Cardioprotection initiated by reactive oxygen species is dependent on activation of PKC ϵ

Alamgir M. N. Kabir, James E. Clark, Masaya Tanno, Xuebin Cao, John S. Hothersall, Semjidmaa Dashnyam, Diana A. Gorog, Mohamed Bellahcene, Michael J. Shattock and Michael S. Marber

Am J Physiol Heart Circ Physiol 291:H1893-H1899, 2006. First published 19 May 2006; doi: 10.1152/ajpheart.00798.2005

You might find this additional info useful...

- This article cites 35 articles, 19 of which you can access for free at: http://ajpheart.physiology.org/content/291/4/H1893.full#ref-list-1
- This article has been cited by 10 other HighWire-hosted articles: http://ajpheart.physiology.org/content/291/4/H1893#cited-by
- Updated information and services including high resolution figures, can be found at: http://ajpheart.physiology.org/content/291/4/H1893.full

Additional material and information about American Journal of Physiology - Heart and Circulatory Physiology can be found at:

http://www.the-aps.org/publications/ajpheart

This information is current as of July 25, 2016.

American Journal of Physiology - Heart and Circulatory Physiology publishes original investigations on the physiology of the heart, blood vessels, and lymphatics, including experimental and theoretical studies of cardiovascular function at all levels of organization ranging from the intact animal to the cellular, subcellular, and molecular levels. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. Copyright © 2006 by the American Physiological Society. ISSN: 0363-6135, ESSN: 1522-1539. Visit our website at http://www.the-aps.org/.

Cardioprotection initiated by reactive oxygen species is dependent on activation of PKCe

Alamgir M. N. Kabir,¹ James E. Clark,¹ Masaya Tanno,¹ Xuebin Cao,¹ John S. Hothersall,² Semjidmaa Dashnyam,¹ Diana A. Gorog,¹ Mohamed Bellahcene,¹ Michael J. Shattock,¹ and Michael S. Marber¹

¹Cardiovascular Division, King's College London, The Rayne Institute, St. Thomas' Hospital, London; and ²Department of Urology, University College London, London, United Kingdom

Submitted 27 July 2005; accepted in final form 11 April 2006

Kabir, Alamgir M. N., James E. Clark, Masaya Tanno, Xuebin Cao, John S. Hothersall, Semjidmaa Dashnyam, Diana A. Gorog, Mohamed Bellahcene, Michael J. Shattock, and Michael S. Marber. Cardioprotection initiated by reactive oxygen species is dependent on activation of PKCE. Am J Physiol Heart Circ Physiol 291: H1893-H1899, 2006. First published May 19, 2006; doi:10.1152/ajpheart.00798.2005.-To examine whether cardioprotection initiated by reactive oxygen species (ROS) is dependent on protein kinase CE (PKCE), isolated bufferperfused mouse hearts were randomized to four groups: 1) antimycin A (AA) (0.1 µg/ml) for 3 min followed by 10 min washout and then 30 min global ischemia (I) and 2 h reperfusion (R); 2) controls of I/R alone; 3) AA bracketed with 13 min of N-2-mercaptopropionylglycine (MPG) followed by I/R; and 4) MPG (200 µM) alone, followed by I/R. Isolated adult rat ventricular myocytes (ARVM) were exposed to AA (0.1 µg/ml), and lucigenin was used to measure ROS production. Murine hearts and ARVM were exposed to AA (0.1 μ g/ml) with or without MPG, and PKC ϵ translocation was measured by cell fractionation and subsequent Western blot analysis. Finally, the dependence of AA protection on PKCE was determined by the use of knockout mice (-/-) lacking PKC ε . AA exposure caused ROS production, which was abolished by the mitochondrial uncoupler mesoxalonitrile 4-trifluoromethoxyphenylhydrazone. In addition, AA significantly reduced the percent infarction-left ventricular volume compared with control I/R (26 \pm 4 vs. 43 \pm 2%; P < 0.05). Bracketing AA with MPG caused a loss of protection (52 \pm 7 vs. $26 \pm 4\%$; P < 0.05). AA caused PKC ε translocation only in the absence of MPG, and protection was lost on the $pkc\epsilon^{-/-}$ background $(38 \pm 3 \text{ vs. } 15 \pm 4\%; P < 0.001)$. AA causes ROS production, on which protection and PKCE translocation depend. In addition, protection is absent in PKCE null hearts. Our results imply that, in common with ischemic preconditioning, PKCE is crucial to ROS-mediated protection.

protein kinase C; ischemic preconditioning

ISCHEMIC PRECONDITIONING describes the phenomenon whereby sublethal ischemia reduces subsequent lethal ischemic injury (22). The phenomenon is highly reproducible and has been shown in many species, probably including humans (19, 21).

Preconditioning has a number of identifiable triggers, including reactive oxygen species (ROS) (7). It has been shown that ischemic preconditioning can be blocked by ROS scavengers (28). Moreover, it has been suggested that ROS contribute to the threshold necessary for the activation of signals leading to cardioprotection (1). Furthermore, it has been shown that ROS are generated during preconditioning cycles in cardiomyocytes (31). In whole organ and whole animal models there is remaining controversy as to the effect of ROS scavengers on cardioprotection (2). Although at high concentrations ROS are detrimental, there is increasing evidence that at lower concentrations they may play a subtle role in initiating protective cell signaling pathways (8).

PKC ε is a member of the protein kinase C family that has been studied extensively in preconditioning and tumorigenesis (12). It has been suggested that the zinc finger motifs present in PKC ε allow for modification of structure and function by ROS (17). Furthermore, targeted disruption of the PKC ε gene leads to loss of the cardioprotective effect of ischemic preconditioning (27).

Antimycin A is thought to generate mitochondrial ROS through an action on site III of the electron transport chain. At this site it blocks the conversion of ubisemiquinone to ubiquinol, thereby causing accumulation of superoxide ROS (9). In support of this action being of relevance to protection is evidence that other mitochondrial ROS generators, such as menadione, also initiate cardioprotection (34). Although in studies using anesthetic as a protective agent, a number of groups have demonstrated that ROS can trigger cardioprotection through activation of PKC; the specific isoform involved is unclear (3, 24).

Based on these findings we examined whether antimycin A could protect the murine Langendorff-perfused heart and, if such protection existed, whether it depended on ROS generation and the activation of PKC ϵ .

METHODS

All animal experiments were carried out in accordance with Home Office regulations as detailed in the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986. HMSO (London).

ROS measurement with lucigenin. Superoxide radical production was measured using lucigenin. Isolated adult rat cardiocytes were prepared as previously described (26) from 250- to 300-g Wistar rats and suspended in respiration buffer (in mM: 70 sucrose, 220 mannitol, 1 EDTA, 2.5 KH₂PO₄, 1 MgCl₂, and 2 HEPES; pH 7.4). A cell suspension of 1 ml (1 in 10 dilution of original cell isolation) was incubated with 2.5 μ M digitonin (to permeabilize cells), 10 μ M NADH (as a substrate), and 10 μ M lucigenin [below the threshold for subrecycling (20)] in 35-mm dishes sealed with polythene film kept at 37°C. Chemiluminescence measurements were made in a photoncounting device comprising a gallium arsenate photomutiplier tube

Address for reprint requests and other correspondence: M. S. Marber, Dept. of Cardiology, The Rayne Institute, St Thomas' Hospital, London SE1 7EH, UK (e-mail: mike.marber@kcl.ac.uk).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

(Hamamatsu R943) thermoelectrically cooled to -20° C. Readings were taken at 20-s intervals and average counts derived. The treatment group had 0.1 µg/ml of antimycin A added. Controls lacked antimycin A. Control and treatment groups had readings taken serially and alternately to minimize artifacts resulting from agitation in the photocounter carousel. Experiments were also conducted with mesoxalonitrile 4-trifluoromethoxyphenylhydrazone (FCCP, 10 µM) to indicate a mitochondrial basis for superoxide production.

Western blot analysis and cell fractionation. Samples were obtained from perfused hearts that were freeze clamped at the end of defined protocols or cultured adult rat ventricular myocytes following treatment with agents. A 10% (wt/vol) solution of tissue in homogenization buffer [100 mM Tris·HCl (pH 7.2) Protease Complete C tablet in 50 ml, 1 mM Na orthovanadate, 1 mM EDTA, and 10 mM NaF] was centrifuged at 100,000 g for 10 min. The soluble cytosolic fraction was removed and resuspended in $2\times$ sample buffer (250 mM/l Tris·HCl, 4% SDS, 10% glycerol, and 2% β-mercaptoethanol; pH 6.8). The pellet was resuspended in 1% Triton before a second centrifugation at 100,000 g, and the membrane and insoluble fractions were removed and resuspended in 2× sample buffer. All fractions were resuspended or diluted to the same final volume (300 µl). Lane loading was confirmed using Ponceau-S after transfer. After being blocked in 5% nonfat milk, the nitrocellulose membranes were exposed to primary antibodies and then appropriate secondary antibodies. ECL (Amersham) detection was used. Autophotographic images of Western blots were scanned and analyzed using National Institutes of Health image software. Antibodies used were the following: total PKC ε 1:1,000 overnight incubation (Transduction Laboratories) and rabbit anti-mouse IgG secondary (DAKO A/S).

PKC ε -*deficient mice.* pkc $\varepsilon^{-/-}$ mice were from a previously generated source as described previously (5, 27). The colony was derived by crossing pkc $\varepsilon^{-/-}$ with outbred C57BL/6 mice. Null and wild-type littermates of pkc $\varepsilon^{-/+}$ and pkc $\varepsilon^{-/+}$ matings were used in experiments whenever possible to reduce background genetic variability (13).

Perfusion of isolated murine hearts. Male mice were euthanized with phenobarbital (300 mg/kg) and heparin (150 units) intraperito-



Fig. 1. A: isolated buffer-perfused mouse hearts were randomized into 4 groups: 1) controls of 30 min global ischemia and 2 h reperfusion; 2) antimycin A (AA) (0.1 µg/ml) for 3 min followed by 10 min washout then ischemia-reperfusion; 3) antimycin A bracketed with 13 min of N-2-mercaptopropionyl-glycine (MPG, 200 µM) followed by ischemia-reperfusion; and 4) MPG (200 µM) alone followed by ischemia-reperfusion. Downward arrow, time of heart removal and freezing for Western blot analysis. B: isolated buffer-perfused mouse hearts were randomized into 4 groups: 1) pkc $\epsilon^{+/+}$ mice perfused with AA (0.1 µg/ml) for 3 min followed by 10 min washout and then 30 min global ischemia and 2 h reperfusion; 2) $pkc\epsilon^{+/+}$ mice perfused with DMSO (0.01%) for 3 min and then 10 min washout followed by ischemia-reperfusion groups; 3 and 4) identical to *protocols 1* and 2 except $pkc\epsilon^{-/-}$ mice were used.

Experimental Groups	Control	Antimycin A	Antimycin A-MPG	MPG
Group size	9	6	6	6
Body weight, g	26.9 ± 0.6	26.0 ± 0.8	25.7 ± 0.7	24.8 ± 0.8
Wet heart weight, mg	124.8 ± 5.3	112.0±6.6	106.8 ± 9.7	110.4 ± 11.3
Heart volume, mm ³	102.3 ± 2.3	92.0±5.7	92.2 ± 4.8	90.9 ± 7.2
Diastolic pressure (baseline), mmHg	4.1 ± 0.6	5.6 ± 0.5	5.2 ± 0.5	4.5 ± 0.6
Coronary flow, ml/min				
Baseline	3.2 ± 0.3	3.3 ± 0.4	3.7 ± 0.4	2.9 ± 0.5
Reperfusion (60 min)	1.8 ± 0.2	2.4 ± 0.2	1.7 ± 0.2	1.6 ± 0.2
Reperfusion (120 min)	1.5 ± 0.2	2.0 ± 0.2	1.2 ± 0.1	1.3 ± 0.2
LVDP, mmHg				
Baseline	66±3	61 ± 2	64 ± 1	63 ± 2
Reperfusion (60 min)	12±3	$25 \pm 4*$	$8 \pm 2 \ddagger$	23 ± 5 ;
Reperfusion (120 min)	15 ± 3	26 ± 3	$11 \pm 3 \ddagger$	26 ± 5 ;

Table 1. Characteristics of antimycin A and MPG groups

Values are means \pm SE. MPG, *N*-2-mercaptopropionyl-glycine; LVDP, left ventricular developed pressure. *P < 0.05 vs. control; $\dagger P < 0.05$ vs. antimycin A; $\ddagger P < 0.05$ vs. antimycin A-MPG.

neally. Hearts were rapidly isolated and perfused as previously described (13, 27, 29). Specific protocols were used to determine whether antimycin A works as a trigger of preconditioning, and if preconditioning was observed, whether the generation of ROS is involved in the mediation of protection. These are depicted by Fig. 1A. Additional protocols were designed to examine the role of the PKC ϵ protein (as depicted by Fig. 1*B*).

Assessment of infarct size in isolated murine hearts. After completion of perfusion protocols, hearts were perfused with triphenyl





Fig. 2. Analysis of infarct size in Langendorff-perfused mouse hearts. A: infarct size (IS) is significantly reduced by AA. Effect is lost with coadministration of MPG (200 μ M). B: heart volume plotted against infarct volume to ensure results are not skewed by an infarct-to-risk volume relationship that does not intersect the origin. There is a significant reduction in infarct size with AA that is lost with coadministration of MPG (AA+MPG). LV, left ventricle; NS, not significant. Statistical analysis is by one-way ANOVA for A (*P < 0.05) between bracketed groups) and analysis of covariance (ANCOVA) for B (P < 0.05).

Fig. 3. Effect of AA on superoxide formation by rat isolated cardiocytes as assessed by lucigenin chemiluminesence. A: significantly higher chemiluminescent signal at each time point in the AA group compared with control. B: effect of FCCP (10 μ M) is shown, which reduces the chemiluminescent signal. Statistical analysis was by one-way ANOVA at each time point (n = 4 per group). *P < 0.05.

PKCε IN ROS-INITIATED PROTECTION

Fig. 4. A: effect of AA (0.1 µg/ml) on distribution of PKCɛ in 3 independently exposed hearts. Exposure to AA causes membrane localization, which is abolished by MPG. B: representative Western blot analysis of lysates from isolated rat ventricular myocytes following treatment with vehicle (Veh), AA (0.1 µg/ml), and PMA (100 nM, an archetypical activator of PKCɛ) showing cellular localization of PKCɛ. *C* and *D*: densitometry of Western blots shows a significant increase (P = 0.02) in the membrane fraction in those cells treated with AA. This effect is abolished by coadministration of MPG (P = 0.01). The cell fractionation process accurately separates the membrane fraction as shown by immunoreactivity of the α-subunit of Na/K+ pump. M, C and I denote membrane, cytosolic and insoluble fractions, respectively. *P < 0.02 and ** P < 0.01.



tetrazolium chloride (TTC, 5% in Krebs buffer), and infarct size was assessed as previously described (13, 27, 29). Infarct volume (TTC-negative tissue) was normalized to total left ventricular volume.

Statistical analysis. All data are presented as means \pm SE. P < 0.05 was considered significant. Chemiluminescence and normalized infarct size (infarct volume-to-risk volume ratio) were measured by one-way ANOVA. Hemodynamic parameters were compared by two-way ANOVA to factor time as well as group. Linear regression was carried out using the SigmaStat statistical package. Infarct volume with respect to total myocardial volume was compared by analysis of covariance (ANCOVA) using an Excel plug-in (Ferris State University).

RESULTS

Effect of antimycin A on cardioprotection and importance of ROS. The protocols are shown in Fig. 1*A*. Table 1 shows the hemodynamic and morphometric characteristics of each group. There is no significant difference in body weight, heart volume, or baseline hemodynamic characteristics. Comparison by twoway ANOVA to factor time as well as group did not show any significant difference in hemodynamic parameters.

In preliminary experiments a range of concentrations of antimycin A was examined. The concentration that resulted in optimal cardioprotection without detrimental effect was 0.1 μ g/ml. At this concentration, developed pressure immediately before ischemia did not differ between antimycin A and DMSO groups. However, the infarct size in the antimycin A-treated group was significantly less than the control group $(26.2 \pm 4.2 \text{ vs. } 43.4 \pm 2.4\%; P = 0.03)$ (Fig. 2). The cardioprotective effect of antimycin A was abolished by the bracketing with MPG (51.6 \pm 6.6 vs. 26.2 \pm 4.2%; *P* < 0.05). There was no significant difference between control and MPG alone $(43.4 \pm 2.4 \text{ vs. } 34.0 \pm 5.2\%; P = 0.175)$. When heart volume is taken into account and plotted against infarct size, there was still a significant difference between the control and the antimycin A-treated group (P = 0.04), and this was again abolished by MPG (ANCOVA, Fig. 2B).

Assessment of antimycin A-induced ROS production by lucigenin. To assess the presence of ROS, adult rat cardiomyocytes were exposed to antimycin A or vehicle control (Fig. 3). There was a significant increase in chemiluminescence with

Experimental Groups	pkcɛ ^{-/-} - DMSO	pkce ^{+/+} - DMSO	pkce ^{-/-} - Antimycin A	pkce ^{+/+} - Antimycin A		
Group size	6	6	6	6		
Body weight, g	23.8±0.8*	27.0 ± 1.0	23.8±1.0†	28.5 ± 1.2		
Wet heart weight, mg	123.0 ± 10.7	140.1 ± 11.1	$105.3 \pm 5.2 \ddagger$	140.0 ± 3.9		
Heart volume, mm ³	78.5 ± 3.4	82.4 ± 3.2	82.8 ± 6.2	91.5 ± 3.0		
Diastolic pressure, mmHg baseline	7.0 ± 1.7	5.8 ± 1.1	6.5 ± 1.6	7.2 ± 1.0		
Coronary flow, ml/min						
Baseline	2.8 ± 0.3	2.2 ± 0.2	2.8 ± 0.3	2.4 ± 0.2		
Reperfusion (60 min)	2.0 ± 0.2	2.0 ± 0.0	2.1 ± 0.2	2.4 ± 0.2		
Reperfusion (120 min)	2.0 ± 0.2	2.0 ± 0.0	2.3 ± 0.3	2.4 ± 0.2		
LVDP, mmHg						
Baseline	63.5 ± 7.4	67.3 ± 5.5	67.9 ± 8.9	60.5 ± 3.5		
Reperfusion (60 min)	4.0 ± 1.4	12.8 ± 7.0	6.8 ± 2.7	8.8 ± 2.9		
Reperfusion (120 min)	5.5 ± 1.7	15.2 ± 6.7	8.2 ± 3.8	11.2 ± 3.9		

Table 2. Characteristics of groups composed of $PKC\varepsilon$ -targeted hearts exposed to antimycin A

Values are means \pm SE. *P < 0.01 vs. pkc $\epsilon^{+/+}$ -antimycin A; †P < 0.05 vs. pkc $\epsilon^{+/+}$ -antimycin A; ‡P < 0.05 vs. pkc $\epsilon^{+/+}$ -antimycin A and vs. pkc $\epsilon^{+/+}$ -DMSO.

antimycin A from the fourth (1254.25 ± 61 vs. 796.75 ± 162 light units; P = 0.039) to the sixth (1249.25 ± 86 vs. 732.5 ± 166 light units; P = 0.033) acquisition window. These windows correspond to 140 and 220 s after the addition of reagents. In separate experiments the addition of FCCP caused a decrease in luminescent signal, and by the sixth window the difference between antimycin A and control was lost (Fig. 3*B*).

Translocation of PKC ε by antimycin A. Translocation of PKC ε is a measure of activation. Membrane fractions were compared against their own cytosolic fraction (Fig. 4). There was a significant increase in the membrane-to-cytosol ratio following antimycin A compared with vehicle (0.51 ± 0.07 vs. 0.22 ± 0.07; P = 0.01), indicating that PKC ε translocates to the membrane fraction in response to antimycin A. The effect of bracketing antimycin A with MPG was to abolish membrane translocation. Thus the antimycin A-MPG group had a significantly lower ratio of PKC ε immunoreactivity in membrane to-cytosol fraction compared with antimycin A treatment alone (0.18 ± 0.02 vs. 0.51 ± 0.07; P = 0.01). These results in isolated mouse hearts were mirrored by those in isolated adult rat ventricular myocytes (Fig. 4*B*).

Dependence of antimycin A-mediated cardioprotection on PKCE. To further test the role of PKCE in antimycin A-mediated cardioprotection, we examined the effect of antimycin A (0.1 μ g/ml) or DMSO vehicle (0.01%) in pkc $\epsilon^{-/-}$ and pkc $\epsilon^{+/+}$ buffer-perfused isolated mouse hearts. The hearts were randomized into four groups (Fig. 1B). Table 2 shows the hemodynamic and morphometric characteristics of each group. There are statistically significant differences in body and heart weight and wet heart weight. The pkc $\epsilon^{-/-}$ -DMSO group had significantly lower body weight than the pkc $\epsilon^{+/+}$ -antimycin A group (23.8 \pm 0.8 vs. 28.5 \pm 1.2 g; P = 0.01). The pkc $\epsilon^{-/-}$ antimycin A group had significantly lower body and heart weight compared with the pkc $\epsilon^{+/+}$ -antimycin A group (23.8 ± 1.0 vs. 28.5 \pm 1.2 g; P = 0.02 and 105.3 \pm 5.2 vs. 140 \pm 3.9 mg; P = 0.02, respectively). The pkce^{+/+}-DMSO group had significantly higher heart weight than the $pkc\epsilon^{-/-}$ -antimycin A group (140.1 \pm 11.1 vs. 105.3 \pm 5.2 mg; P = 0.04). However, there was no statistical difference in heart volume or baseline hemodynamic characteristics. Comparison by twoway ANOVA to take into account time as well as group did not

show any significant difference in flow or hemodynamic parameters.

The pkc $\epsilon^{-/-}$ mice were not protected by antimycin A, whereas the pkc $\epsilon^{+/+}$ mice were protected (37.5 ± 3 vs. 15.0 ± 4.4%; P < 0.001) (Fig. 5A). ANCOVA shows that the effect is



Fig. 5. Analysis of infarct size in Langendorff-perfused hearts from a pkcɛtargeted line. A: cardioprotective effect of AA is lost in $pkce^{-/-}$ (knock-out, KO), but not in $pkce^{+/+}$ (wild-type, WT), hearts. ***P < 0.001. B: confirmation that this finding persists in an ANCOVA of heart volume versus infarct volume, which is insensitive to the chance differences in heart sizes between groups indicated within Table 2.

maintained when heart volume is taken into account. Again $pkc\epsilon^{-/-}$ mice were not protected by antimycin A compared with $pkc\epsilon^{+/+}$ mice (P = 0.036) (Fig. 5B).

DISCUSSION

We have shown that antimycin A initiates cardioprotection that is blocked by MPG, suggesting a role for ROS in the mechanism of protection. Yue et al. (34) have shown previously that another mitochondrial ROS generator menadione can cause cardioprotection through a pathway involving p38 mitogen-activated protein kinase (MAPK) activation, a finding that is similar to our own in a related study (16). We further examined cardioprotection by looking at the effect of antimycin A on rat cardiomyocytes. We have shown that lucigenin chemiluminescence is significantly enhanced by antimycin A and depends on continued mitochondrial electron transport. Furthermore, these increases in chemiluminescence were seen at concentrations of antimycin A that were cardioprotective in murine Langendorff experiments. This implies a role for mitochondrially derived ROS. We have then further shown that PKCe translocates to the membrane in response to antimycin A, an effect blocked by MPG. This phenomenon of PKCE translocation being mediated via ROS production has been shown previously in cardiac tissue in response to anestheticmediated preconditioning as well as in other tissues (3, 24). Jung et al. (15) demonstrated that PKCE translocation in neuronal tissue can be blocked by the antioxidant trolox. Taken together, this suggests that antimycin A causes mitochondrially derived ROS generation that triggers PKCE activation. Furthermore, to test the role of PKCE in cardioprotection, we have used a targeted mouse line and demonstrated that PKCE is crucial to protection. In this series of experiments the cardioprotective effect of antimycin A seems to be manifest predominantly as a reduction of infarct size. We cannot disregard any effect of antimycin A on postischemic left ventricular function in the PKCE-targeted mouse line, because postischemic recovery was poor in all groups causing no discernable differences in left ventricular function. Similar effects have been reported by others (27, 33) and in their models attributed to ROS production increased stunning in viable myocardium (25).

At high concentrations, ROS are detrimental to cells. However, there is increasing evidence that at lower concentrations they have a role to play in the signaling mechanisms of preconditioning (30). Antimycin A is thought to act at site III of the electron transport chain to cause superoxide accumulation. There is evidence to support site III as the principal point of ROS generation during oxidation of complex I substrates (6). Moreover, there is evidence to suggest that novel PKCs have many potential sites sensitive to redox modification (12), in particular within the cysteine-rich (11) and zinc finger regions (17). The relative position of ROS in relation to p38-MAPK has been suggested by others (35). The relative position of ROS in relation to PKC is under debate because there is evidence to support a role upstream as well as downstream of PKC activation. There is evidence to suggest that PKC is an initiator of ROS production rather than its target. In zebra fish it has been demonstrated that PKC activation leads to a ROS burst (14). There is evidence that ROS production lies upstream of PKC activation in anesthetic preconditioning (4, 24). This is a finding that mirrors that of the current study. However, in a related study, we have also shown that antimycin A-triggered protection is dependent on the preischemic activation of p38-MAPK (16), a finding also supported by other investigators (18, 35). The relationship between PKCE activation and the downstream target p38-MAPK is given more credence by studies showing that xenon-preconditioning results in both PKCE and p38-MAPK activation, which is sensitive to inhibitors of both PKC and p38-MAPK (32), whereas with ischemic preconditioning disruption of microtubules by colchicine prevents translocation of PKCE as well as the activation of p38-MAPK (23). In this study, we show that the mitochondrial ROS generator-antimycin A can precondition the myocardium. Moreover, this cardioprotection is dependent on mitochondrial ROS production and the subsequent activation of PKCE. A putative mechanism of PKCE activation by ROS is through direct tyrosine phosphorylation because regulatory and catalytic domains of PKC are susceptible to oxidative modification by H₂O₂ (12), a mechanism previously described in a number of PKC isoforms including PKC ε (10, 11). However, this is an area that requires further study to elucidate the exact mechanism(s) by which ROS activates PKC ε and leads to subsequent cardiac protection in our model.

ACKNOWLEDGMENTS

We thank Peter Parker and Michael Owen of Cancer Research United Kingdom for the PKCe-targeted mouse line.

GRANTS

This study was supported by grants from the Wellcome Trust (0645447 and 074653) and British Heart Foundation (02/105/14432).

REFERENCES

- Baines CP, Goto M, and Downey JM. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. J Mol Cell Cardiol 29: 207–216, 1997.
- 2. Becker LB. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovasc Res* 61: 461–470, 2004.
- Bouwman RA, Musters RJ, van Beek-Harmsen BJ, de Lange JJ, and Boer C. Reactive oxygen species precede protein kinase C-delta activation independent of adenosine triphosphate-sensitive mitochondrial channel opening in sevoflurane-induced cardioprotection. *Anesthesiology* 100: 506–514, 2004.
- 4. Bouwman RA, Musters RJ, van Beek-Harmsen BJ, de Lange JJ, and Boer C. Reactive oxygen species precede protein kinase C-delta activation independent of adenosine triphosphate-sensitive mitochondrial channel opening in sevoflurane-induced cardioprotection. *Anesthesiology* 100: 506–514, 2004.
- Castrillo A, Pennington DJ, Otto F, Parker PJ, Owen MJ, and Bosca L. Protein kinase Cepsilon is required for macrophage activation and defense against bacterial infection. *J Exp Med* 194: 1231–1242, 2001.
- Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, and Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem 278: 36027–36031, 2003.
- 7. Das DK, Engelman RM, and Maulik N. Oxygen free radical signaling in ischemic preconditioning. *Ann NY Acad Sci* 874: 49–65, 1999.
- Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47–95, 2002.
- Duranteau J, Chandel NS, Kulisz A, Shao Z, and Schumacker PT. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J Biol Chem 273: 11619–11624, 1998.
- Gopalakrishna R and Anderson WB. Ca²⁺- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain. *Proc Natl Acad Sci USA* 86: 6758–6762, 1989.
- Gopalakrishna R, Chen ZH, and Gundimeda U. Modifications of cysteine-rich regions in protein kinase C induced by oxidant tumor promoters and enzyme-specific inhibitors. *Methods Enzymol* 252: 132– 146, 1995.

- Gopalakrishna R and Jaken S. Protein kinase C signaling and oxidative stress. Free Radic Biol Med 28: 1349–1361, 2000.
- Gorog DA, Tanno M, Kabir AM, Kanaganayagam GS, Bassi R, Fisher SG, and Marber MS. Varying susceptibility to myocardial infarction among C57BL/6 mice of different genetic background. *J Mol Cell Cardiol* 35: 705–708, 2003.
- Hermann AC, Millard PJ, Blake SL, and Kim CH. Development of a respiratory burst assay using zebrafish kidneys and embryos. *J Immunol Methods* 292: 119–129, 2004.
- Jung YS, Ryu BR, Lee BK, Mook-Jung I, Kim SU, Lee SH, Baik EJ, and Moon CH. Role for PKC-epsilon in neuronal death induced by oxidative stress. *Biochem Biophys Res Commun* 320: 789–794, 2004.
- Kabir AM, Cao X, Gorog DA, Tanno M, Bassi R, Bellahcene M, Quinlan RA, Davis RJ, Flavell RA, Shattock MJ, and Marber MS. Antimycin A induced cardioprotection is dependent on pre-ischemic p38-MAPK activation but independent of MKK3. J Mol Cell Cardiol 39: 709–717, 2005.
- Korichneva I, Hoyos B, Chua R, Levi E, and Hammerling U. Zinc release from protein kinase C as the common event during activation by lipid second messenger or reactive oxygen. *J Biol Chem* 277: 44327– 44331, 2002.
- Kulisz A, Chen N, Chandel NS, Shao Z, and Schumacker PT. Mitochondrial ROS initiate phosphorylation of p38 MAP kinase during hypoxia in cardiomyocytes. *Am J Physiol Lung Cell Mol Physiol* 282: L1324–L1329, 2002.
- Lambiase PD, Edwards RJ, Cusack MR, Bucknall CA, Redwood SR, and Marber MS. Exercise-induced ischemia initiates the second window of protection in humans independent of collateral recruitment. *J Am Coll Cardiol* 41: 1174–1182, 2003.
- Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, and Trush MA. Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem* 273: 2015–2023, 1998.
- Marber M, Walker D, and Yellon D. Ischaemic preconditioning. BMJ 308: 1–2, 1994.
- Murry CE, Jennings RB, and Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124–1136, 1986.
- Nakamura Y, Miura T, Nakano A, Ichikawa Y, Yano T, Kobayashi H, Ikeda Y, Miki T, and Shimamoto K. Role of microtubules in ischemic preconditioning against myocardial infarction. *Cardiovasc Res* 64: 322– 330, 2004.
- 24. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, and Stowe DF. Reactive oxygen species precede the epsilon isoform of protein

kinase C in the anesthetic preconditioning signaling cascade. Anesthesiology 99: 421–428, 2003.

- Puett DW, Forman MB, Cates CU, Wilson BH, Hande KR, Friesinger GC, and Virmani R. Oxypurinol limits myocardial stunning but does not reduce infarct size after reperfusion. *Circulation* 76: 678–686, 1987.
- Roberts NA, Haworth RS, and Avkiran M. Effects of bisindolylmaleimide PKC inhibitors on p90RSK activity in vitro and in adult ventricular myocytes. *Br J Pharmacol* 145: 477–489, 2005.
- Saurin AT, Pennington DJ, Raat NJ, Latchman DS, Owen MJ, and Marber MS. Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. *Cardiovasc Res* 55: 672–680, 2002.
- Tanaka M, Fujiwara H, Yamasaki K, and Sasayama S. Superoxide dismutase and N-2-mercaptopropionyl glycine attenuate infarct size limitation effect of ischaemic preconditioning in the rabbit. *Cardiovasc Res* 28: 980–986, 1994.
- 29. Tanno M, Bassi R, Gorog DA, Saurin AT, Jiang J, Heads RJ, Martin JL, Davis RJ, Flavell RA, and Marber MS. Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: evidence for MKK-independent activation by a TAB1-associated mechanism contributing to injury during myocardial ischemia. *Circ Res* 93: 254–261, 2003.
- Tritto I and Ambrosio G. Role of oxidants in the signaling pathway of preconditioning. *Antioxid Redox Signal* 3: 3–10, 2001.
- vanden Hoek TL, Becker LB, Shao Z, Li C, and Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* 273: 18092– 18098, 1998.
- 32. Weber NC, Toma O, Wolter JI, Obal D, Mullenheim J, Preckel B, and Schlack W. The noble gas xenon induces pharmacological preconditioning in the rat heart in vivo via induction of PKC-epsilon and p38 MAPK. *Br J Pharmacol* 144: 123–132, 2005.
- Xi L, Hess ML, and Kukreja RC. Ischemic preconditioning in isolated perfused mouse heart: reduction in infarct size without improvement of post-ischemic ventricular function. *Mol Cell Biochem* 186: 69–77, 1998.
- 34. Yue Y, Krenz M, Cohen MV, Downey JM, and Critz SD. Menadione mimics the infarct-limiting effect of preconditioning in isolated rat hearts. *Am J Physiol Heart Circ Physiol* 281: H590–H595, 2001.
- 35. Yue Y, Qin Q, Cohen MV, Downey JM, and Critz SD. The relative order of mK(ATP) channels, free radicals and p38 MAPK in preconditioning's protective pathway in rat heart. *Cardiovasc Res* 55: 681–689, 2002.