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Role of the Protein Kinase C-ε–Raf-1–MEK-1/2–p44/42 MAPK Signaling Cascade in the Activation of Signal Transducers and Activators of Transcription 1 and 3 and Induction of Cyclooxygenase-2 After Ischemic Preconditioning

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Abstract

Background—Although Janus kinase (JAK)–mediated Tyr phosphorylation of signal transducers and activators of transcription (STAT) 1 and 3 is essential for the upregulation of cyclooxygenase-2 (COX-2) and the cardioprotection of late preconditioning (PC), the role of Ser phosphorylation of STAT1 and STAT3 in late PC and the upstream signaling mechanisms responsible for mediating Ser phosphorylation of STAT1 and STAT3 remain unknown.

Methods and Results—In mice preconditioned with six 4-minute coronary occlusion/4-minute reperfusion cycles, we found that (1) ischemic PC activates the Raf1–mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase kinase (MEK) 1/2–p44/42 MAPK signaling pathway, induces phosphorylation of STAT1 and STAT3 on the Ser-727 residue, and upregulates COX-2 expression; (2) pSer-STAT1 and pSer-STAT3 form complexes with pTyr-p44/42 MAPKs in preconditioned myocardium, supporting the concept that Ser phosphorylation of these 2 factors is mediated by activated p44/42 MAPKs; and (3) activation of the Raf-1-MEK-1/2–p44/42 MAPK-pSer-STAT1/3 pathway and induction of COX-2 during ischemic PC are dependent on protein kinase C (PKC)-ε activity, as determined by both pharmacological and genetic inhibition of PKCε.

Conclusions—To our knowledge, this is the first study to demonstrate that ischemic PC causes Ser phosphorylation of STAT1 and STAT3 and that this event is governed by PKCε via a PKCε–Raf1-MEK1/2-p44/42 MAPK pathway. Furthermore, this is the first report that COX-2 expression in the heart is controlled by PKCε. Together with our previous findings, the present study implies that STAT-dependent transcription of the genes responsible for ischemic PC is modulated by a dual signaling mechanism that involves both JAK1/2 (Tyr phosphorylation) and PKCε (Ser phosphorylation).

Keywords

ischemia; myocardial infarction; signal transduction

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The online-only Data Supplement, which contains Figures I through VII and Table I, can be found at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.561522/DC1.

The late phase of ischemic preconditioning (PC) is a delayed protective adaptation whereby brief episodes of ischemia enhance the resistance of the heart to ischemia/reperfusion injury 12 to 72 hours later.[1] It is now appreciated that the development of late PC after the ischemic PC challenge (on day 1) occurs via activation of various signaling molecules, including protein kinase C (PKC)[2–4] and Janus Tyr kinases (JAKs),[5] which in turn activate latent transcription factors, including nuclear factor-κB and signal transducers and activators of transcription (STATs),[1,5,6] leading to the upregulation of cardioprotective genes such as inducible nitric oxide synthase and cyclooxygenase-2 (COX-2).[5,7–11] Activation of the JAK-STAT pathway is essential for the development of late PC, as inhibition of this pathway results in complete loss of protection against infarction.[5,10,11] However, the exact mechanism responsible for the recruitment of STATs after the PC ischemia remains incompletely understood. Furthermore, little is known about the mechanism by which ischemic PC regulates COX-2.

Our previous studies have shown that pretreatment with the JAK inhibitor AG-490 before ischemic PC blocked both the Tyr phosphorylation and activation of STAT1 and STAT3 and the subsequent upregulation of COX-2 protein, indicating a necessary role for STAT1 and STAT3 Tyr phosphorylation in the induction of COX-2.[5,10] Tyr phosphorylation of STAT1/3 is known to result in dimerization, nuclear transport, and transactivation of STAT-responsive genes.[12] However, full transcriptional activation of STATs requires not only Tyr phosphorylation (Tyr-701 in STAT1 and Tyr-705 in STAT3) but also Ser phosphorylation (Ser-727 in both STAT1 and STAT3).[12–14] At present, nothing is known about the role of Ser-727 phosphorylation of STAT1/3 in the upregulation of cardiac COX-2 and in the delayed cardioprotection of late PC. More generally, the role of Ser-727 phosphorylation in STAT-dependent transactivation in the heart is unknown.

The ε isoform of PKC is known to play a crucial role in the protective effects of late PC[2–4] and to induce activation of p44/42 mitogen-activated protein kinases (MAPKs) in conscious rabbits.[15] The Ser-727 residue of STAT1/3 is a recognition site for p44/42 MAPKs,[16] suggesting a link among activation of PKCκ and p44/42 MAPKs and Ser-727 phosphorylation of STAT1/3. The Ser/Thr kinase Raf-1 is known to phosphorylate MAPK/extracellular signal–regulated kinase kinase (MEK) 1/2, leading to activation of p44/42 MAPKs.[17] Because the promoter of the mouse *COX-2* gene contains the interferon-γ activation site (GAS) consensus sequence for the binding of STATs[5,18] and because our previous studies have shown that ischemic PC activates PKCε,[2] JAK1/2,[5,10] and STAT1/3,[5,10] we hypothesized that ischemic PC upregulates COX-2 protein expression via rapid activation of PKCε, which in turn activates a downstream pathway that includes Raf-1, MEK-1/2, and p44/42 MAPKs, leading to Ser phosphorylation of STAT1 and STAT3, transcription of the *COX-2* gene, and cardioprotection.

The overall objective of the present study was to test this hypothesis. The following specific questions were addressed: (1) Does ischemic PC induce Ser-727 phosphorylation of STAT1/3? (2) If so, does ischemic PC activate the Raf-1–MEK-1/2–p44/42 MAPK pathway? (3) Does pharmacological or genetic inhibition of PKCε prevent activation of the Raf-1–MEK-1/2–p44/42 MAPK pathway? The results demonstrate, for the first time, that ischemic PC causes Ser phosphorylation of STAT1/3 and activation of the Raf-1–MEK-1/2–p44/42 MAPK signaling pathway and that both of these events, as well as the subsequent induction of COX-2, are dependent on PKCε.

Methods

Animal Care

PKCε$^{-/-}$ mice and their wild-type (WT) littermates were generated by intercrossing 129SvJae×C57BL/6 hybrid PKCε$^{-/-}$ mice.[19] All mice were maintained in sterile microisolator cages under pathogenfree conditions. Food and water were autoclaved, and all handling was done under a laminar-flow hood according to standard procedures for maintaining pathogen-free transgenic mice. The mice were genotyped by polymerase chain reaction, as previously described, with DNA prepared from tissue samples taken at the end of the experiments.[9,10]

Experimental Preparation

The murine model of late PC has been previously described in detail.[5,9] In brief, mice were anesthetized and ventilated. After administration of antibiotics, the chest was opened through a midline sternotomy, and a nontraumatic balloon occluder was implanted around the mid-left anterior descending coronary artery by using an 8-0 nylon suture. Ischemic PC was elicited by a sequence of six 4-minute coronary occlusion/4-minute reperfusion (O/R) cycles.[5,9] To prevent hypotension, blood from a donor mouse was given during surgery.[5,9] Rectal temperature was maintained close to 37°C throughout the experiment.[5,9] The investigation consisted of 2 successive phases (A and B). The objective of phase A was to determine whether ischemic PC activates the Raf-1–MEK1/2– p44/42 MAPK signaling pathway and induces Ser phosphorylation of STAT1/3 and subsequent upregulation of COX-2. The objective of phase B was to determine whether the activation of this pathway is dependent on PKCε.

Phase A

Mice were assigned to 12 groups (Data Supplement Figure I; see http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.561522/DC1). Control groups (I, IV, VIII, and X) underwent 1 hour of the open-chest state without coronary occlusion. The PC groups (II, V, and IX) (PC-30′) underwent a sequence of six 4-minute coronary O/R cycles (a protocol that induces late PC[5,9]). Groups III and VI (CHE+PC-30′) received the PKC inhibitor chelerythrine (0.5 mg/kg IV dissolved in dimethyl sulfoxide) 5 minutes before the first occlusion, whereas group VII (dimethyl sulfoxide+PC-30′) received dimethyl sulfoxide 5 minutes before the first occlusion. All mice in groups I to IX were euthanized 30 minutes after the last reperfusion. Group XI (PC-24 hours) underwent six 4-minute coronary O/R cycles with no treatment, whereas group XII (CHE+PC-24 hours) received chelerythrine 5 minutes before the first occlusion. Mice in groups X to XII were euthanized 24 hours after the last occlusion or after sham coronary occlusion. Myocardial samples were rapidly removed from the ischemic/reperfused region or the left ventricle and frozen in LN_2 until used.

Phase B

Mice were assigned to 8 groups (Data Supplement Figure II). Groups XIV (WT PC-30′), XVI (PKCε$^{-/-}$ PC-30′), XVIII (WT PC-24 hours), and XX (*PKC*ε$^{-/-}$ PC-24 hours) underwent a sequence of six 4-minute coronary O/R cycles, whereas groups XIII (WT control-30′), XV (PKCε$^{-/-}$ control-30′), XVII (WT control-24 hours), and XIX (PKCε$^{-/-}$ control-24) underwent 1 hour of the open-chest state without coronary occlusion. Mice were euthanized either 30 minutes (groups XIII–XVI) or 24 hour (groups XVII–XX) after the sham coronary occlusion or the last reperfusion. In all groups, myocardial samples were rapidly removed from the ischemic/reperfused region or the left ventricle and frozen in LN_2 until used.

Preparation of Cytosolic, Membranous, and Nuclear Fractions

Cytosolic, membranous, and nuclear fractions were prepared from heart samples as previously described.[5,9,10]

Western Immunoblotting

Western immunoblotting analysis was performed with standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis Western immunoblotting techniques as previously described.[5,9,10] Details about the antibodies are provided in Data Supplement Table I. Equal loading was confirmed by staining with Ponceau-S.[5,9,10]

Coimmunoprecipitation

Myocardial homogenates (600 μg) were incubated with specific monoclonal anti-pTyr-p44/42 MAPK antibodies for 4 hours, followed by addition of protein G PLUS-Agarose-S (Santa Cruz) overnight at 4°C as per a previously described method.[5,10] After extensive washing, the anti-pTyr-p44/42 MAPK precipitates were subjected to immunoblotting with anti-pSer727-STAT1 or antipSer727-STAT3 antibodies.

Electrophoretic Mobility Shift Assays

The DNA binding activity of STAT1/3 was measured with electrophoretic mobility shift assays (EMSAs) as previously described.[5] A synthetic double-stranded probe with the sequence 5′-GATCAGCTTCAT*TT*C*C*CGT*AA*ATCCCTA-3′ (Gibco) was end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase and purified on a G-25 Sephadex column (Pharmacia). This oligonucleotide has the consensus sequence for GAS elements, as indicated by italics.[5,10,20]

Statistical Analysis

Data are reported as mean±SEM. Measurements were analyzed by ANOVA followed by unpaired Student *t* tests with the Bonferroni correction. In all Western blot analyses, the content of the specific protein of interest was expressed as a percentage of the corresponding protein in the anterior left ventricular wall of control mice.[5,9,10]

Results

A total of 132 mice (20 groups) were used.

Ischemic PC Induces Phosphorylation of Raf-1, MEK-1/2, and p44/42 MAPKs; Ser Phosphorylation and DNA Binding of STAT1/3; and Expression of COX-2

The sequence of six 4-minute coronary O/R cycles resulted in a marked increase, 30 minutes later (30 minutes after the sixth reperfusion), in pSer(338)-Raf-1 (190±8% of control [Figure 1A and 1B]), pSer-MEK-1/2 (274±32% of control [Figure 1C and 1D]), and pThr(202)/Tyr(204)-p44/42 MAPKs, as detected both with an anti-pTyr(204)-p44/42 MAPK antibody (393±63% of control [Figure 1E and 1F]) and an antipThr(202)/Tyr(204)-p44/42 MAPK antibody that recognizes the dually phosphorylated form of the kinases (676±148% of control). These data indicate that ischemic PC activates the Raf-1–MEK-1/2–p44/42 MAPK signaling pathway. The six 4-minute coronary O/R cycles did not change the total levels of Raf-1, MEK-1/2, and p44/42 MAPKs (Figure 1A through 1F). We could not detect phosphorylation of Raf-1 on Tyr-341 by either immunoblotting with anti-pTyr(341)-Raf-1 antibodies (Data Supplement Figure III) or immunoprecipitation with anti–Raf-1 antibodies followed by immunoblotting with anti-pTyr(341)-Raf-1 antibodies (Data Supplement Figure IV). The anti-pSer(338)-Raf-1 antibody (Cell Signaling) did not react with

immunoprecipitated A-Raf (Data Supplement Figure V), indicating that the immunoreactivity observed in the samples cannot be ascribed to pSer(338)-A-Raf.

In addition, the six 4-minute coronary O/R cycles caused a marked increase, 30 minutes later, in the Ser-phosphorylated forms of STAT1 and STAT3 in the nuclear fraction: pSer(727)-STAT1, 331±37% of control ($P<0.05$ [Figure 2A and 2B]) and pSer(727)-STAT3, 449±64% of control ($P<0.05$ [Figure 2C and 2D]). The total nuclear levels of STAT1 (243±17% of control, $P<0.05$ [Figure 2A and 2B]) and STAT3 (304±25% of control, $P<0.05$ [Figure 2C and 2D]) were also increased 30 minutes after the six 4-minute coronary O/R cycles, indicating nuclear translocation of these transcription factors. This translocation was associated with a striking increase in the STAT1/3-GAS complex in the nuclear fraction (637±30% of control, $P<0.05$) (Figure 2E and 2F), indicating increased DNA binding activity of these factors, and with a marked increase in myocardial COX-2 expression 24 hours later (374±36% of control, $P<0.05$) (Figure 2G and 2H). In the whole homogenate, ischemic PC increased pSer(727)-STAT1 (Data Supplement Figure VIA) and pSer(727)-STAT3 (Data Supplement Figure VIIA) but did not change total STAT1 or STAT3 content (Data Supplement Figures VIB and VIIB), confirming increased Ser-727 phosphorylation of STAT1 and STAT3.

Physical Association of p44/42 MAPKs With STAT1/3

Myocardial cytosolic fractions from control and preconditioned mice were immunoprecipitated with anti-pTyr-p44/42 antibodies, and the resulting immunoprecipitates were immunoblotted with anti-pSer(727)-STAT1 and anti-pSer(727)-STAT3 antibodies, respectively. As shown in Figure 3A through 3D, Tyr-phosphorylated p44/42 MAPKs coprecipitated with pSer(727)-STAT1 (334±36% of control [Figure 3A and 3B]) and pSer(727)-STAT3 (310±37% of control [Figure 3C and 3D]), supporting a direct interaction between phosphorylated (activated) p44/42 MAPKs and Ser-phosphorylated STAT1/3 in the PC myocardium.

Chelerythrine Suppresses Activation of the Raf-1–MEK-1/2–p44/42 MAPK-pSer-STAT1/3 Pathway and COX-2 Induction

As a first step to interrogate the role of PKC, we used the broad PKC inhibitor chelerythrine. Pretreatment with chelerythrine 5 minutes before the six 4-minute coronary O/R cycles blocked the increases in pSer(338)-Raf-1 (Figure 1A and 1B), pSer-MEK-1/2 (Figure 1C and 1D), and pTyr(204)-p44/42 MAPKs (Figure 1E and 1F), suggesting that the phosphorylation of Raf-1–MEK-1/2-p44/42 MAPKs by ischemic PC is mediated by PKC.

In addition, pretreatment with chelerythrine markedly blunted the increase in nuclear pSer(727)-STAT1 and pSer(727)-STAT3 (Figure 2A through 2D), the nuclear translocation of these factors (Figure 2A through 2D), the increase in STAT1/3-DNA binding activity (Figure 2E and 2F), and the increase in COX-2 protein (Figure 2G and 2H), suggesting that phosphorylation of STAT1 and STAT3 on Ser-727, activation of STAT1 and STAT3, and upregulation of COX-2 are also PKC dependent. Finally, the formation of complexes between pTyr-p44/42 and pSer-STAT1/3 was also inhibited by pretreatment with chelerythrine (Figure 3).

Deletion of PKCε Blunts Activation of the MEK-1/2–p44/42 MAPK-pSer-STAT1/3 Pathway and the Upregulation of COX-2

To specifically interrogate the role of the ε isoform of PKC in the recruitment of the Raf-1–MEK-1/2–p44/42 MAPK signaling pathway, we examined the effect of deletion of the *PKC*ε gene (*PKC*$\varepsilon^{-/-}$ mice). Targeted ablation of PKCε blunted the increase in pSer(338)-

Raf-1 (Figure 4A and 4B), pSer-MEK-1/2 (Figure 4A and 4C), and pThr(202)/Tyr(204)-p44/42 MAPK (Figure 4A and 4D) 30 minutes after ischemic PC.

Deletion of PKCε also inhibited the increase in total STAT1 and pSer(727)-STAT1 (Figure 5A, 5C, and 5E) and in total STAT3 and pSer(727)-STAT3 (Figure 6A, 6C, and 6E) in the nuclear fraction. Furthermore, deletion of PKCε inhibited the increase in STAT1/3-DNA binding activity in the nuclear fraction 30 minutes after PC (Figure 7A and 7B) and the subsequent upregulation of COX-2 protein expression 24 hours later (Figure 7C and 7D). However, deletion of PKCε did not block the increase in pTyr(701)-STAT1 (Figure 5A and 5D) and pTyr(705)-STAT3 (Figure 6A and 6D). Taken together, these data indicate an obligatory role of PKCε in the activation of Raf-1, MEK-1/2, and p44/42 MAPKs; in the Ser (but not Tyr) phosphorylation and activation of STAT1/3; and in the induction of COX-2 after ischemic PC.

Discussion

Inhibition of COX-2 activity during the late phase of PC results in complete loss of protection against infarction, demonstrating that upregulation of this enzyme after the initial ischemic stress is necessary for late PC to become manifest.[1,7,8,10,11] However, the signaling mechanism by which ischemic PC induces the synthesis of COX-2 protein remains incompletely understood. We have recently found that activation of STAT1 and STAT3 via JAK-dependent Tyr phosphorylation is essential for both PC-induced protection[5] and PC-induced upregulation of COX-2.[10] However, it is unknown whether ischemic PC also leads to phosphorylation of STAT1 and STAT3 on the Ser-727 residue (which is essential for STAT-dependent transcriptional activation in other systems[12–14]) and, if so, which upstream signaling mechanism leads to Ser phosphorylation of these 2 transcription factors and what role Ser phosphorylation of STAT1/3 plays in the upregulation of COX-2 after ischemic PC.

The present study provides new information pertaining to these issues in an in vivo murine model of myocardial ischemia and reperfusion. The salient findings can be summarized as follows: (1) Ischemic PC activates the Raf-1– MEK-1/2–p44/42 MAPK signaling pathway, induces phosphorylation of STAT1 and STAT3 on the Ser-727 residue, and upregulates COX-2; (2) pSer-STAT1 and pSer-STAT3 form complexes with pTyr-p44/42 MAPKs in PC myocardium, supporting the concept that Ser phosphorylation of these 2 factors is mediated by activated p44/42 MAPKs; (3) activation of the Raf-1–MEK-1/2–p44/42 MAPK pathway, the Ser phosphorylation of STAT1 and STAT3, and the upregulation of COX-2 after ischemic PC are suppressed by broad pharmacological inhibition of the PKC family (chelerythrine) or isoform-specific deletion of PKCε (*PKCε*$^{-/-}$ mice), indicating that they are dependent on PKCε activity; and (4) in contrast, deletion of PKCε has no effect on Tyr phosphorylation of STAT1/3. Previous studies have documented that Tyr phosphorylation of STAT1 and STAT3 via JAK activity is a crucial mechanism for the development of late PC.[5,10] To our knowledge, this is the first study to demonstrate that ischemic PC also causes Ser phosphorylation of STAT1 and STAT3 and that this event is governed by PKCε. Furthermore, this is the first report that COX-2 expression in the heart is controlled by PKCε. Together with our previous findings,[5,10] the present study implies that STATdependent transcription after ischemic PC is modulated by a dual signaling mechanism that involves both JAK1/2 (Tyr phosphorylation) and PKCε (Ser phosphorylation).

The Ser-Thr kinases p44/42 MAPKs have been reported to be involved in Ser phosphorylation of STATs[14,21,22] and are required for phosphorylation of STAT3 on Ser-727 in noncardiac cells.[14,21] Activation of p44/42 MAPKs, in turn, could be secondary to PKC activation. PKC, and specifically its ε isoform, is known to play a crucial role in the

development of late PC[2–4] and to activate p44/42 MAPKs in a number of cell types.[23,24] In the heart, ischemic PC has been shown to activate p44/42 MAPKs and their activators MEK-1/2 via a PKCε-dependent mechanism.[15] Accordingly, we postulated that Ser phosphorylation of STAT1 and STAT3 is mediated by a PKCε–Raf-1–MEK-1/2-p44/42 MAPK signaling cascade and examined each component of this pathway. We found that six 4-minute coronary O/R cycles led to a rapid increase in pSer(338)-Raf-1 (Figures 1A, 1B, 4A, and 4B), pSer-MEK-1/2 (Figure 1C and 1D), pTyr(204)-p44/42 MAPKs (Figure 1E and 1F), and pSer-STAT1/STAT3 (Figure 2A through 2D), all of which are inhibited by pretreatment with chelerythrine (a broad inhibitor of the entire family of PKCs) (Figure 1A through 1F and 2A–2D) or by targeted genetic disruption of PKCε (Figure 4A through 4D, 5A, 5C, 6A, and 6C), demonstrating that the entire Raf-1–MEK-1/2– p44/42 MAPK–pSer-STAT1/3 signaling pathway is PKCε dependent. Our data show that ischemic PC rapidly phosphorylates Raf-1 on Ser-338 but not on Tyr-341 (Figure 4A and 4B and Data Supplement Figures III and IV), demonstrating that Raf-1 activation by ischemic PC is mediated primarily or exclusively by phosphorylation on Ser-338. Deletion of PKCε blocks the Ser-338 phosphorylation of Raf-1 (Figure 4A and 4B), indicating that Ser-338 phosphorylation is PKCε dependent. This is consistent with the previous finding that PKCε is necessary for Raf-1 activation in mouse C3H10T1/2 fibroblasts.[24] Furthermore, we found that the Ser-phosphorylated STAT1 and STAT3 coprecipitated with phosphorylated (activated) p44/42 MAPKs and that this was also inhibited by chelerythrine (Figure 3A through 3D). In the aggregate, these data suggest that PKCε (which is known to be rapidly activated by ischemic PC[2–4]) plays a critical role in the activation of the downstream Raf-1–MEK-1/2–p44/42 MAPK signaling cascade and in the resulting Ser phosphorylation of STAT1 and STAT3. Of note, deletion of PKCε had no effect on Tyr phosphorylation of STAT1 or STAT3 (Figures 5A, 5D, 6A, and 6D), indicating that this event is mediated by a distinct, PKCε-independent signaling pathway.

Thus, we propose that the activation of STAT1 and STAT3 after ischemic PC is modulated via 2 parallel pathways, namely (1) activation of JAK1/2 and subsequent Tyr phosphorylation of STATs[5,10] and (2) activation of PKCε and subsequent recruitment of the Raf-1–MEK-1/2–p44/42 MAPK cascade, leading to Ser phosphorylation of STATs (Figure 8). We suggest that these 2 pathways are activated simultaneously, and both of them are necessary for STATdependent transcription after ischemic PC.

We found no evidence of Tyr-341 phosphorylation of Raf-1, as determined by both Western blot analysis (Data Supplement Figure III) and immunoprecipitation followed by immunoblotting (Data Supplement Figure IV). Despite the use of immunoprecipitation, it is still possible that phosphorylation of Tyr-341 occurred after ischemic PC but that its level was below detection. Several previous studies in various systems have failed to detect pTyr-341 in active Raf-1.[25–28] Activation of Raf-1 is a very complex process, in which phosphorylation of Ser-338 and Tyr-341 occurs in different proportions, depending on the stimulus and cell type.[28,29] King et al[29] have proposed that different degrees of Raf-1 activation are achieved by Ser-338 and/or Tyr-341 phosphorylation occurring individually or in combination.

By documenting a new pathway through which ischemic PC activates STATs (the PKCε–Raf-1–MEK-1/2–p44/42 MAPK axis), the present findings expand our understanding of the molecular mechanisms whereby these transcription factors contribute to the upregulation of COX-2, to the development of late PC, and to the response of the heart to stress in general. Furthermore, our findings reveal that PKCε controls COX-2 expression in the heart and suggest a new mechanism whereby this PKC isoenzyme may be involved in late PC, namely, the modulation of STAT1 and STAT3 activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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CLINICAL PERSPECTIVE

Ischemic heart disease is the leading cause of morbidity and mortality in all industrialized nations. As the population grows older and risk factors become more prevalent, the enormous public health burden caused by ischemic heart disease is likely to increase even further. Preconditioning (PC) is one of the most powerful cardioprotective interventions identified to date. It consistently limits infarct size in every animal model and species examined, and evidence suggests that it is effective in protecting human myocardium as well. Thus, PC represents an attractive strategy for inducing cardioprotection. Because of its sustained nature, late PC offers the potential to afford long-lasting protection against myocardial cell death and therefore may have great clinical relevance. The elucidation of the endogenous signaling mechanisms used by this phenomenon has major pathophysiological and therapeutic implications. The mechanisms regulating ischemic PC are known to involve many proteins, including protein kinase C and the JAK/signal transducer and activator of transcription (STAT) pathway, which result in the eventual upregulation of cardioprotective genes such as inducible nitric oxide synthase and cyclooxygenase-2. However, the exact mechanisms supporting these interactions are unknown. This article establishes for the first time that ischemic PC causes Ser phosphorylation of STAT1/3 and activation of the Raf-1–extracellular signal–regulated kinase kinase-1/2–p44/42 mitogen-activated protein kinase signaling pathway and that both of these events, as well as the subsequent induction of cyclooxygenase-2, are dependent on protein kinase C-ε. By continuing to unravel the mechanisms underlying ischemic PC in a clinically relevant murine model of ischemia/reperfusion, we hope to establish the groundwork for novel therapies that will one day be used for patients suffering from ischemic heart disease.

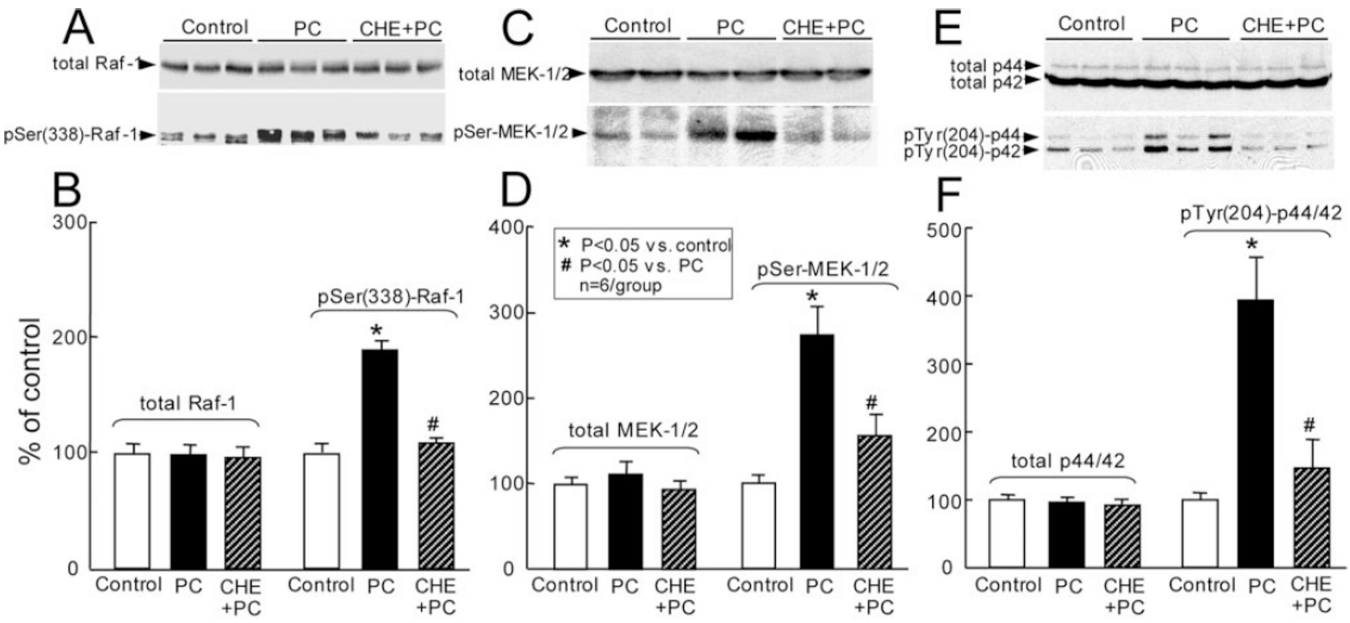

Figure 1.

Effect of ischemic PC on phosphorylation of Raf-1, MEK-1/2, and p44/42 MAPKs. Cytosolic proteins of myocardial samples were prepared from control mice that underwent 1 hour of open-chest state without coronary O/R (control group) or from the ischemic/reperfused region of PC mice that received either no treatment (PC-30′) or chelerythrine (CHE+PC-30′) 5 minutes before 6 cycles of 4-minute coronary O/R. All mice were euthanized 30 minutes after the sham operation or the sixth reperfusion. The figure illustrates representative immunoblots (A, C, and E) and densitometric analysis of total and Ser-phosphorylated Raf-1 (B), total and Ser-phosphorylated MEK-1/2 (D), and total and Tyr-phosphorylated p44/42 MAPKs (F). Data are mean±SEM, n=6/group.

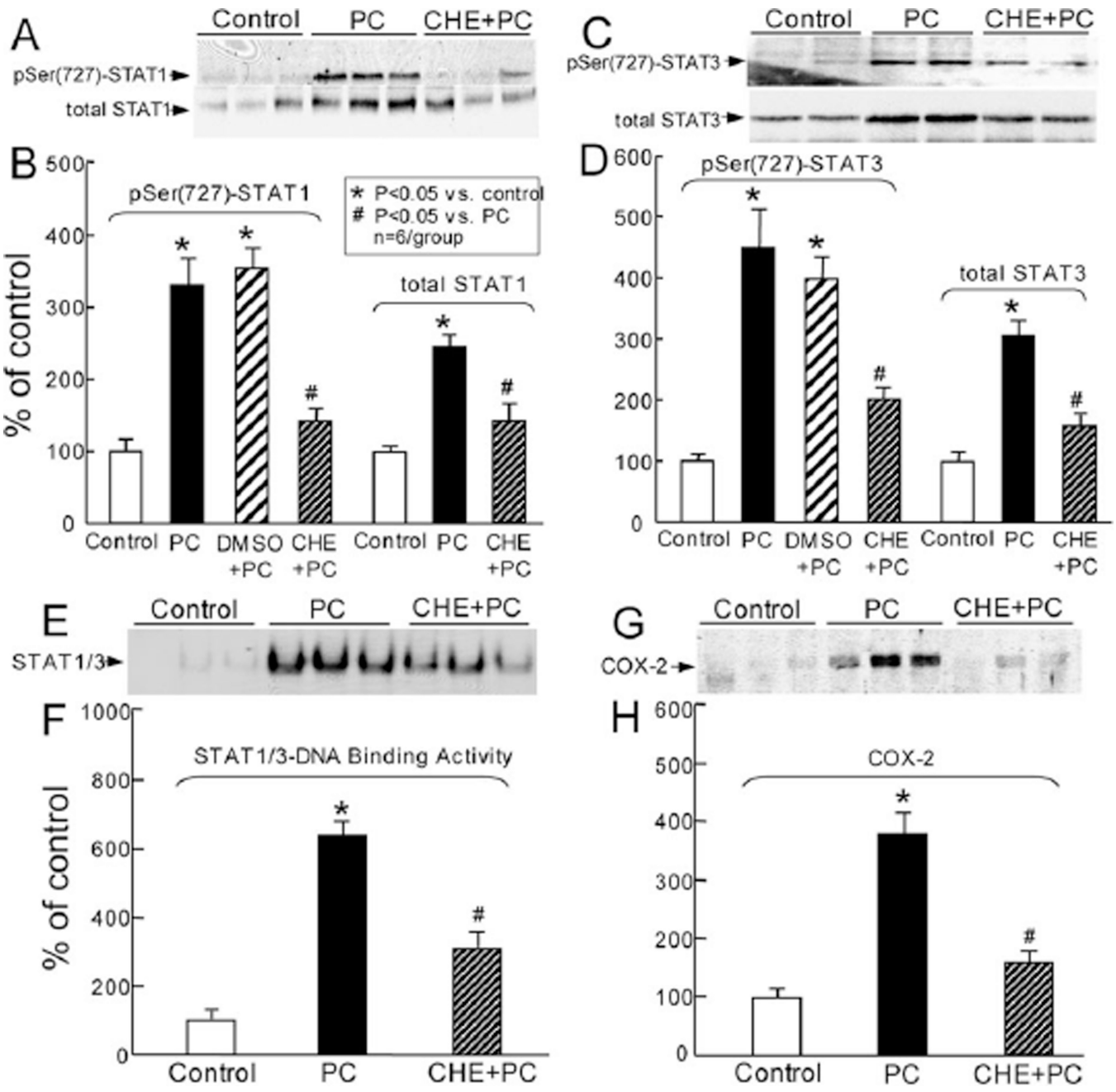

Figure 2.

A–D, Effect of ischemic PC on Ser phosphorylation of STAT1 and STAT3. Nuclear proteins of myocardial samples were prepared for immunoblotting. E and F, Effects of CHE on the ischemic PC-induced increase in DNA binding activity of STAT1/3. Nuclear proteins were subjected to EMSA for analysis of STAT1/3-DNA binding activity with the ^{32}P-labeled GAS probe. G and H, Effect of CHE on the ischemic PC-induced upregulation of COX-2. Myocardial samples were obtained from mice that underwent sham operation (control) or from the ischemic/reperfused region of PC mice that received no treatment (PC-24 hours) or CHE 5 minutes before the 6 coronary O/R cycles (CHE+PC-24 hours). Membranous proteins were prepared for determination of COX-2 expression. Data are mean ±SEM, n=6/group.

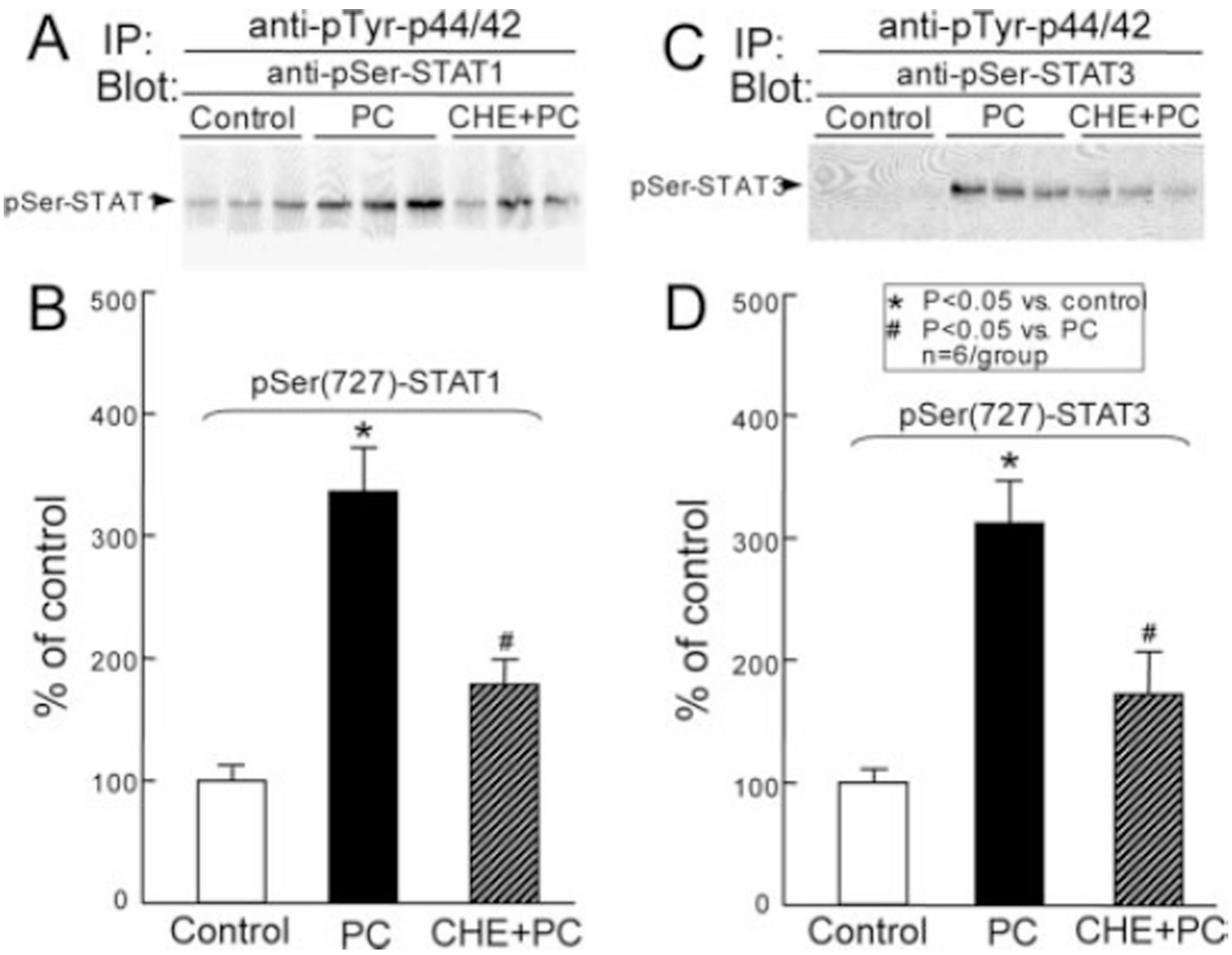

Figure 3.

p44/42 MAPKs interact with Ser-phosphorylated STAT1 and STAT3 in PC myocardium. Cytosolic proteins of myocardial samples were prepared 30 minutes after sham operation (control) or 30 minutes after 6 cycles of 4-minute coronary O/R with no treatment (PC-30′) or with prior treatment with CHE (CHE+PC-30′). The cytosolic proteins were then immunoprecipitated with anti-phosphorylated p44/42 MAPK antibodies followed by immunoblotting with anti–pSer727-STAT1 or anti– pSer727-STAT3 antibodies. The figure illustrates representative immunoblots (A and C) and densitometric analyses of the Serphosphorylated forms of STAT1 and STAT3 (B and D). There was increased coprecipitation of pSer-STAT1 and pSer-STAT3 with pTyr-p44/42 MAPKs 30 minutes after ischemic PC. Data are mean±SEM, n=6/group.

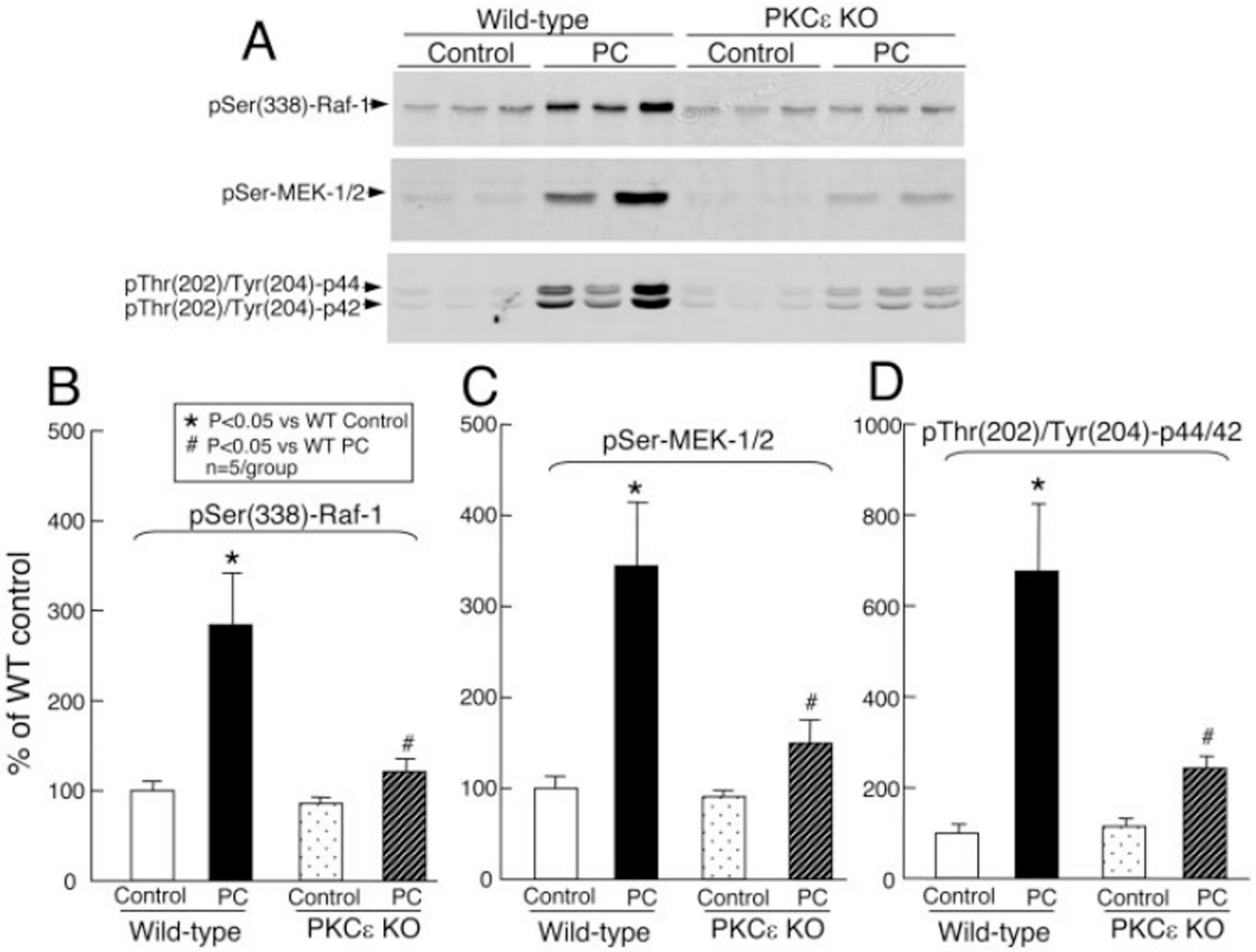

Figure 4.

Targeted deletion of the *PKC*ε gene inhibits the phosphorylation of MEK-1/2 and p44/42 MAPKs by ischemic PC. Myocardial samples were taken 30 minutes after 1 hour of open-chest state without ischemia (control group) or 30 minutes after ischemic PC in WT and *PKC*ε$^{-/-}$ mice. Cytosolic proteins were used for immunoblotting analysis of the phosphorylated forms of Raf-1, MEK-1/2, and p44/42 MAPKs. The anti–pSer(338)-Raf-1 antibody was from Cell Signaling. Western blots (A) and densitometric analysis (B–D) demonstrate that the ischemic PC-induced increase in pSer(338)-Raf-1, pSer-MEK-1/2, and pThr(202)/Tyr(204)-p44/42 MAPKs was inhibited in *PKC*ε$^{-/-}$ mice. Ischemic PC had no effect on Tyr-341 phosphorylation of Raf-1 (Data Supplement Figures III and IV). Data are mean±SEM, n=5/group.

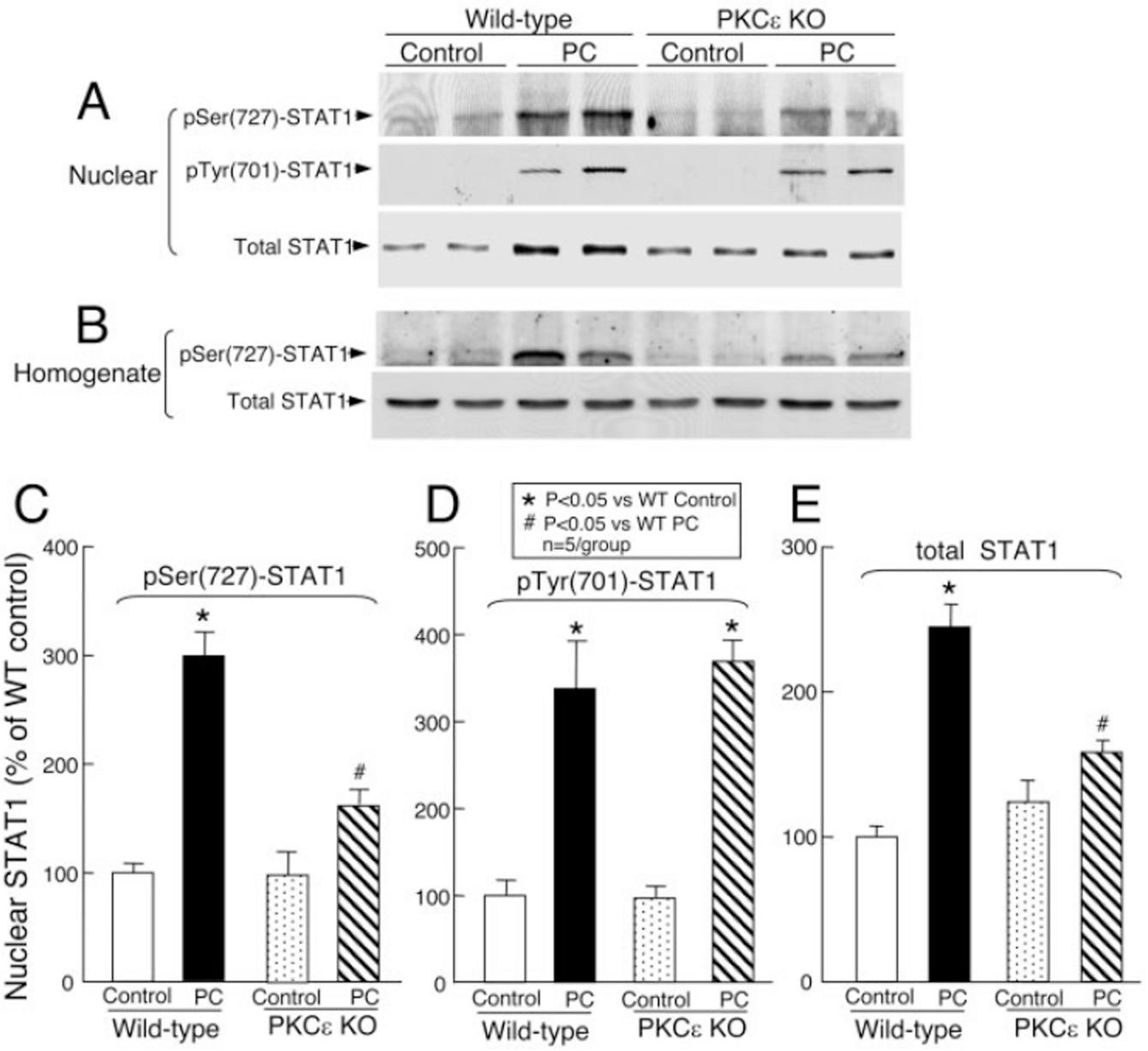

Figure 5.

Deletion of the *PKCε* gene (KO) inhibits the Ser phosphorylation of STAT1 by ischemic PC. Homogenates and nuclear extracts were isolated from myocardial samples taken 30 minutes after 1 hour of an open-chest state without ischemia (control group) or 30 minutes after ischemic PC in WT and $PKC\varepsilon^{-/-}$ mice. Data are mean±SEM, n=5/group.

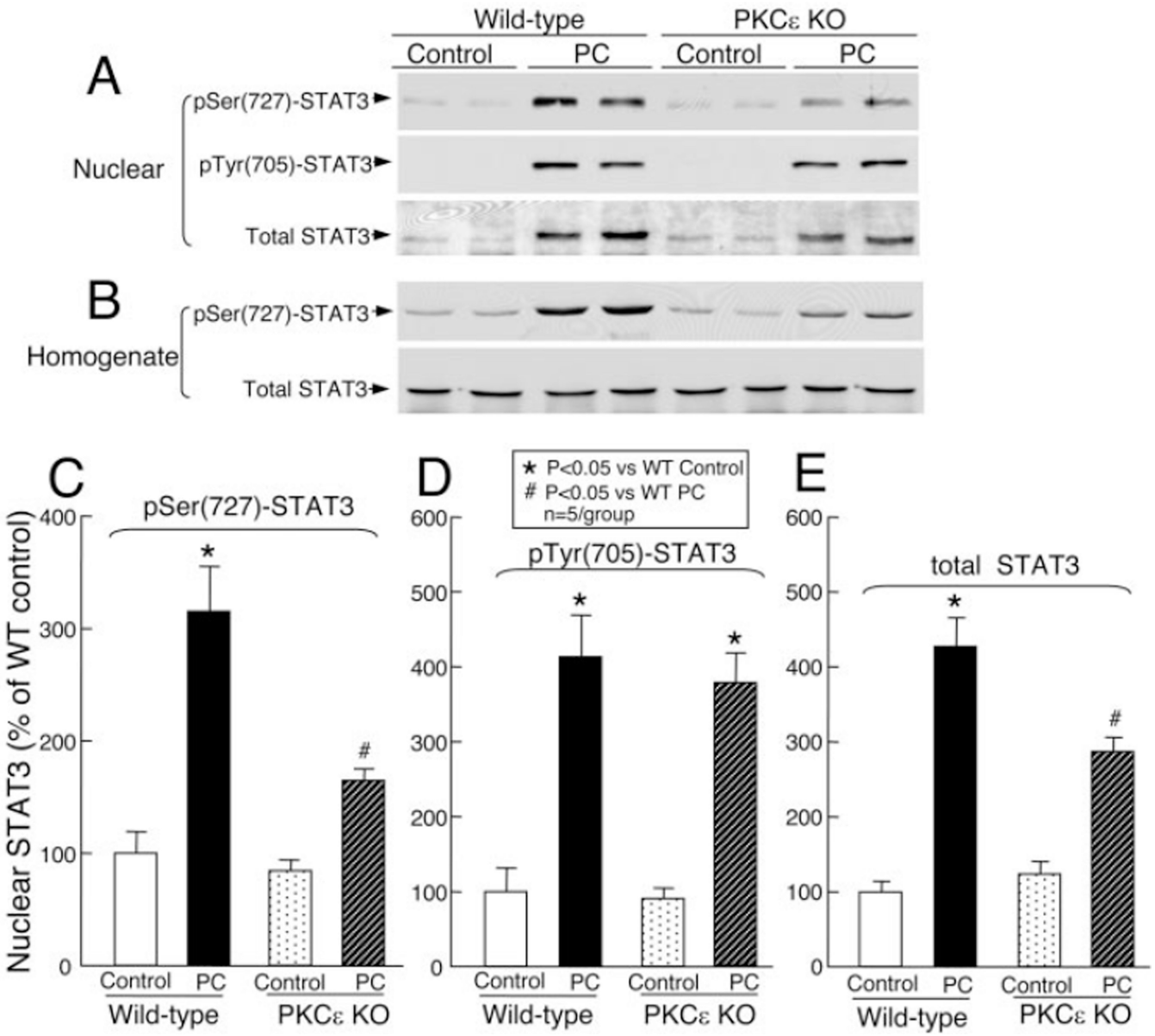

Figure 6.

Deletion of the *PKCε* gene blocks Ser phosphorylation of STAT3 by ischemic PC. Homogenates and nuclear extracts were obtained as described in Figure 5. Data are mean ±SEM (n=5/group).

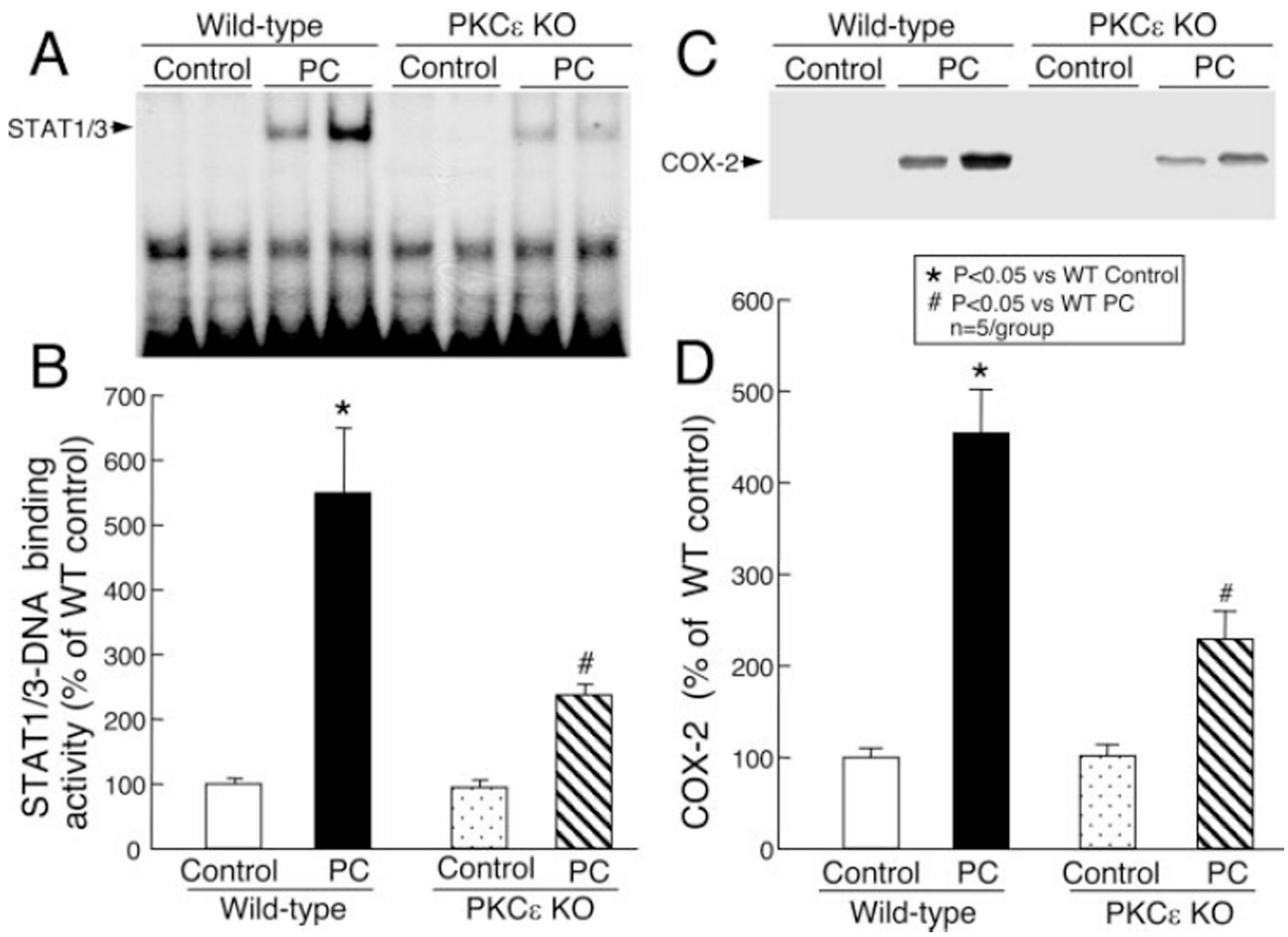

Figure 7.

Deletion of the *PKCε* gene blocks the ischemic PC-induced increase in STAT1/3-DNA binding activity and COX-2 upregulation. A and B, Nuclear extracts were obtained as described in Figure 5 and subjected to EMSA for analysis of STAT1/3-DNA binding activity with a ^{32}P-labeled GAS probe. C and D, Myocardial samples were obtained from WT and *PKCε*$^{-/-}$ mice that underwent sham operation (WT control and *PKCε*$^{-/-}$ control, respectively) or from the ischemic/reperfused region of WT and *PKCε*$^{-/-}$ mice that were preconditioned with six 4-minute coronary O/R cycles. All mice were euthanized 24 hours after sham operation or after the sixth reperfusion. Data are mean±SEM, n=5/group.

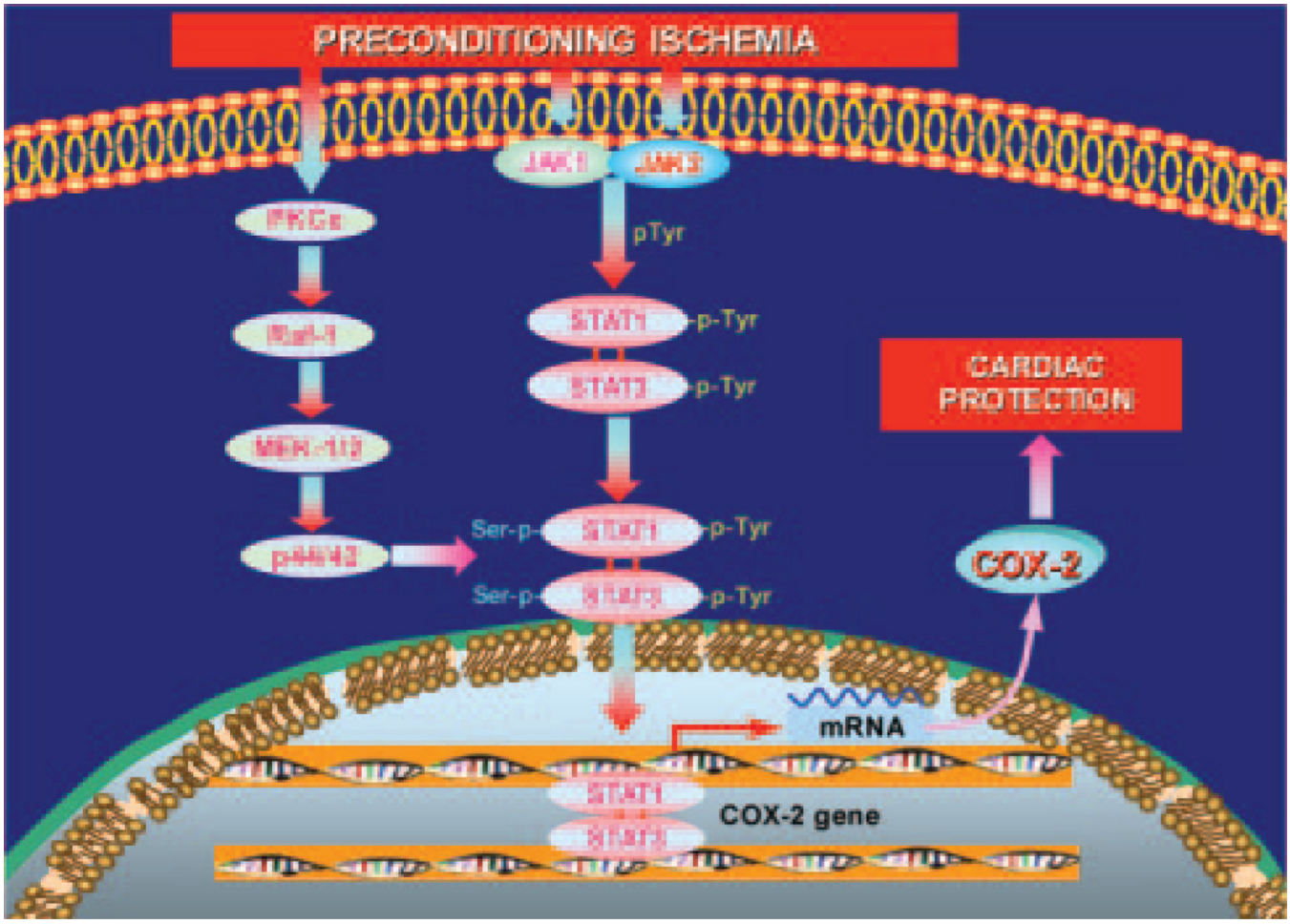

Figure 8.

Proposed signaling mechanism controlling COX-2 protein expression during ischemic PC. A sublethal ischemic stress (PC stimulus) induces phosphorylation of STAT1 and STAT3 on both Tyr and Ser residues. Tyr phosphorylation of STAT1/3 is mediated by a JAK-dependent mechanism, whereas Ser phosphorylation of these transcription factors is modulated by p44/42 MAPKs via PKCε-dependent activation of the Raf-1– MEK-1/2–p44/42 MAPK pathway during PC ischemia. On activation, phosphorylated STAT1/3 translocate to the nucleus, where they promote transcription of the *COX-2* gene, leading to the synthesis of COX-2 protein that is necessary for the development of delayed protection against myocardial infarction.