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

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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 PKCε. *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 Cε (PKCε), isolated buffer-perfused 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 μg/ml) with or without MPG, and PKCε translocation was measured by cell fractionation and subsequent Western blot analysis. Finally, the dependence of AA protection on PKCε was determined by the use of knockout mice (−/−) lacking PKCε. 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 ± 4 vs. 43 ± 2%; $P < 0.05$). Bracketing AA with MPG caused a loss of protection (52 ± 7 vs. 26 ± 4%; $P < 0.05$). AA caused PKCε translocation only in the absence of MPG, and protection was lost on the pkcε$^{-/-}$ background (38 ± 3 vs. 15 ± 4%; $P < 0.001$). AA causes ROS production, on which protection and PKCε translocation depend. In addition, protection is absent in PKCε null hearts. Our results imply that, in common with ischemic preconditioning, PKCε 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).

PKCε 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 PKCε allow for modification of structure and function by ROS (17). Furthermore, targeted disruption of the PKCε 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 PKCε.

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 KH_2PO_4, 1 $MgCl_2$, 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 μM digitonin (to permeabilize cells), 10 μM NADH (as a substrate), and 10 μM 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 photon-counting device comprising a gallium arsenate photomutiplier tube

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(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× 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 PKCε 1:1,000 overnight incubation (Transduction Laboratories) and rabbit anti-mouse IgG secondary (DAKO A/S).

PKCε-deficient mice. pkcε$^{-/-}$ mice were from a previously generated source as described previously (5, 27). The colony was derived by crossing pkcε$^{-/-}$ with outbred C57BL/6 mice. Null and wild-type littermates of pkcε$^{-/+}$ and pkcε$^{-/+}$ 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-

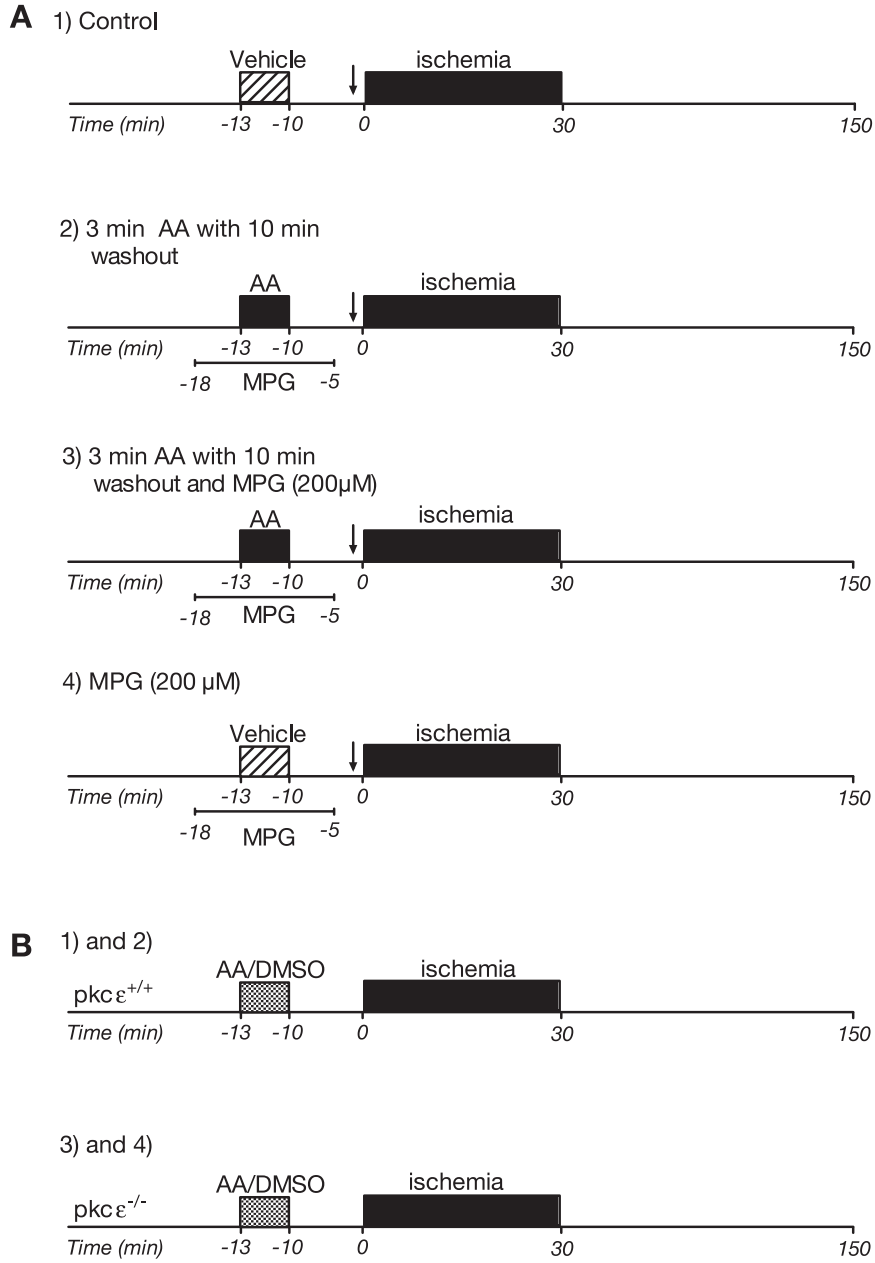

Fig. 1. *A*: isolated buffer-perfused mouse hearts were randomized into 4 groups: *1*) controls of 30 min global ischemia and 2 h reperfusion; *2*) antimycin A (AA) (0.1 μg/ml) for 3 min followed by 10 min washout then ischemia-reperfusion; *3*) antimycin A bracketed with 13 min of *N*-2-mercaptopropionyl-glycine (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*) pkcε$^{+/+}$ 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*) pkcε$^{+/+}$ 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 pkcε$^{-/-}$ mice were used.

Table 1. *Characteristics of antimycin A and MPG groups*

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±4*	8±2†	23±5‡
Reperfusion (120 min)	15±3	26±3	11±3†	26±5‡

Values are means ± SE. MPG, *N*-2-mercaptopropionyl-glycine; LVDP, left ventricular developed pressure. *$P < 0.05$ vs. control; †$P < 0.05$ vs. antimycin A; ‡$P < 0.05$ 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. 1*A*. Additional protocols were designed to examine the role of the PKCε 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

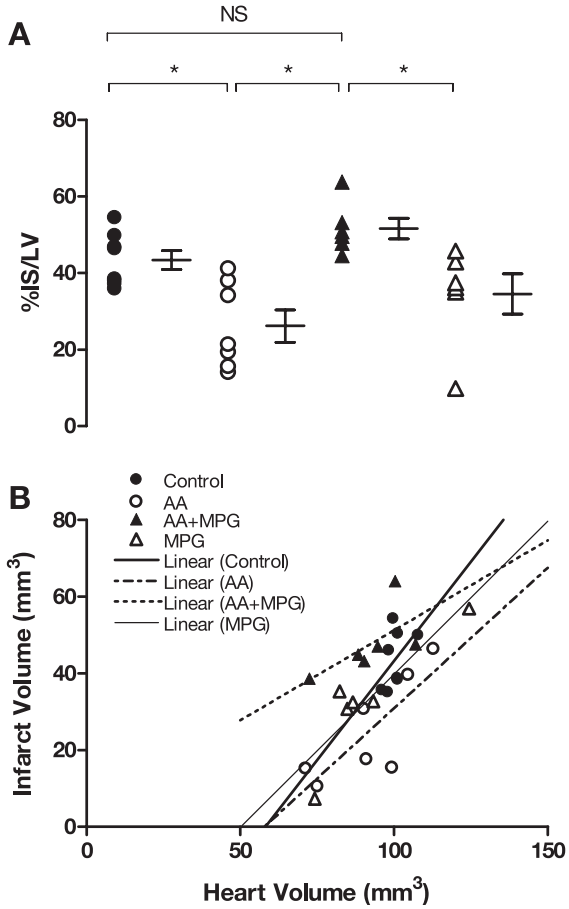

Fig. 2. Analysis of infarct size in Langendorff-perfused mouse hearts. *A*: infarct size (IS) is significantly reduced by AA. Effect is lost with coadministration of MPG (200 μM). *B*: heart volume plotted against infarct volume to ensure results are not skewed by an infarct-to-risk volume relationship that does not intersect the origin. There is a significant reduction in infarct size with AA that is lost with coadminstration 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* ($P < 0.05$).

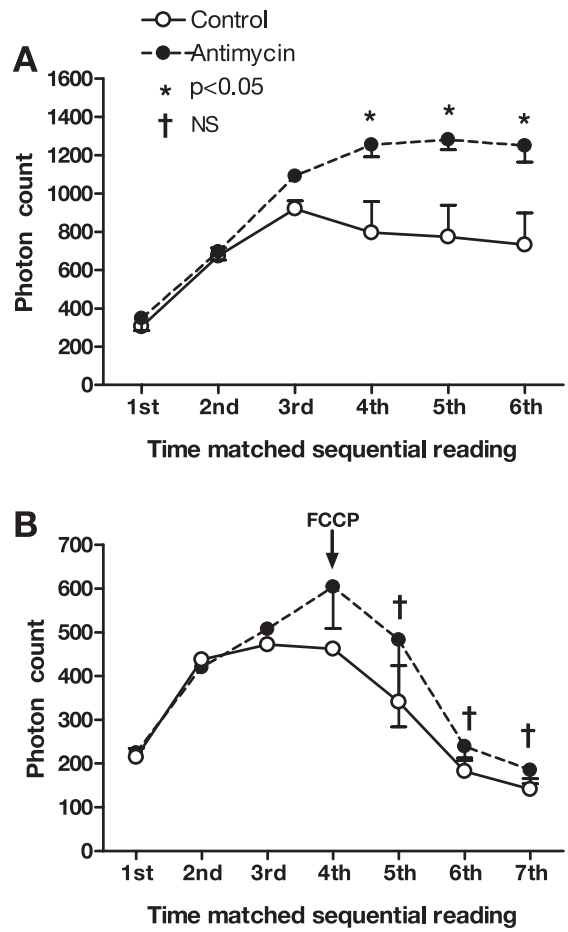

Fig. 3. Effect of AA on superoxide formation by rat isolated cardiocytes as assessed by lucigenin chemiluminesence. *A*: significantly higher chemiluminescent signal at each time point in the AA group compared with control. *B*: effect of FCCP (10 μM) is shown, which reduces the chemiluminescent signal.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

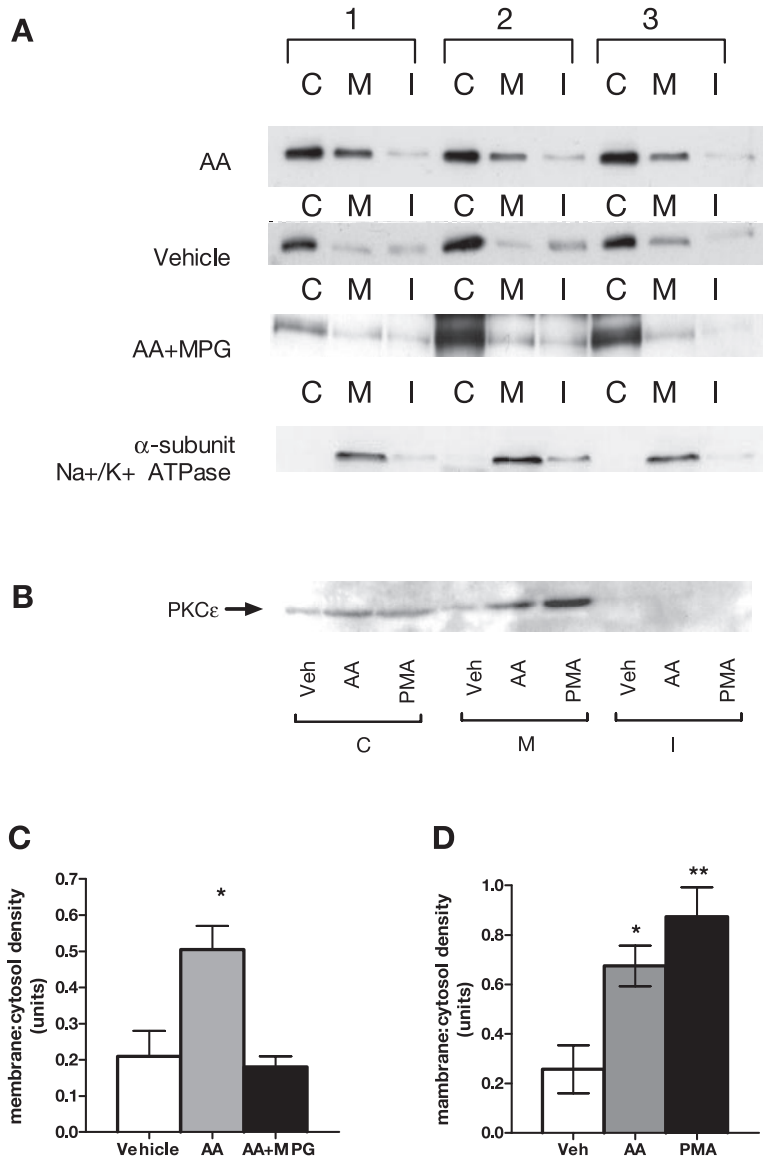

Fig. 4. *A*: effect of AA (0.1 μg/ml) on distribution of PKCε in 3 independently exposed hearts. Exposure to AA causes membrane localization, which is abolished by MPG. *B*: representative Western blot analysis of lysates from isolated rat ventricular myocytes following treatment with vehicle (Veh), AA (0.1 μg/ml), and PMA (100 nM, an archetypical activator of PKCε) showing cellular localization of PKCε. *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 ± 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 two-way 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 μg/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 ± 4.2 vs. 43.4 ± 2.4%; $P = 0.03$) (Fig. 2). The cardioprotective effect of antimycin A was abolished by the bracketing with MPG (51.6 ± 6.6 vs. 26.2 ± 4.2%; $P < 0.05$). There was no significant difference between control and MPG alone (43.4 ± 2.4 vs. 34.0 ± 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. 2*B*).

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

Table 2. *Characteristics of groups composed of PKCε-targeted hearts exposed to antimycin A*

Experimental Groups	pkcε$^{-/-}$-DMSO	pkcε$^{+/+}$-DMSO	pkcε$^{-/-}$-Antimycin A	pkcε$^{+/+}$-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±5.2‡	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

Values are means ± SE. *$P < 0.01$ vs. pkcε$^{+/+}$-antimycin A; †$P < 0.05$ vs. pkcε$^{+/+}$-antimycin A; ‡$P < 0.05$ vs. pkcε$^{+/+}$-antimycin A and vs. pkcε$^{+/+}$-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 PKCε by antimycin A. Translocation of PKCε 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 PKCε 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 PKCε 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 PKCε. To further test the role of PKCε in antimycin A-mediated cardioprotection, we examined the effect of antimycin A (0.1 μg/ml) or DMSO vehicle (0.01%) in pkcε$^{-/-}$ and pkcε$^{+/+}$ buffer-perfused isolated mouse hearts. The hearts were randomized into four groups (Fig. 1*B*). 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 pkcε$^{-/-}$-DMSO group had significantly lower body weight than the pkcε$^{+/+}$-antimycin A group (23.8 ± 0.8 vs. 28.5 ± 1.2 g; $P = 0.01$). The pkcε$^{-/-}$-antimycin A group had significantly lower body and heart weight compared with the pkcε$^{+/+}$-antimycin A group (23.8 ± 1.0 vs. 28.5 ± 1.2 g; $P = 0.02$ and 105.3 ± 5.2 vs. 140 ± 3.9 mg; $P = 0.02$, respectively). The pkcε$^{+/+}$-DMSO group had significantly higher heart weight than the pkcε$^{-/-}$-antimycin A group (140.1 ± 11.1 vs. 105.3 ± 5.2 mg; $P = 0.04$). However, there was no statistical difference in heart volume or baseline hemodynamic characteristics. Comparison by two-way ANOVA to take into account time as well as group did not show any significant difference in flow or hemodynamic parameters.

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

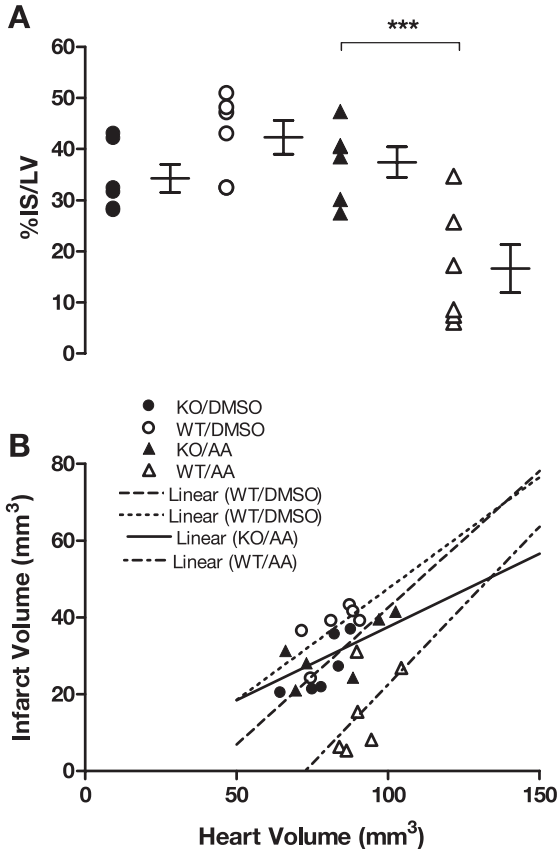

Fig. 5. Analysis of infarct size in Langendorff-perfused hearts from a pkcε-targeted line. *A*: cardioprotective effect of AA is lost in pkcε$^{-/-}$ (knock-out, KO), but not in pkcε$^{+/+}$ (wild-type, WT), hearts. ***$P < 0.001$. *B*: confirmation that this finding persists in an ANCOVA of heart volume 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 pkcε$^{-/-}$ mice were not protected by antimycin A compared with pkcε$^{+/+}$ mice ($P = 0.036$) (Fig. 5*B*).

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 PKCε translocates to the membrane in response to antimycin A, an effect blocked by MPG. This phenomenon of PKCε translocation being mediated via ROS production has been shown previously in cardiac tissue in response to anesthetic-mediated preconditioning as well as in other tissues (3, 24). Jung et al. (15) demonstrated that PKCε 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 PKCε activation. Furthermore, to test the role of PKCε in cardioprotection, we have used a targeted mouse line and demonstrated that PKCε 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 PKCε-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 PKCε activation and the downstream target p38-MAPK is given more credence by studies showing that xenon-preconditioning results in both PKCε 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 PKCε 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 PKCε. A putative mechanism of PKCε activation by ROS is through direct tyrosine phosphorylation because regulatory and catalytic domains of PKC are susceptible to oxidative modification by H_2O_2 (12), a mechanism previously described in a number of PKC isoforms including PKCε (10, 11). However, this is an area that requires further study to elucidate the exact mechanism(s) by which ROS activates PKCε and leads to subsequent cardiac protection in our model.

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GRANTS

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