase-3, caspase-8, and caspase-9 was also seen in frozen samples processed by means of Western blotting. After 40 to 55 minutes of cardioplegic arrest followed by 10 minutes of reperfusion (group A), the proportion of TUNEL-positive myocytes was

$3.3\% \pm 0.5\%$ ($P < .001$). TUNEL- and cleaved caspase-3-positive staining was consistently colocalized, although not all cells labeled by the anti-active caspase-3 antibody were TUNEL positive (Figure 1, C and D). In patients from group B exposed to roughly twice the duration of cardioplegic arrest followed by 10 minutes of reperfusion, the number of cardiac myocytes labeled by means of TUNEL staining was significantly increased compared with that in group A ($5.1\% \pm 0.9\%$, $P < .001$). A significant correlation ($P < .001$) was observed between the magnitude of myocyte cell apoptosis and the length of aortic crossclamp time. A parallel increase in the proportion of cardiac myocytes showing activation of initiator caspases was also observed. By means of immunocytochemistry, processing of caspase-9 was more pronounced than that of caspase-8 both in group A and group B (Figure 2). However, the level of caspase-8 activation was consistently higher in the hearts from group B, which were exposed to a longer cardioplegia time compared with those of group A (Table 2). Similarly, by means of Western blotting, processing of caspase-3 was observed in all the biopsy samples exposed to cardioplegia-reperfusion. Additionally, in the same specimens, cleavage of caspase-9 was more marked than that of caspase-8 (Figure 3).

The basal level of urocortin expression detected in control specimens was less than 3% in both groups (Table 2). However, cardioplegia followed by reperfusion greatly enhanced urocortin expression in a time-dependent manner. In tissue samples from group A and group B, $17\% \pm 2.3\%$ and $29\% \pm 3.5\%$ of cardiac myocytes, respectively, exhibited cytoplasmic positive staining for urocortin, with the increased expression in group B being significantly ($P < .001$) greater than that in group A.

Myocytes expressing urocortin at the protein level showed no TUNEL-positive staining, suggesting that endogenous urocortin effectively protects those myocytes in which it is produced (Figure 4, A and B). Among a total of 156 urocortin-positive cells examined, only 2 showed weak TUNEL positivity. Correspondingly, none of the 83 TUNEL-positive myocytes observed expressed urocortin. In addition, urocortin-positive, TUNEL-negative myocytes were surrounded by a cuff of urocortin-negative, TUNEL-negative cells (Figure 4, C and D).

In specimens from group B only, the myocytes overexpressing urocortin were also labeled by an antibody specific for the Kir6.1 cardiac K_{ATP} channel subtype. In these samples, urocortin-positive cells were surrounded by a cuff of TUNEL- and urocortin-negative myocytes but that also expressed the Kir6.1 cardiac K_{ATP} channel subunit (Figure 5).

Discussion

This study demonstrates that effector caspase activation and DNA fragmentation are produced after cardiopulmonary bypass in human myocytes despite warm blood cardioplegia and that the degree of myocardial apoptosis is dependent on the duration of cardioplegic arrest. Apoptosis appears to mostly reflect mitochondrial injury because activation of caspase-9 was predominant over that of caspase-8, irrespective of the length of cardioplegia. Urocortin expression was increased in human cardioplegic hearts, and cells expressing urocortin were nonapoptotic. Moreover, viable urocortin-positive myocytes were surrounded by a ring of urocortin-negative cells, which, however, expressed the Kir6.1 component of the K_{ATP} channel.

The finding that all TUNEL-positive cells were also stained with an antibody recognizing the active form of caspase-3 confirms that these cells were apoptotic because they exhibited 2 distinct hallmarks of apoptosis: DNA fragmentation and caspase activation. However, other active caspase-3-positive cells were TUNEL negative. It is likely that these latter cells, sampled in a single time window, were undergoing apoptosis but had not yet developed the final stage of DNA fragmentation, and this appears to be even more verisimilar after shorter periods of cardioplegic arrest. The numbers of TUNEL-positive cells reported here might therefore underestimate the true level of apoptosis

Figure 5. Serial atrial sections from a postcardioplegic heart (group B). Induction of urocortin in cardiac myocytes (B, bright red cytosolic staining) is associated with TUNEL negativity (A, TUNEL-positive nuclei appear yellow) and overexpression of the Kir6.1 potassium channel (bright green cytosolic staining), which is detected not only in urocortin-positive cells but also in urocortin-negative neighboring cells (C). Cardiac myocytes are labeled by an anti-desmin antibody (A). (Original magnification 400 $\times$.)

caused by cardioplegic arrest. Ventricular myocytes from patients with ischemic cardiomyopathy have been shown to be more susceptible than are normal myocytes to caspase-3 cleavage induced by hypoxia in vitro,¹⁴ and thus preceding ischemic pathology might have contributed to the levels of apoptosis observed in the present study.

This study confirms that in patients undergoing on-pump cardiac surgery, cardioplegia might ameliorate but does not totally inhibit myocyte apoptosis. Indeed, DNA fragmentation, mitochondrial cytochrome c release, and morphologic changes characteristic of apoptosis have previously been demonstrated in atria from human hearts exposed to cold cardioplegia,^{2,3} although atrial changes might not fully reflect cell death in ventricular myocytes. Indeed, atrial tissue might be suboptimally protected and therefore at greater risk of cellular injury and death. However, the initiating pathways sustaining myocyte apoptosis have not been defined. We have previously shown, in the ischemic-reperfused rat heart, that endothelial cell apoptosis precedes that of myocytes⁸ and is exclusively mediated by activation of caspase-9.⁹ Subsequent myocyte apoptosis during reperfusion is also initially mediated by caspase-9 activation, although cleavage of caspase-8 consistently increases with longer reperfusion times. Although the apparent differential kinetics of caspase-9 and caspase-8 activation in the human heart exposed to cardioplegic IRI might reflect different affinities of the antibodies used, the earlier activation of caspase-9 in human hearts is consistent with the rat data, during the accumulation of which different caspase antibodies were used.

In neonatal cultured rat cardiac myocytes, ischemia increases urocortin mRNA abundance, and the peptide is released into the culture medium.¹³ Ischemia-preconditioned media are themselves cardioprotective, and the protective effect is abrogated by a urocortin receptor antagonist.¹³ Moreover, both in cultured myocytes and in the isolated perfused rat heart, exogenous urocortin reduces cell death and infarct size, respectively,^{13,15} and, in the intact heart, promotes hemodynamic and bioenergetic recovery.¹⁶ These cardioprotective effects of endogenous and exogenous urocortin are mediated by several mechanisms, including activation of p42/44 mitogen-activated protein kinase,¹⁵ induction of hsp90,¹⁷ and increased expression of Kir 6.1 K_{ATP} channels.¹⁸ Mitochondrial K_{ATP} channels have been shown to play a crucial role in ischemic preconditioning¹⁹ and in cardioprotection after IRI, including cardioplegic arrest,²⁰ and recent evidence suggests that Kir6.1 is localized in mitochondria.²¹

Enhanced expression of urocortin and its concomitant colocalization with TUNEL-negative cells after cardioplegic arrest (Figure 4, B) might suggest that in the human subject, as in the rat, IRI results in self-limitation of its consequences by inducing enhanced cardiac expression of

endogenous urocortin. However, other explanations of the above-mentioned findings (eg, a more rapid degradation of urocortin in apoptotic cells) cannot be currently excluded and therefore should be considered. In addition, although the human data are necessarily associative and do not prove causation, the findings that viable urocortin-positive cells express the Kir6.1 K_{ATP} channel and are surrounded by viable urocortin-negative but Kir6.1-positive cells are suggestive that autocrine and paracrine enhancement of K_{ATP} channel expression by endogenously released urocortin is also one mechanism of its cardioprotective function in the human subject. However, although this hypothesis is well supported by experimental data in the rat, further studies are needed to prove such a causative protective pathway in the human heart.

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