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Authors

Lu, Xiyuan

Moore, Peter G

Liu, Hong

et al.

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Phosphorylation of ARC Is a Critical Element in the Antiapoptotic Effect of Anesthetic Preconditioning

Xiyuan Lu, MS,* Peter G. Moore, MD, PhD,† Hong Liu, MD,† and Saul Schaefer, MD*‡

BACKGROUND: Transient exposure to volatile anesthetics before cardiac ischemia/reperfusion (I/R), termed *anesthetic preconditioning*, limits myocardial injury and inhibits apoptosis. Apoptosis repressor with caspase recruitment domain (ARC) is a novel protein that has been demonstrated to protect cardiomyocytes from apoptosis induced by I/R and is regulated by phosphorylation. We therefore hypothesized that the antiapoptotic effect of anesthetic preconditioning is, in part, mediated by phosphorylation of ARC.
METHODS: In the experiments we used a perfused rat heart model of sevoflurane anesthetic preconditioning and I/R. In addition to measures of left ventricular function, phosphorylation of ARC was measured with and without anesthetic preconditioning. Because the phosphorylation status of ARC is determined by calcineurin and protein kinase CK2, the role of ARC was defined by measuring calcineurin activity and using the calcineurin inhibitor FK506 and the ARC phosphorylation inhibitor 4,5,6,7-tetrabromobenzotrizole (TBB).
RESULTS: I/R without anesthetic preconditioning increased calcineurin and reduced ARC phosphorylation levels, whereas anesthetic preconditioning significantly improved functional recovery, decreased ischemic injury, limited the increase in calcineurin activity, increased the phosphorylation level of ARC, reduced cytochrome *c* release, and blocked the increase in caspase-8 after I/R. The effects of anesthetic preconditioning were mirrored by FK506 and abolished by TBB.
CONCLUSION: This study has identified a novel cardiac pathway in which anesthetic preconditioning prevents the increase in calcineurin after I/R, resulting in increased phosphorylated ARC and decreased markers of apoptosis. (Anesth Analg 2011;112:525–31)

Anesthetic preconditioning (APC), defined as exposure to volatile anesthetics such as sevoflurane before ischemia/reperfusion (I/R), has been reported to provide myocardial protection in animal models.[1–3] This cardioprotection has been demonstrated by relative preservation of myocardial function, adenosine triphosphate levels, mitochondrial integrity, and reduction in apoptosis after I/R.[2,4] Apoptosis repressor with caspase recruitment domain (ARC) is a recently described protein that represses apoptosis[5] by directly binding to and inhibiting caspase-8 activity,[5–7] interacting with Fas, FADD, or Bax,[8] and inhibiting cytochrome *c* release.[9] ARC is regulated by protein kinase CK2 (CK2), which can phosphorylate ARC at threonine-149 (T149) and thereby enable ARC to translocate from the cytoplasm to mitochondria where it directly binds to caspase-8 or caspase-2 (Fig. 1).[6,10] Tan et al.[11] reported that ARC can be dephosphorylated by calcineurin during cardiac apoptosis, suggesting that the phosphorylation status, and hence activity, of ARC is regulated by the balance of CK2 and calcineurin.

Calcineurin is a serine/threonine protein phosphatase that can be activated by sustained increases in cytosolic-free Ca^{2+}.[12,13] Because previous work showed that APC limited the increase in intracellular and mitochondrial Ca^{2+} in ischemic myocardium,[3] we hypothesized that the antiapoptotic effect of APC is, in part, mediated by a reduction in calcineurin activity and greater phosphorylation of ARC. This hypothesis was tested in an established perfused rat heart model of sevoflurane APC.

METHODS

The study protocol was approved by the Animal Care Committee of the University of California, Davis (Davis, CA), protocol number 07-13113, and the investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication No. 85 to 23, revised 1996).

All experiments used an isolated perfused rat heart model, as has been previously described.[14] Briefly, hearts were obtained from male Sprague–Dawley rats (weight, 250 to 300 g). Anesthesia was first induced with an intraperitoneal injection of sodium thiopental (50 to 75 mg/kg) along with 1000 U heparin for anticoagulation. Sodium thiopental was chosen for initial anesthesia because this drug has been shown not to influence preconditioning.[17] The heart was excised and placed in an ice-cold solution of

From the *Department of Internal Medicine, Division of Cardiovascular Medicine, and †Department of Anesthesiology and Pain Medicine, University of California, Davis, Davis, California; and ‡Cardiology Section, Department of Veteran Affairs, Northern California Health Care System, Mather, California.

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The authors declare no conflict of interest.

Address correspondence to Saul Schaefer, MD, Department of Internal Medicine, Division of Cardiovascular Medicine, University of California, Davis, One Shields Avenue, TB 172, Davis, CA 95616. Address e-mail to sschaefer@ucdavis.edu.

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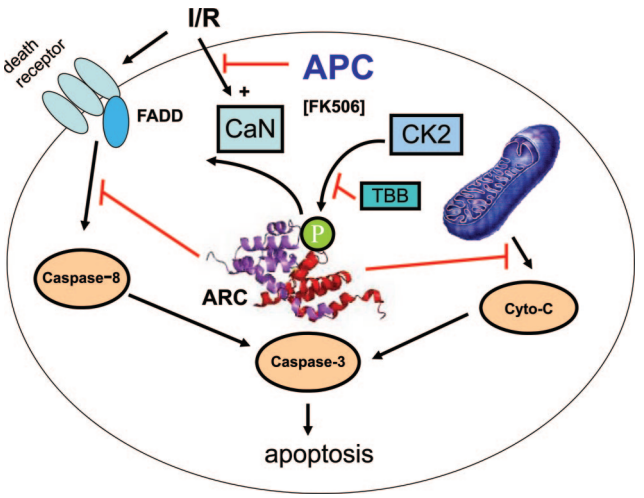

Figure 1. Schematic diagram of the regulation of ARC (apoptosis repressor with caspase recruitment domain). ARC is regulated by phosphorylation by protein kinase CK2 and dephosphorylation by calcineurin (CaN). Ischemia/reperfusion (I/R) increases calcineurin and initiates extrinsic and intrinsic apoptotic cascades through the death receptor and mitochondrial release of cytochrome *c*, respectively. Anesthetic preconditioning (APC) limits the increase in calcineurin and maintains the phosphorylation status of ARC, reducing mitochondrial release of cytochrome *c* and activation of caspase-8. The protective effects of APC are largely recapitulated by the calcineurin inhibitor FK506, and blocked by TBB (4,5,6,7-tetrabromobenzotrizole), an inhibitor of protein kinase CK2.

Krebs–Henseleit buffer. It was then cannulated and perfused in a Langendorff preparation with Krebs–Henseleit buffer (127 mM NaCl, 4.7 mM KCl, 1.25 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose) at a constant perfusion pressure of 80 ± 10 mm Hg at 37°C ± 0.5°C. The perfusion was continuously oxygenated with 95% O_2–5% CO_2 except during no-flow ischemia.

APC was performed using sevoflurane delivered at 2.5% to the gas mixture via a standard Sevotec5 variable bypass vaporizer (Datex-Ohmeda, Milwaukee, WI) with a final concentration of 0.4 ± 0.02 mM, corresponding to 0.9 minimum alveolar concentration.[1] After equilibration, preconditioned hearts were exposed to sevoflurane for 10 minutes followed by perfusion with plain Krebs–Henseleit buffer for 20 minutes.

Experimental Grouping

Rats were randomly assigned to 1 of 9 experimental groups, as is shown in Figure 2.

Functional Measurements

Left ventricular pressure was measured continuously using a latex balloon filled with water connected to a pressure transducer (Medex, Dublin, CA). The balloon was inserted into the left ventricle via the left atrial appendage through the mitral valve. The balloon volume was adjusted during the equilibration period to yield a left ventricular end-diastolic pressure (LVEDP) of 5 to 10 mm Hg. Pressures were recorded using Powerlab 4/20 hardware with an

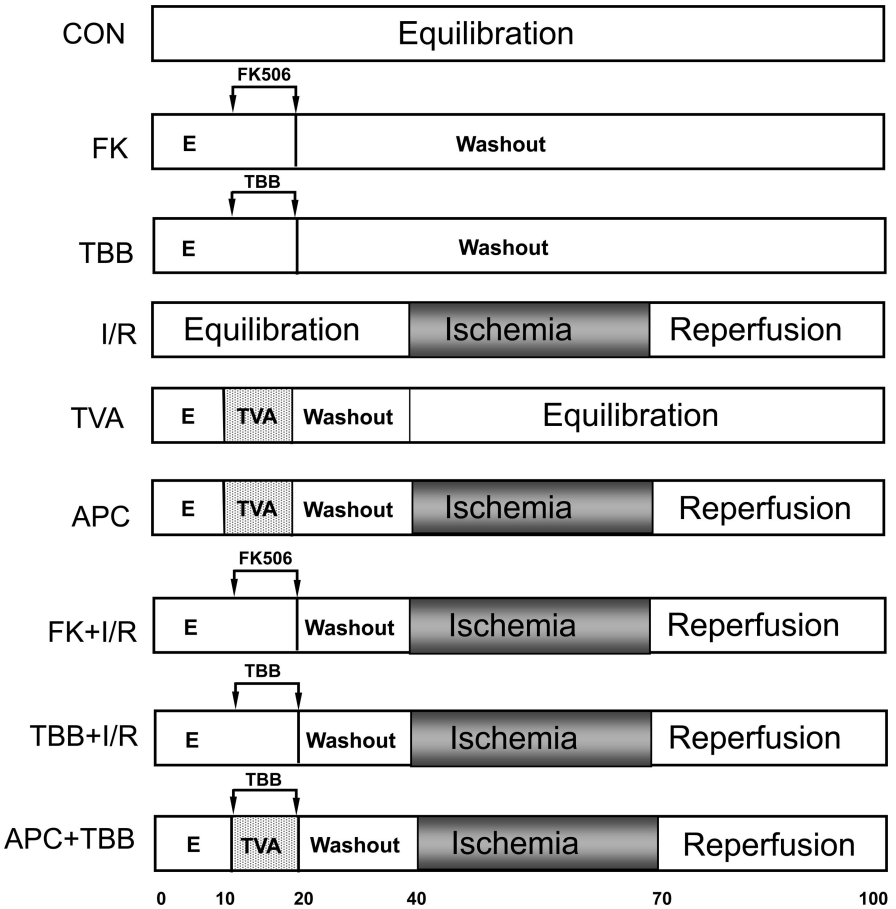

Figure 2. Overview of the experimental groups and protocols. Functional measurements were made continuously throughout the protocols, and hearts were frozen and protein isolated for measurements at the end of each protocol. Separate experiments were performed to measure CK release on reperfusion in the different protocols. CON = control, FK = FK506, TBB = 4,5,6,7-tetrabromobenzotrizole, I/R = ischemia/reperfusion, TVA = transient volatile anesthetic (i.e., exposure to sevoflurane without subsequent ischemia), APC = anesthetic preconditioning.

amplifier and Chart for Windows version 4.0.4 software (AD Instruments, Colorado Springs, CO).

Ischemic Injury Measurement

Creatine kinase release was measured from effluent collected in the first 10 minutes after reperfusion and assayed using an EnzyChrom Creatine Kinase Assay Kit (BioAssay Systems, Hayward, CA) and UV–VIS recording photospectrometer (Microplate Reader, BioTek, Winooski, VT), as has been previously described.[18] Units are expressed as IU per gram of wet weight.

Protein Isolation

At the end of each experimental protocol, hearts were frozen in liquid nitrogen and subsequently homogenized in ice-cold lysis buffer (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/mL each of leupeptin, aprotinin, and pepstatin A) containing 250 mM sucrose with homogenizer PowerGen 1800D (Fisher Scientific, Pittsburg, PA). The homogenates were centrifuged twice at 750 g for 5 minutes at 4°C to collect nuclei and debris. The supernatants were centrifuged at 10,000 g for 15 minutes at 4°C to collect the HM pellet. The resulting supernatants were centrifuged at 100,000 g for 1 hour at 4°C to yield light membrane (LM) pellets. The final supernatants are referred to as *cytosolic fractions*.[19]

Protein Analysis

Western blot analyses were used to measure the levels of phosphorylated ARC (pARC), ARC, caspase-8, and cytochrome *c* in the hearts. Heart cytosolic proteins were loaded and separated on 12% SDS-PAGE, followed by transblotting to an ImmunBlot PVDF membrane (Bio-Rad, Hercules, CA). The membrane was subsequently probed with primary ARC, pARC, caspase-8, and cytochrome *c* antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000. Horseradish peroxidase–conjugated secondary antibody was added at 1:3000 dilution. The blots were subsequently developed using an enhanced chemiluminescence detection kit (Thermo, Waltham, MA). After exposure on autoradiography film, immunoreactive protein bands were quantified by densitometry.

Calcineurin Analysis

Calcineurin activity was analyzed using 4 μg of total protein with the Calcineurin Cellular Assay Kit (Biomol International, Plymouth Meeting, PA). Calcineurin phosphatase activity was measured spectrophotometrically by detecting free-phosphate released from the calcineurin-specific RII phosphopeptide using a Microplate Reader (BioTek, Winooski, VT).

Capase-8 Activity Assay

A microwell colorimetric assay was performed to determine the enzymatic activity of the caspase-8 class of proteases. Briefly, 4 mg/mL protein extracts was analyzed by using Caspase-8 Colorimetric Assay according to the manufacturer's procedure (R&D Systems, Inc., Minneapolis, MN). Absorbance was read on a microplate reader using 405-nm wavelength light.

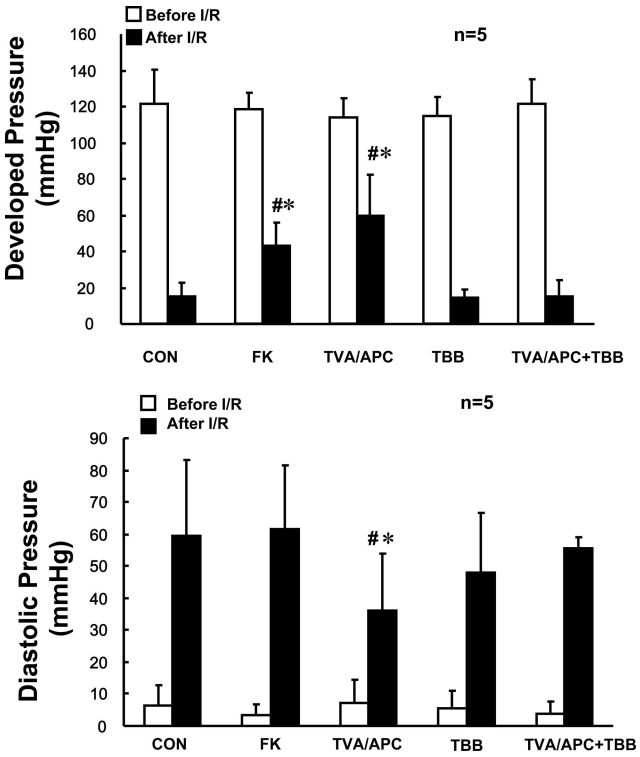

Figure 3. Functional recovery of the hearts as determined by left ventricular developed pressure (LVDP) (top) and left ventricular end-diastolic pressure (LVEDP) (bottom) before and after ischemia/reperfusion (I/R), as a function of experimental conditions. Anesthetic preconditioning (APC) and FK506 protected hearts, with significantly higher LVDP than CON + I/R. APC, but not FK506, preserved LVEDP. The protective effect of APC was abolished by 4,5,6,7-tetrabromobenzotrizole (TBB). TVA = transient volatile anesthetic. *$P < 0.05$ experimental groups versus CON. #$P < 0.05$ experimental groups versus APC+TBB after I/R.

Statistics

Data are presented as mean ± SD. On the basis of reductions in creatine kinase release seen in prior studies of APC in this laboratory,[2,3,20] a power analysis was performed using a 30% treatment effect, α of 0.05, and a power of 0.80, yielding a minimum sample size of 5. Outcome measures for each experimental group were compared using an analysis of variance (ANOVA) for repeated measures with Holm correction for the Bonferroni method. Version 13 of the statistical program SPSS (SPSS, Inc., Chicago, IL) was used. A $P < 0.05$ was used to test the null hypothesis.

RESULTS

Hemodynamic results for the experimental groups are shown in Figure 3. FK506 and 4,5,6,7-tetrabromobenzotrizole (TBB) in the absence of I/R had no significant effect on any of the measured variables. APC improved the recovery of left ventricular developed pressure after I/R (APC, 59 ± 24 mm Hg versus control (CON), 15 ± 8 mm Hg; $P < 0.05$). Exposure of APC hearts to TBB for 10 minutes, followed by 20 minutes washout, resulted in diminished recovery in comparison with APC alone (15 ± 9 mm Hg, Fig. 3A). However, exposure of control hearts to FK506 resulted in improved cardiac function after I/R (43 ± 12 mm Hg, $P < 0.05$) in comparison with control

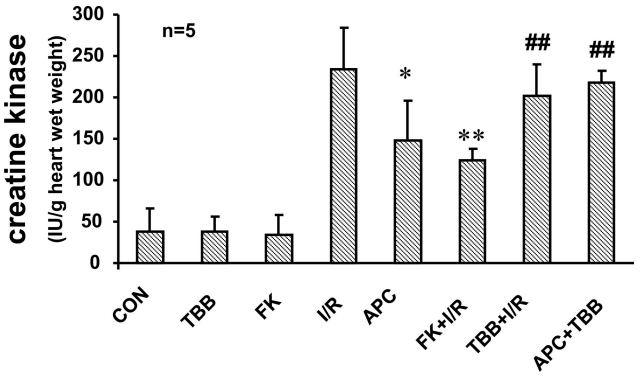

Figure 4. Myocyte injury evaluated by creatine kinase release upon reperfusion. Anesthetic preconditioning (APC) significantly reduced CK release after ischemia/reperfusion (I/R) in comparison with control hearts (CON) ($P < 0.05$), an effect that was paralleled by exposure of I/R hearts to FK506. The protective effect of APC was abolished by treatment with 4,5,6,7-tetrabromobenzotrizole (TBB). *$P < 0.05$ vs. I/R. **$P < 0.01$ vs. I/R . ##$P < 0.01$ vs. APC.

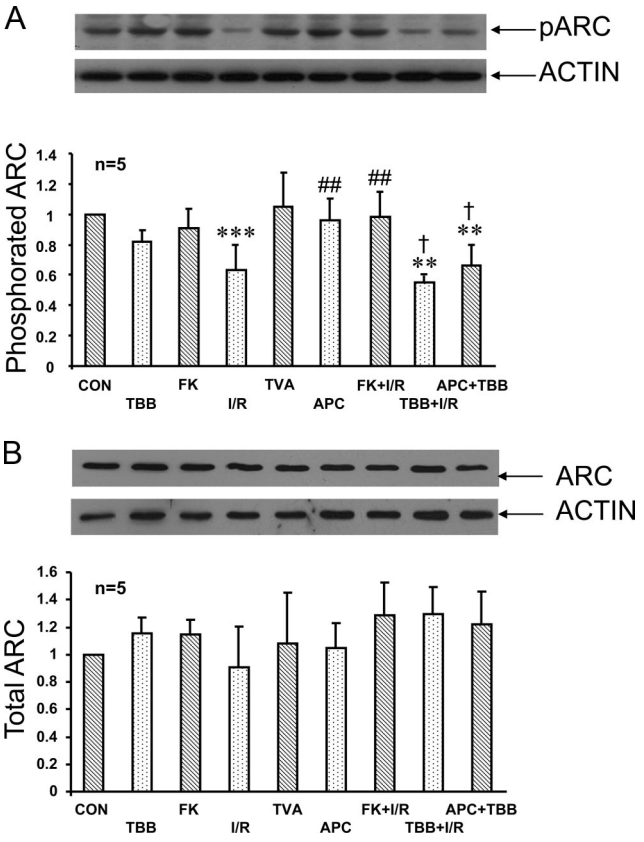

Figure 5. Anesthetic preconditioning inhibits the decreases in apoptosis repressor with caspase recruitment domain (ARC) phosphorylation levels. A, representative Western blots for phosphorylated ARC (pARC) and β-actin for the experimental groups (top) and group data for pARC (relative units) (bottom). B, representative Western blots for total ARC and β-actin for the experimental groups (top); total ARC (relative units) (bottom). **$P < 0.01$, ***$P < 0.001$ experimental groups versus control. ##$P < 0.01$ experimental groups versus I/R. CON = controls; TBB = 4,5,6,7-tetrabromobenzotrizole; I/R = ischemia/reperfusion; TVA = transient volatile anesthetic; APC = anesthetic preconditioning.

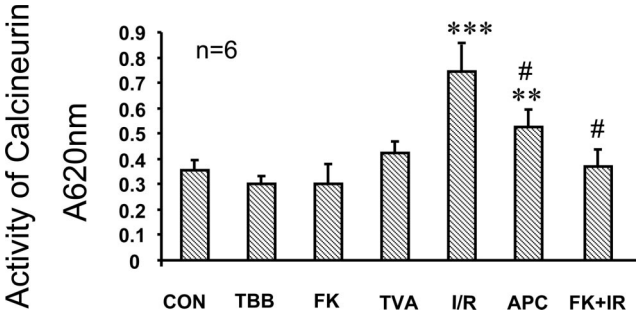

Figure 6. Calcineurin activity after ischemia/reperfusion (I/R) in the experimental groups. Activity of calcineurin after I/R was significantly greater than baseline. Calcineurin activity was markedly reduced by anesthetic preconditioning (APC) and FK506. CON = controls; TBB = 4,5,6,7-tetrabromobenzotrizole; TVA = transient volatile anesthetic. **$P < 0.01$, ***$P < 0.001$ experimental groups versus control; #$P < 0.05$ APC and FK+I/R versus I/R.

hearts. LVEDP was significantly reduced by APC in comparison with controls (37 ± 7 mm Hg vs. 59 ± 6 mm Hg; $P < 0.05$, Fig. 3B). However, as with systolic function, APC hearts concurrently exposed to TBB lost the protective effect of APC on LVEDP (56 ± 4 mm Hg; $P < 0.05$ vs. APC). In contrast to the findings in systolic pressure, there was no beneficial effect of FK506 in control hearts after I/R, such that LVEDP was similar in I/R and FK + I/R.

Figure 4 demonstrates that cardiac injury, as measured by creatine kinase release, was significantly reduced by APC when compared with I/R ($P < 0.05$). This protection was similar to that provided by FK506, and was eliminated by TBB.

Figure 5 shows the effect of APC on expression and the phosphorylation of ARC. Although total ARC was not different between groups (Fig. 5B), I/R decreased pARC by ~37.6% when compared with control hearts ($P < 0.001$, Fig. 5A). Transient volatile anesthetic alone did not affect pARC, whereas APC restored the phosphorylation status of ARC equal to control levels without I/R. FK506 prevented the decrease in pARC seen with I/R. TBB alone did not affect pARC after I/R, but it did counter the preservation of pARC seen with APC.

The results of calcineurin measurements are shown in Figure 6. I/R increased the activity of calcineurin by ~108% ($P < 0.001$), whereas APC significantly inhibited the increase in activity of calcineurin ($P < 0.05$ vs. I/R). Consistent with its effects on pARC, FK+I/R resulted in lower levels of calcineurin that were similar to those seen with APC. Together, these data indicate that APC limited the I/R-related increase in calcineurin activity and preserved the phosphorylation status of ARC.

Figure 7 shows the effects of experimental interventions on cytochrome *c* release and activity of caspase-8. Cytochrome *c* release after APC was reduced by ~40% when compared with I/R ($P < 0.05$), an effect eliminated by TBB. Moreover, FK506 also reduced cytochrome *c* release compared with CON + I/R ($P < 0.05$). As is seen in Figure 7B, the activity of capspase-8 was increased by ~165% after I/R ($P < 0.05$ vs. CON). However, the increase in caspase-8 activity was significantly inhibited by interventions, which preserved the phosphorylation status of ARC, namely, APC and FK506 treatment ($P < 0.05$ vs. I/R). Moreover,

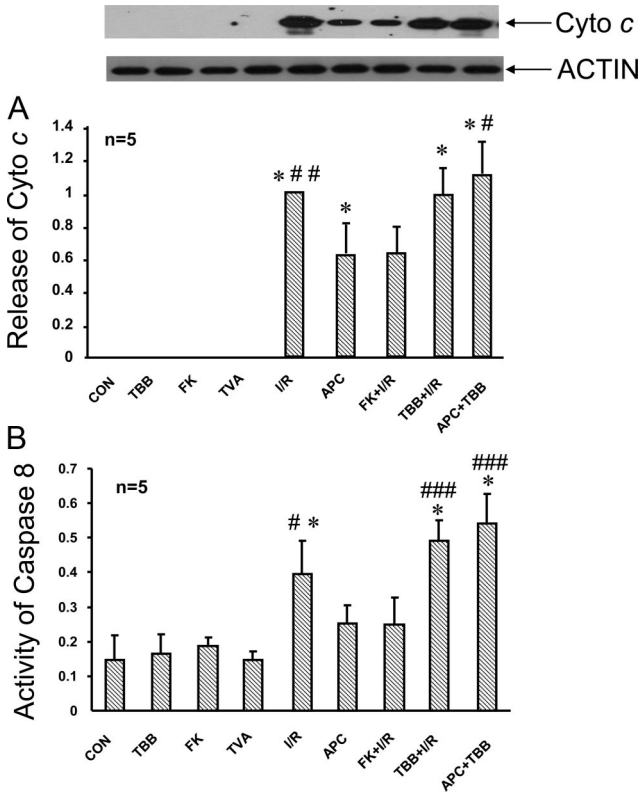

Figure 7. Cytochrome *c* (cyto *c*) release and activity of caspase-8 in the experimental groups. A, representative Western blots for cytochrome *c* with β-actin controls (CON) (top) and group data (relative units) (bottom). Anesthetic preconditioning (APC) and FK506 decreased cytochrome *c* release caused by ischemia/reperfusion (I/R), and this protective effect was abrogated by 4,5,6,7-tetrabromobenzotrizole (TBB). B, I/R resulted in increased caspase-8 activity, which was inhibited by APC and FK506. TVA = transient volatile anesthetic. $*P < 0.001$, experimental groups versus control; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ experimental groups versus APC.

concurrent TBB treatment with APC also increased the activity of caspase-8 by ~234%, indicating that inhibition of the phosphorylation of ARC diminished the effect of APC on caspase-8.

DISCUSSION

APC has profound positive effects on myocardial functional recovery and infarct size after global ischemia in animal models.[16,21–24] Since the initial descriptions of a protective effect of volatile anesthetics against myocardial I/R injury,[25,26] numerous studies have demonstrated that APC can protect the heart from reperfusion injury, in part by suppressing cardiomyocyte apoptosis.[2,27]

A number of investigators, including those in this laboratory, have shown that I/R results in an increase in $[Ca^{2+}]_i$, resulting in subsequent Ca-dependent events leading to cell damage.[3,28,29] In particular, sustained increases in cytosolic-free Ca^{2+} ($[Ca^{2+}]_i$) lead to activation of the serine–threonine phosphatase calcineurin and subsequent apoptosis in susceptible cells.[12,13] Activation of calcineurin may result in dephosphorylation of many apoptosis-related proteins.[11,13] Accumulating evidence strongly demonstrates that inhibiting calcineurin activity could have protective effects on I/R-induced cardiac injury. For example, Cai et al.[15] reported that FK506 and cyclosporine A, both calcineurin inhibitors, could protect ischemic myocardium and reduce infarct size. As with pharmacologic inhibition of calcineurin, the current experiments demonstrate that APC can inhibit calcineurin activity. Because prior studies have shown that APC limited the increase of myocardial intracellular and mitochondrial Ca^{2+} in during I/R,[3,21] it is plausible that the lower $[Ca^{2+}]_i$ seen with APC resulted in the observed decreased activity of calcineurin.

As an apoptosis repressor, ARC has been shown to block apoptotic cascades in several tissues, such as the heart[30] and embryonic kidney cells.[6] The antiapoptotic activity of ARC depends on its phosphorylation state,[11] which is a balance between the effects of protein kinase CK2 and the dephosphorylation by calcineurin.[6,10] In the present study, I/R did not change the expression of total ARC, but significantly down-regulated phosphorylation of ARC (Fig. 5) in parallel with an increase in calcineurin. The increased activity of calcineurin after I/R appeared to play an important role in regulating the phosphorylation status of ARC, because FK506, a pharmacologic inhibitor of calcineurin, attenuated the decrease in ARC phosphorylation levels caused by I/R. APC, as was hypothesized, reduced calcineurin and increased the levels of pARC, an effect abrogated by concurrent exposure to TBB. These data indicate that I/R-induced dephosphorylation of ARC is secondary to activation of calcineurin and that APC counters these effects of I/R.

The effects of APC on calcineurin and $[Ca^{2+}]$, in addition to preventing the decrease in pARC, may also be protective by modulating translation of nuclear factor-κ-B (NF-κB) into the nucleus. We have previously shown that APC has multiple effects on NF-κB, including activation by reactive oxygen species during anesthetic exposure and decreased translation during reperfusion, resulting in both translation of Bcl-2 to mitochondria and reduction of inflammatory cytokines.[2,20] Because an increase in calcineurin dephosphorylates the regulatory kinase IκBβ,[31] the decreased calcineurin activity after reperfusion seen with APC (Fig. 5) could explain, in part, the decreased NF-κB activity at this time.

One mechanism of protection by APC is reduction of apoptotic signaling. ARC has been shown to reduce apoptosis by reducing the release of cytochrome *c* and activity of caspase-8.[7,9,10,30] Under conditions of APC, the decreased calcineurin activity and higher levels of pARC were associated with reduced cytochrome *c* release and decreased caspase-8 activity, thus demonstrating the expected effects of higher pARC. These changes in cytochrome *c* and caspase-8, as well as translation of the antiapoptotic protein Bcl-2 to mitochondria, were previously shown to be reduced by transient pharmacologic inhibition of NF-κB during anesthetic exposure,[2] indicating the presence of multiple antiapoptotic effects of APC.

Limitations

First, although the myocardial-protective effects of APC may involve many different signaling pathways and mediators—such as protein kinase C, adenosine receptor, potassium channel opening, and NF-κB[2,20]—this study only addressed phenomena related to the ARC pathway

and the effects of APC that we observed during I/R in this specific isolated perfused heart model. The interaction of ARC with these other pathways and potential mediators was not defined by these experiments. Second, the anti-apoptotic effect of APC was assessed by both release of cytochrome *c* and activity of caspase-8, because these are known targets of ARC.[5,10,30] Although direct histologic measurements were not done, prior studies have shown that caspase-8 activity and histologic measures of apoptosis parallel each other.[32] Third, despite limiting CK release, FK506 did not improve LVEDP after I/R. This may be due to a failure of FK506, unlike APC, to limit calcium overload and myocardial stunning.[33,34] Finally, pharmacologic inhibition of calcineurin and phosphorylation of ARC by FK506 and TBB depends on the dose and specificity of these agents. It is noted that in addition to inhibition of calcineurin, FK506 can affect CREB transcriptional activity.[35] With respect to TBB, its inhibition of CK2 at the concentrations used is 1 to 2 orders of magnitude more than inhibition of phosphorylase kinase, glycogen synthase kinase 3 β, and cyclin-dependent kinase 2/cyclin A.[16] Because only 1 concentration of each agent was studied, it is possible that lower or higher doses would have different effects.

CONCLUSION

The present work provides novel evidence that ARC phosphorylation levels can be maintained by APC under conditions of I/R. APC prevents the increase in calcineurin after I/R, resulting in preserved levels of pARC, and decreased cytochrome *c* release and caspase-8 activation. FK506 has similar effects on calcineurin, pARC, and markers of apoptosis. In addition to APC, interventions that limit calcineurin activation, preserve phosphorylation of ARC, or both are potential strategies for limiting the consequences of I/R.

DISCLOSURES

Name: Xiyuan Lu, MS.
Contribution: This author helped conduct the study and analyze the data.
Attestation: Xiyuan Lu has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Name: Peter G. Moore, MD, PhD.
Contribution: This author helped analyze the data.
Attestation: Peter G. Moore has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Name: Hong Liu, MD.
Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.
Attestation: Hong Liu has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Name: Saul Schaefer, MD.
Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.
Attestation: Saul Schaefer has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.

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