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Cardiac release of urocortin precedes the occurrence of irreversible myocardial damage in the rat heart exposed to ischemia/reperfusion injury

Richard A. Knight[a,*], Carol Chen-Scarabelli[b], Zhaokan Yuan[c], Roy B. McCauley[c], J. Di Rezze[c], Gabriele M. Scarabelli[c], Paul A. Townsend[a], David Latchman[a], Louis Saravolatz[c], Giuseppe Faggian[d], Alessandro Mazzucco[d], Hardial S. Chowdrey[e], Anastasis Stephanou[a], Tiziano M. Scarabelli[c]

[a] *Medical Molecular Biology Unit, Institute of Child Health, University College London, 30 Guilford Street, London WC1N 1EH, United Kingdom*
[b] *Division of Cardiology, Veteran Affairs Ann Arbor, University of Michigan, Ann Arbor, MI, USA*
[c] *Division of Cardiovascular Disease, Department of Internal Medicine, Wayne State University Medical School, Detroit, USA*
[d] *Divisione di Cardiochirurgia, Universita' degli Studi di Verona, Verona, Italy*
[e] *Department of Biomedical Sciences, University of Westminster, New Cavendish Street, London W1W 6UW, United Kingdom*

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Abstract **This study evaluates whether cardiac ischemia induces release of urocortin, before and independently from myocyte cell death. Urocortin levels rose after 5-min ischemia and peaked after 10-min ischemia, when cell death was not detected. However, myocyte apoptosis and/or necrosis occurred following 20- and 30-min ischemia, which paralleled a fall in urocortin levels, suggesting that urocortin expression and release are mainly sustained by metabolically challenged, though still viable myocytes. Hence, since cardiac release of urocortin, unlike that of conventional biomarkers, occurs before and apart from cell death, urocortin levels may be clinically useful in the diagnosis of sublethal myocardial ischemia.**

Keywords: Sublethal ischemia; Biomarker; Apoptosis; Urocortin

1. Introduction

Urocortin, urocortin II and urocortin III are homologous peptides related to corticotropin releasing hormone (CRH) that have all been shown to have potent cardioprotective properties against ischemia/reperfusion (I/R) injury [1]. There are two G-protein-coupled receptors for this family, CRH-R1 and CRH-R2, both of which can be expressed as alternatively spliced forms. Urocortin binds to both receptor subtypes (though with higher affinity to CRH-R2), while urocortins II and III are specific for CRH-R2 [1].

The protective effects of urocortin are mediated by a variety of mechanisms. For example, urocortin activates both the PI3-kinase/Akt and p42/p44 MAPK signal transduction pathways [2,3]. Many of the downstream protective mechanisms converge on mitochondria, organelles whose function is severely compromised by I/R, leading to both apoptotic and necrotic cell death [4]. Thus, urocortin increases mitochondrial expression and activity of the Kir6.1 K_{ATP} channel [5], causes translocation of PKCε to the mitochondrial membrane [6] and reduces the levels of $iPLA_2$ and its toxic metabolite, LPC, in mitochondria [7].

Cardiac myocytes express urocortin, urocortin II and urocortin III together with one spliced isoform of CRH-R2, depending on species [1]. This suggests that endogenous urocortins may also act via an autocrine/paracrine cardioprotective mechanism. Indeed, supernatants from primary cultures of rat cardiomyocytes exposed to I/R are cardioprotective and these cardioprotective effects of ischemia-preconditioned media are inhibited by CRH-R antagonists [8]. In addition, we have shown that both myocyte apoptosis and urocortin expression are increased in human hearts exposed to cardioplegic arrest, a common iatrogenic form of I/R, and all urocortin positive cells are viable, while apoptotic cells are urocortin-negative [9]. Moreover, urocortin-positive myocytes are surrounded by a cuff of viable urocortin-negative cells which, however, express the Kir6.1 ATP-dependent potassium channel, whose opening has been implicated in urocortin-mediated cardioprotection. Since we previously showed that urocortin induced Kir6.1 overexpression in the rat heart [5], this finding seems to corroborate the hypothesis that autocrine/paracrine enhancement of K_{ATP} channel expression by endogenously-released urocortin is also one mechanism of its cardioprotective function in the human heart.

The evaluation of patients with chest pain is often challenging and may be misleading [10]. The incorrect diagnosis of acute cardiac ischemia, with subsequent admission to costly emergency and observation units, incurs redundant expenses. On the other hand, the misinterpretation of cardiac chest pain, with discharge of patients with acute myocardial infarction, considerably increases the risk of adverse outcomes. The recent development and characterization of precise imaging techniques, as well as analytically dependable biomarkers to confirm or exclude cardiac ischemia, has certainly refined the diagnosis of acute myocardial infarction, but only limitedly facilitated the diagnosis of coronary events due to transient

*Corresponding author. Fax: +44 207 905 2301.
E-mail address: r.knight@ich.ucl.ac.uk (R.A. Knight).

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ischemia and leading to reversible cardiac damage [11]. These patients are often admitted to the hospital only after the remission of their symptoms, and therefore exhibit at the same time negative necrosis biomarkers and a normalized EKG trace.

As previously stated, urocortin is a cardioprotective peptide, which is released by cardiac cells exposed to I/R injury. Hence, based on these premises, we have assessed whether myocyte release of urocortin is merely a local event, or whether myocyte-derived urocortin is also released systemically and could therefore act as a circulating biomarker for I/R injury. Hence, we have assayed urocortin in the perfusate of Langendorff-perfused Sprague–Dawley rat hearts exposed to increasing periods of zero-flow global ischemia followed by reperfusion, as well as in the circulation of Sprague–Dawley rats exposed in vivo to identical periods of regional ischemia and reperfusion.

2. Materials and methods

2.1. Ex vivo ischemia/reperfusion

Isolated Langendorff-perfused hearts from adult male Sprague–Dawley rats were exposed to I/R and their mechanical function was monitored, as described previously [12]. At least six hearts were used

Table 1
Overview of the different time points of I/R, used for the ex vivo and in vivo sets of experiments

Ex vivo ischemia/reperfusion		
Control hearts (6 units)		
Stabilization (10 m)		Simple perfusion (2 h)
I/R groups (6 units per group)		
Stabilization (10 m)	5 m zero-flow global ischemia	2 h reperfusion
Stabilization (10 m)	10 m zero-flow global ischemia	2 h reperfusion
Stabilization (10 m)	20 m zero-flow global ischemia	2 h reperfusion
Stabilization (10 m)	30 m zero-flow global ischemia	2 h reperfusion
In vivo ischemia/reperfusion		
Control hearts (6 units)		
Open thoracotomy for 2 h		Closure of thoracotomy
I/R groups (6 units per group)		
5 m regional ischemia		2 h reperfusion
10 m regional ischemia		2 h reperfusion
20 m regional ischemia		2 h reperfusion
30 m regional ischemia		2 h reperfusion

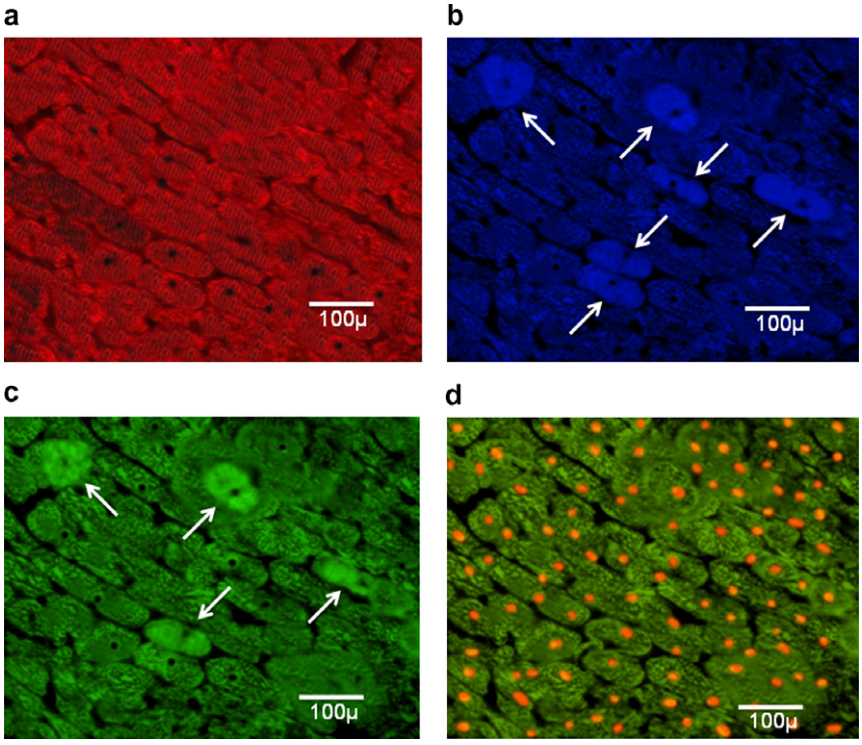

Fig. 1. Four adjacent myocardial sections serially cut from the same isolated Langendorff-perfused control heart. (Panel a) Desmin-positive myocytes, as identified by the ''red banding'' running perpendicularly to their long axis. (Panels b and c) Same myocyte population stained for urocortin mRNA and protein. White arrows indicate urocortin-positive myocytes. (Panel d) Among the matching myocyte population counterstained with propidium iodide (orange nuclei), no cell shows TUNEL-positive staining (for details of TUNEL positive labeling see Fig. 5d). Magnification: 400×.

in each group. After 10-min stabilization, the non-ischemic control group was simply perfused with Krebs–Heinsleit buffer for 2 h, while the treated groups were exposed to 5, 10, 20 or 30 min of zero-flow global ischemia and subsequently to 2 h reperfusion (Table 1).

2.2. Immunohistochemistry, fluorescent in situ hybridization, and TUNEL staining

Four adjacent 4 μm myocardial sections were serially cut from the same wax blocks of each group, and stained with either an anti-desmin antibody (used as a marker of cardiac cells), or an antisense urocortin cRNA probe synthesized with a 3′-biotinylated tail, or an anti-urocortin antibody, or a fluorescein-conjugated TUNEL labeling, with propidium iodide used as a counterstaining [9,13,14]. The urocortin probe consisted of 48 bases (AAA-TAA-CCA-CTC-AGA-GTA-TTC-AGG-GTG-ACT-CTG-GGC-GCG-CTG-CAC-CGC) and was complementary to the rat mRNA encoding base pairs 571–618 of the urocortin message (GenBank accession number NM021290).

A rhodamine-conjugated secondary antibody was used to label desmin, while Cy5-conjugated streptavidin and a fluorescein-conjugated secondary antibody were used to label urocortin messenger and protein, respectively. Sections were finally analyzed by confocal fluorescent microscopy. Data were expressed as the means of 12–15 high power fields, ±S.D.

2.3. In vivo ischemia/reperfusion

Adult male Sprague–Dawley rats were anesthetised, ventilated using room air through a tracheotomy, and exposed to thoracotomy crossing the fifth intercostal space [15]. At least six rats were used in each group. In control sham-operated animals, the surgical breach was kept patent for 2 h, before being sutured. In treated animals, the left coronary artery was occluded for 5, 10, 20 or 30 min and subsequently reperfused for 2 h (Table 1).

2.4. Urocortin and creatine phosphokinase (CPK) assays

Urocortin concentrations were evaluated by competitive radioimmunoassay [16], using a commercially available kit (Phoenix Pharmaceutical; sensitivity range: from 0.2 to 27 pmol/L), according to the recommended protocol, while CPK levels were evaluated spectrophotometrically [12]. For the ex vivo groups, the perfusate was collected from the control hearts before (after stabilization) and after the buffer perfusion, and from the treated hearts before ischemia (after stabilization) and after reperfusion. For the in vivo groups, 1 ml of blood was collected from the tail vein of both sham-operated and treated rats before the execution of the thoracotomy and after the closure of the thoracotomy breach.

The Langendorff perfusate was collected in chilled glass and subsequently concentrated by means of a centrifugal concentrator (SpeedVac). Blood samples were collected into Lavender Vacutainer tubes, containing EDTA, transferred into centrifuge tubes containing aprotinin and subsequently centrifuged at $1600 \times g$ for 15 min, to allow collection of plasma. Peptide extraction from collected perfusate and plasma was completed by means of pre-treated C18 separation columns, before measurement by competitive radioimmunoassay.

2.5. Statistics

Comparisons between different groups were made using repeated measures two-way ANOVA with appropriate post-doc analysis: P-values <0.05 were considered significant.

Table 2
Occurrence of apoptosis and percentage of myocytes expressing urocortin at the mRNA and protein level in the intact heart exposed to ex vivo ischemia/reperfusion injury

	% Myocytes expressing urocortin mRNA	% Myocytes expressing urocortin protein	% TUNEL positive myocytes
Ex vivo ischemia/reperfusion			
Control hearts	15 ± 2.5%	12 ± 2.2%	<0.1
5-min ischemia + 2 h reperfusion	41 ± 4.2**	35 ± 3.8**	<0.1
10-min ischemia + 2 h reperfusion	58 ± 6.8**	52 ± 6.2**	<0.1
20-min ischemia + 2 h reperfusion	22 ± 1.4*	24 ± 1.8*	6.3 + 1.2**
30-min ischemia + 2 h reperfusion	20 + 3.7%*	18 ± 3.2*	8.9 ± 2.3%**

* $P < 0.05$.
** $P < 0.01$, versus control hearts.

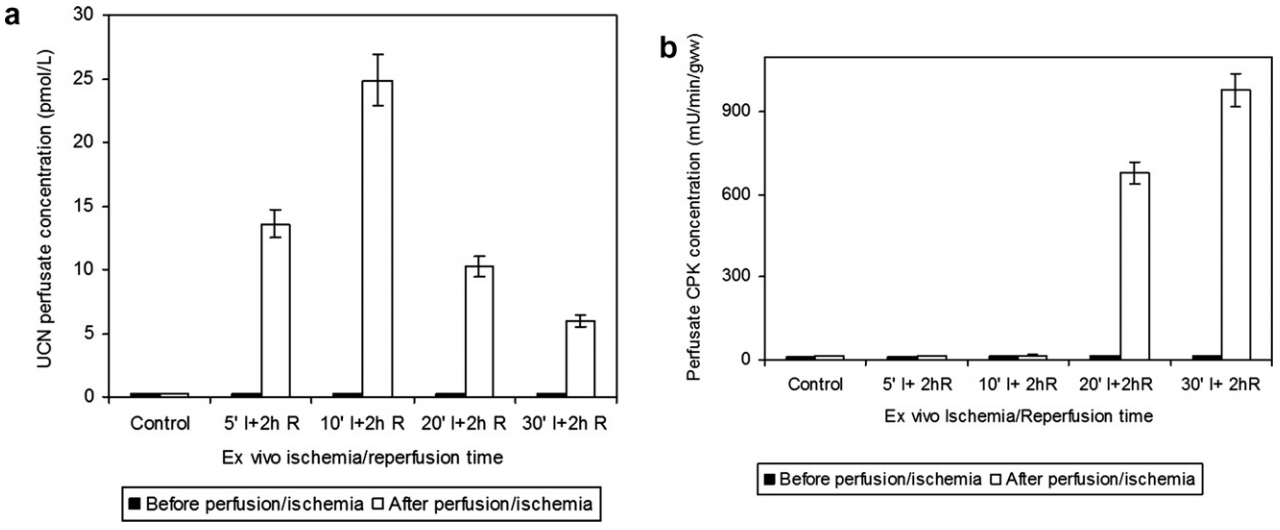

Fig. 2. Concentration of urocortin (Panel a) and CPK (Panel b) in the perfusate from isolated Langendorff-perfused control and treated hearts (for explanation see Section 2).

3. Results

In serial adjacent sections from control hearts unexposed to I/R, among the population of desmin-positive myocytes (Fig. 1a), only a small percentage (Table 2) expressed urocortin at the mRNA (Fig. 1b) and protein level (Fig. 1c), and none showed evidence of apoptosis, as assessed by TUNEL staining (Fig. 1d). Furthermore, no urocortin (Fig. 2a) or CPK (Fig. 2b) were released in the perfusate from the same control hearts, either before or after perfusion. Hence, control hearts showed low basal expression of urocortin, no release of urocortin in the perfusate and absence of both apoptosis and necrosis. Conversely, a significantly increased proportion of desmin-positive cardiomyocytes (Fig. 3a) from hearts exposed to 5- and 10-min ischemia showed enhanced expression of urocortin (Table 2) at the mRNA (Fig. 3b) and protein (Fig. 3c) level, although none of them were apoptotic (Fig. 3d). In these two groups, urocortin was also released into the perfusate after reperfusion in amounts proportional to the duration of ischemia (Fig. 2a). More specifically, perfusate urocortin levels, which were undetectable at the end of stabilization, significantly rose in hearts receiving 5-min ischemia (13.6 ± 0.7 pmol/L), and almost doubled following 10-min ischemia, reaching a mean concentration of 24.86 ± 1.3 pmol/L (both P-values <0.001, versus control). However, no significant release of CPK into the perfusate was seen from these hearts (Fig. 2b), exhibiting a complete hemodynamic recovery following ischemia (Fig. 4a and b). Hence, in heart exposed to short ischemia, increased transcription, translation and release of urocortin were associated with neither apoptotic nor necrotic cell death.

With longer periods of ischemia (20 and 30 min) followed again by a 2 h reperfusion period, the proportion of desmin-positive myocytes (Fig. 5a) overexpressing urocortin mRNA (Fig. 5b) and protein (Fig. 5c) fell dramatically and proportionally to the length of ischemia (Table 2). Moreover, in the same myocyte population, indeed TUNEL-positive nuclear staining (Fig. 5d) was observed (Table 2), and, of note, all myocytes undergoing apoptosis were negative for urocortin mRNA (Fig. 5b) as well as for urocortin protein (Fig. 5c). In line with this finding, cardiac release of urocortin in the perfusate from hearts exposed to 20 and 30 min of ischemia was also significantly reduced (Fig. 2a), while that of CPK was dramatically heightened (Fig. 2b), which paralleled a significant impairment of postischemic recovery of cardiac function (Fig. 4c and d). The reduced transcription and translation of urocortin in viable myocytes, the exclusive occurrence of apoptosis in urocortin-negative cardiac cells, and the pattern of cardiac release of urocortin, which, in correlation with postischemic recovery of cardiac function, diminishes with increasing ischemic times, unlike that of CKP, which progressively augments, suggest that cardiac expression and release of urocortin are mainly sustained by metabolically challenged, but still viable myocytes.

To assess whether a similar pattern of cardiac release of urocortin into the circulation could also be observed following

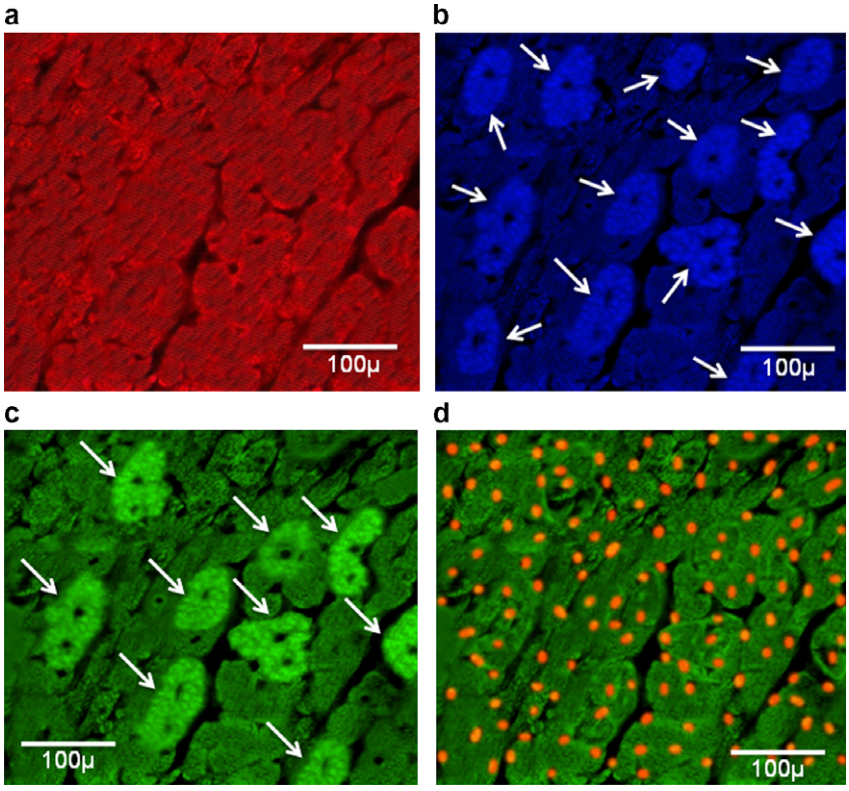

Fig. 3. Four adjacent myocardial sections serially cut from the same isolated Langendorff-perfused heart exposed to 10-min ischemia and 2 h reperfusion. (Panel a) Desmin-positive myocytes with identifying "red banding". (Panels b and c) Same myocyte population showing enhanced expression (white arrows) of urocortin mRNA and protein, as compared to control hearts. (Panel d) Matching myocyte population counterstained with propidium iodide (orange nuclei), lacking TUNEL-positive staining (for details of TUNEL positive labeling compare with Fig. 5d). Magnification: 400×.

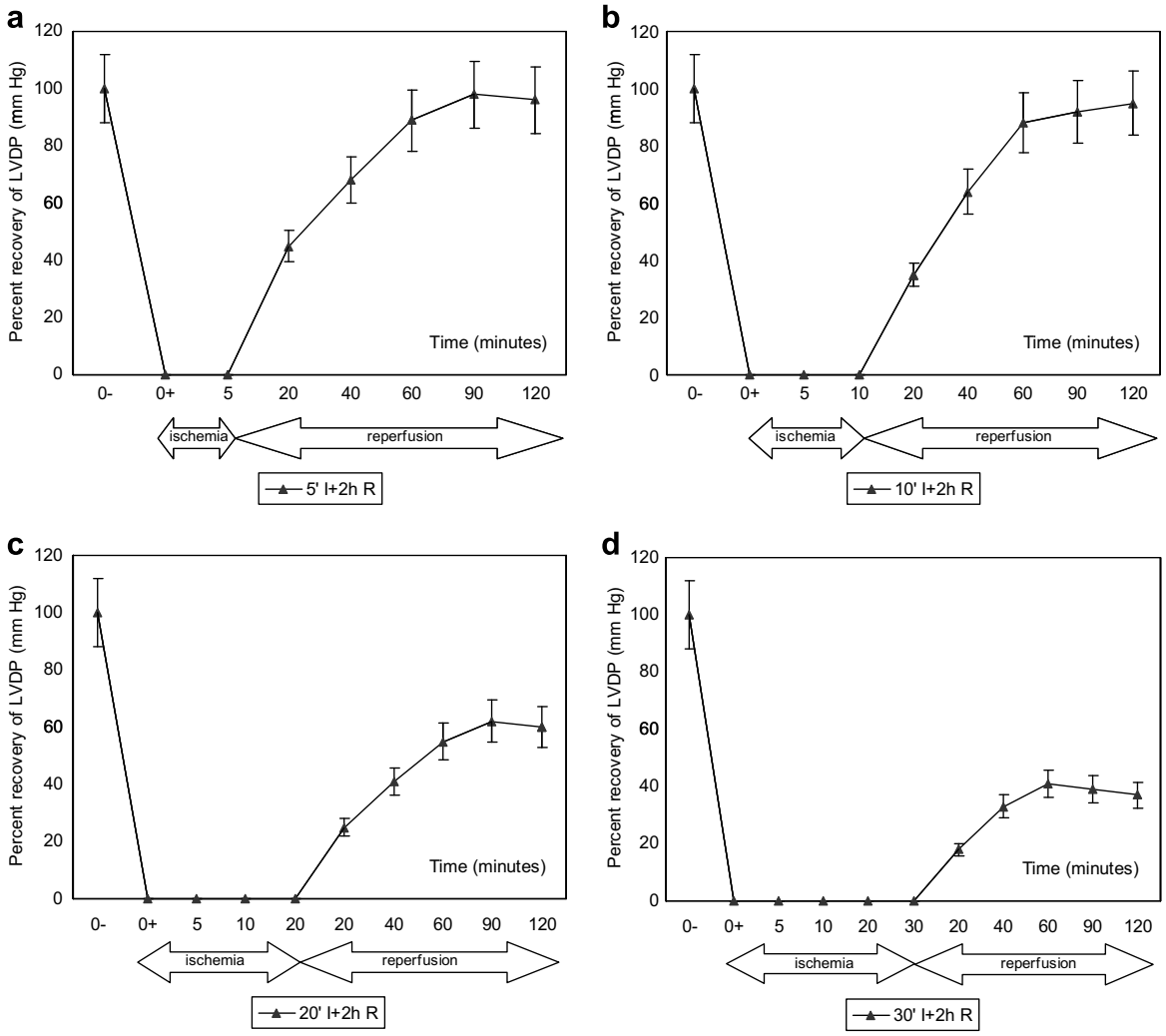

Fig. 4. Recovery of left ventricular developed pressure (LVDP) in the isolated Langendorff-perfused rat heart exposed to 5- (Panel a), 10- (Panel b), 20- (Panel c) and 30 (Panel d)-min ischemia, followed by 2 h reperfusion.

in vivo I/R injury, we assayed plasma levels of urocortin in rats exposed to either 5, 10, 20 or 30 min of in vivo regional ischemia, followed by 2 h reperfusion. In parallel with the ex vivo studies, urocortin plasma levels, assessed after the closure of the thoracotomy, were significantly higher in the rats subjected to short ischemia (5 and 10 min, Fig. 6a; all P-values <0.05 versus controls), whose groups showed no elevation of CPK plasma levels, as compared to sham-operated animals (Fig. 6b; P-values <0.05, versus sham-operated controls, only for groups given 20- and 30-min ischemia).

4. Discussion

Although basal urocortin levels were previously reported to be around 14 and 20 pmol/L in normal females and males, respectively [17], in our study, perfusate and plasma urocortin concentrations were below the limits of assay detection. This apparent discrepancy might be explained with differences in heart mass between diverse species. Moreover, plasma urocortin levels, which were significantly increased in patients with heart failure, especially in its early stages, increasingly fell with progression of the disease [17]. This drop in concentration paralleled a significant correlation between urocortin levels and values of ejection fraction, a powerful indicator of cardiac function, suggesting that cardiac release of urocortin is mainly achieved by functionally competent, rather than dysfunctional, cardiac myocytes.

In our previous study, we documented that the mild, though metabolically challenging, form of ischemia iatrogenically given to the human heart during cardioplegic arrest, is "per se" sufficient to induce increased cardiac expression and potentially release of urocortin. This was confirmed in the present study, where urocortin was detected in the perfusate and the plasma of rats exposed to ex vivo and in vivo I/R injury, respectively. The absence of urocortin from the plasma of sham-operated rats, both before the execution of the thoracotomy and after the closure of the thoracotomy breach, suggests that neither anesthesia nor surgery alone increase circulating urocortin levels, although small but significant rises in plasma CRH have been reported in patients undergoing cholecystectomy [18].

The highest release of urocortin into both perfusate and plasma was seen with 5- and 10-min ischemia, when the post-ischemic functional recovery was complete and there was neither myocyte apoptosis nor release of conventional markers

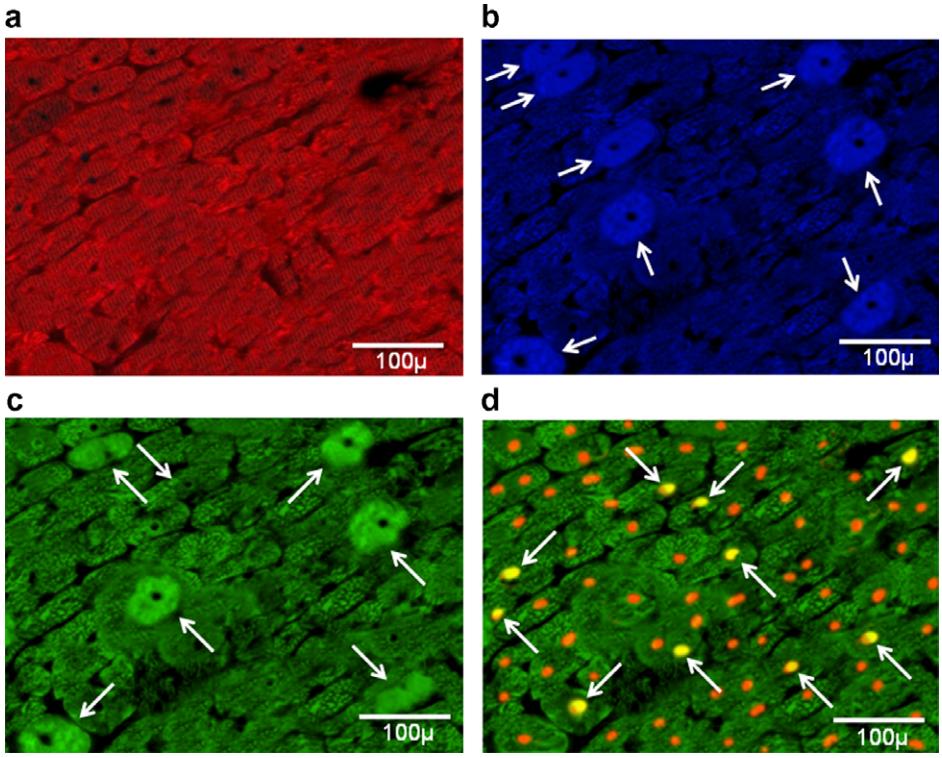

Fig. 5. Four adjacent myocardial sections serially cut from the same isolated Langendorff-perfused heart exposed to 30-min ischemia and 2 h reperfusion. (Panel a): Desmin-positive myocytes with typical "red banding". (Panels b and c) Same myocyte population showing reduced expression (white arrows) of urocortin mRNA and protein, as compared to hearts exposed to short ischemia. (Panel d) Matching myocyte population counterstained with propidium iodide (orange nuclei), showing nuclear TUNEL-positive staining (yellow nuclei pointed by white arrows). Magnification: 400×.

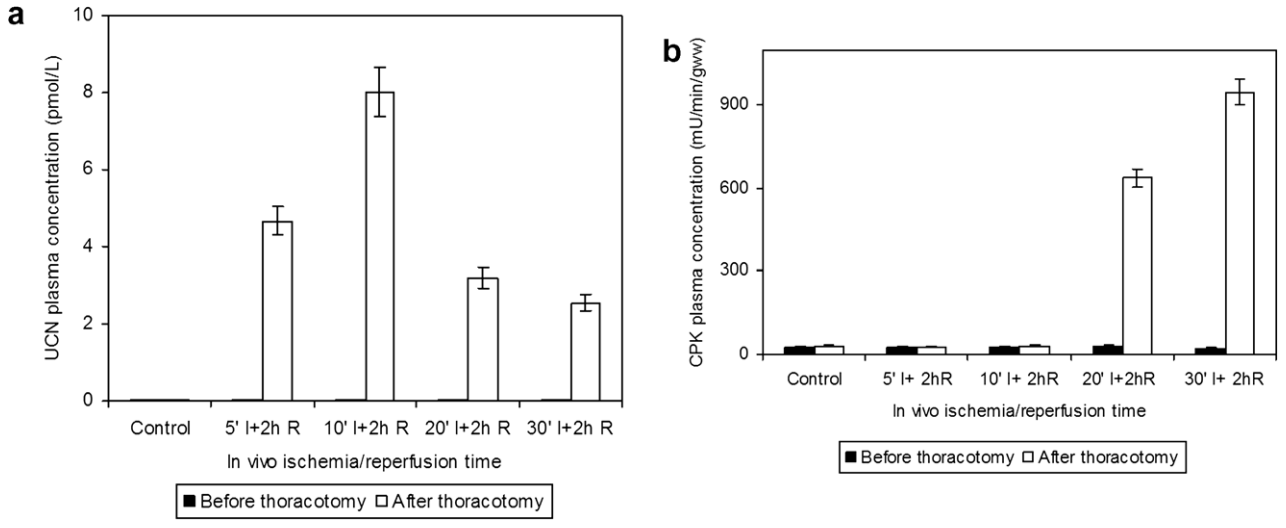

Fig. 6. Plasma concentration of urocortin (Panel a) and CPK (Panel b) in sham-operated and treated rats exposed to in vivo I/R injury (for explanation see Section 2).

of cardiac necrosis, such as CPK. Therefore, although the animal data presented in this study are preliminary and need extensive confirmation in the clinical setting, measurement of serum urocortin may be a potentially useful and relatively inexpensive biochemical indicator of mild sublethal ischemia, where significant myocyte loss has not occurred. In particular, it may be valuable in the differential diagnosis of brief episodes of chest pain, especially when other specific, but later, clinical parameters of cardiac injury, such as CPK and ECG changes are negative. Similarly, during cardiac surgery, the measurement of serum urocortin may help in the identification of peri- and post-operative cardiac injury. In particular, serum urocortin may be a more specific marker of ischemic injury, since the elevation of other current biochemical markers, such as

cardiac troponin and CPK, may be a consequence of iatrogenic injury from the surgery itself, whereas serum urocortin is unaffected either by anaesthesia or surgery.

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