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Cardiac remodelling hinders activation of cyclooxygenase-2, diminishing protection by delayed pharmacological preconditioning: role of HIF1α and CREB

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Aims We tested whether delayed pharmacologic preconditioning elicited by isoflurane is protective in infarct-remodelled hearts.

Methods and results Male Wistar rats were treated with the preconditioning drug isoflurane 6 weeks after permanent ligation of the left anterior descending coronary artery. Twenty-four and 48 h later, hearts were perfused on the Langendorff system and treated with cyclooxygenase-2 or 12-lipoxygenase inhibitors before exposure to 40 min of ischaemia followed by 90 min of reperfusion. Infarct size was determined by triphenyltetrazolium chloride staining and lactate dehydrogenase release. Cyclooxygenase-2 expression and activity were measured by Western blotting and colorimetric assay. Nuclear translocation of cyclooxygenase-2-inducing transcription factors HIF1α, CREB, STAT3, and NFκB was determined. Post-infarct, remodelled hearts exhibit alterations in cellular signalling, time course and extent of isoflurane-induced late protection. While remodelled, preconditioned hearts exhibited protection exclusively at 24 h, healthy hearts showed sustained protection for up to 48 h, which correlated with cyclooxygenase-2 protein expression and enzymatic activity. The cyclooxygenase-2 inhibitors celecoxib and NS-398, but not the 12-lipoxygenase inhibitor cinnamyl-3,4-dihydroxycyanocinnamate, abolished delayed protection in both healthy and remodelled hearts, identifying cyclooxygenase-2 as a key mediator of late protection in both models. Isoflurane induced nuclear translocation of HIF1α in all hearts, but CREB was exclusively activated in healthy but not remodelled myocardium, which expressed higher levels of the CREB antagonist ICER. Delayed protection by isoflurane in remodelled hearts was more vulnerable to inhibition by celecoxib.

Conclusion Isoflurane failed to mobilize cyclooxygenase-2-inducing CREB in ICER-overexpressing, remodelled hearts, which was associated with a shortening of the second window of protection.

1. Introduction

In 1993, Marber *et al.*[1] and Kuzuya *et al.*[2] described a remarkable phenomenon called delayed ischaemic preconditioning of the heart, which reflects a late 'window of protection' against ischaemia, usually occurring 12–72 h after brief repetitive ischaemic episodes. The resulting protective phenotype of the heart is due to a profound transcriptional reprogramming with *de novo* protein synthesis, and is of great clinical relevance, as it is 30-times longer than the early window, which only lasts 2–3 h.[3] Today, we know that early and delayed preconditioning of the heart can be elicited more safely by pharmacologic means such as halogenated ethers or opioids than by ischaemic episodes.[4,5] This is of particular relevance for the already jeopardized diseased myocardium. Isoflurane-induced delayed protection was first reported in 2003 in a rabbit model by Toncovic-Capin *et al.*[6] In healthy rabbit myocardium, celecoxib, a specific

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cyclooxygenase-2 (COX-2) inhibitor, abolished delayed isoflurane preconditioning,[7] while late isoflurane protection was dependent on 12-lipoxygenase activity in healthy murine hearts.[8] Recently, molecular evidence of delayed anaesthetic preconditioning after sevoflurane inhalation has been also reported in humans.[9]

Most experimental studies evaluated the phenomenon of early and delayed preconditioning in healthy juvenile hearts, but this is far from clinical reality, as diseased myocardium would benefit most from this protection. Some studies reported reduced protection in the early phase of preconditioning in diseased hearts.[10,11] Since the early phase of preconditioning is based on similar signalling pathways as the late phase, it could be speculated that protection during the late phase would be impaired in remodelled hearts. Post-infarct remodelled myocardium exhibits marked structural changes,[12] alterations in energy metabolism[13] and cellular signalling,[14] which put the heart at particular risk for further ischaemic damage. In contrast to this notion are our own recent findings that post-infarct remodelled hearts remain receptive to protection by early isoflurane preconditioning via the same signalling pathways, as observed in healthy hearts.[15] Nonetheless, no data exist as to whether delayed anaesthetic protection retains its effectiveness in post-infarct myocardium. Using an established rat model of ventricular remodelling after permanent coronary artery ligation, we investigated whether ventricular remodelling would affect late isoflurane protection. Specifically, we hypothesized that remodelling would abolish late isoflurane protection and increase the vulnerability of the heart to ischaemia-reperfusion injury. This study also investigated possible mechanisms underlying the postulated refractoriness to late anaesthetic protection in remodelled hearts.

The current study shows that the isoflurane-induced second window of protection shrinks to a short-lived phase in remodelled myocardium, which would require repetition of the preconditioning stimulus every 24 h to maintain the heart in the protected state.

2. Methods

This animal study was performed according to the guidelines of the Animal Care and Use Committee of the University of Zurich, Switzerland. All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85-23, revised 1996).

2.1 Post-infarct remodelled hearts

Myocardial infarction ($\sim$35% of left ventricular mass) and subsequent hypertrophic remodelling was induced in male adult (180–200 g, 8–9 weeks old) Wistar rats by permanent ligation of the left anterior descending coronary artery (LAD) under anaesthesia, as previously described in detail.[16] Some animals served as control and underwent the same procedure except that the suture was passed under the coronary artery without ligation. Rats were sacrificed 6 weeks after surgery, and the body weight and heart weight were measured. Hearts were further evaluated for their function on a Langendorff apparatus (see Supplementary material online, *Table S1*).

2.2 Protocol for delayed isoflurane preconditioning

Healthy age-matched rats and infarct rats 6 weeks after coronary artery ligation were placed on a thermoregulating pad and exposed to 2.1 vol% [1.5 minimum alveolar concentration (MAC) in rats] isoflurane (Abbott, Bar, Switzerland) in oxygen for 90 min by inhalation (*Figure 1*). Rats exposed to 100% oxygen for 90 min served as sham control. The concentration of applied isoflurane was continuously monitored using a gas monitor. In separate experiments, a catheter was inserted in the femoral artery and arterial blood was withdrawn for blood gas analysis (see Supplementary material online, *Table S2*). The animals subsequently recovered in room air (21 vol% oxygen). Twenty-four and 48 h later, delayed protection against ischaemia-reperfusion injury was assessed on a Langendorff apparatus (*Figure 1*).

2.3 Isolated perfused rat heart experiments

Rats were heparinized (500 units i.p.) and 15 min later decapitated without prior anaesthesia. Hearts were mounted on a non-circulating Langendorff apparatus and perfused with Krebs–Henseleit buffer gassed with 95% O_2 and 5% CO_2 at pH 7.4 and 37°C, as previously described.[17] Five hearts were assigned to each experimental group (*Figure 1*). The selective COX-2 inhibitors celecoxib (CEL, Pfizer AG, Zurich, Switzerland, $IC_{50} = 0.04$ μM, low dose used 0.1 μM, high dose used 1 μM), N-2-cyclohexyloxy-4-nitrophenyl-methanesulphonamide (NS-398, Cayman Chemical, Ann Arbor, MI, $IC_{50} = 1.77$ μM, used concentration 5 μM) and the selective 12-lipoxygenase inhibitor cinnamyl-3,4-dihydroxycyanocinnamate (CDC, USBiological, Swampscott, MA, $IC_{50} = 0.063$ μM, used concentration 0.5 μM) were dissolved in 0.1% dimethyl sulphoxide (DMSO) vehicle and used to perfuse the hearts for 10 min before induction of global ischaemia (*Figure 1*). Infarct size was determined by 1% 2,3,5-triphenyltetrazolium chloride staining after 90 min of reperfusion, as previously described.[16,18] In addition, myocardial damage was estimated by measuring the release of lactate dehydrogenase (LDH) from necrotic tissue, as previously described.[16] Briefly, the perfusate was collected and LDH activity was determined by the Roche/Hitachi 917 kit (sensitivity 6 U/L, intra- and interassay coefficients of variance $<$1%).

2.4 Western blot analysis

Separate experiments served to determine expression of 12-lipoxygenase, 5-lipoxygenase, and COX-2 in healthy and remodelled hearts 24 and 48 h after isoflurane exposure by Western blot analysis. The antibodies were from the following sources: COX-2, Cell Signaling Technology, Beverly, CA, USA; 12-lipoxygenase, Cayman Chemical; 5-lipoxygenase, BD Biosciences, San Diego, CA, USA; α-tubulin, Sigma, St Louis, MO, USA.

2.5 COX-2 activity assay

Left ventricular tissue was homogenized in cold homogenization buffer (0.1 M Tris-HCl, pH 7.8 containing 1 mM EDTA). After centrifugation at 10 000 g for 20 min, the supernatant was collected for enzyme assay. COX-2 activity was measured in the presence of a potent and selective cyclooxygenase-1 inhibitor SC-560 (Cayman Chemical, IC_{50} for cyclooxygenase-1 = 9 nM and for cyclooxygenase-2 =

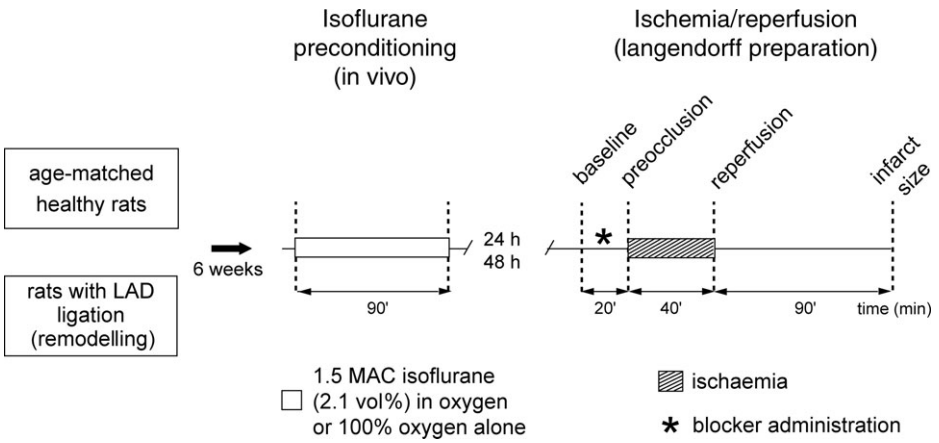

Figure 1 Treatment protocols. Six weeks after ligation of the left anterior descending coronary artery (LAD), rats were exposed *in vivo* to 2.1 vol% (1.5 MAC) isoflurane in oxygen or oxygen alone for 90 min. Age-matched healthy rats served as control group. Twenty-four and 48 h after isoflurane preconditioning, Langendorff perfused healthy and remodelled hearts were exposed to 40 min of ischaemia and 90 min of reperfusion. Blockers were administered 10 min before index ischaemia. MAC: minimum alveolar concentration.

6.3 μM) using a chromogenic cyclooxygenase activity assay kit according to the manufacturer's instruction (Cayman Chemical). This assay measures the colour reaction of cyclooxygenase-generated hydroperoxides.[19]

2.6 Electrophoretic mobility shift assays

Nuclear proteins were extracted as previously described in detail.[20] Electrophoretic mobility shift assays (EMSAs) were performed with Odyssey® IRDye® 700 infrared dye labelled double-stranded oligonucleotides coupled with the EMSA buffer kit (LI-COR Bioscience, Bad Homburg, Germany) according to the manufacturer's instructions. Briefly, 10–20 μg of nuclear extract was incubated with 1–1.5 μL of IRDye® 700 infrared dye labelled oligonucleotides, 2 μL of 10× binding buffer, 2.5 mM DTT, 0.25% Tween, and 1 μg of poly (dI-dC) in a total volume of 20 μL for 30 min at room temperature. For CREB binding assay a final concentration of 0.05% NP-40 was added, and for STAT3 binding assay a final concentration of 0.05% NP-40 and 5 mM MgCl were added. Samples were separated on a 4% polyacrylamide gel in 0.5× tris-borate-EDTA running buffer for 2 h at 200 V. The gel was then scanned by direct infrared fluorescence detection on the Odyssey® Imaging System (LI-COR Bioscience). Oligonucleotide sequences of the double-stranded DNA probes used were as follows: HIF-1α, 5′-AGC TTG CCC TAC GTG CTG TCT CAG A-3′; CREB, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′; NFκB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; STAT3, 5′-GAT CCT TCT GGG AAT TCC TAG ATC-3′. The specificity of the DNA-protein binding signal was confirmed by competition with 200-fold molar excess of the unlabelled consensus competitor oligonucleotide.

2.7 Real-time quantitative polymerase chain reaction

Details are provided in the Supplementary Materials and Methods.

2.8 Statistical analysis

Data are presented as mean ± SD. For cardiac functional data, repeated-measures analysis of variance was used to evaluate differences over time between groups. Unpaired *t*-test was used to compare groups at identical time points, and paired *t*-test to compare within groups over time. *P*-values were multiplied by the number of comparisons (Bonferroni correction). Post-hoc Tukey test was applied for multiple comparisons of the one-way analysis of variance for all other data. $P < 0.05$ was considered as significant. SigmaStat (version 2.0; SPSS Science, Chicago, IL, USA) was used for the analyses.

3. Results

3.1 Post-infarct remodelling narrows the second window of protection after isoflurane preconditioning

From the 106 rats used for coronary artery ligation, 10 animals were lost intraoperatively due to intractable ventricular fibrillation. Heart weight over body weight ratios determined 6 weeks after surgery were markedly higher in post-infarct hearts compared with age-matched healthy or sham-operated hearts (remodelled: 5.52 ± 0.70 g/kg, healthy: 3.58 ± 0.57 g/kg, sham-operated: 3.63 ± 0.55 g/kg, $P < 0.05$; see Supplementary material online, *Table S1*), indicating significant ventricular remodelling. In addition, left ventricular developed pressure, coronary flow, and heart rate were determined *ex vivo* on the Langendorff apparatus. Left ventricular developed pressure was lower in remodelled hearts compared with age-matched healthy hearts. No changes were observed between groups for coronary flow and heart rate (see Supplementary material online, *Table S1*).

Preconditioning elicited by isoflurane inhalation at 1.5 MAC for 90 min significantly improved functional recovery (see Supplementary material online, *Table S3 and S4*) and decreased infarct size after ischaemia-reperfusion in both healthy and remodelled hearts (*Figures 2A* and *3A*). However, protection in infarct hearts was exclusively present at 24 h after preconditioning, while in healthy hearts the protection extended from 24 to 48 h after isoflurane exposure. Infarct-size reduction was corroborated with measurements of LDH release into the perfusate during reperfusion in healthy and remodelled hearts (*Figures 2B*

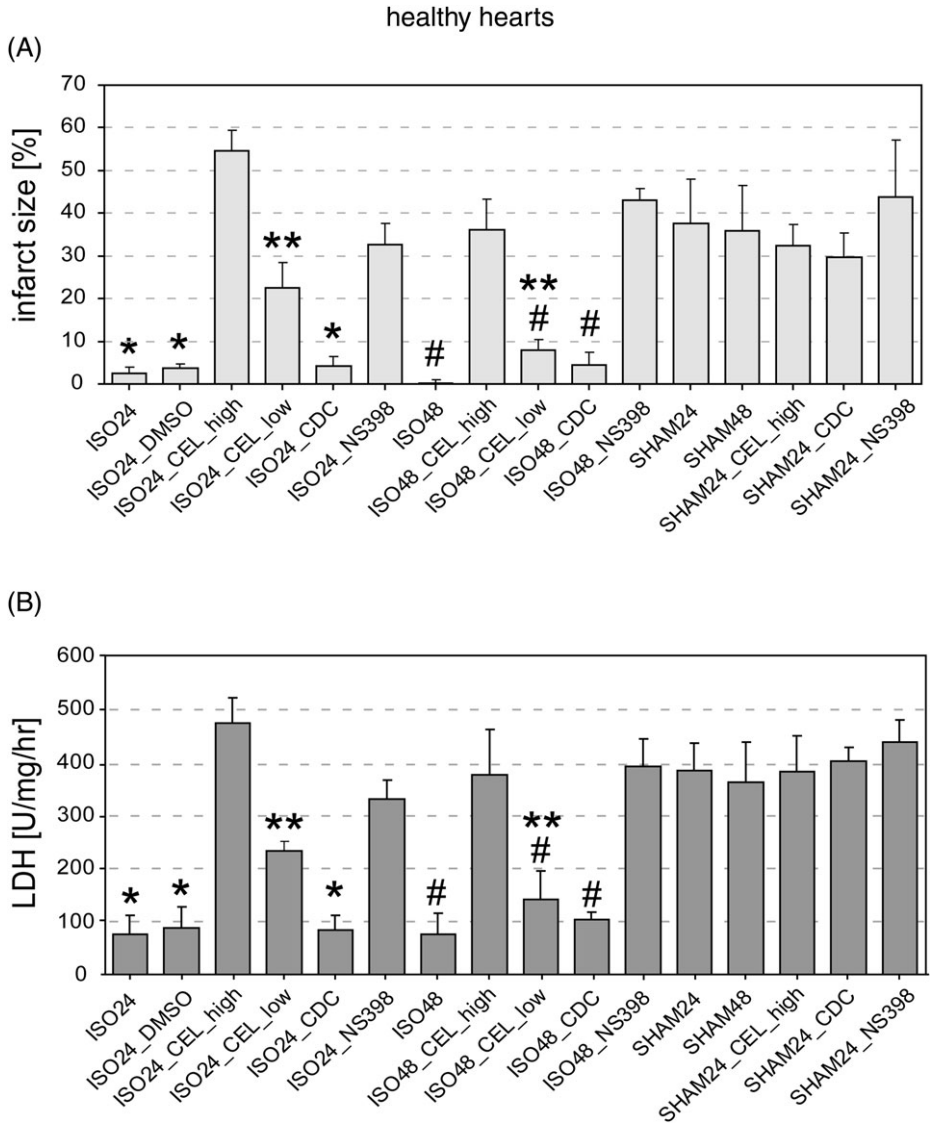

Figure 2 Infarct size (*A*) and lactate dehydrogenase (LDH) release (*B*) in healthy hearts. ISO24/48: hearts exposed to ischaemia-reperfusion 24 or 48 h after isoflurane preconditioning. ISO24/48_CEL_high: preconditioned hearts treated with 1 μM celecoxib. ISO24/48_CEL_low: preconditioned hearts treated with 0.1 μM celecoxib. ISO24/48_CDC: preconditioned hearts treated with 0.5 μM cinnamyl-3,4-dihydroxy-cyanocinnamate (CDC). ISO24/48_NS398: preconditioned hearts treated with 5 μM N-2-cyclohexyloxy-4-nitrophenyl-methane-sulphonamide (NS-398). Final concentration of dimethyl sulphoxide (DMSO) was <0.1%. The prefix SHAM in group names indicates respective groups without isoflurane preconditioning (oxygen alone). $^{*}P < 0.05$ vs. SHAM24 groups. $^{\#}P < 0.05$ vs. SHAM48 groups. $^{**}P < 0.05$ vs. high celecoxib concentration. Data are mean ± SD ($n = 5$ per group).

and *3B*). Sham groups (oxygen inhalation alone) did not exhibit protection 24 or 48 h later in either model. The recovery of developed pressure after delayed preconditioning was lower by ~20% in remodelled hearts when compared with healthy hearts, and end-diastolic pressure reached baseline values after 90 min of reperfusion in healthy but not remodelled hearts (see Supplementary material online, *Tables S3 and S4*). Groups with celecoxib administration before ischaemia-reperfusion exhibited increased coronary flow consistent with previous reports.[21] Of note, during triggering of isoflurane preconditioning, arterial blood gas measurements did not show significant differences compared with animals treated with oxygen alone (see Supplementary material online, *Table S2*). These results provide evidence that post-infarct remodelling narrows the second window of protection after isoflurane preconditioning.

3.2 Late protection by isoflurane preconditioning is more vulnerable to cyclooxygenase-2 inhibition in remodelled myocardium

COX-2 and 12-lipoxygenase were shown to be important in mediating delayed cardioprotection by isoflurane.[7,8] Here we show that delayed protection by isoflurane is abolished or attenuated by inhibitors of COX-2, but not of 12-lipoxygenase (*Figures 2* and *3*). While delayed protection was more resistant to celecoxib in healthy hearts, remodelled hearts completely lost protection in the presence of even lower celecoxib concentrations (see Supplementary material online, *Tables S3 and S4*). Dimethyl sulphoxide alone or blockers alone had no effect on infarct size. Taken together, the data provide evidence that delayed protection by isoflurane is mediated by COX-2 in both healthy and remodelled hearts and further underscore the

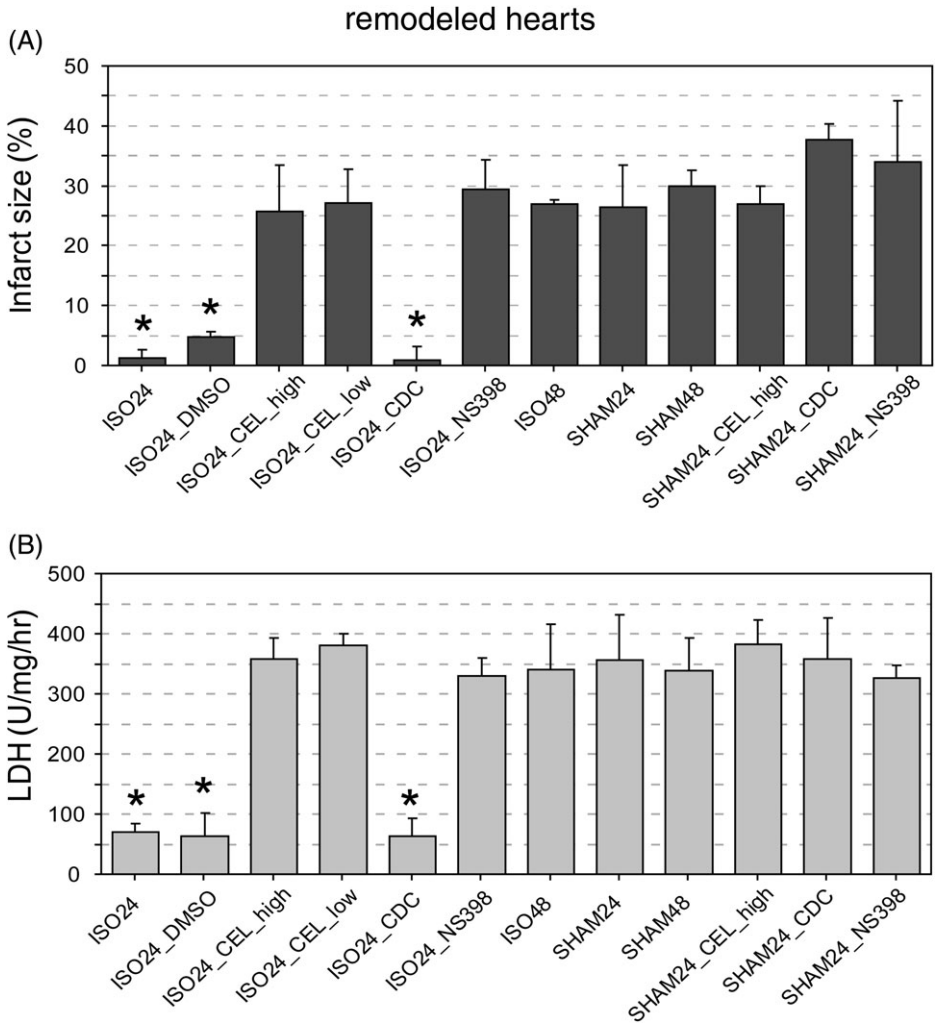

Figure 3 Infarct size (*A*) and lactate dehydrogenase (LDH) release (*B*) in remodelled hearts. ISO24/48: hearts exposed to ischaemia-reperfusion 24 or 48 h after isoflurane preconditioning. ISO24/48_CEL_high: preconditioned hearts treated with 1 μM celecoxib. ISO24/48_CEL_low: preconditioned hearts treated with 0.1 μM celecoxib. ISO24/48_CDC: preconditioned hearts treated with 0.5 μM cinnamyl-3,4-dihydroxy-cyanocinnamate (CDC). ISO24/48_NS398: preconditioned hearts treated with 5 μM N-2-cyclohexyloxy-4-nitrophenyl-methane-sulphonamide (NS-398). Final concentration of dimethyl sulphoxide (DMSO) was $<0.1\%$. The prefix SHAM in group names indicates corresponding groups without isoflurane preconditioning (oxygen alone). $^{*}P < 0.05$ vs. SHAM24 group. Data are mean $\pm$ SD ($n = 5$ per group).

remarkable vulnerability of the diseased myocardium to COX-2 inhibition.

3.3 Isoflurane-induced cyclooxygenase-2 expression and activity show alterations in remodelled hearts

As expected, COX-2 was more abundant in infarct hearts (see Supplementary material online, *Figure S1*).[22] To characterize the expression and activity of COX-2 in delayed protection by isoflurane preconditioning, Western blot analyses (*Figure 4*) and activity measurements (*Figure 5*) were performed in isoflurane- and sham-treated rat hearts 24 and 48 h after triggering with isoflurane. In healthy hearts, COX-2 expression was increased 24 (1.5-fold) and 48 h (2.7-fold) after isoflurane exposure, while in remodelled hearts, COX-2 expression was exclusively increased at 24 h (three-fold) (*Figure 4*). Oxygen inhalation in sham groups did not affect COX-2 expression or activity. COX-2 activity closely paralleled COX-2 expression (*Figure 5*). No changes in 12-lipoxygenase and 5-lipoxygenase expression were observed (see Supplementary material online, *Figure S2*). Together, isoflurane-induced COX-2 showed a shortened activation profile in remodelled hearts, which closely paralleled structural and functional protection.

3.4 Isoflurane induces nuclear translocation of HIF1α but not CREB in post-infarct remodelled hearts

To determine the contribution of putative transcription factors in the observed isoflurane-induced COX-2 expression in healthy and remodelled hearts, nuclear translocation of NFκB, STAT3, HIF1α, and CREB was measured 30 min after termination of isoflurane inhalation (2.1 vol% for 90 min) in additional experiments. Isoflurane induced nuclear translocation of HIF1α in healthy and remodelled hearts (*Figure 6*). In contrast, CREB was exclusively translocated to nuclei in healthy hearts. None of the other transcription factors was activated by isoflurane compared with sham

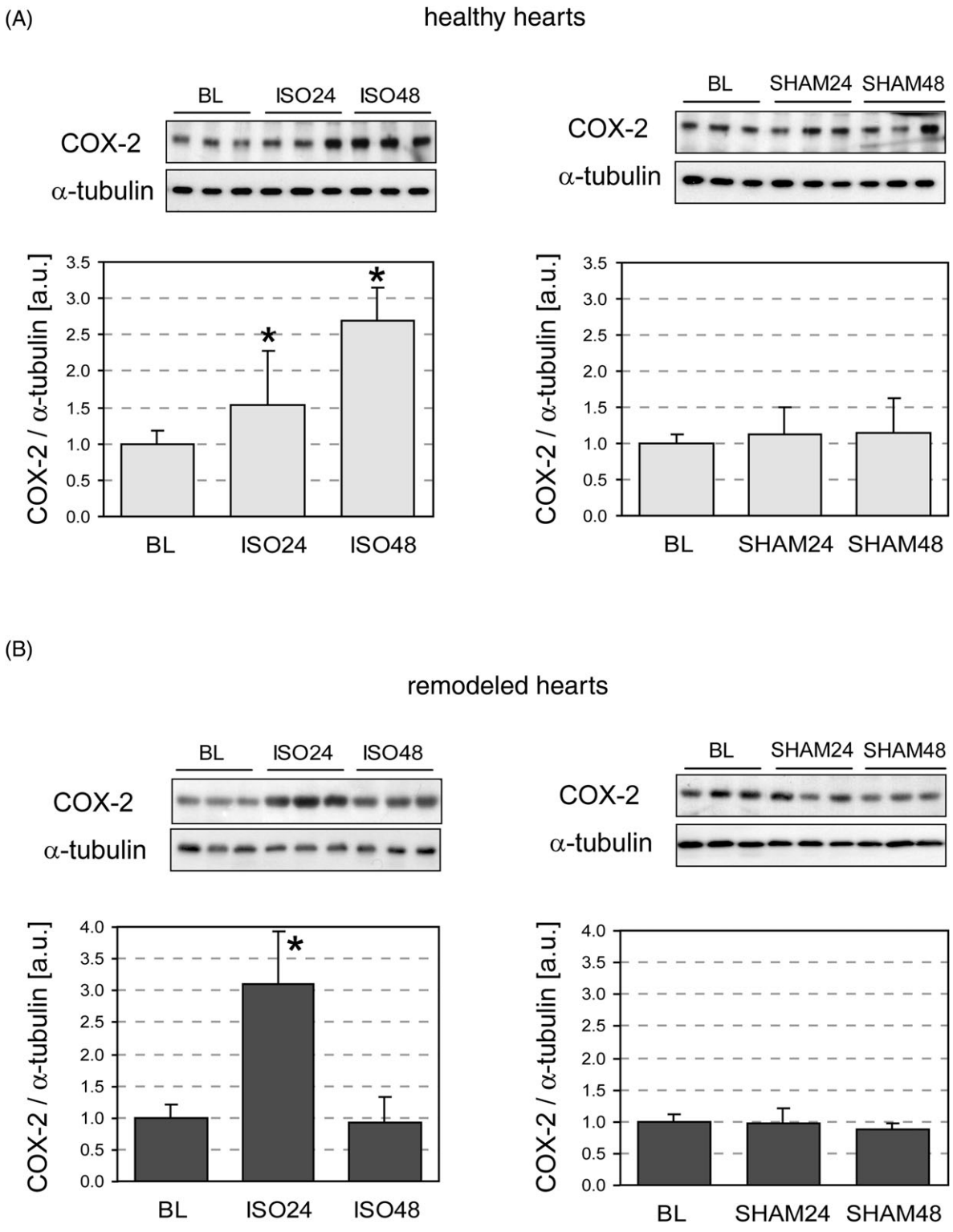

Figure 4 Representative Western blots of cyclooxygenase-2 (COX-2) expression (72 kDa) in healthy (*A*) and remodelled (*B*) hearts 24 and 48 h after isoflurane exposure expressed as average density ratio of COX-2 to α-tubulin (57 kDa) normalized to baseline (BL). ISO24/48: hearts exposed to isoflurane and harvested 24 or 48 h later. SHAM24/48: time-matched hearts exposed to oxygen alone. Data are mean ± SD ($n = 4$ per group). *$P < 0.05$ vs. BL.

treatment (data not shown). In search of possible mechanisms for the lack of CREB activation by isoflurane in remodelled myocardium, we determined the mRNA level of the CREB-antagonist inducible cAMP early repressor (ICER), which was increased by 63% in remodelled myocardium: 0.026 ± ;0.005 vs. 0.016 ± ;0.004 (arbitrary units relative to α-tubulin, $P = 0.009$). These data suggest that the failure of isoflurane to activate CREB in remodelled myocardium may be due to overexpression of the CREB-antagonist ICER in infarct hearts and thus may contribute to the observed narrowing of the second window of protection.

4. Discussion

The salient findings of this study can be summarized as follows. First, isoflurane induced a robust but narrow second window of protection in hearts with marked post-infarct ventricular remodelling. In these diseased hearts, reduction in infarct size and improved functional recovery were only present 24 h after isoflurane application, while in healthy hearts protection extended for up to 48 h. Secondly, delayed protection by isoflurane in rat hearts was mediated by COX-2 but not 12-lipoxygenase in both remodelled and healthy myocardium, and closely correlated with increased COX-2 expression and activity levels. Hence, isoflurane elicits late protection in remodelled rat myocardium via similar signalling pathways as previously reported in healthy rabbit hearts.[7] While COX-2-inducing HIF-1α was activated by isoflurane in healthy and remodelled hearts, isoflurane failed to mobilize COX-2-inducing CREB in post-infarct myocardium. Overexpression of the CREB-antagonist ICER offers an explanation for the observed narrowing of the second window of protection, a hypothesis that needs validation. Thirdly, despite increased expression of COX-2, remodelled preconditioned hearts exhibited a remarkable vulnerability to inhibition by the clinically used anti-inflammatory drug celecoxib, which completely abolished delayed isoflurane protection in these hearts, but only partly diminished the protection in healthy hearts. Together, we present for the first time evidence that delayed protection by isoflurane preconditioning varies with the disease state of the heart and concomitant medication (*Figure 7*). Since recruitment of COX-2 is a critical innate mechanism, whereby the heart protects itself from ischaemia,[3] our findings may be of relevance for the medical management of patients at risk of cardiovascular complications.

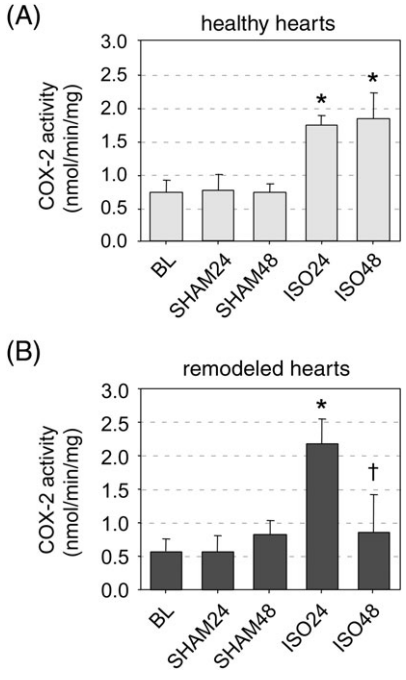

Figure 5 Cyclooxygenase-2 (COX-2) activity in healthy (*A*) and remodelled (*B*) hearts 24 and 48 h after isoflurane exposure. ISO24/48: hearts exposed to isoflurane and harvested 24 or 48 h later. SHAM24/48: time-matched hearts exposed to oxygen alone. Data are mean $\pm$ SD ($n = 4$ per group). *$P < 0.05$ vs. baseline (BL).

Delayed preconditioning is a second window of protection whereby stimulation by ischaemia or chemical agents enhances the resistance of the heart to subsequent potentially lethal stimuli 24–72 h later. It is a universal response

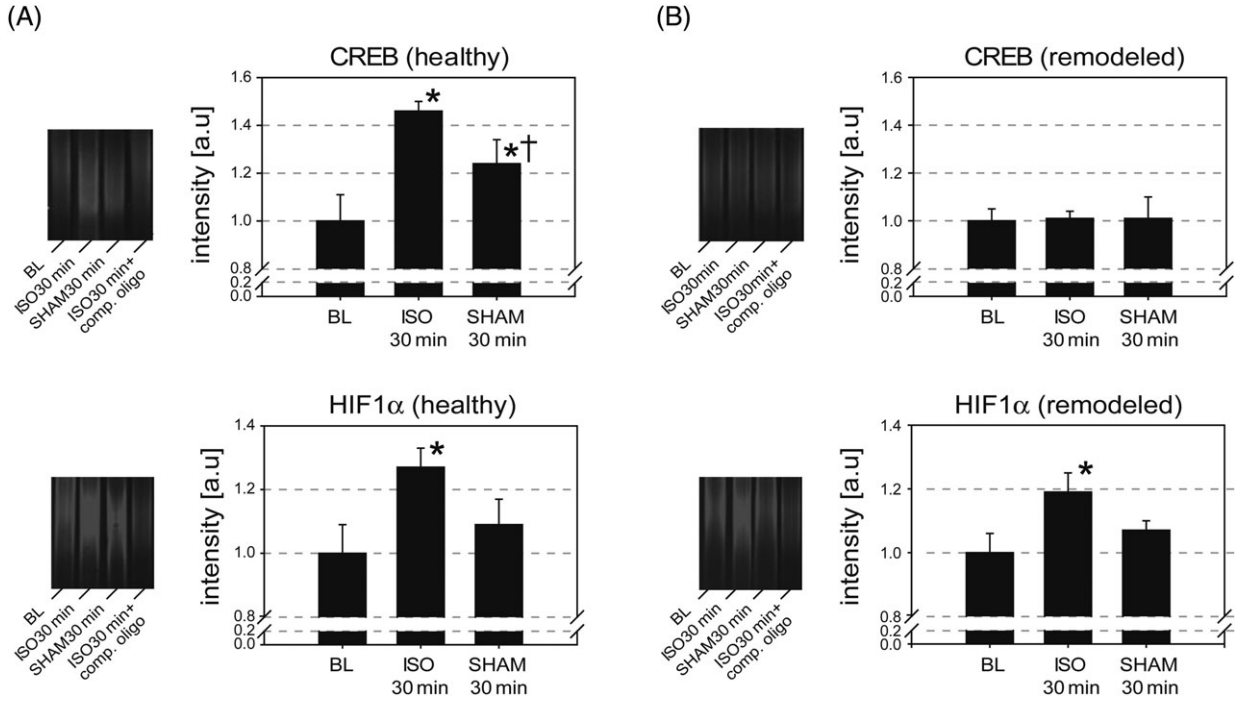

Figure 6 Electrophoretic mobility shift assays of HIF1α and CREB in healthy (*A*) and remodelled (*B*) hearts (gels with corresponding densitometry). ISO30 min: nuclear extracts from hearts exposed to isoflurane preconditioning harvested 30 min after termination of inhalation (2.1 vol% for 90 min). SHAM30 min: nuclear extracts from respective sham group (oxygen alone). Gels show additional lines with ISO30 min plus 200-fold excess of unlabelled consensus competitor oligonucleotide (comp. oligo) confirming specificity of the detected fluorescence signal for HIF1α and CREB, respectively. Data are mean $\pm$ SD ($n = 4$ per group). *$P < 0.05$ vs. baseline (BL) and healthy hearts, respectively. $^{\dagger}P < 0.05$ vs. ISO30 min.

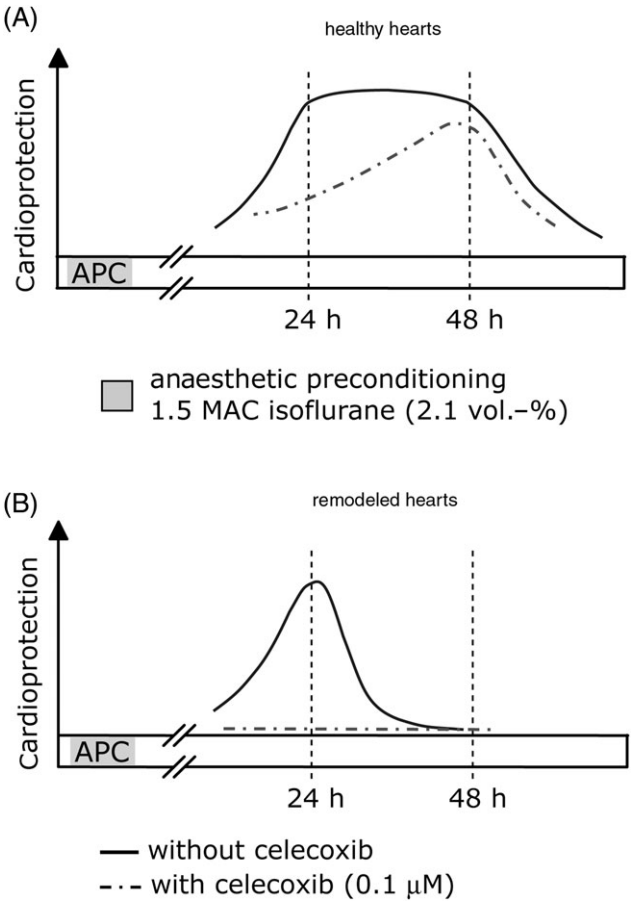

Figure 7 'Second window of protection' in healthy and remodelled hearts. The panels show the second window of protection at 24 and 48 h after isoflurane exposure (APC) in healthy (*A*) and infarct remodelled (*B*) hearts in the absence (solid line) and presence (dashed line) of celecoxib (0.1 μM).

of the heart to stress.[23] In contrast to early preconditioning, delayed preconditioning has strong and more reliable anti-stunning and anti-infarct effects making it more clinically relevant. It causes an increase in prostaglandin biosynthesis in cardiac tissue via the enhanced expression of COX-2 catalysing the conversion of arachidonic acid to prostanoids.[24] COX-2 is constitutively expressed in the heart at lower levels[25] but rapidly induced through the activation of several transcription factors.[3,23] Prostanoids such as prostacyclin (PGI_2) open mitochondrial K_{ATP} channels,[26] key players in isoflurane-induced cardioprotection.[27] Inhibition of the rate-limiting enzyme COX-2 abolishes the infarct-sparing effect of delayed preconditioning,[24] but also the protection by early preconditioning.[28] Using healthy animal models from different species, previous studies reported on late cardioprotection after exposure to volatile anaesthetics,[29,30] and identified reactive oxygen and nitrogen species released by nitric oxide synthase as triggers,[31] inducible nitric oxide synthase,[32] 12-lipoxygenase,[8] and endothelial nitric oxide synthase[33] as mediators, and sarcolemmal and mitochondrial K_{ATP} channels as end effectors[6] of delayed anaesthetic preconditioning. Hitherto, however, only one study with healthy rabbit hearts investigated the role of COX-2 in isoflurane-induced delayed preconditioning using the COX-2 inhibitor celecoxib.[7] Our study now confirms COX-2 as an obligatory mediator in isoflurane-induced delayed preconditioning of healthy rat hearts, and further extends its critical role in cardioprotection to diseased post-infarct remodelled myocardium.

Structural changes due to loss of viable tissue and scar formation,[12] alterations in metabolism,[13] and cellular signalling[14,34,35] put the remodelled myocardium at risk for further ischaemic damage. Experiments from muscle slices of right atrial appendices of patients with failing hearts indicate that remodelled myocardium is less amenable to innate protection by preconditioning.[36] At the molecular level, a gene program resembling the foetal program is activated and protective signalling pathways were reported to be lost in various disease models associated with remodelling.[37] Complete refractoriness to early ischaemic preconditioning as opposed to pharmacologic preconditioning by diazoxide, was reported in a rabbit infarct model, for which interruption of signal transduction between G-protein coupled receptors and protein kinase C was found to be responsible.[38] Conversely, erythropoietin-mediated preconditioning was preserved in post-infarct remodelled rat myocardium despite the disruption of the erythropoietin receptor–PI3K–PKB pathway via up-regulation of the suppressor of cytokine signalling protein-1.[39] In this case, compensatory PI3K-independent activation of ERK upstream of the guanylyl cyclase-mitochondrial K_{ATP} channel pathway restored the protective phenotype. To date, no data is available with respect to delayed protection after pharmacologic or ischaemic preconditioning in post-infarct remodelled hearts. In contrast to our previous findings on early isoflurane-induced preconditioning[15] and postconditioning in remodelled myocardium,[16] where protection against ischaemia-reperfusion was fully preserved, our current study on delayed isoflurane preconditioning clearly indicates that ventricular remodelling alters protective signalling rendering the heart more vulnerable to ischaemia-reperfusion injury. In search of mechanisms for this remarkable vulnerability, we tested a number of COX-2-inducing transcription factors to see whether their nuclear translocation would be impaired in the diseased state. Increased HIF1α protein levels were previously reported after isoflurane exposure in rat myocardium[40] and were similarly regulated in the current study in healthy and diseased hearts. HIF1α is an oxygen-dependent transcription factor activating an array of genes required for the adaptation to hypoxia, including COX-2. In contrast, CREB was exclusively mobilized in healthy but not remodelled hearts, which showed increased expression of the transcriptional repressor and CREB-antagonist ICER.[41] ICER represses transcription either by heterodimerization with activating forms of CREB and other bZIP domain-containing transcription factors, or by competing with these proteins for DNA binding.[42] Due to the homology between ICER and CREB, we cannot completely rule out that both CREB and ICER bind to the CRE-consensus oligos used in our experiments. However, the DNA-binding activity is lower in remodelled hearts than in healthy hearts indicating that ICER may not or only weakly bind to CREB-oligos (data not shown). The results of our experiments raise the possibility that the observed lack of CREB-DNA binding in the remodelled hearts might be due to heterodimerization of CREB with other transcriptional factors including ICER that thereby could antagonize CREB. This hypothesis should be proven in future experiments.

COX-2 inhibitors increase the number of cardiovascular complications specifically in patients with preexisting heart disease.[43] A recent pig study impressively showed that peri-infarct inhibition of COX-2 by celecoxib decreased myocardial function and increased left ventricular remodelling and mortality.[44] In the current study, we also evaluated the effects of low and higher concentrations of the clinically used celecoxib on late isoflurane protection. The IC_{50} of celecoxib for COX-2 (0.04 μM) is 375-times lower than for cyclooxygenase-1,[45] but celecoxib has additional unspecific inhibitory effects on other key enzymes.[21] A single oral administration of 200 mg celecoxib results in a plasma concentration of 700–1100 μg/L, whereby only a small amount (3%) of the drug is unbound and biologically active.[45] Hence, the effective tissue concentration under clinical conditions is close to 0.1 μM, as used in our study in the protein-free buffer perfusing the isolated hearts. Higher concentrations of unbound drug in the range of 1 μM are unusual, but were reported in patients with CYP2C9 deficiency due to genetic polymorphisms.[45] Our results clearly show that even low concentrations of celecoxib suffice to abolish delayed protection by isoflurane. Increased expression of ICER was previously reported to downregulate prosurvival Bcl-2,[41,46] and thus might well be responsible for the higher susceptibility of infarct hearts to celecoxib. From a translational point of view, our data suggest that the use of selective COX-2 inhibitors should be minimized in at-risk patients with significant ventricular remodelling. Finally, our results imply that diseased myocardium requires more frequent intermittent preconditioning stimuli to maintain the protected state.

Although previous studies showed the significance of CREB in establishing delayed preconditioning,[47] our experiments cannot exclude that other factors contributed to the shorter duration of COX-2 upregulation. Our study focused on COX-2 in healthy and diseased rat hearts, and did not investigate molecular cross-talk between iNOS and COX-2 or other kinases such as protein kinase B. Since ischaemic postconditioning was recently reported to enhance delayed preconditioning by further up-regulation of COX-2,[48] future studies should test whether ischaemic and pharmacologic postconditioning could fully restore delayed protection by preconditioning in post-infarct hearts. Some previous studies showed divergent mechanisms during the early, late and final stage of delayed preconditioning.[49] Although our findings suggest that delayed isoflurane preconditioning is mediated via the same mechanisms throughout its duration, we cannot exclude that other or additional mechanisms may be involved in the final stage (at 72 h) of late isoflurane protection. Finally, timing of protection is often species-dependent and may be different in humans.

In summary, our study shows that post-infarct remodelling in rat hearts hinders sustained COX-2 expression and activity after isoflurane preconditioning and thus narrows the second window of protection. Isoflurane induces nuclear translocation of HIF1α in both healthy and remodelled hearts, but fails to mobilize the transcription factor CREB in diseased ICER-overexpressing hearts. The study further demonstrates that innate protection of remodelled myocardium is exceptionally vulnerable to COX-2 inhibition. Hence, protection by delayed isoflurane preconditioning varies with the disease state of the heart and concomitant medication.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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