



# Peroxynitrite induces HMGB1 release by cardiac cells *in vitro* and HMGB1 upregulation in the infarcted myocardium *in vivo*

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**Aims** High-mobility group box 1 (HMGB1) is a nuclear protein actively secreted by immune cells and passively released by necrotic cells that initiates pro-inflammatory signalling through binding to the receptor for advanced glycation end-products. HMGB1 has been established as a key inflammatory mediator during myocardial infarction, but the proximal mechanisms responsible for myocardial HMGB1 expression and release in this setting remain unclear. Here, we investigated the possible involvement of peroxynitrite, a potent cytotoxic oxidant formed during myocardial infarction, on these processes.

**Methods and results** The ability of peroxynitrite to induce necrosis and HMGB1 release *in vitro* was evaluated in H9c2 cardiomyoblasts and in primary murine cardiac cells (myocytes and non-myocytes). *In vivo*, myocardial HMGB1 expression and nitrotyrosine content (a marker of peroxynitrite generation) were determined following myocardial ischaemia and reperfusion in rats, whereas peroxynitrite formation was inhibited by two different peroxynitrite decomposition catalysts: 5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrinato iron (III) (FeTPPS) or Mn(III)-tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP). In all types of cells studied, peroxynitrite (100  $\mu$ M) elicited significant necrosis, the loss of intracellular HMGB1, and its passive release into the medium. *In vivo*, myocardial ischaemia–reperfusion induced significant myocardial necrosis, cardiac nitrotyrosine formation, and marked overexpression of myocardial HMGB1. FeTPPS reduced nitrotyrosine, decreased infarct size, and suppressed HMGB1 overexpression, an effect that was similarly obtained with MnTBAP.

**Conclusion** These findings indicate that peroxynitrite represents a key mediator of HMGB1 overexpression and release by cardiac cells and provide a novel mechanism linking myocardial oxidative/nitrosative stress with post-infarction myocardial inflammation.

**Keywords** Myocardial infarction • Peroxynitrite • High-mobility group box 1 • Inflammation • Cardiomyocytes

## 1. Introduction

High-mobility group box 1 (HMGB1) is a nuclear protein implicated in the binding of transcription factors to their cognate DNA sequences. Furthermore, HMGB1 acts as a cytokine secreted by activated immune cells and is also passively released by cells undergoing necrotic cell death, triggering inflammatory responses in neighbour cells

through its ability to bind the receptor for advanced glycation end-product (RAGE) and to activate nuclear factor kappa B (NF- $\kappa$ B).<sup>1,2</sup> A series of recent investigations indicated that HMGB1 is upregulated and released by ischaemic tissues *in vivo*, including brain,<sup>3</sup> liver,<sup>4</sup> and heart.<sup>5</sup> With respect to the heart, elevated plasma levels of HMGB1 have been detected in patients after myocardial infarction and appear correlated with adverse clinical outcomes.<sup>6</sup> The emerging

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role of HMGB1 in the pathophysiology of myocardial infarction remains controversial, with studies reporting detrimental pro-inflammatory and cardiodepressive actions of HMGB1 in this setting,<sup>5</sup> and others indicating instead that HMGB1 may promote the proliferation of cardiac stem cells, improve the healing process, and reduce post-infarction myocardial dysfunction.<sup>6–9</sup>

A major mechanism contributing to cell death during myocardial ischaemia and reperfusion is the development of myocardial oxidative stress and nitrosative stress, related to the formation of reactive oxygen and reactive nitrogen species, most notably peroxynitrite, formed from nitric oxide (NO) and superoxide ( $O_2^-$ ).<sup>10</sup> Peroxynitrite represents a key effector of cardiomyocyte cell death during myocardial infarction via multiple mechanisms, including diffuse biomolecular injury, DNA damage and secondary activation of poly(ADP-ribose) polymerase, activation of metalloproteinases, and mitochondrial inhibition as reviewed in Ferdinandy and Schulz<sup>11</sup> and Pacher *et al.*<sup>12</sup> The toxicity of peroxynitrite towards cardiomyocytes suggests that it might contribute to the release of HMGB1 during myocardial infarction. This would represent an important novel mechanism linking peroxynitrite not only with the acute myocardial damage triggered by ischaemia and reperfusion, but also with the molecular signals involved in the process of post-ischaemic inflammation, regeneration, and healing. The present study was therefore designed to explore a possible role of peroxynitrite in the process of HMGB1 release by cardiac cells, using both *in vitro* and *in vivo* approaches. We found that peroxynitrite induces the release of significant amounts of HMGB1 by cardiac cells *in vitro* (myocytes and non-myocytes) and also represents a key regulator of HMGB1 expression in the infarcted myocardium. These data identify a previously unrecognized role of peroxynitrite in the complex pathophysiology of myocardial ischaemia–reperfusion injury.

## 2. Methods

All the animal experiments were approved by the State Government Veterinary Office (Lausanne, Switzerland) and were performed according to the University of Lausanne Medical School institutional guidelines. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.1 Cell culture conditions and stimulation

H9c2 cells, a clonal line derived from rat heart,<sup>13,14</sup> were grown (5% CO<sub>2</sub>, 37°C) in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Invitrogen, Basel, Switzerland) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. H9c2 cells retain several morphological and metabolic characteristics of embryonic cardiocytes and are recognized as a well-suited model to study cardiomyocyte biology.<sup>15</sup> However, given the inherent limitations of cell lines, we performed most *in vitro* experiments in primary murine cardiac cells. Neonatal C57BL/6 mice (Charles River Laboratories) were used as a source of ventricular cardiomyocytes and mesenchymal non-myocyte cells (NMCs), according to previously published procedures.<sup>16</sup> Briefly, ventricles were separated from the atria, minced, and digested in buffer containing 0.45 mg/mL collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 1 mg/mL pancreatin (Invitrogen Life Technologies, Basel, Switzerland). Cardiomyocytes and NMCs were separated by two steps of differential plating at 45 min each. Adherent cells, reflecting the NMC fraction, were expanded in culture in a 3:1 mixture of DMEM and Medium 199 (Invitrogen Life Technologies, Basel, Switzerland) supplemented with 10% horse serum (Serotech Ltd, Oxford, UK), 5% FBS

(Serotech Ltd), 100 U/mL penicillin G, and 100 g/mL streptomycin. Cardiomyocytes were plated on gelatinized plates in the same medium, allowed to adhere overnight, and then maintained in serum-free medium with antibiotics.

Peroxynitrite (Calbiochem, Calbiochemi, Merck Chemicals, Darmstadt, Germany) was synthesized from isoamyl nitrite and hydrogen peroxide followed by removal of hydrogen peroxide over a manganese dioxide column, as described,<sup>17</sup> and was stored in 0.4 M NaOH at –80°C. Prior to experimentation, the concentration of peroxynitrite was determined from its absorption at 302 nm (extinction coefficient of 1670/M/cm). Stimulations were performed in phosphate-buffered saline (PBS) to avoid reactions of peroxynitrite with media constituents.<sup>13,18</sup> Peroxynitrite was delivered as a single bolus at a 1:100 dilution, at a final concentration of 100 μM. This concentration was used on the basis of pilot experiments, showing that it was sufficient to induce significant cell necrosis. This concentration has physiological relevance, since it has been estimated that rates of peroxynitrite production up to 50–100 μM/min could be reached in specific compartments *in vivo*.<sup>19</sup> It is also worth mentioning that the actual effective concentration of peroxynitrite experienced by cells should be expressed as a concentration × time product.<sup>20</sup> Therefore, sustained production of low micromolar concentrations of peroxynitrite would produce effects comparable to a single bolus addition of higher concentrations of peroxynitrite, as performed in the current study. After 40 min of stimulation, the cells were replaced in the culture medium and were then examined after 1–4 h.

### 2.2 Cytotoxicity assays and evaluation of necrosis and apoptosis

The release of lactate dehydrogenase (LDH) in the culture medium was determined as an indicator of cell necrosis,<sup>21</sup> using a commercially available kit (Roche Molecular Biochemicals, Basel, Switzerland), as per manufacturer's protocol. The release of troponin I by cardiac myocytes was further used as an indicator of necrosis, using the ACCESS<sup>®</sup> 2 cardiac troponin I immunoassay system from Beckman Coulter (Brea, CA, USA). In a subset of experiments, necrosis was also measured in H9c2 cells by the propidium iodide (PI) staining method. At the end of stimulation, cells were stained with 1 μg/mL PI (Molecular Probes, Eugene, OR, USA) for 30 min and washed twice with PBS+2% FBS, and necrosis was then analysed by a FACS Calibur flow cytometer (Beckton–Dickinson, San Jose, CA, USA), as described previously.<sup>22</sup>

The influence of peroxynitrite on the development of apoptosis was also evaluated in a subset of cardiac cells (non-myocytes). At the end of stimulation, cells were washed (PBS+2% FBS), and incubated with PI and Annexin V conjugated to the fluorochrome Cy<sup>TM</sup>5 (Pharmingen, Allschwil, Switzerland) for 15 min, and then fluorescence activated cell sorting (FACS) sorted. Living, apoptotic, and necrotic cells were then used to evaluate the intracellular expression of HMGB1 by western blotting, as exposed below.

Contrast-phase microscopic evaluation was performed for the morphological assessment of cytotoxicity.

### 2.3 Immunoblot detection of HMGB1, RAGE, and TLR4

Cells were scraped in lysis buffer (Tris–HCl 10 mM, NP40 0.5%, NaCl 0.15 M, Na<sub>3</sub>VO<sub>4</sub> 1 mM, NaF 10 mM, phenylmethanesulfonyl fluoride (PMSF) 1 mM, EDTA 1 mM, aprotinin 10 μg/mL, leupeptin 10 μg/mL, and pepstatin 1 μg/mL). Proteins (20 μg) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20. The membrane was then incubated overnight at 4°C with primary antibodies raised against HMGB1 (Assay Designs, Ann Arbor, MI, USA), RAGE (ABCAM, Cambridge, MA, USA), or toll-like receptor

4 (TLR4) (a kind gift from Dr Thierry Roger, Service of Infectious Diseases, Lausanne University Hospital, Switzerland), followed by incubation for 1 h with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA).

## 2.4 HMGB1 assay in cell medium

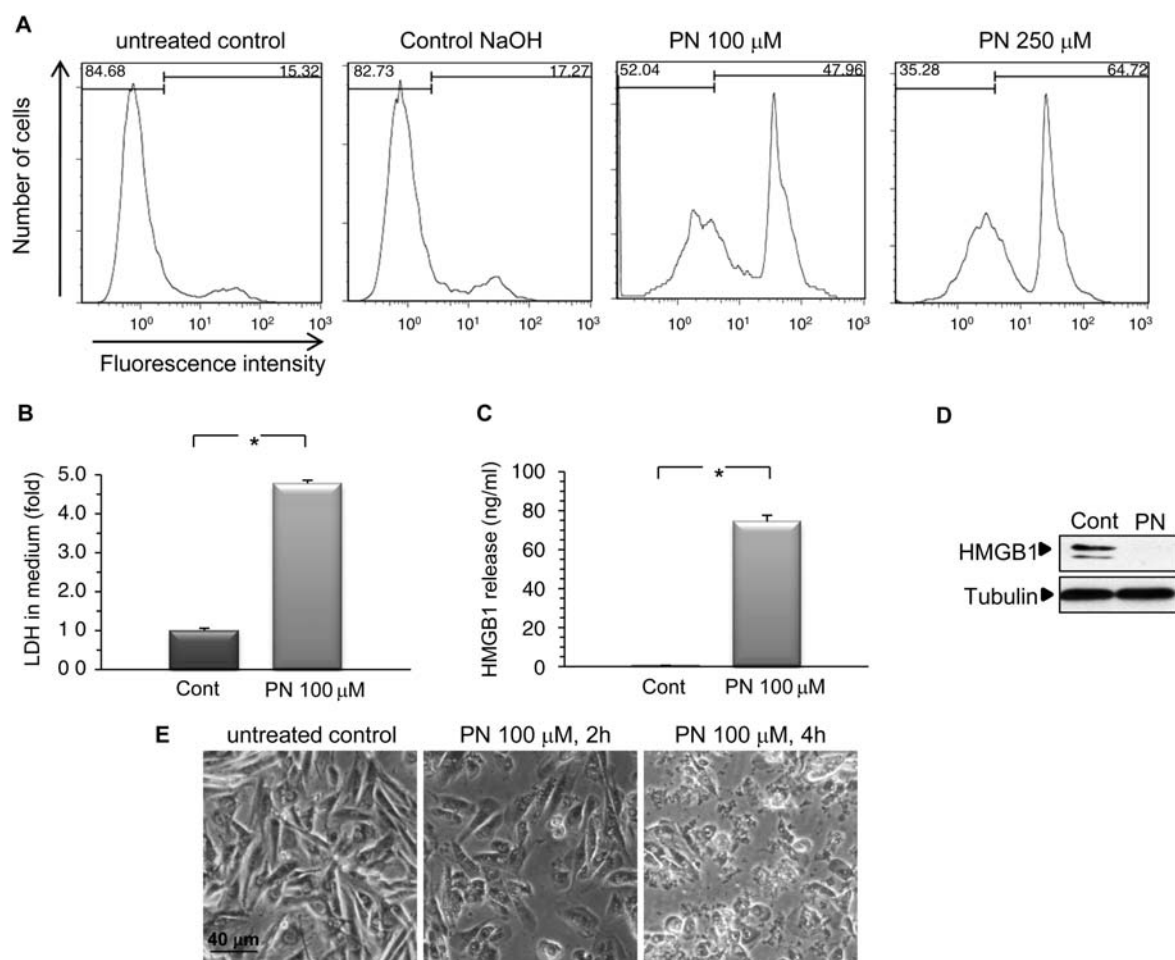
After stimulation, the cell medium was aspirated, cleared by centrifugation, and assayed for the release of HMGB1 using a commercially available ELISA (Shino-Test Corporation, Tokyo), according to the instructions provided by the manufacturer.

## 2.5 RNA isolation and reverse transcriptase–polymerase chain reaction

RNA isolation and reverse transcription were performed as described,<sup>13</sup> using the following primer pairs: RAGE (sense, 5'-GAAGGCTCTGTGGGTGAGTC-3'; antisense, 5'-ATTCAGCTCTGCACGTTCCCT-3') and TLR4 (sense, 5'-CAGCAAAGTCCCTGATGACA-3'; anti-sense, 5'-AGAGGTGGTGAAGCCATGC-3').

## 2.6 Myocardial ischaemia–reperfusion in rats

Adult male Wistar rats (300–350 g) were anaesthetized with intraperitoneal thiopentone sodium (60 mg kg<sup>-1</sup>), tracheostomized, and mechanically ventilated (Harvard Model 683 rodent respirator, Holliston, MA, USA). Core temperature was maintained at 37°C. Following a left thoracotomy, the left anterior descending coronary artery was ligated by an intramural 5.0 silk suture and a snare occluder. After 45 min of ischaemia, reperfusion was allowed for 1, 2, or 6 h by removing the coronary occluder. Rats were treated 10 min before reperfusion with 10 mg/kg 5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrinato iron (III) chloride (FeTPPS), a metalloporphyrin peroxynitrite decomposition catalyst,<sup>23,24</sup> or isotonic saline (0.5 mL), given intravenously. A group of sham rats not exposed to myocardial ischaemia–reperfusion was used for control purpose. At the end of the reperfusion period, hearts were removed for the determination of myocardial infarct size (2 h reperfusion) and for the evaluation of the expression levels of HMGB1 and nitrotyrosine (1 h reperfusion) within the cardiac tissue (discussed subsequently). In addition, cardiac HMGB1 expression was also determined (after 2 h reperfusion) in cardiac samples from a previous experimental study,<sup>14</sup> in which rats exposed to the same protocol of myocardial ischaemia–



**Figure 1** Peroxynitrite induces necrosis of H9c2 cardiomyoblasts and the passive release of HMGB1. H9c2 cells were exposed to 100–250 μM peroxynitrite for 40 min and then replaced in the culture medium for a total of 4 h. Cell necrosis was evaluated by propidium iodide staining and FACS analysis (A) and by the release of LDH (B). The release of HMGB1 into the cell medium was determined by enzyme-linked immunosorbent assay (C). The cellular content of HMGB1 was determined by western blotting (D). Tubulin is shown as a loading control. Morphological evaluation of cells was performed by a phase-contrast microscope 2 and 4 h after peroxynitrite (100 μM) treatment (E). Cont: control, unstimulated cells. Graphs show means ± SEM of at least three independent observations. \**P* < 0.05, *t*-test.

reperfusion were treated with Mn(III)-tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP), a stable and cell-permeable superoxide dismutase mimetic and peroxynitrite decomposition catalyst (1 mg/kg intraperitoneally 20 min before and 30 and 60 min after reperfusion).

### 2.6.1 Determination of myocardial infarct size

Area at risk (AAR) and infarct size were determined using the triphenyl-tetrazolium chloride-Evans Blue technique, as described.<sup>14</sup> The AAR and the infarcted area were expressed as percentage values according to conventional methods (AAR/left ventricle and infarcted area/AAR).

### 2.6.2 Protein extraction and immunoblot detection of HMGB1 and nitrotyrosine

Heart tissue was homogenized (50 mg/mL) in lysis buffer (Tris-HCl 10 mM, NP40 0.5%, NaCl 0.15 M, Na<sub>3</sub>VO<sub>4</sub> 1 mM, NaF 10 mM, PMSF 1 mM, EDTA 1 mM, aprotinin 10 µg/mL, leupeptin 10 µg/mL, and pepstatin 1 µg/mL). Western immunoblotting (30 µg myocardial proteins) was performed as described earlier, using anti-HMGB1 (Assay Designs) or mouse monoclonal anti-nitrotyrosine (Cayman Chemical, Ann Arbor, MI, USA) primary antibodies. Densitometric analysis of enhanced chemiluminescence autoradiographs was performed using a Personal Densitometer and TotalLab Software.

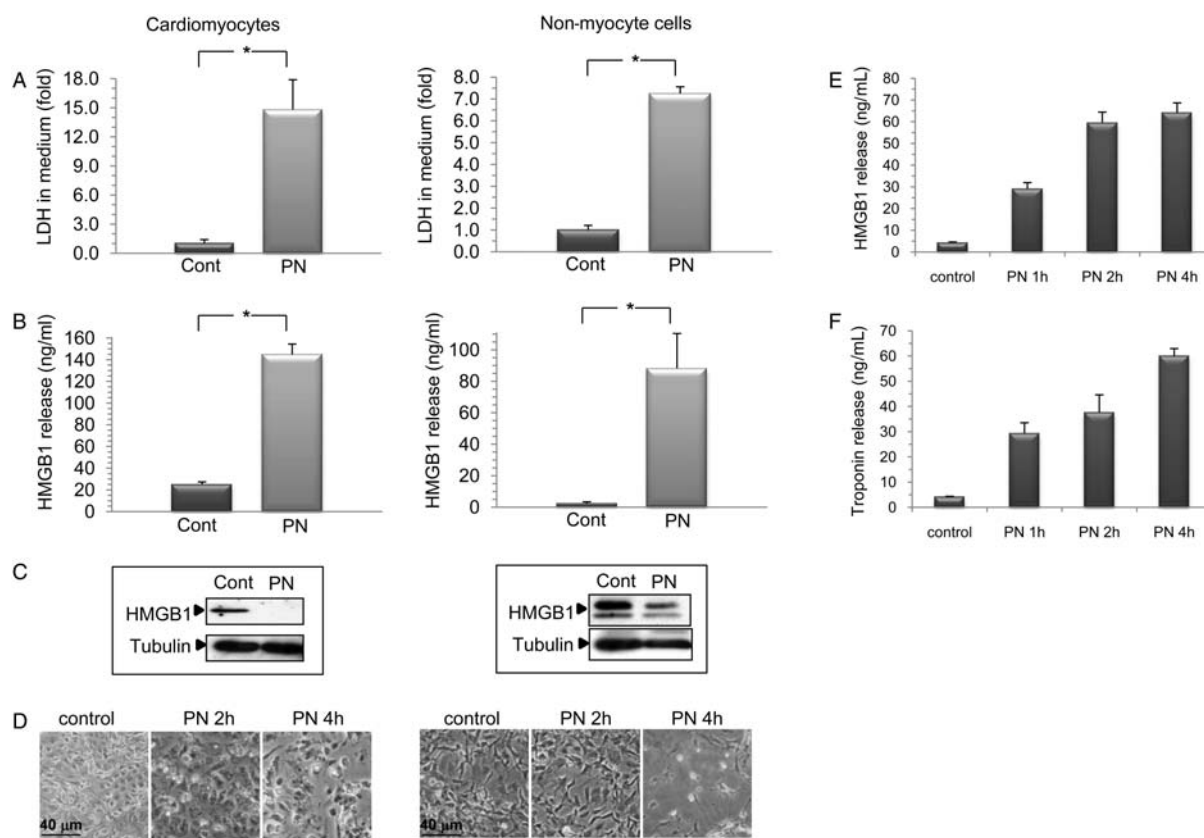
## 2.7 Presentation of data and statistical analysis

All graphs present data as means ± SE. When only two conditions were compared, analysis was performed by Student's *t*-test. In experiments with more than two conditions, statistical analysis was performed using analysis of variance (ANOVA), followed by Tukey's adjustments when appropriate. Statistical significance was ascribed to  $P < 0.05$ .

## 3. Results

### 3.1 Peroxynitrite provokes necrosis of cardiac cells and their passive release of HMGB1 *in vitro*

H9c2 cardiomyoblasts exposed to 100–250 µM peroxynitrite disclosed significant PI staining as shown by the FACS analysis, with the proportion of necrotic cells reaching 48 and 65%, respectively (Figure 1A). In contrast, cells treated with NaOH (PN vehicle) did not show any significant increase in PI staining when compared with untreated control cells. The concentration of 100 µM peroxynitrite was then used for all other *in vitro* experiments. As shown in Figure 1B, such concentrations induced significant release of LDH



**Figure 2** Necrosis and passive release of HMGB1 are induced by peroxynitrite in primary murine cardiomyocytes and non-myocyte cells. Neonatal murine cardiomyocytes (left panel) and non-myocyte cells (middle panel) exposed to 100 µM peroxynitrite for 40 min and then replaced in the culture medium for a total of 4 h disclosed significant necrosis, as shown by LDH release (A), as well as by passive release of HMGB1, as shown by the liberation of HMGB1 within the cell medium [(B) ELISA] and the disappearance of intracellular HMGB1 [(C), western immunoblotting]. Tubulin is shown as a loading control. Morphological evaluation of cells was performed by a phase-contrast microscope 2 and 4 h after peroxynitrite (D). The kinetics of HMGB1 (E) and troponin I (F) release by cardiomyocytes was evaluated from 1 to 4 h after peroxynitrite stimulation. Cont: control, unstimulated cells. Graphs show means ± SEM of at least three independent observations. \* $P < 0.05$ , *t*-test.

within the cell medium, and this was associated with a marked increase in HMGB1 in the cell medium (Figure 1C), occurring in parallel to a profound reduction in the cellular expression of HMGB1 (Figure 1D). Thus, the enhanced HMGB1 signal in the cell medium was due to passive release, but not to increased HMGB1 expression. The expression of  $\alpha$ -tubulin, used as a loading control (Figure 1D), did not change between untreated and peroxynitrite-treated cells, implying that  $\alpha$ -tubulin remained confined within the cellular protein pool and that the release of HMGB1 was not a general process affecting all cellular proteins. Morphological evaluation of H9c2 cells treated with peroxynitrite is shown in Figure 1E. Obvious signs of cytotoxicity were detected after 2 h and further increased after 4 h.

We then evaluated the effects of peroxynitrite on primary cardiac cells obtained from neonatal mice, which contain both a myocyte and a non-myocyte fraction. Both types of cells disclosed significant necrosis upon peroxynitrite treatment (LDH release, Figure 2A), accompanied by the passive release of HMGB1 within the cell medium (HMGB1 in medium, Figure 2B; HMGB1 cellular expression, Figure 2C). Of note, cardiac myocytes appeared more sensitive to the toxic effects of peroxynitrite, as shown by a much greater increase in LDH in the medium. Morphological evaluation showed marked signs of cytotoxicity of peroxynitrite, both after 2 and 4 h, as illustrated in Figure 2D. Finally, the kinetics of HMGB1 release by cardiac myocytes was compared with that of troponin I. As shown in Figure 2E and F, both HMGB1 and troponin I disclosed a progressive and comparable increase in the cell medium after peroxynitrite stimulation, further demonstrating the link between necrosis and the release of HMGB1.

### 3.2 Cardiac cells express the HMGB1 receptors: RAGE and TLR4

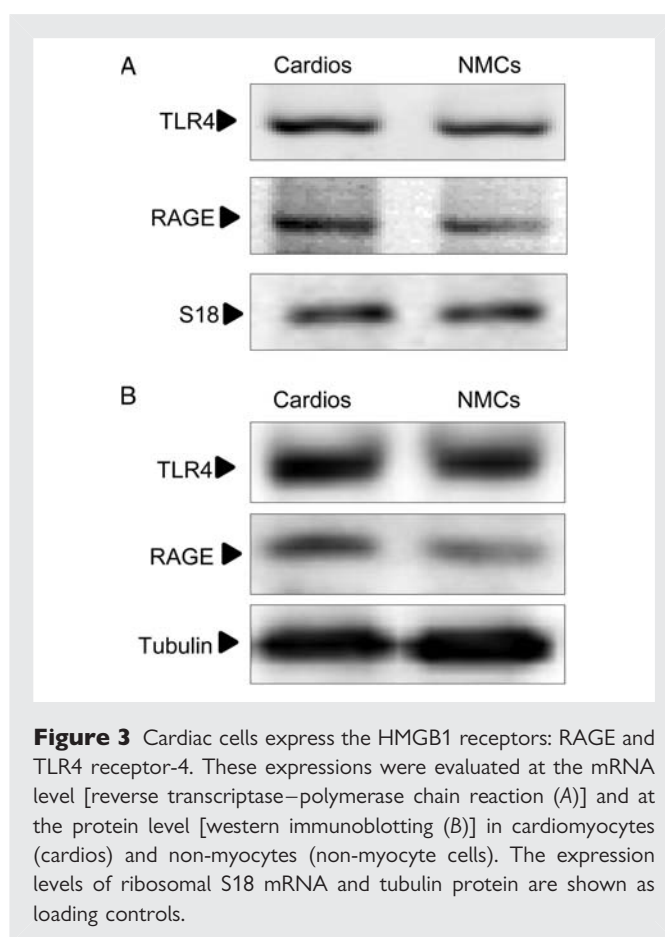
As indicated in Figure 3A (reverse transcriptase–polymerase chain reaction) and B (western immunoblotting), cardiomyocytes and NMCs express RAGE and TLR4, both at the mRNA and protein level, implying that these cells might have the ability to be targeted by HMGB1.

### 3.3 Peroxynitrite-induced apoptosis does not trigger the release of HMGB1 by cardiac cells *in vitro*

NMCs treated with peroxynitrite were stained with PI and Cy<sup>TM</sup>5 Annexin V and were then FACS sorted as indicated in Figure 4A. Viable cells were both Annexin V- and PI-negative, apoptotic cells were Annexin V-positive and PI-negative, whereas necrotic cells were PI-positive. The expression level of HMGB1 was then determined by western immunoblotting in each cell subpopulation. As shown in Figure 4B, HMGB1 expression could not be detected in necrotic cells, indicative of its passive release. In contrast, HMGB1 expression was still observed in apoptotic cells similar to viable cells, implying that cells dying by apoptosis retained HMGB1 intracellularly.

### 3.4 Myocardial necrosis triggered by ischaemia–reperfusion is associated with an increased expression of HMGB1 within the myocardium

Figure 5A illustrates the large area of myocardial necrosis (whitish zone within the left ventricular free wall) induced by 45 min ischaemia,

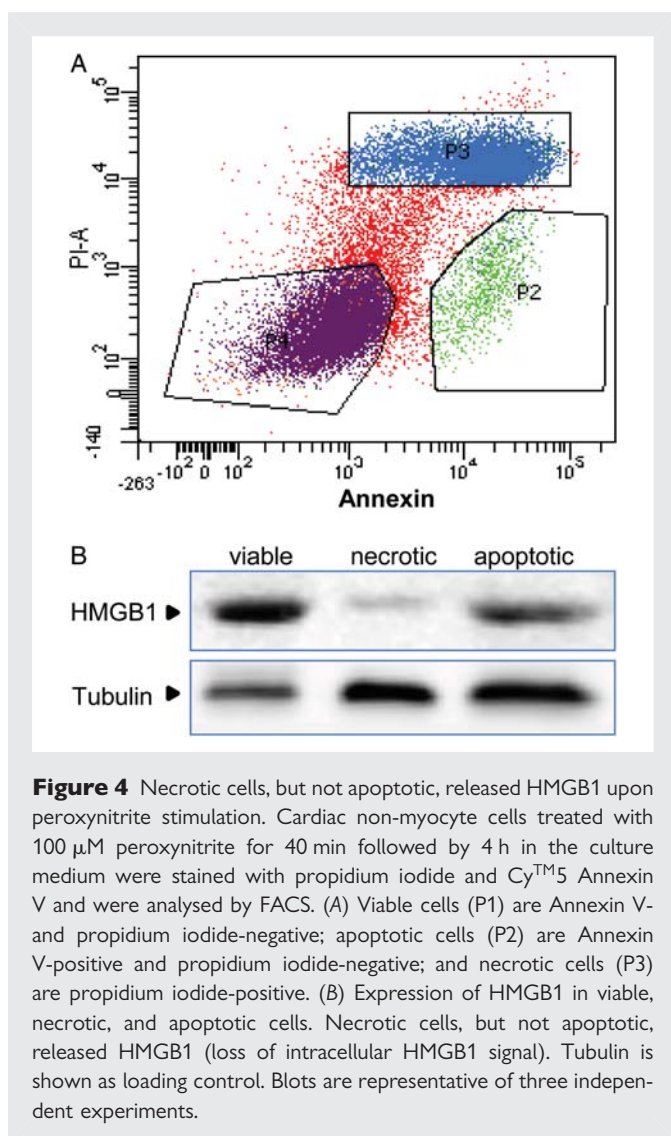


**Figure 3** Cardiac cells express the HMGB1 receptors: RAGE and TLR4 receptor-4. These expressions were evaluated at the mRNA level [reverse transcriptase–polymerase chain reaction (A)] and at the protein level [western immunoblotting (B)] in cardiomyocytes (cardios) and non-myocytes (non-myocyte cells). The expression levels of ribosomal S18 mRNA and tubulin protein are shown as loading controls.

followed by 2 h reperfusion in the rat. The expression of HMGB1 is shown in Figure 5B. In sham rats not exposed to ischaemia, HMGB1 was detected at a low level, but this level increased significantly after 1 h reperfusion, further increased after 2 h reperfusion, and then persisted at an elevated level after 6 h, with no further increase in comparison to 2 h. Compared with baseline conditions, the expression levels of HMGB1 increased by  $2.6 \pm 0.4$ -,  $6.9 \pm 1.7$ -, and  $4.1 \pm 0.5$ -fold after 1, 2, and 6 h reperfusion, respectively.

### 3.5 Peroxynitrite decomposition catalysts reduce infarct size, decrease cardiac nitrotyrosine accumulation, and suppress HMGB1 upregulation in the reperfused myocardium

There was no difference in the ischaemic area (AAR) between control animals and animals treated with FeTPPS (Figure 6A). In contrast, FeTPPS provided significant protection against myocardial injury, as shown by a reduction in infarct size (in per cent of the AAR) from  $46 \pm 1$  to  $25 \pm 6\%$  ( $P < 0.05$ , Figure 6A), which compares well with the protective effect of MnTBAP ( $13 \pm 4\%$ ) reported in our previous works.<sup>14</sup> Myocardial ischaemia–reperfusion also resulted in a significant increase in the myocardial nitrotyrosine content (used as a marker for the generation of peroxynitrite), which was significantly reduced with FeTPPS (Figure 6B), to an extent similar to that reported earlier with MnTBAP.<sup>14</sup> In addition, the increased expression of HMGB1 triggered by 1 and 2 h reperfusion could be markedly



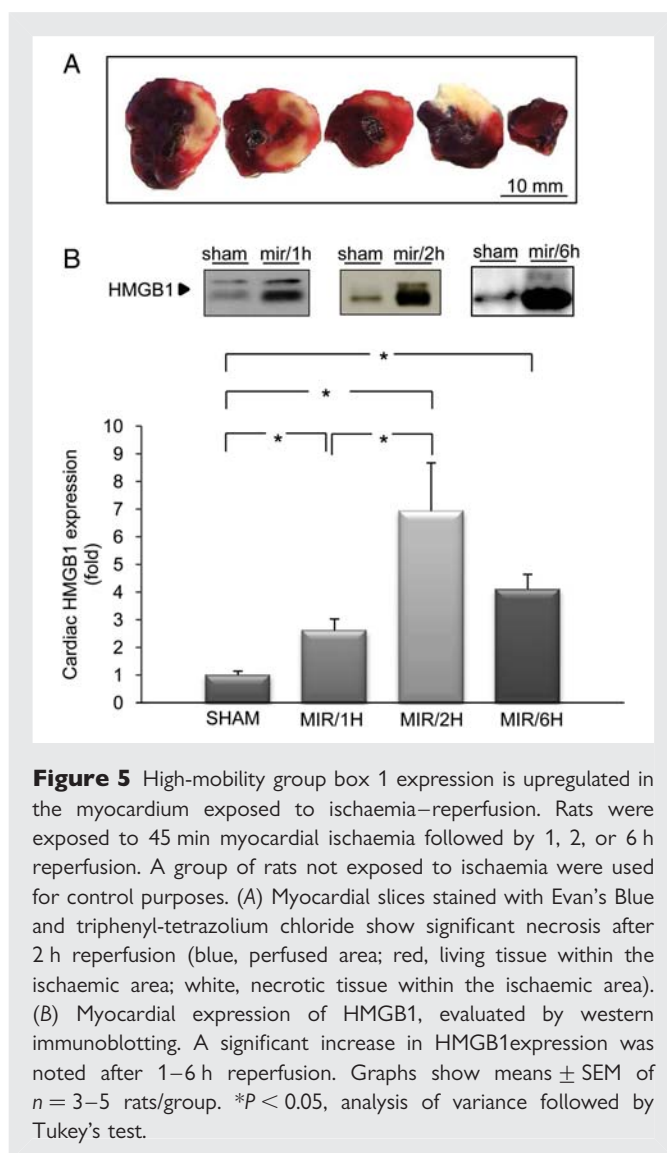
**Figure 4** Necrotic cells, but not apoptotic, released HMGB1 upon peroxynitrite stimulation. Cardiac non-myocyte cells treated with 100  $\mu$ M peroxynitrite for 40 min followed by 4 h in the culture medium were stained with propidium iodide and Cy<sup>TM</sup>5 Annexin V and were analysed by FACS. (A) Viable cells (P1) are Annexin V- and propidium iodide-negative; apoptotic cells (P2) are Annexin V-positive and propidium iodide-negative; and necrotic cells (P3) are propidium iodide-positive. (B) Expression of HMGB1 in viable, necrotic, and apoptotic cells. Necrotic cells, but not apoptotic, released HMGB1 (loss of intracellular HMGB1 signal). Tubulin is shown as loading control. Blots are representative of three independent experiments.

suppressed by FeTPPS (1 h reperfusion, *Figure 6B*) and MnTBAP (2 h reperfusion, *Figure 6C*). The myocardial expression of Akt (*Figure 6B*) and tubulin (*Figure 6C*), used as internal controls, did not show any variations in the different conditions studied.

## 4. Discussion

The major novel finding of this study is that peroxynitrite promotes significant release of HMGB1 by cardiac cells *in vitro* and also represents a key effector of increased HMGB1 expression in the myocardium exposed to ischemia–reperfusion.

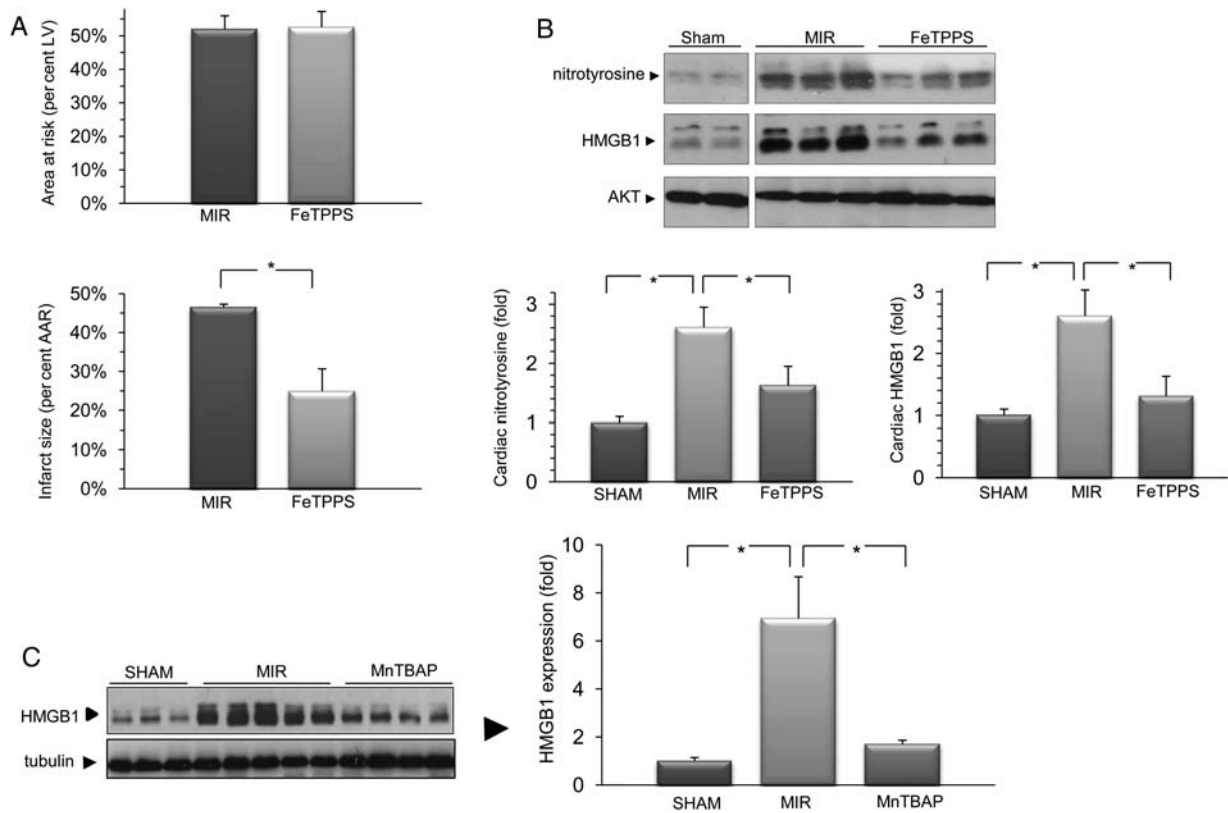
Emerging evidence indicates that molecules released by damaged tissues act as internal danger signals, triggering innate immune defence mechanisms comparable to those triggered by external signals released by invading pathogens.<sup>25</sup> HMGB1, a ubiquitous nuclear protein promoting transcriptional activation, represents a major member of such ‘danger-associated molecular patterns’, which is passively released by cells undergoing necrotic (but not apoptotic) cell death.<sup>2</sup> HMGB1 exerts its pro-inflammatory activity by binding to the RAGE, as well as to TLR4.<sup>26,27</sup>



**Figure 5** High-mobility group box 1 expression is upregulated in the myocardium exposed to ischaemia–reperfusion. Rats were exposed to 45 min myocardial ischaemia followed by 1, 2, or 6 h reperfusion. A group of rats not exposed to ischaemia were used for control purposes. (A) Myocardial slices stained with Evan's Blue and triphenyl-tetrazolium chloride show significant necrosis after 2 h reperfusion (blue, perfused area; red, living tissue within the ischaemic area; white, necrotic tissue within the ischaemic area). (B) Myocardial expression of HMGB1, evaluated by western immunoblotting. A significant increase in HMGB1 expression was noted after 1–6 h reperfusion. Graphs show means  $\pm$  SEM of  $n = 3$ –5 rats/group. \* $P < 0.05$ , analysis of variance followed by Tukey's test.

Recent observations support an important role of HMGB1, RAGE, and TLR4 in promoting innate immune defence mechanisms and inflammatory responses in the heart exposed to ischaemia–reperfusion (reviewed in Volz *et al.*<sup>28</sup>). Indeed, HMGB1 is released by hypoxic cardiomyocytes *in vitro*<sup>5</sup> and is upregulated in the infarcted myocardium *in vivo*,<sup>5,6</sup> and strategies neutralizing HMGB1 or its receptors attenuate myocardial inflammation after myocardial infarction.<sup>5,29,30</sup> Importantly, HMGB1 may not only foster detrimental pro-inflammatory effects in the myocardium, but might also be important for the successful adaptation of the heart to injury, as supported by studies reporting beneficial actions of HMGB1 on the process of adverse cardiac remodelling.<sup>6,7</sup> Besides HMGB1, it is also worth noting that additional candidate molecules might be able to trigger TLR (or RAGE)-dependent innate immune response after myocardial infarction, including S100 proteins,<sup>31</sup> the heat shock protein (HSP) 70,<sup>32</sup> as well as the extracellular matrix protein fibronectin EDA.<sup>33</sup>

One of the earliest pathophysiological mechanisms of myocardial reperfusion injury is the generation of significant amounts of oxygen- and nitrogen-centred free radicals and oxidants, triggered by the re-oxygenation of the previously anoxic myocardium.<sup>34</sup> Accumulating evidence indicates that peroxynitrite, formed from



**Figure 6** Peroxynitrite decomposition catalysts reduce myocardial necrosis and suppress myocardial HMGB1 overexpression induced by ischaemia–reperfusion. Rats challenged with 45 min myocardial ischaemia followed by reperfusion (1–2 h) were left untreated or were treated with FeTPPS (10 mg/kg i.v.), given 10 min before reperfusion, or with MnTBAP (1 mg/kg i.p. 20 min before and 30 and 60 min after reperfusion). Sham rats not exposed to ischaemia–reperfusion were used for control purposes. (A) Reduction of infarct size by FeTPPS (2 h reperfusion). Infarct size is expressed in per cent of the area at risk. Means  $\pm$  SEM of  $n = 4$  rats/group.  $*P < 0.05$ ,  $t$ -test. (B) Reduction of myocardial nitrotyrosine and HMGB1 expression by FeTPPS (1 h reperfusion). Means  $\pm$  SEM of  $n = 3–4$  rats/group.  $*P < 0.05$ , analysis of variance followed by Tukey's test. (C) Suppression of myocardial overexpression of HMGB1 by MnTBAP (2 h reperfusion). Means  $\pm$  SEM of  $n = 3–5$  rats/group.  $*P < 0.05$ , analysis of variance followed by Tukey's test.

the diffusion-controlled reaction between NO and the superoxide radical  $O_2^{\cdot-}$ ,<sup>10,35</sup> represents a major oxidant and nitrating species triggering significant myocardial damage in this setting.<sup>11,12</sup> Accordingly, therapeutic strategies reducing peroxynitrite have been associated with significant cardioprotection during myocardial ischaemia and reperfusion.<sup>14,36,37</sup>

In the present study, we report a previously unrecognized link between peroxynitrite and HMGB1, which may be important to understand the connections between oxidative/nitrosative stress and inflammation in the reperfused myocardium. *In vitro*, peroxynitrite induced significant necrosis of H9c2 cells, which then released large amounts of HMGB1, as indicated by the reduction in cellular HMGB1 levels concomitant to a large increase in extracellular HMGB1. More importantly, primary mouse cardiomyocytes, as well as the non-myocyte fraction of cardiac cells (which includes fibroblasts, endothelial and smooth muscle vascular cells, as well as cardiac progenitor cells) exhibited a response similar to peroxynitrite, that is, cell necrosis and the complete release of HMGB1 in the extracellular milieu. Interestingly, the release of HMGB1 and LDH by cardiomyocytes was approximately twice higher than that of non-myocytes, suggesting that cardiac myocytes may be more sensitive to the cytotoxic actions of peroxynitrite. This may be consistent

with the reported higher sensitivity of cardiomyocytes to anoxo/ischaemic injury in comparison to the much greater resistance of non-myocytes, especially fibroblasts.<sup>38</sup>

Peroxynitrite also induced apoptotic cell death, as evaluated in the non-myocyte fraction, in agreement with our previous report on peroxynitrite-induced cytotoxicity in cardiac cells.<sup>14</sup> However, at variance with necrotic cells, apoptotic cells retained HMGB1 intracellularly, implying that the passive release of HMGB1 was restricted to cells dying by necrosis, but not by apoptosis, which perfectly concurs with previous findings obtained in unrelated types of cells (reviewed in Raucci et al.<sup>39</sup>). These findings clearly indicate that an important consequence of peroxynitrite exposure in all subtypes of cardiac cells is the massive liberation of HMGB1, in line with the concept of passive HMGB1 release by cells dying by necrosis.<sup>2,4,5</sup> Finally, we also found that both cardiac myocytes and non-myocytes express RAGE and TLR4, the two major cell surface receptors interacting with extracellular HMGB1.<sup>26,27</sup> This supports the contention that HMGB1 released by necrotic cardiac cells might induce HMGB1-dependent signalling in neighbour viable cells, a hypothesis that should be investigated in future studies.

We then determined whether peroxynitrite might also influence HMGB1 *in vivo*, using a rat model of myocardial ischaemia–

reperfusion injury. In such conditions, we first noticed that the expression level of HMGB1 within the myocardium was significantly increased after reperfusion. HMGB1 expression increased already after 1 h, was maximal after 2 h reperfusion, and then persisted at comparable levels after prolonged reperfusion (6 h). These findings are in line with a previous observation in mice, showing that both HMGB1 mRNA and protein levels were upregulated as early as 30 min after myocardial reperfusion.<sup>5</sup> Also, it is worth mentioning that such an increased expression of HMGB1 has been reported in the liver in a murine model of hepatic ischaemia/reperfusion.<sup>4</sup> These data suggest that both passive release and upregulated expression of HMGB1 concur to promote post-ischaemic inflammation in reperfused organs. Since we did not determine which cell population contributed to the increased HMGB1 level in the heart, we cannot rule out that overexpression of HMGB1 was due, at least in part, to inflammatory cells. Indeed, in rats exposed to permanent coronary occlusion, HMGB1 expression was enhanced not only in cardiomyocytes but also in infiltrating inflammatory cells.<sup>6</sup> However, cardiac myocytes might represent a major source of HMGB1, as suggested by a recent study in mice showing marked overexpression of HMGB1 in these cells upon treatment with endotoxin, implying that cardiomyocytes can be converted to an immune/inflammatory phenotype under stressful conditions *in vivo*.<sup>40</sup>

Our second key observation *in vivo* was that FeTPPS suppressed myocardial upregulation of HMGB1 observed after 1 h reperfusion, an effect correlated with the reduced accumulation of nitrotyrosine, a marker of nitrative stress, and more specifically peroxynitrite generation,<sup>10,41</sup> and a similar reduction of HMGB1 expression was obtained with MnTBAP after 2 h reperfusion. In addition, FeTPPS significantly reduced myocardial infarct size to an extent comparable to our previous findings with MnTBAP<sup>14</sup> and to those of other investigators using two unrelated peroxynitrite decomposition catalysts, FP15<sup>42</sup> and INO4885.<sup>37</sup> Coupled to our *in vitro* findings, these data imply a causative role of peroxynitrite not only as an effector of myocardial cell death, but also as a proximal mediator triggering cardiac HMGB1 expression and release during myocardial infarction.

Although the pharmacological reduction of peroxynitrite has been associated with reduced inflammation in reperfused organs,<sup>14,43,44</sup> the mechanisms underlying such link between peroxynitrite and post-ischaemic inflammation remain incompletely understood. It has been proposed that peroxynitrite (and, more generally, reactive oxygen and nitrogen species) might directly activate the pro-inflammatory transcription NF- $\kappa$ B,<sup>45,46</sup> but this issue remains highly controversial.<sup>47,48</sup> Indeed, recent findings have indicated that, under some conditions, oxidants (including peroxynitrite) do not activate, but instead inhibit NF- $\kappa$ B.<sup>18,49</sup> On the basis of our current results, we may therefore propose an alternative mechanism connecting peroxynitrite and myocardial inflammation, based on HMGB1 release and upregulation during myocardial reperfusion.

In conclusion, this study provides the first experimental demonstration implicating peroxynitrite as a key mediator of HMGB1 release by cardiac cells (myocytic and non-myocytic) *in vitro* and of myocardial HMGB1 upregulation in ischaemia/reperfusion. In view of the emerging pro-inflammatory role of HMGB1 in such conditions, our findings provide a novel insight into the mechanisms linking oxidative/nitrosative stress and post-ischaemic inflammation in the myocardium.

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