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Ischemic Preconditioning: A Potential Role for Protein *S*-Thiolation?

PHILIP EATON,[1] ROBERT M. BELL,[2] ALISON C. CAVE,[1] and MICHAEL J. SHATTOCK[2]

ABSTRACT

Oxidant stress plays a crucial role in the triggering of cardioprotection involving ischemic preconditioning (IPC). We have used biotin-tagged cysteine to probe for redox-modified proteins in IPC protocols. Cysteine was biotinylated and introduced into isolated rat hearts. *S*-Thiolated proteins were detected and quantified using nonreducing western blots probed with streptavidin–horseradish peroxidase. Controls (15 min of aerobic perfusion plus 5 min of 0.5 m*M* biotin-cysteine plus 5 min of aerobic perfusion) showed low-level protein *S*-thiolation. Hearts preconditioned with 5 min of ischemia and reperfused for 5 min with biotin-cysteine plus 5 min of aerobic perfusion showed increased thiolation (160%) that was fully blocked by the antioxidant mercaptopropionylglycine, which is also known to block IPC. "Preconditioning" agonists (phorbol 12-myristate 13-acetate or phenylephrine) or oxidants (hydrogen peroxide or diamide) administered during aerobic preparations to biotin-cysteine-loaded hearts induced efficient protein *S*-thiolation. Preconditioning agonist-induced *S*-thiolation was significantly attenuated by diphenyleneiodonium (a flavoprotein inhibitor) or by the protein kinase C inhibitor bisindolylmaleimide I. Additional studies testing the role of a Nox2-containing NAD(P)H oxidase as the source of the oxidant stress essential to the triggering IPC showed that protein *S*-thiolation was the same in wild-type and Nox2 knockout mice. *Antioxid. Redox Signal* 7, 882–888.

INTRODUCTION

THE CELLULAR MECHANISMS underlying the powerful protection afforded by ischemic preconditioning (IPC) have been the center of intense research. Many of the components of the signaling pathways have been identified although the "end-effector" remains elusive. Although there is much controversy surrounding the exact order of the signaling components, the weight of evidence is consistent with a scheme in which short ischemic durations lead to G protein-coupled receptor activation and the subsequent activation of protein kinase C (PKC). This in turn leads to the opening of the ATP-sensitive mitochondrial potassium channel ($^{mito}K_{ATP}$). For some time, the $^{mito}K_{ATP}$ was considered by many to be the end-effector of protection, but now evidence suggests the $^{mito}K_{ATP}$ is simply another link in a complex signaling cascade. For example, Downey and colleagues (32) suggest that opening of $^{mito}K_{ATP}$ channels "short-circuits" the proton gradient across the inner mitochondrial membrane, which, through some as yet unspecified mechanism, generates reactive oxygen species (RDS) that, in turn, activate a variety of kinase pathways leading to an as yet unidentified end-effector. Garlid and others have also suggested that this might involve the further activation of PKC, leading to enhanced $^{mito}K_{ATP}$ channel opening (23).

Although its position in the above cascade may be uncertain, the weight of evidence suggests that PKC activation is an essential prerequisite for cardioprotection in most, but not all, species (5). Activation of PKC by phorbol esters or agonists of receptors that couple to this kinase can mimic protection (10). Conversely, blockers of PKC (bisindolylmaleimide or Ro compounds) during the trigger of IPC can block protection (3, 5, 31, 39). Another component of this pathway that is widely accepted is oxidative stress. The generation of free radicals is known to be integral to the trigger phase of preconditioning, as antioxidants given at this time block protection

[1]Department of Cardiology and [2]Cardiac Physiology, Cardiovascular Division, King's College London, London, U.K.
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(20). Similarly, prooxidants can replace the trigger phase and induce protection (1, 37, 40). Thus, it is clear that protection by preconditioning has a dual requirement for PKC, as well as oxidative stress.

Recently, we have shown that biotinylated cysteine (biotin-cysteine), when administered to the isolated rat heart, acts as a probe for proteins that are *S*-thiolated during times of oxidative stress. In this study, we investigate protein *S*-thiolation during the trigger phase of IPC, as well as by established pharmacological interventions that both activate PKC and induce preconditioning-like cardioprotection. We also test the hypothesis that a Nox2-containing NAD(P)H oxidase is the molecular source of IPC-induced oxidant stress leading to protein *S*-thiolation by using a Nox2 knockout mouse.

MATERIALS AND METHODS

Chemicals

These were obtained from Sigma Chemical (U.K.) or BDH (U.K.) unless stated and were of AnalaR grade or above. Biotin-cysteine was prepared as described previously (17).

Animals

Male Wistar rats (200–250 g) were used throughout this study and were obtained from BK Universal. The animals were maintained humanely in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and *Guide for Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 85-23, revised 1985). Male Nox2 knockout mice ($Nox2^{-/-}$) and wild-type controls with the same genetic background (C57BL/6J) were obtained from our in-house animal breeding facility. $Nox2^{-/-}$ mice were originally generated by Dinauer and colleagues (33) and obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.).

Isolated heart preparations

Male rats (220–250 g) or mice (20–30 g) were anesthetized with sodium pentobarbitone (40 mg/kg i.p.) and rats injected with sodium heparin (200 IU) via the femoral vein. Hearts were then rapidly excised, placed in cold (4°C) bicarbonate buffer, and cannulated via the aorta. Hearts were then perfused in nonrecirculating Langendorff mode using a peristaltic pump and feedback system (STH Pump Controller, AD Instruments, Castle Hill, Australia). Using this system, rat hearts were perfused at a constant flow of 12 ml/g of tissue/min (and perfusion pressure monitored) and mouse hearts at a constant pressure of 80 ± 1 mm Hg (and coronary flow monitored). The bicarbonate buffer contained the following (in m*M*): NaCl 118.5, KCl 3.1, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgCl_2$ 1.2, $CaCl_2$ 1.4, and glucose 10, pH 7.4 when gassed with 95% O_2 and 5% CO_2 at 37°C.

Perfusion protocols

Control levels of protein *S*-thiolation were measured by aerobically perfusing isolated hearts for 15 min, introducing biotin-cysteine (0.5 m*M*) for 5 min during aerobic perfusion, with a further 5 min of aerobic perfusion alone (to fully wash out biotin-cysteine from the vascular space). To investigate *S*-thiolation during IPC, rat hearts were aerobically perfused for 15 min, made globally ischemic (by zero flow), reperfused with biotin-cysteine (0.5 m*M*) for 5 min, followed by 5 min of washout. In studies investigating the role of the Nox2-containing NAD(P)H oxidase, mouse hearts were subjected to a similar protocol apart from the preconditioning stimulus involving two cycles of 5 min of ischemia and 5 min of reperfusion.

The effect of mercaptopropionylglycine (MPG; 1 m*M*) on preconditioning-induced *S*-thiolation was assessed by preloading the isolated rat hearts with MPG for 5 min immediately before the onset of ischemia. Phorbol 12-myristate 13-acetate (PMA; 200 n*M*)-, phenylephrine (10 μ*M*)-, hydrogen peroxide (10 μ*M*)-, or diamide (0.5 m*M*)-induced protein *S*-thiolation was assessed by aerobically perfusing isolated hearts for 15 min, introducing biotin-cysteine (0.5 m*M*) for 5 min, and then treating with either of the test compounds for 5 min. The ability of the flavoprotein inhibition or PKC inhibition to modulate protein *S*-thiolation induced by PMA or phenylephrine was investigated by loading either diphenyleneiodonium (DPI; 1 μ*M*) or bisindolylmaleimide I (10 μ*M*) into the hearts for 5 min prior to agonist treatment (at the same time as biotin-cysteine), with continued inhibitor treatment simultaneously with the agonist. At the end of each protocol, hearts were frozen and stored in liquid nitrogen until subsequent analysis.

Protein analysis

Ventricular tissue was homogenized (10 ml of buffer/g of cardiac tissue) on ice in 100 m*M* Tris-HCl, 5 m*M* EGTA, 5 m*M* EDTA, benzamidine (10 μg/ml), leupeptin (100 ng/ml), aprotinin (100 ng/ml), pH 7.0, using a Polytron tissue grinder. A sample of the homogenate was reconstituted in sodium dodecyl sulfate (SDS) buffer without a reducing agent. SDS–polyacrylamide gel electrophoresis was carried out using the Bio-Rad Mini Protean II system. In some samples, to confirm that *S*-thiolated/biotinylated proteins were modified via disulfide formation, 20 m*M* dithiothreitol was added to the SDS buffer. After electrophoresis, samples were transferred to polyvinylidene difluoride using a Pharmacia system semi-dry blotter. *S*-Thiolated proteins were identified by virtue of their biotin tag using streptavidin–horseradish peroxidase (HRP) (Amersham, U.K.) and the enhanced chemiluminescence reagent HRP (Amersham, U.K.). Polyvinylidene difluoride membranes were stained with Coomassie Blue to confirm blotting integrity. Western blots were digitized using a flat-bed scanner (HP Scanjet 11C). The digitized image was then quantitatively analyzed for total protein *S*-thiolation in each lane using the NIH Image software (Freeware, NIH, Baltimore, MD, U.S.A.).

Statistics

Results are presented as means ± SEM. Differences between groups were assessed using ANOVA, followed by a Bonferroni *t* test. Differences were considered significant at the 95% confidence level.

RESULTS

IPC and exogenous oxidants induce protein S-*thiolation*

Figure 1 shows a series of western blots probed with streptavidin-HRP used to detect protein *S*-thiolation in isolated rat hearts. Quantitative analysis (Fig. 2) showed IPC induced a significant (160%, $p < 0.05$) increase in protein *S*-thiolation. This IPC-induced increase in protein *S*-thiolation was fully attenuated, back to basal levels, in hearts that were loaded with the thiol-containing compound MPG. The thiol-selective oxidant was notably effective in inducing robust protein *S*-thiolation. Comparable large increases were also achieved by treatment of biotin-cysteine-loaded hearts with hydrogen peroxide for 5 min at 10 μ*M* (data not shown).

Agonists that stimulate PKC induce protein S-*thiolation*

Figure 3 shows that, in the absence of ischemia and reperfusion, the well-established "preconditioning agonists" PMA and phenylephrine induced significant protein *S*-thiolation ($p < 0.05$). The extent of protein *S*-thiolation induced by these agonists under aerobic conditions was, in fact, quantitatively comparable to that induced by IPC. The PMA- and phenylephrine-induced protein *S*-thiolation was efficiently blocked by both the NAD(P)H oxidase inhibitor DPI and the PKC inhibitor bisindolylmaleimide.

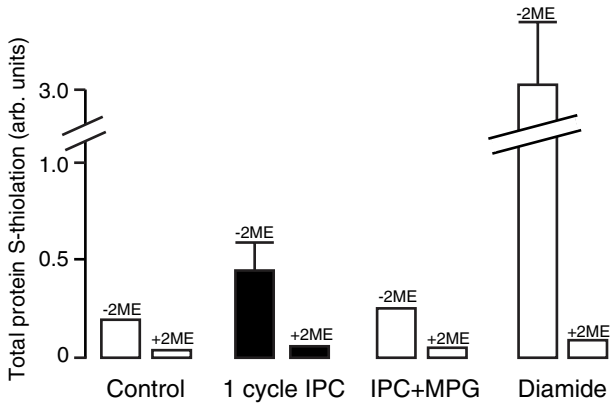

FIG. 2. Quantitation of western immunoblot data presented in Fig. 1 ($n = 4$). Clearly IPC induced a significant ($p < 0.05$) increase in protein *S*-thiolation, which was normalized to control levels when the experiment was repeated in the presence of MPG. The extensive protein *S*-thiolation present in Fig. 1 is confirmed by the quantitative analysis.

Protein S-*thiolation during IPC in the Nox2 knockout mouse*

Figure 4 shows western blots probed with streptavidin-HRP to detect protein *S*-thiolation in hearts from wild-type or Nox2 knockout mice loaded with biotin-cysteine. The blots were quantitated (Fig. 5), which showed IPC induced signifi-

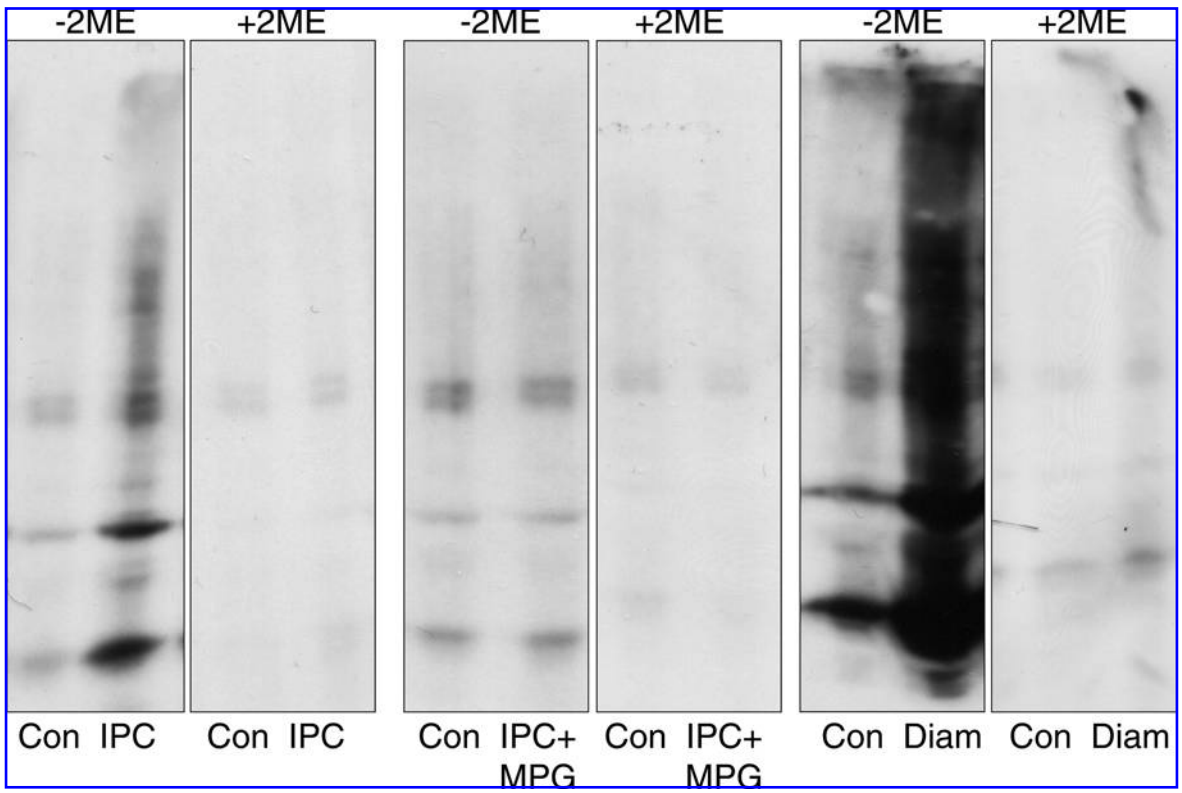

FIG. 1. Western blot probed with streptavidin-HRP shows protein *S*-thiolation during IPC, which can be blocked when the heart is loaded with MPG. The thiol-selective oxidant diamide induced very high levels of protein *S*-thiolation, as would be expected, and serves as a good positive control. The signals were abolished following treatment of the sample with 2-mercaptoethanol (2ME), consistent with the signal being dependent on a disulfide bond.

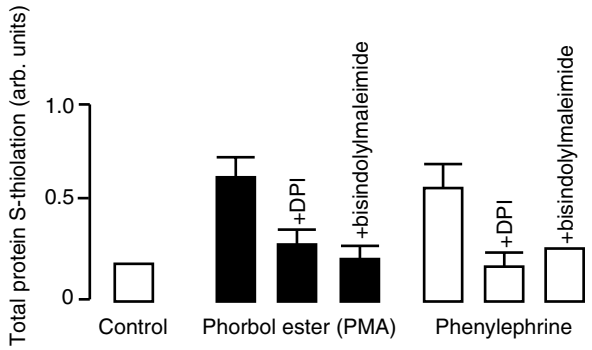

FIG. 3. PMA and phenylephrine induced significant ($p < 0.05$) protein *S*-thiolation, comparable to that following the trigger phase of IPC. PMA- and phenylephrine-induced protein *S*-thiolation was attenuated by either DPI or treatment with the PKC inhibitor bisindolylmaleimide 1.

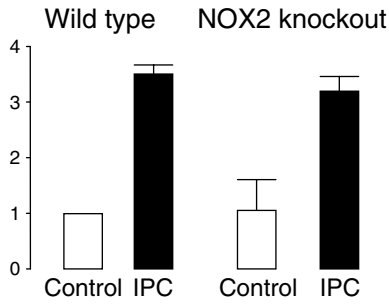

FIG. 5. Quantitation of western immunoblot data presented in Fig. 4. As in the isolated rat heart studies, IPC induced significant protein *S*-thiolation in murine myocardium, which was unaffected by the absence of the NAD(P)H oxidase subunit protein Nox 2 ($n = 4$).

cant protein *S*-thiolation in murine myocardium, as it did in the rat heart. This IPC-induced *S*-thiolation was achieved regardless of the presence of the Nox2 isoform of NAD(P)H oxidase.

DISCUSSION

Oxidative stress during the trigger of IPC is crucial to the genesis of protection. Protein *S*-thiolation is an established oxidative modification of proteins, which occurs in tissues during oxidative stress. We previously showed that cardiac protein *S*-thiolation occurs during ischemia and reperfusion and identified a number of the oxidized proteins (17), as well as some the functional consequences of this reversible, oxidative modification (15, 16). A number of the proteins that became *S*-thiolated during cardiac ischemia and reperfusion have obvious cardioprotective potential, as discussed below.

Cardioprotective interventions also promote protein S*-thiolation*

A lot of the evidence for the involvement of a specific mechanism in IPC is based on pharmacological studies. Agonists are used to activate a specific process, independently of preconditioning cycles, to test whether they initiate protection. Of course, these antiischemic interventions could provide protection by a mechanism not involving preconditioning. Perhaps stronger evidence comes from studies where inhibitors of the implicated process are given during the trigger cycles of preconditioning to test if they block protection. By using this logic, it is clear that protein *S*-thiolation could have a role in IPC. This is because protein *S*-thiolation occurs not only during the trigger of IPC, but also following treatment with agonists (phorbol ester, phenylephrine) or oxidants, all of which can induce the preconditioned state. In addition, preconditioning-induced *S*-thiolation is blocked by the thiol antioxidant compound MPG. This is of note because antioxidants, including MPG, are known to block protection when given during the trigger phase of preconditioning.

It is clear that there is a close association between agents or mechanisms that promote oxidative stress and those that induce protection. The key question is whether protein *S*-thiolation during the preconditioning trigger is functionally important in establishing protection, or is it just an epiphenomenon? It is possible that *S*-thiolation simply functions as an index of cellular redox status, and the fact that MPG administered during the trigger abolishes both protection and *S*-thiolation is simply coincidental. However, we believe there is a strong case for protein *S*-thiolation via oxidase-dependent free radical production being an integral component in establishing protection, as considered below.

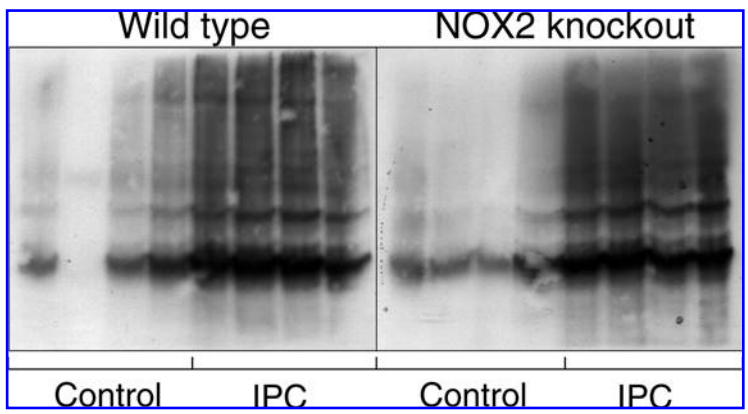

FIG. 4. Western blot probed with streptavidin-HRP to detect protein *S*-thiolation in wild-type or Nox2 knockout mice subjected to either aerobic perfusion (control) or IPC.

A potential role for protein S*-thiolation in IPC*

S-Thiolation of proteins during the trigger of IPC may be important to the establishment of protection in three ways: (a) Oxidative damage is known to contribute to ischemia and reperfusion injury, as highlighted by studies that show antioxidants given at this time can block injury measured in terms of infarction and postischemic electromechanical recovery. Protein cysteines are targets of this oxidative burden and may become irreversibly or terminally oxidized to sulfonic acid. If this happened to functionally critical cysteines, which tends to happen, as they are generally the most reactive, these proteins would become permanently dysfunctional: *de novo* synthesis would then be needed to replace them, which is time- and energy-consuming and could compromise tissue survival. However, if the proteins become oxidized by a reversible oxidation, such as *S*-thiolation, protein function can be rapidly restored when basal redox status returns. As protein *S*-thiolation occurs during preconditioning, this provides a mechanism for protecting critical protein thiols that might otherwise be terminally oxidized during a subsequent damaging period of ischemia and reperfusion. (b) The integral role of oxidative stress in the generation of protection is clear, but it is not known how redox changes transduce into a downstream protective effect. Reversible cysteine-targeted oxidations, such as *S*-thiolation, provide a *sensor mechanism* whereby the oxidative stress can be transduced into a functional response, including the initiation of phosphorylation-dependent signaling. Many classes of protein are regulated by cysteine-targeted oxidation, including metabolic enzymes (8, 34), ion translocators (19, 24), structural proteins (7), and signaling molecules. A variety of signaling molecules are regulated by *S*-thiolation, and some have been implicated in preconditioning, including receptors (28, 36), G proteins (25, 30), kinases (21, 38), phosphatases (29), and transcription factors (9). Previously, we showed that a number of signaling molecules are subject to protein *S*-thiolation in cardiac tissue following ischemia and reperfusion, including protein-tyrosine phosphatase 1B, PKC, and the small G protein ras (17). (c) In earlier studies, we also showed *S*-thiolation of a number of cardiac proteins that could directly influence myocardial ischemic tolerance, including the stress proteins heat shock protein 27 (HSP27) and HSP60 (15, 17, 18). HSP27 *S*-thiolation plays an important regulatory role, controlling the aggregate size of this protein. Thus, protein *S*-thiolation not only transduces an oxidative signal into a phosphorylation cascade, but may also directly regulate cardioprotective proteins. There are an increasing number of proteins that are known to be regulated by reversible cysteine oxidation by *S*-thiolation, nitrosylation or nitrosation, reactive lipids, or oxygen (13, 14, 27, 35). In addition, interprotein disulfide bond formation may also be important in the response of tissues and cells to oxidative stress (4, 12). It is clear therefore that protein *S*-thiolation may provide an important link between oxidative stress and cardioprotection by directly affecting protein function, as well as initiating kinase-signaling events.

The role of Nox2-containing NAD(P)H oxidase

Although it is accepted that oxidative stress plays a key signaling role in cardioprotection by IPC, neither the source nor the targets of these free radicals have been equivocally identified. One suggestion is that the oxidative stress results from leakage of electrons from mitochondrial electron transport following the opening of the $^{mito}K_{ATP}$ channel. Much of the evidence for this comes from studies using diazoxide and 5-hydroxydecanoate (5-HD) as putative selective openers and blockers of the mitochondrial channel (6, 11, 32). Unfortunately, these agents are far from specific: diazoxide blocks succinate dehydrogenase and 5-HD can be metabolized to 5-HD CoA, a mitochondrial β-oxidation substrate (26). What is clear, however, is that PKC and oxidant stress are both intimately involved in protection. An alternative mechanism of PKC-dependent oxidant production involves the PKC-dependent stimulation of oxidases, such as NAD(P)H oxidase, and as such we have recently shown that in the mouse heart, NAD(P)H oxidase is an essential prerequisite for IPC (2).

In the present study, we have shown that DPI, a flavoprotein inhibitor that efficiently prevents the function of many oxidases, including NAD(P)H oxidase, limits phorbol ester- and phenylephrine-induced protein oxidation to a similar extent as the PKC inhibitor bisindolylmaleimide. These data suggest that the radical production that is crucial to IPC could originate from the activation of NAD(P)H oxidase by PKC. It is important to note that the subcellular location of NAD(P)H oxidase in the myocardium is unknown and, if it resides in the mitochondria, it could interact with mitochondrial components and drugs in a way that is entirely consistent with the literature implicating this organelle in the signaling pathway. We therefore considered a scenario where surface receptor stimulation during IPC activates PKC, which phosphorylates NAD(P)H oxidase, promoting increased radical production and protein *S*-thiolation. To investigate the role of NAD(P)H oxidase in IPC directly, we made use of knockout mice engineered to be deficient in Nox2, a crucial subunit of the enzyme complex. However, IPC-induced protein *S*-thiolation was identical in both wild-type and the $Nox2^{-/-}$ mice.

As we have previously demonstrated NAD(P)H oxidase to be crucial to the oxidative signaling pathway in IPC (2), the lack of a gross difference in protein *S*-thiolation implies either (a) protein *S*-thiolation is an epiphenomenon that reports cellular redox state, but is not causally involved in the IPC signaling pathway, or (b) both *S*-thiolation and NAD(P)H oxidase are essential components of the signaling pathway, but the quantitative contribution of the Nox2-containing NAD(P)H oxidase-mediated *S*-thiolation must be small in comparison with that from other sources of ROS. That is, "knocking out" this small fraction of protein *S*-thiolation is crucial to the signaling pathway, but this small but important change cannot be seen against the high background of IPC-induced *S*-thiolation. It is possible that, in the knockout mice, although the remaining sources of ROS are sufficient to induce a generalized protein *S*-thiolation, this is not produced in the appropriate compartment to induce a protective response. Indeed this concept is supported by studies in pressure overload model of left ventricular hypertrophy in which loss of Nox2-derived ROS specifically protects against contractile dysfunction despite similar overall levels of hypertrophy and NAD(P)H oxidase activity (22).

In summary, this study demonstrates that both IPC and its pharmacological mimetics can induce protein *S*-thiolation of

cardiac proteins. The source of these radicals is DPI-sensitive, indicating the involvement of flavoprotein oxidase. Although the current studies indicate that the contribution of Nox2-containing NAD(P)H oxidase to overall *S*-thiolation is small, its functional impact has yet to be defined. *S*-Thiolation of cardiac proteins may play a role in IPC either by directly protecting key cellular proteins from terminal oxidation or by activating signaling pathways or end-effectors that limit injury following ischemia and reperfusion.

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ABBREVIATIONS

biotin-cysteine, biotinylated cysteine; DPI, diphenyleneiodonium; 5-HD, 5-hydroxydecanoate; HRP, horseradish peroxidase; HSP, heat shock protein; IPC, ischemic preconditioning; $^{mito}K_{ATP}$, ATP-sensitive mitochondrial potassium channel; MPG, mercaptopropionylglycine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

REFERENCES

1. 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. Bell R, Cave A, Johar S, Shah AM, and Shattock MJ. The role of a gp91phox-containing NADPH oxidase in early ischemic preconditioning. *Circulation* 108: 212, 2003.
3. Belosjorow S, Schulz M, Gre P, Jansen J, Michel M, and Heusch G. Blockade of p38 MAP kinase abolishes ischemic preconditioning in pigs. *Circulation* 104: 484, 2001.
4. Brennan JP, Wait R, Begum S, Bell JR, Dunn MJ, and Eaton P. Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J Biol Chem* 279: 41352–41360, 2004.
5. Brooks G and Hearse DJ. Role of protein kinase C in ischemic preconditioning: player or spectator? *Circ Res* 79: 627–630, 1996.
6. Carroll R, Gant VA, and Yellon DM. Mitochondrial K-ATP channel opening protects a human atrial-derived cell line by a mechanism involving free radical generation. *Cardiovasc Res* 51: 691–700, 2001.
7. Chai YC, Ashraf SS, Rokutan K, Johnston RB, and Thomas JA. *S*-Thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst—evidence against a role for glutathione disulfide. *Arch Biochem Biophys* 310: 273–281, 2001.
8. Cheung P-Y, Danial H, Jong J, and Schultz R. Thiols protect the inhibition of myocardial aconitase by peroxynitrite. *Arch Biochem Biophys* 350: 104–108, 1998.
9. Choi HJ, Kim SJ, Mukhopadhyay P, Cho S, Woo JR, Storz G, and Ryu SE. Structural basis of the redox switch in the OxyR transcription factor. *Cell* 105: 103–113, 2001.
10. Cohen MV, Baines CP, and Downey JM. Ischemic preconditioning: from adenosine receptor to K-ATP channel. *Annu Rev Physiol* 62: 79–109, 2000.
11. Cohen MV, Yang XM, Liu GS, Heusch G, and Downey JM. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K-ATP channels. *Circ Res* 89: 273–278, 2001.
12. Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, and Schubert D. Protein disulfide bond formation in the cytoplasm during oxidative stress. *J Biol Chem* 279: 21749–21758, 2004.
13. Eaton P, Li JM, Hearse DJ, and Shattock MJ. Formation of 4-hydroxy-2-nonenal-modified proteins in ischemic rat heart. *Am J Physiol* 45: H935–H943, 1999.
14. Eaton P, Hearse DJ, and Shattock MJ. Lipid hydroperoxide modification of proteins during myocardial ischemia. *Cardiovasc Res* 51: 294–303, 2001.
15. Eaton P, Fuller W, and Shattock MJ. *S*-Thiolation of HSP27 regulates its multimeric aggregate size independently of phosphorylation. *J Biol Chem* 277: 21189–21196, 2002.
16. Eaton P, Wright N, Hearse DJ, and Shattock MJ. Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. *J Mol Cell Cardiol* 34: 1549–1560, 2002.
17. Eaton P, Byers HL, Leeds N, Ward MA, and Shattock MJ. Detection, quantitation, purification and identification of cardiac proteins *S*-thiolated during ischemia and reperfusion. *J Biol Chem* 277: 9806–9811, 2002.
18. Eaton P, Leeds N, Campbell J, Leung K-Y, Byers H, Ward M, and Shattock MJ. Proteomic analysis identifies mitochondrial proteins which are *S*-thiolated during ischemia and reperfusion. *Circulation* 106: 247, 2002.
19. Eu JP, Sun J, Xu L, Stamler JS, and Meissner G. The skeletal muscle calcium release channel: coupled O^2 sensor and NO signaling functions. *Cell* 102: 499–509, 2000.
20. Fryer RM, Hsu AK, Eells JT, Nagase H, and Gross GJ. Opioid-induced second window of cardioprotection: potential role of mitochondrial KATP channels. *Circ Res* 84: 846–851, 1999.
21. Gopalakrishna R and Jaken S. Protein kinase C signaling and oxidative stress. *Free Radic Biol Med* 28: 1349–1361, 2000.
22. Grieve DJ, Byrne JA, Cave AC, and Shah AC. Pivotal role of NADPH oxidase in cardiac contractile dysfunction and fibrosis induced by chronic pressure-overload. *Eur Heart J* 24: 93.A458, 2003.
23. Grover GJ and Garlid KD. ATP-sensitive potassium channels: a review of their cardioprotective pharmacology. *J Mol Cell Cardiol* 32: 677–695, 2000.
24. Haddock PS, Shattock MJ, and Hearse DJ. Modulation of cardiac Na^+-K^+ pump current—role of protein and nonprotein sulfhydryl redox status. *Am J Physiol* 38: H297–H307, 1995.

25. Hallak HM and Fox PL. Oxidant stress induces covalent glutathiolation of hetrotrimeric G-proteins: a novel post-translational modification of Gq(alpha). *Circulation* 98: 1142, 1998.
26. Hanley PJ, Mickel M, Löffler M, Brandt U, and Daut J. K_{ATP} channel-independent targets of diazoxide and 5-hydroxydecanoate in the heart. *J Physiol* 542: 735–741, 2002.
27. Kuncewicz T, Sheta EA, Goldknopf IL, and Kone BC. Proteomic analysis of *S*-nitrosylated proteins in mesangial cells. *Mol Cell Proteomics* 2: 156–163, 2003.
28. Liu W, Akhand AA, Kato M, Miyate T, Kurakawa K, Uchida K, and Nakashima I. 4-Hydroxynonenal triggers an epidermal growth factor receptor-linked signal pathway for growth inhibition. *J Cell Sci* 112: 2409–2417, 1999.
29. Mahadev K, Zilbering A, Zhu L, and Goldstein BJ. Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1B in vivo and enhances the early insulin action cascade. *J Biol Chem* 276: 21938–21942, 2001.
30. Mallis RJ, Buss JE, and Thomas JA. Oxidative modification of H-ras: *S*-thiolation and S-nitrosylation of reactive cysteines. *Biochem J* 355: 145–153, 2001.
31. Nakano A, Cohen MV, Critz S, and Downey JM. SB203580, an inhibitor of p38 MAPK, abolishes infarct-limiting effect of ischemic preconditioning in isolated rabbit hearts. *Basic Res Cardiol* 95: 466–471, 2000.
32. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, and Downey JM. Opening of mitochondrial K-ATP channels triggers the preconditioned state by generating free radicals. *Circ Res* 87: 460–466, 2000.
33. Pollock JD, Williams DA, Gifford MAC, Li LL, Du X, Fisherman J, Orkin SH, Doerschuk CM, and Dinauer MC. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet* 9: 202–209, 1995.
34. Reddy S, Jones AD, vander Vliet A, and Cross CE. Glutathiolation of creatine kinase during oxidative stress. *Free Radic Biol Med* 25: 223, 1998.
35. Saurin AT, Neubert H, Brennan JP, and Eaton P. Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. *Proc Natl Acad Sci U S A* 101: 17982–17987, 2004.
36. Suc I, Meilhac O, Lajoie-Mazenc I, Vandaele J, Jurgens G, Salvayre R, and Negre-Salvayre A. Activation of EGF receptor by oxidized LDL. *FASEB J* 12: 665–671, 1998.
37. Vanden Hoek TL, Becker LB, Shao ZH, Li CQ, and Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* 273: 18092–18098, 1998.
38. Ward NE, Pierce DS, Chung SE, Gravitt KR, and O'Brian CA. Irreversible inactivation of protein kinase C by glutathione. *J Biol Chem* 273: 12558–12566, 1998.
39. Weinbrenner C, Liu GS, Cohen MV, and Downey JM. Phosphorylation of tyrosine 182 of p38 mitogen activated protein kinase correlates with the protection of preconditioning in the rabbit heart. *J Mol Cell Cardiol* 29: 2383–2391, 1997.
40. Zhou XB, Zhai XL, and Ashraf M. Direct evidence that initial oxidative stress triggered by preconditioning contributes to second window of protection by endogenous antioxidant enzyme in myocytes. *Circulation* 93: 1177–1184, 1996.

Address reprint requests to:
Philip Eaton, Ph.D.
Department of Cardiology
Cardiovascular Division
The Rayne Institute
St. Thomas' Hospital
London, SE1 7EH, U.K.

E-mail: philip.eaton@kcl.ac.uk

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