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

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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 IkBα 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 IkBα 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 IkBα 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 NH2-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 relief of reactive oxygen species (ROS) (2, 3, 19, 20, 32, 33), activation of mitochondrial ATP-sensitive K+ (KATP) 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, IκBα 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-
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 IkBα 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 to not 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 MgCl2, 2.5 CaCl2, 25 NaHCO3, and 10 glucose at a constant perfusion pressure of 80 ± 10 cmH2O at 37 ± 0.5°C. The perfusion was continuously oxygenated with 95% O2-5% CO2.

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 mg heart nuclear extracts were diluted by the complete lysis buffer and subsequently homogenized in ice-cold Tris containing 25 mm Tris, 1 mm EDTA, 10% glycerol, and 1 mm DTT (pH 8.0). The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant (cytosolic fraction) was aspirated 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 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 aspirated 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 IkBα and activation of NF-κB were examined by Western blot assay and NF-κB-DNA binding activity assay, respectively.
When compared with control hearts, phosphorylated IκBα was increased by 125 ± 50% (Fig. 2A) 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.

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. 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.

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. 3A) and activation of NF-κB (P < 0.05; Fig. 3B). 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
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. 6A). 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. 6B). 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. 7A, 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 I/R 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 KATP 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-
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).

**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).

Although the difference in p- IkBα 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.
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 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
or during I/R. The current data show that despite the question of whether gene expression was modulated before I/R was not measured in either study, leaving open the hypothesis that Bcl-2 expression preceded I/R (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 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 and SC-514 with washout, is not well defined. SC-514 has been well characterized as a specific inhibitor of IKK-2 with an IC<sub>50</sub> of 14.4 μM and a half-life of 12 min (21). In addition, the much higher IC<sub>50</sub> 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 IkBα 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.
REFERENCES


