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Activation of NF-κB is a critical element in the antiapoptotic effect of anesthetic preconditioning

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Departments of [1]Internal Medicine, Division of Cardiovascular Medicine, and [2]Anesthesiology and Pain Management, University of California, Davis; and [3]Cardiology Section, Department of Veteran Affairs, Northern California Health Care System, Mather, California; and [4]Department of Anesthesiology and Pain Medicine, University of Alberta, Edmonton, Alberta, Canada

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Lu X, Liu H, Wang L, Schaefer S. Activation of NF-κB is a critical element in the antiapoptotic effect of anesthetic preconditioning. *Am J Physiol Heart Circ Physiol* 296: H1296–H1304, 2009. First published March 20, 2009; doi:10.1152/ajpheart.01282.2008.—Anesthetic preconditioning (APC), defined as brief exposure to inhalational anesthetics before cardiac ischemia-reperfusion (I/R), limits injury in both animal models and in humans. APC can result in the production of reactive oxygen species (ROS), and prior work has shown that APC can modify activation of NF-κB during I/R, with consequent reduction in the expression of inflammatory mediators. However, the role of NF-κB activation before I/R is unknown. Therefore, these experiments tested the hypothesis that APC-induced ROS results in activation of NF-κB before I/R, with consequent increased expression of antiapoptotic proteins such as Bcl-2 and decreased apoptosis. Experiments utilized an established perfused heart rat model of sevoflurane APC and I/R. The role of NF-κB was defined by a novel method of transient inhibition of the regulatory kinase IKK using the reversible inhibitor SC-514. In addition to functional measures of left ventricular developed and end-diastolic pressure, phosphorylation of IκBα and activation of NF-κB were measured along with cytosolic protein content of Bcl-2, release of cytochrome *c*, and degradation of caspase-3. APC resulted in ROS-dependent phosphorylation of IκBα and activation of NF-κB before I/R. APC also increased the expression of Bcl-2 before I/R. In addition to functional protection following I/R, APC resulted in lower release of cytochrome *c* and caspase-3 degradation. These protective effects of APC were abolished by transient inhibition of IκBα phosphorylation and NF-κB activation by SC-514 followed by washout. ROS-dependent activation of NF-κB by APC before I/R is a critical element in the protective effect of APC. APC reduces apoptosis and functional impairment by increasing Bcl-2 expression before I/R. Interventions that increase NF-κB activation before I/R should protect hearts from I/R injury.

nuclear factor-κB; reactive oxygen species

PROTECTION OF ISCHEMIC myocardium, either in patients with acute coronary syndromes or patients undergoing surgical procedures, is becoming increasingly important as our population ages. In animal models, anesthetic preconditioning (APC) using inhaled anesthetic agents such as sevoflurane has demonstrated beneficial effects on myocardial function, ATP levels, and mitochondrial integrity as measured by cytosolic and mitochondrial calcium accumulation (1, 27, 45) and cytochrome *c* release (37). APC has also been shown to reduce cardiac injury during surgery in humans (23, 24) and reduce NH_2-terminal pro-brain natriuretic peptide (a marker of left ventricular stretch) (22) in patients undergoing coronary artery bypass surgery (15). Therefore, understanding the mechanisms of APC could be critical in improving the surgical outcomes of patients.

Our laboratory and others have elucidated some of the important mechanisms of APC, both in vivo and in vitro (44), as well as in newborn and young animals (27). Proposed mechanisms of inhaled anesthetic protection on the myocardium include the anesthetic-mediated release of reactive oxygen species (ROS) (2, 3, 19, 20, 32, 33), activation of mitochondrial ATP-sensitive K^+ (K_{ATP}) channels (27, 42), and, most recently, attenuation of NF-κB activation after ischemia-reperfusion (I/R) (54).

NF-κB is a pivotal inducible transcription factor that regulates the expression of many genes involved in important biological processes including inflammatory stress responses and cell survival (4). It is activated and translocated to the nucleus by stimuli such as interleukin-1, TNF-α, LPS, UV irradiation, ROS, and oxidative stress (7). Normally, NF-κB is maintained in the inactive form in the cytoplasm by the inhibitory protein IκB, mainly IκBα (40). Upon stimulation by ROS, IκB is rapidly phosphorylated by IKK and subsequently undergoes ubiquitination and degradation by a proteasome, thereby releasing NF-κB (40). The released NF-κB complex (consisting of p50 and p65 subunits) then translocates to the nucleus and activates target gene transcription (35). Negative feedback inhibition is provided by nuclear transcription of IκBα, which is then dependent on NF-κB binding to DNA promoter sequences.

NF-κB activation can be either detrimental or protective, depending on both the timing and degree of activation. Activation of NF-κB on reperfusion has been observed in models of I/R, with destructive effects in part due to the expression of inflammatory cytokines (54). However, paradoxically, a modest increase in NF-κB before ischemia can be protective in myocardial tissue. For example, activation of NF-κB by *Trypanosoma cruzi* (36), morphine-induced preconditioning (50), brief ischemia (26), hyperoxia (47), and viral-mediated delivery of IKK-β (39) all reduced injury and limited apoptosis with hypoxia or ischemia. The mechanism of NF-κB protection may depend on the upregulation of a number of protective genes, including genes for antiapoptotic proteins such as Bcl-2, thereby limiting apoptosis (30, 39, 49).

Since most studies have only examined the effect of APC on changes occurring after I/R (19), the effect of APC on NF-κB activation before I/R, and the subsequent changes in gene expression dependent on NF-κB, has not been elucidated. Therefore, these experiments tested the hypothesis that APC-

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induced ROS production results in NF-κB activation and expression of antiapoptotic proteins before I/R, resulting in improved functional recovery and reduced I/R injury. This hypothesis was tested in an isolated perfused rat heart model of APC using a technique of reversible inhibition of IκBα phosphorylation and NF-κB activation during APC. The findings indicate that ROS-mediated NF-κB activation is a critical element of APC and that increased expression of antiapoptotic proteins such as Bcl-2 secondary to NF-κB activation by APC before I/R limits apoptosis.

METHODS

The study protocol was approved by the Animal Care Committee of the University of California, Davis (Davis, CA), and all experiments were conducted in accordance with guidelines of animal care from the National Institutes of Health.

All experiments used an isolated perfused rat heart model as previously described (27). Briefly, hearts were obtained from male Sprague-Dawley rats (weight, 250–300 g). Anesthesia was first induced with an intraperitoneal injection of sodium thiopental (50–75 mg/kg) along with 1,000 U heparin. Sodium thiopental was chosen for initial anesthesia because this drug has been shown not to influence preconditioning (31). The heart was excised and placed in an ice-cold solution of Krebs-Henseleit buffer. It was then cannulated and Langendorff perfused with Krebs-Henseleit buffer containing (in mm) 127 NaCl, 4.7 KCl, 1.25 $MgCl_2$, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 10 glucose at a constant perfusion pressure of 80 ± 10 cmH_2O at 37 ± 0.5°C. The perfusion was continuously oxygenated with 95% O_2-5% CO_2.

APC was obtained 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 measured at the cannula (46), corresponding to one minimum alveolar concentration (17). After 10 min treatment with sevoflurane, the hearts were reperfused with Krebs-Henseleit buffer for 20 min.

Experimental protocols. Protocol A was designed to assess the effects of APC-induced production of ROS in phosphorylation of IκBα (p-IκBα) and activation of NF-κB before I/R. Rats were randomly assigned to one of four groups: control perfusion or APC with or without concurrent exposure to the ROS scavenger 2-mercaptoproprinylglycine (2-MPG; 1 mM), each followed by 20 min of washout. In each group, hearts were immediately freeze-clamped following the experiment for further analysis of p-IκBα and NF-κB.

The goal of experiments in *protocol B* (Fig. 1) was to develop and validate a strategy that would reversibly inhibit p-IκBα and activation of NF-κB during exposure to sevoflurane yet permit a normal response to I/R. In brief, we used the reversible selective IKK-2 inhibitor SC-514 (100 μM; Calbiochem, San Diego, CA) with concurrent exposure to APC and defined the restoration of a normal response using LPS as a pharmacological inducer of IκBα and NF-κB (21). The first set of experiments was similar to those in *protocol A*, except that SC-514 or placebo was concurrently administered with APC and p-IκBα and NF-κB were measured after either APC or APC plus washout. The second set of experiments then used LPS following the above protocols, and p-IκBα and NF-κB were measured after LPS exposure.

Protocol C utilized the model developed in *protocol B* above to determine the effects of reversible inhibition of IκBα and NF-κB on I/R protection on functional recovery by measuring left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), myocyte injury [creatine kinase (CK) release], and apoptosis (caspase-3). Following the preparatory periods illustrated in *protocol C*, the hearts underwent I/R, defined as 30 min total global ischemia produced by shutting off flow to the heart, followed by 30 min reperfusion. Hearts were freeze-clamped at the end of I/R.

Experiments in *protocol D* (not shown) examined the effect of APC with and without reversible inhibition by SC-514 on cytochrome *c* release and Bcl-2 expression before and following I/R. Protocols were identical to those in *group C*, except that hearts were frozen at time points immediately before and after I/R for analysis.

Functional measurements. To measure left ventricular pressures, a latex balloon was filled with water and 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 LVEDP of 5–10 mmHg. Pressures were recorded using Powerlab 4/20 hardware with an amplifier and Chart for Windows version 4.0.4 software (ADInstruments, Colorado Springs, CO).

CK analysis. The runoff from the coronary sinus was collected for the first 5 min of reperfusion. This was placed in aliquots and stored at −80°C until analysis. The amount of CK was determined using an EnzyChrom Creatine Kinase Assay Kit (BioAssay Systems) and UV-VIS recording photospectrometer. Units are expressed as units per gram of wet weight (Microplate Reader; BioTek).

Protein analysis. Western blot analyses were used to measure the levels of p-IκBα, Bcl-2, caspase-3, and cytochrome *c* in the hearts. Heart cytosolic proteins were loaded and separated on 12% SDS-PAGE, followed by transblotting to an immunblot polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). The membrane was subsequently probed with primary p-IκBα, Bcl-2, caspase-3, and cytochrome *c* antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1,000. Horseradish peroxidase-conjugated secondary antibody was added at 1:3,000 dilution. The blots were subsequently developed using an enhanced chemiluminescence detection kit (Thermo). After exposure on autoradiography film, immunoreactive protein bands were quantified by densitometry.

NF-κB-DNA binding activity. A microwell colorimetric assay was performed to determine NF-κB-DNA binding activity (41). Briefly, 20 μg heart nuclear extracts were diluted by the complete lysis buffer per well, and the activity of the NF-κB-DNA was determined using TransAM NF-κB transcription factor assay kits (Active Motif). Absorbance was read on a spectrophotometer within 5 min at 450 nm with a reference wavelength of 655 nm.

Protein isolation. At the time points indicated in the experimental protocols, hearts from each group were frozen in liquid nitrogen and subsequently homogenized in ice-cold Tris·HCl buffer containing 25 mm Tris, 1 mm EDTA, 10% glycerol, and 1 mm DTT (pH 7.4) with homogenizer (PowerGen 1800D; Fisher Scientific) (54). The homogenate was centrifuged at 10,000 *g* for 20 min at 4°C. The supernatant (cytosolic fraction) was aspired and stored at −80°C. The crude nuclear fraction in the low-speed centrifugation was collected and washed three times with homogenate buffer containing Triton X-100, followed by washing one time without Triton X-100. Nuclear protein was extracted with buffer containing 20 mm HEPES, 25% glycerol, 0.42 m NaCl, and 1 mm EDTA by centrifuging at 50,000 *g* for 30 min. Protease inhibitor cocktail was added into the homogenizing and extract buffer. Protein concentrations of the extracts were measured by means of a modified Bradford assay according to the manufacturer's instructions (Bio-Rad) using BSA as a standard.

Statistics. Data are presented as means ± SE. Outcome measures for each experimental group were compared using two-tailed ANOVA for repeated measures with Holm-Sitek or Dunn's posttest as appropriate on the statistical program SPSS (SPSS, Chicago, IL). A $P < 0.05$ was used to test the null hypothesis.

RESULTS

APC phosphorylation of IKBα and activation of NF-κB requires ROS. The effect of APC on the phosphorylation of IκBα and activation of NF-κB were examined by Western blot assay and NF-κB-DNA binding activity assay, respectively.

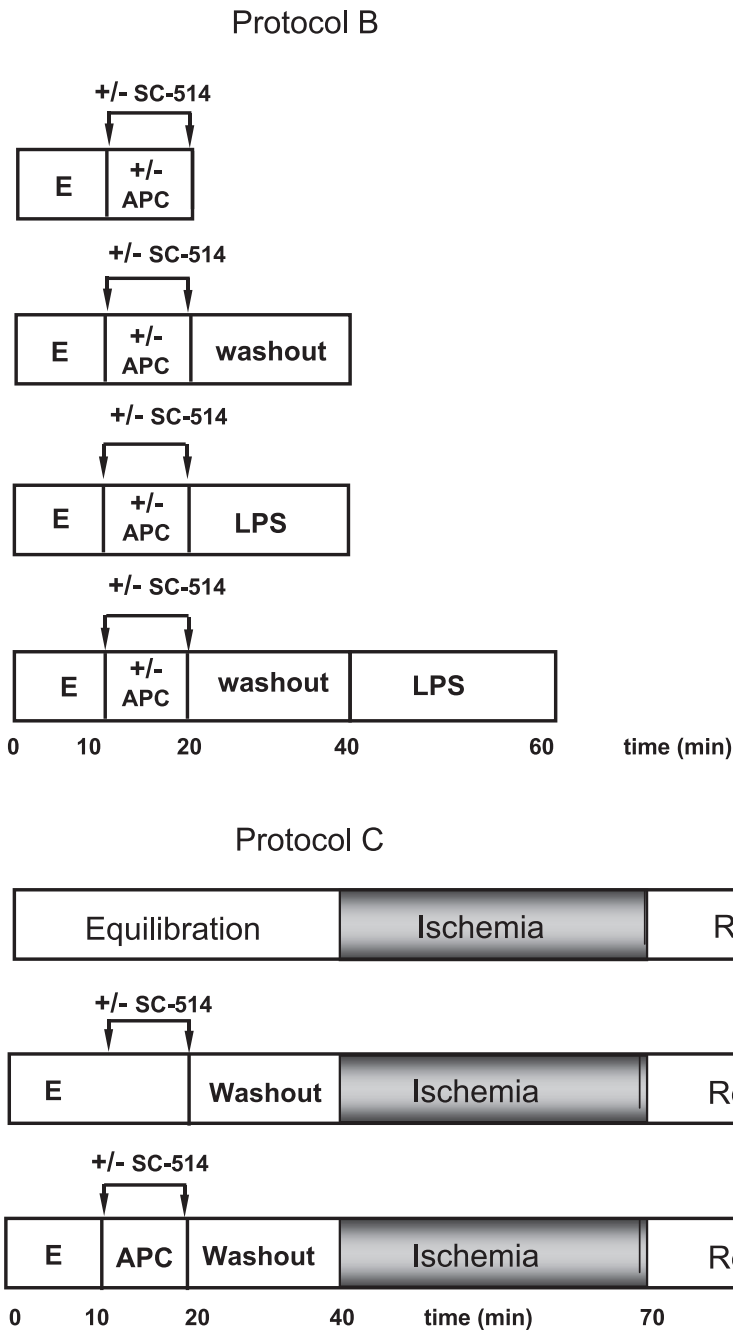

Fig. 1. Schematic of the experimental design. *Protocol B* validated the effect of SC-514 and subsequent washout on phosphorylation of IκBα (p-IκBα) and NF-κB with anesthetic preconditioning (APC) and/or LPS. In each experiment, p-IκBα and NF-κB were measured at the end of the experiment. In addition, measurement of Bcl-2, caspase-3, and cytochrome *c* was done at the end of washout in the first and third groups in *protocol B*. *Protocol C* involved the ischemia-reperfusion (I/R) experiments, with measurement performed at the end of reperfusion ($n = 4–6$ in all experiments). E, equilibration period.

When compared with control hearts, phosphorylated IκBα was increased by 125 ± 50% (Fig. 2*A*) and NF-κB-DNA binding activity (Fig. 2*B*) was increased by 72 ± 2% at the end of APC alone ($P < 0.05$). Exposure of hearts to the ROS scavenger 2-MPG only during the APC episode inhibited the activation caused by APC, such that NF-κB activation was significantly reduced ($P < 0.05$; Fig. 2*B*).

SC-514 with washout reversibly inhibits phosphorylation of IκBα and activation of NF-κB. The effects of SC-514 without and with 20 min washout on the activity of NF-κB and phosphorylation of IκBα were examined using LPS exposure as a stimulus known to induce phosphorylation of IκBα and activation of NF-κB. Figure 3 shows that, when compared with control, APC alone caused a significant increase in both the phosphorylation of IκBα ($P < 0.05$; Fig. 3*A*) and activation of NF-κB ($P < 0.05$; Fig. 3*B*). Exposure to SC-514 during APC blocked this activation, independent of washout following APC.

Following either control perfusion or APC, hearts then exposed to 100 μg/ml LPS demonstrated a large increase in p-IκBα and NF-κB, an effect completely inhibited by the presence of SC-514. Thus, SC-514, in this model, was an effective inhibitor of IκBα phosphorylation and NF-κB activation induced by either APC or LPS.

Critically, washout of SC-514 for 20 min after control perfusion or APC restored the response to LPS (phosphorylation of IκBα and activation of NF-κB) without any exposure to SC-514. These data demonstrate that although SC-514 is an

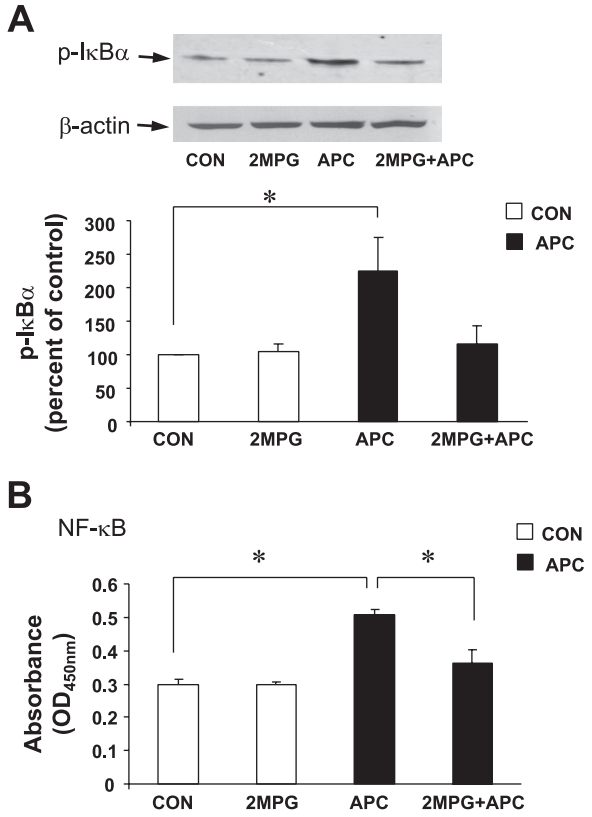

Fig. 2. Experiments showing the effect of the reactive oxygen species scavenger 2-mercaptoproprinylglycine (2-MPG; hearts exposed for 10 min) on p-IκBα and NF-κB (*protocol A*). *A*, *top*: typical Western blot of p-IκBα under the 4 experimental conditions. *A*, *bottom*: p-IκBα as a fraction of control. *B*: results of the assay for activation of NF-κB. Only the increase in NF-κB was significantly blocked by concurrent exposure to 2-MPG. Control (Con) hearts were perfused for 40 min. For APC, hearts had 10 min exposure to sevoflurane before 20 min washout. For 2-MPG + APC, hearts had concurrent exposure to 2-MPG during APC. *$P < 0.05$ between indicated experimental groups. OD_{450nm}, optical density at 450 nm.

effective inhibitor of p-IκBα and NF-κB activation, washout of this inhibitor restores the response of hearts to stimuli to control levels.

Activation of NF-κB is a critical element of APC protection on cardiac functional recovery, injury, and apoptosis. As previously shown (27), APC improved myocardial systolic function following I/R as measured by recovery of LVDP following I/R [APC, 78 ± 4 mmHg after I/R (baseline, 85 ± 20 mmHg) vs. control, 13 ± 11 mmHg after I/R (baseline, 86 ± 7 mmHg); $P < 0.05$; Fig. 4]. Exposure of control hearts to SC-514 for 10 min, followed by 20 min washout, had no effect on recovery of LVDP (11 ± 7 mmHg after I/R), whereas exposure of APC hearts to SC-514 with washout also resulted in diminished recovery compared with APC alone (29 ± 4 mmHg; $P < 0.05$).

LVEDP, a measure of contracture, was significantly reduced by APC [APC, 15 ± 2 mmHg (baseline, 10 ± 1 mmHg) vs. 49 ± 8 mmHg (baseline, 8 ± 4); $P < 0.05$]. However, APC hearts concurrently exposed to SC-514 followed by washout lost the protective effect of APC on LVEDP (40 ± 9 mmHg in APC with SC-514 with washout; $P < 0.05$ vs. APC).

Cardiac myocyte injury, as measured by the release of CK immediately on reperfusion, was reduced by APC (91 ± 13 IU/g wet wt APC vs. 298 ± 22 control; $P < 0.05$; Fig. 5). Although SC-514 with washout had no effect on CK release in control I/R hearts, SC-514 with washout had a modest effect on CK release in the APC-I/R hearts (APC + SC-514 + washout, 144 ± 5 IU) compared with APC-I/R alone ($P < 0.05$).

Cardiac myocyte apoptosis following I/R, as measured by cleavage of caspase-3, was reduced by 69% by APC compared with control hearts ($P < 0.05$; Fig. 6*A*). This beneficial effect of APC on apoptosis was eliminated by concurrent exposure to SC-514 with washout, resulting in caspase-3 equivalent to that of untreated control hearts (Fig. 6*B*). Similarly, cytochrome *c* release following I/R was reduced by APC ($P < 0.05$), and this effect was eliminated by SC-514 with washout. Interestingly, APC alone caused an increase in cytochrome *c* release before I/R ($P < 0.05$), possibly due to the release of ROS by APC.

Activation of NF-κB by APC results in increased cytosolic levels of Bcl-2 before and following I/R. One mechanism by which activation and translocation of NF-κB can protect against subsequent I/R is the induction of one or more antiapoptotic genes. To test this hypothesis, the cytosolic protein levels of Bcl-2, an antioxidant oncogene that is inversely related to apoptosis (16), were measured before and after I/R (Fig. 7). As seen in Fig. 7*A*, APC alone resulted in significantly increased levels of Bcl-2 before I/R compared with control perfusion ($P < 0.05$), an effect that was blocked by concurrent exposure to SC-514. Bcl-2 protein levels following I/R were similarly elevated in APC + I/R hearts ($P < 0.05$). However, Bcl-2 protein levels following I/R in SC-514 washout hearts were similar to control hearts, indicating that inhibition of NF-κB activation by APC prevented Bcl-2 expression both before and after I/R.

DISCUSSION

These experiments validate the protective role of APC in limiting the detrimental consequences of cardiac I/R and support the hypothesis that APC-induced ROS is an essential element of the protective pathway. Importantly, using a novel technique of reversible inhibition of IκBα phosphorylation and NF-κB activation, these experiments show that NF-κB activation is a pivotal element in the mechanism of APC protection and that this pathway involves the upregulation of Bcl-2 before I/R and subsequent reduction of cytochrome *c* release and decreased caspase-3 activity.

Mechanisms of APC. APC has profound effects on myocardial functional recovery and injury after I/R in animal models (5, 29, 38, 42, 54). Numerous mechanisms for APC have been supported by both in vitro and in vivo studies, including an increase in ROS during anesthetic exposure (32), induction of heat shock protein (34) opening of K_{ATP} channels before I/R (18, 27), reduced mitochondrial calcium accumulation (27), and lower NF-κB activation and release of inflammatory cytokines after I/R (8, 54).

APC induces ROS. There are substantial data showing that enhanced generation of ROS due to anesthetic-induced attenuation of mitochondrial electron transport is required as an initiating factor to trigger APC protection (14, 32, 43), findings similar to the role that ROS have in initiating ischemic preconditioning (9). The subsequent cell-signaling and ROS-

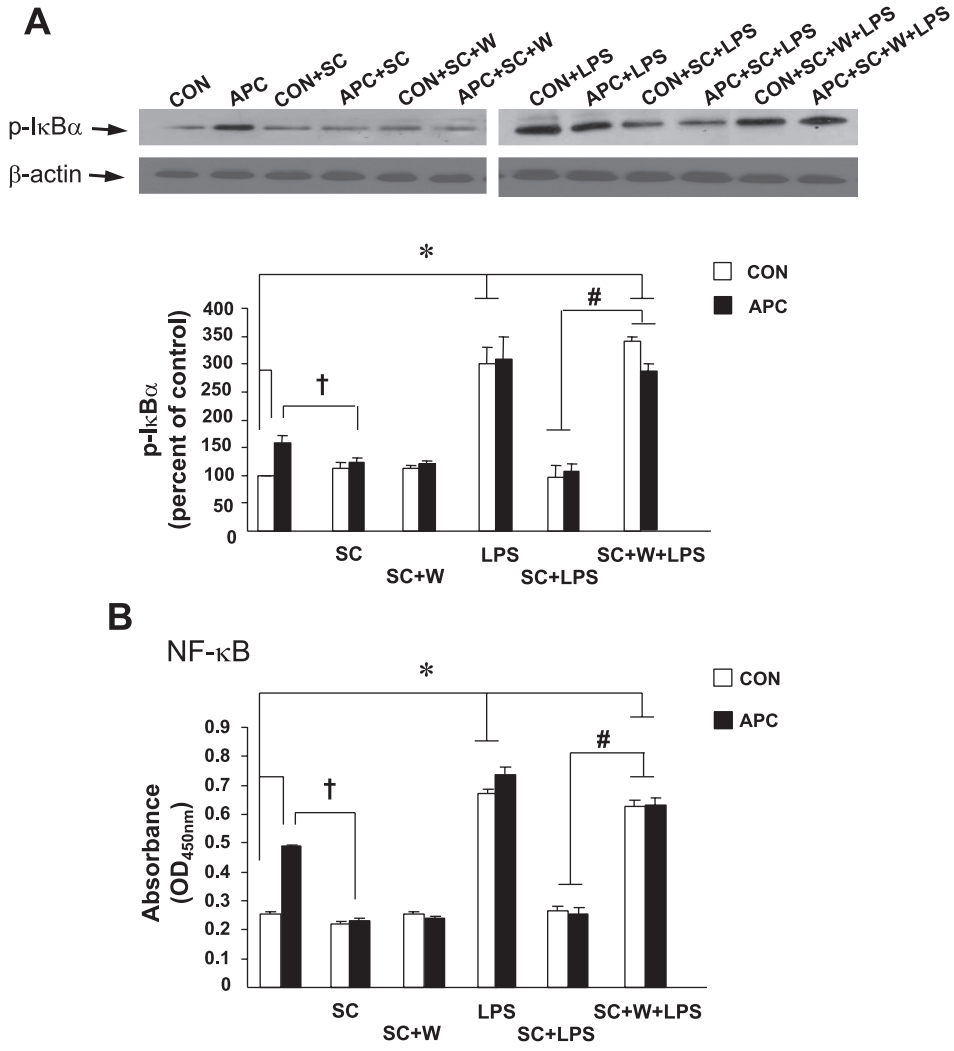

Fig. 3. Effect of SC-514 (SC) on p-IκBα and NF-κB (*protocol B*). *A*, *top*: Western blots for p-IκBα for the various experimental conditions. *A*, *bottom*: group data for p-IκBα as a fraction of control perfusion. *B*: activation of NF-κB under the same conditions. APC alone increased p-IκBα and NF-κB moderately, an effect blocked by SC-514. However, with SC-514 with washout, hearts stimulated with LPS responded appropriately, independent of APC. W, washout. $*P < 0.05$ experimental groups vs. control; $\#P < 0.05$ SC + W + LPS vs. SC + LPS; $\dagger P < 0.05$, APC + SC vs. APC.

mediated pathways include modulation of ion channels and activation of enzyme systems, transcription factors, and kinases (20). However, the degree of anesthetic exposure is critical, since prolonged exposure to inhalational anesthetics in cultured cells can result in apoptosis (52).

APC induces ROS-dependent phosphorylation of IκBα and activation of NF-κB. As noted earlier, ROS stimulation, in general, causes phosphorylation of IκB by IKK, leading to ubiquitination and degradation by a proteasome, thereby releasing NF-κB (11). The released NF-κB complex (consisting of p50 and p65 subunits) then translocates to the nucleus and activates target gene transcription. Consistent with the hypothesis, the current data show that using a scavenger of ROS during the exposure period to sevoflurane abolished the effect of APC on both phosphorylation of IκBα and activation of NF-κB. Although the difference in p-IκBα between APC alone and 2-MPG + APC was not statistically different (likely due to a type II error), the difference in NF-κB between these two groups was statistically different. Combined with prior data showing that an ROS scavenger abrogates the protective effect of APC (43), these findings support ROS activation of NF-κB as a critical element in APC.

NF-κB as double-edged sword. Depending on timing and amount, activation of NF-κB can be either protective or harmful. I/R alone result in activation of NF-κB, with deleterious consequences such as induction of inflammatory cytokines and cleavage of pro-caspases (11, 25). As expected, pharmacological inhibition of NF-κB before and during I/R has been shown to reduce injury and apoptosis, with the expected reduction in the release of cytokines (12).

Despite the injury caused by activation of NF-κB by I/R, NF-κB activation at a lower level before I/R may be protective. Pharmacological activation of NF-κB by means such as recombinant human erythropoietin (28), adenosine (53), opiods (13), or adenovirus-mediated delivery of wild-type IKK-β (39) has shown protection with subsequent I/R or hypoxia. Similarly, preconditioning stimuli such as hyperoxia (6) can activate NF-κB. Thus there are substantial data that modest NF-κB activation can be protective if occurring prior to a subsequent event, such as I/R, which results in greater NF-κB activation.

SC-514 reversibly inhibits phosphorylation of IκBα. These experiments showed that the reversible IKK inhibitor SC-514, used concurrently with sevoflurane exposure, inhibited both phosphorylation of IκBα and activation of NF-κB with either

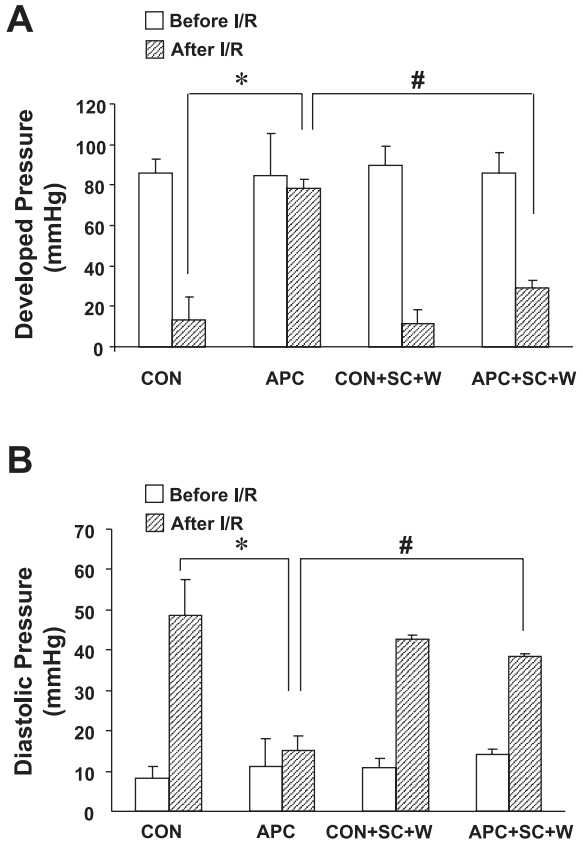

Fig. 4. Functional recovery of hearts, as determined by left ventricular developed pressure (LVDP; *A*) and left ventricular end-diastolic pressure (LVEDP; *B*) before and after I/R, as a function of experimental conditions in *protocol C*. APC protected hearts, with LVDP and LVEDP not different than control perfusion. However, SC-514 (SC) with washout eliminated the beneficial effect of APC on both LVDP and LVEDP. *$P < 0.05$ APC vs. control after I/R; #$P < 0.05$ APC + SC + W vs. APC alone.

APC or LPS exposure. However, when followed by a 20-min washout period, hearts transiently exposed to SC-514 were able to respond normally to LPS stimulation independent of sevoflurane exposure. These data indicate the inhibition by SC-514 was both complete (preventing the normal response to

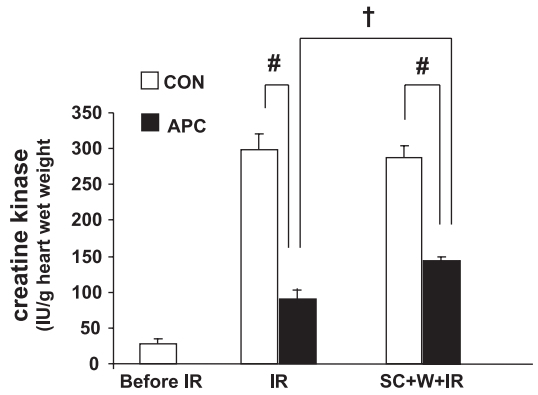

Fig. 5. Creatine kinase (CK) release following I/R (IR) in the experimental groups. CK release after I/R was significantly greater than baseline in all groups (symbols not shown). CK release was markedly reduced by APC, an effect only partially blocked by SC-514 (SC) with washout. #$P < 0.05$ vs. corresponding control after I/R; †$P < 0.05$ APC + SC + W vs. APC alone.

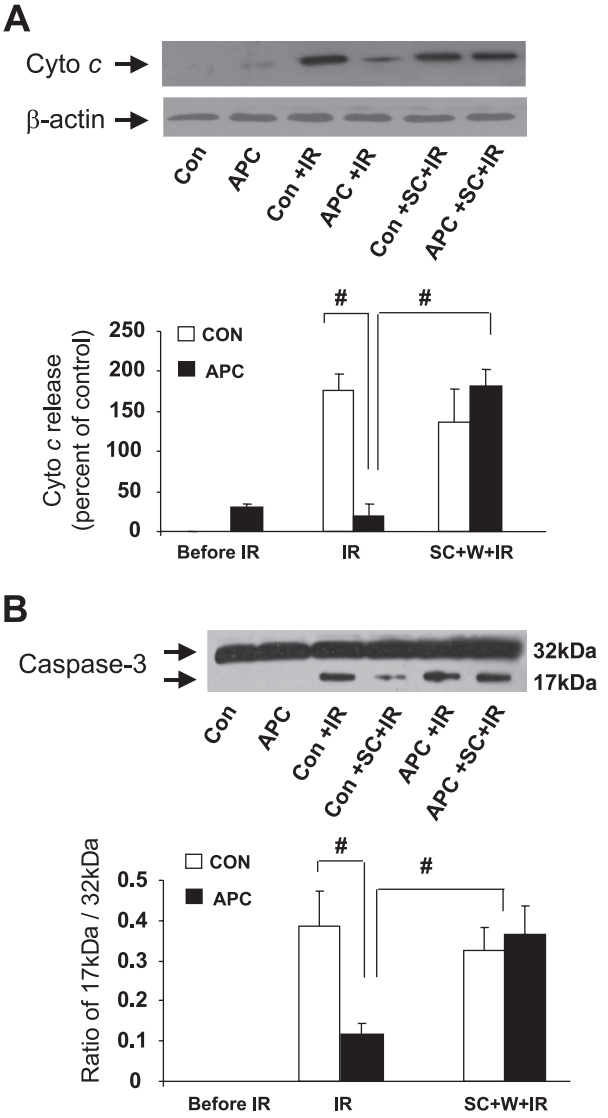

Fig. 6. Cytochrome *c* (cyto *c*) release and 17-kDa caspase-3 degradation before and after I/R (IR). *A* and *B*, *top*: representative Western blot for cytochrome *c* with β-actin controls. *A* and *B*, *bottom*: group data. APC caused a small but significant increase in cytochrome *c* release before I/R. The increase in cytochrome *c* release following I/R in control hearts was significantly reduced by APC. This protective effect of APC was abrogated by SC-514 (SC) with washout. A similar response is seen in the degradation of caspase-3, although no caspase-3 was detected before I/R. All values of cytochrome *c* and caspase-3 were significantly greater than control before I/R ($P < 0.05$, symbols not shown; #$P < 0.05$, comparisons as shown).

LPS) and reversible (allowing the normal response to LPS after washout).

The protective effects of APC are blunted by transient inhibition of NF-κB activation. Consistent with prior data (54), APC reduced the detrimental effects of I/R on LVDP and LVEDP (Fig. 4) and limited the release of CK on reperfusion. Transient inhibition of NF-κB by SC-514 eliminated the protective effect of APC on developed pressure and end-diastolic pressure, with a more modest, but still significant, effect on the release of CK. This discordance between functional measures and infarct size has been previously noted by others (10) and likely reflects beneficial

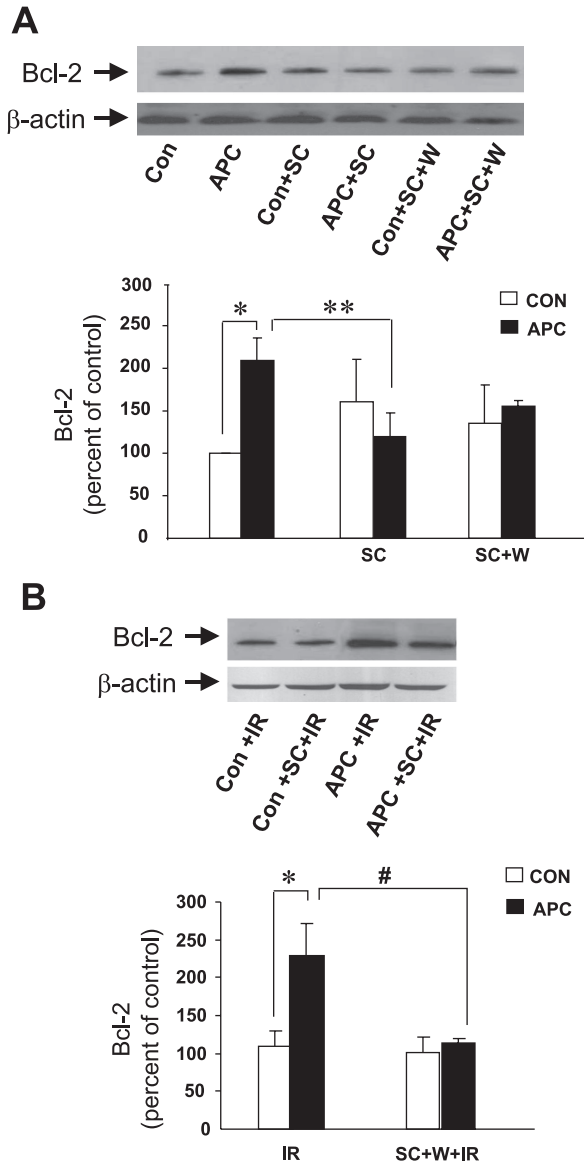

Fig. 7. Expression of Bcl-2 before (*A*) and following (*B*) I/R (IR). *A* and *B*, *top*: representative Western blots. *A* and *B*, *bottom*: group data. APC alone caused a significant increase in Bcl-2 before I/R, an effect blocked by SC-514 (SC) alone or with washout. The increase in Bcl-2 was maintained following I/R, again blocked by SC-514 with washout. *$P < 0.05$ vs. corresponding control; **$P < 0.05$ APC vs. APC + SC; #$P < 0.05$ APC vs. APC + SC + W.

effects of APC on stunning versus myocyte injury (20) and/or a greater effect of APC on apoptosis versus injury.

APC induces Bcl-2 expression before and following I/R. APC alone resulted in a twofold increase in Bcl-2, an effect blocked by concurrent exposure to SC-514. Following I/R, the increase in Bcl-2 was similar to that seen without I/R, namely a twofold increase over control conditions.

The increases in Bcl-2 expression are consistent with those reported following I/R in APC-treated rabbit hearts (38) and hyperoxic preconditioned rat hearts (6). However, in contrast with the present study, Bcl-2 expression preceding I/R was not measured in either study, leaving open the question of whether gene expression was modulated before or during I/R. The current data show that despite the relatively brief time period (30 min for APC + washout), expression of Bcl-2 was increased significantly.

In addition to increased Bcl-2 expression following I/R, the study by Raphael et al. (38) also showed that APC was dependent on the activation of the PI3K survival pathway and increased Akt phosphorylation at the end of I/R. Since this signaling pathway would serve to inhibit NF-κB activation, it is likely that the measurements made reflected the effect of APC in reducing NF-κB activation during and following I/R, similar to the results reported by Zhong et al. (54). Since the current experiments examined events after APC but before I/R, it is not known whether activation of the survival pathway occurs with anesthetic exposure in the absence of I/R.

APC reduces cytochrome c release and caspase-3 following I/R. In parallel with the increase in Bcl-2 expression by APC, markers of apoptosis (cytochrome *c* release and caspase-3 degradation) were reduced by APC and restored by transient inhibition of NF-κB. The protective effects of APC on apoptosis parallel the findings of Qian et al. (37) and demonstrate that NF-κB activation by APC has a profound antiapoptotic effect.

Limitations. These findings must be considered in light of the perfused heart model used. Although several studies have demonstrated efficacy of APC in human surgical conditions (23, 24), it is unknown whether NF-κB activation occurs in human APC. In addition, these findings are applicable to early APC, not delayed APC in which I/R occurs 24–48 h following anesthetic exposure. The specificity of the intervention to limit NF-κB activation during APC, namely SC-514 and washout, is not well defined. SC-514 has been well characterized as a specific inhibitor of IKK-2 with an IC_{50} of 14.4 μM and a half-life of 12 min (21). In addition, the much higher IC_{50} for SC-514 against an array of tyrosine and serine-threonine kinases, and, in the current experiments, the absence of an inhibitory effect on LPS stimulation after 20 min of washout, support the specificity of this intervention on IKK. ROS production by APC in this model was not directly measured; however, there are ample data in prior studies showing that anesthetic exposure, as used in this study, results in ROS production (2, 3, 19, 20, 32, 33). Finally, histological determination of infarct size was not performed due to the need for tissue analysis. However, CK release is an accepted marker of pathological cell necrosis in dogs and perfused rat hearts (48, 51).

Conclusions

APC is a powerful mechanism to limit injury from I/R. With the use of a novel technique of transient inhibition of IKK in the perfused rat heart, these data show that ROS-mediated phosphorylation of IκBα and activation of NF-κB before I/R are critical elements of the protective mechanism of APC. One mechanism of this effect is the increased expression of the antiapoptotic protein Bcl-2 by APC before ischemia and a resultant decrease in cytochrome *c* release and caspase-3 degradation with I/R. Interventions (such as APC) that increase ROS and appropriately activate NF-κB could limit I/R injury in the human.

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