In Vivo Hyperoxic Preconditioning Prevents Myocardial Infarction by Expressing Bcl-2

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Preconditioning with oxidative stress has been demonstrated *in vitro* to stimulate the cellular adaptation to subsequent severe oxidative stress. However, it is uncertain whether this preconditioning works *in vivo*. In the present study, we examined *in vivo* the beneficial effect of oxidative preconditioning. After rats were pretreated with whole-body hyperoxygenation (100% O_2 at 3 atmosphere for 20 mins, four cycles with 20-min intermission), isolated hearts were subjected to 45-min ischemia followed by 90-min reperfusion. This hyperoxic preconditioning significantly reduced infarct size, cytochrome-*c* release, DNA fragmentation, and terminal deoxynucleotidyl transferase-mediated dUTD nick-end labeling-positive cell frequency in the left ventricle, biphasically with an early (30-min) and a delayed (48-hr) effect after the hyperoxygenation. Mechanistically, the NF-κB activity and Bcl-2 expression were enhanced in the hearts, and a NF-κB inhibitor, pyrrolidine dithiocarbamate, abolished the Bcl-2 induction as well as the infarct-limiting effect. An antioxidant, N-acetylcysteine, and protein kinase C (PKC) inhibitors chelerythrine and Gö 6983 also blocked the preconditioning effects. These results indicate that hyperoxia induces myocardial tolerance against ischemia-reperfusion injury in association with Bcl-2 induction by NF-κB activation through reactive oxygen species and PKC-dependent signaling pathway. Exp Biol Med 231:463–472, 2006

Key words: hyperoxia; preconditioning; reperfusion injury; heart; NF-κB, Bcl-2

Introduction

Ischemia-reperfusion (I/R) injury is a major complication occurring in heart stroke, cardiopulmonary bypass surgery, and heart transplantation. During the early reperfusion stage, oxidative stress results in destruction of essential components in heart cells (1). Paradoxically, oxidative stress can be a profitable stimulus to enable hearts to withstand I/R injury. Many oxidative stimuli, such as transient ischemia (2), inflammatory mediators (3), and heat shock (4), have been demonstrated to induce myocardial tolerance to I/R injury. This indication is strongly supported by a number of studies demonstrating that antioxidants treated before these stimuli block the development of I/R tolerance (5–7). An experiment showing I/R tolerance in rabbit hearts pretreated with reactive oxygen species (ROS) also supports this suggestion (8). Thus, it is considered that pretreatment of hearts with oxidative stress may elicit a protective effect against the cellular damage produced by subsequent I/R. However, these experiments were done in cultured cells or in isolated heart tissues because of the technical limitation of *in vivo* oxidative stress.

Hyperbaric oxygen therapy, which involves intermittent inhalation of 100% oxygen under a pressure greater than 1 atmosphere (atm) absolute, can increase arterial and tissue oxygen tension up to 2000 and 400 mm Hg, respectively. Such doses of oxygen have a number of beneficial biochemical, cellular, and physiologic effects. Indeed, hyperbaric oxygen has successfully been employed in the management of hypoxia-aggravated clinical conditions, including poorly healing wounds, chronic osteomyelitis, diabetic and pressure ulcers, necrotizing fascitis, crush injuries, radiation-induced hemorrhagic cystitis, optic neuritis, and carbon monoxide intoxication (9, 10).

Hyperoxic treatment can be safely used for human beings if it is applied according to established protocols, with oxygen pressure not exceeding 3.0 atm and a duration limited to a maximum of 2 hrs. However, since the ROS production depends on the oxygen tension, most tissues may undergo oxidative stress during whole-body treatment. Fortunately, the oxidative stress is endurable and does not produce serious adverse effects. Therefore, hyperoxic treatment can be an appropriate method for introducing an oxidative stress *in vivo* (11). Therefore, we here used whole-

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body hyperoxygenation as an *in vivo* oxidative stress. Although we previously used a long-term hyperoxia to induce catalase in rat hearts (12), the 4-day protocol could be inconvenient for application in clinical settings. Thus, we tried an easier method to reduce I/R injury using a short-term hyperoxia. This new method prevented the apoptosis of cardiac cells by stimulating the NF-κB/Bcl-2 pathway rather than by inducing antioxidant enzymes. As the hypoxic pretreatment for I/R tolerance is named "hypoxic preconditioning," the hyperoxic pretreatment is here designated "hyperoxic preconditioning."

Materials and Methods

Animals and Hyperoxic Pretreatment (HOP) Protocol. Specific-pathogen-free adult male Sprague-Dawley rats weighing 200–250 g were used. Rats were exposed to 100% O_2 at 3 atm for 20 mins; four consecutive cycles with 20 mins of normobaric, normoxic intermission in a hyperbaric chamber. Compression and decompression were carried out at a rate of 0.2 atm/min. The gas in the chamber was continuously ventilated to minimize pCO_2 changes, and the temperature of the chamber was maintained at a range of 22°–25°C. All animal procedures were performed according to the established procedures of the *Seoul National University Laboratory Animal Maintenance Manual.* Total numbers of animals used for this study are 276 (i.e., control, 11; I/R only, 34; 0.5 hrs after HOP, 96; 3 hrs after HOP, 6; 6 hrs after HOP, 7; 12 hrs after HOP, 6; 24 hrs after HOP, 8; 48 hrs after HOP, 94; 72 hrs after HOP, 8; 96 hrs after HOP, 6). The animal numbers used for experiments are described in the figures.

I/R Protocol and Analysis of Infarct Size. Rats were anesthetized by an intravenous administration of pentobarbital sodium (30 mg/kg). Hearts were excised and immediately connected to an aortic cannula and perfused at a constant pressure (80 cm H_2O) in a Langendorff apparatus. The perfusion solution was Krebs-Henseleit buffer containing 118 m*M* NaCl, 4.7 m*M* KCl, 1.25 m*M* $CaCl_2$, 1.2 m*M* $MgSO_2$, 10 m*M* glucose, 25 m*M* $NaHCO_3$, and 1.2 m*M* KH_2PO4, and the solution was saturated with a 95% O_2–5% CO_2 mixture at 37°C to obtain a pH of 7.4. Each heart was stabilized for 20 mins and then subjected to global ischemia by stopping the perfusion for 45 mins, which was then followed by reperfusion for 90 mins. During ischemia the hearts were immersed in the Krebs-Henseleit buffer saturated with 95% N_2:5% CO_2 mixture at 37°C.

After completion of the perfusion, left and right ventricles were weighed, frozen at –70°C for 10 mins, and sliced from apex to base into 3-mm transverse sections. Sliced sections were incubated at 37°C in 1% buffered 2,3,4-triphenyltetrazolium chloride (TTC, pH 7.4) for 10 mins to allow the demarcation of the infarcted region and were then fixed in 10% formalin. The infarct area in the left ventricle was determined using a Sony XC-77 CCD camera attached to a Microcomputer Imaging Device (MCID-M4) (Tokyo, Japan). The infarct volume was calculated by multiplying the measured area by the slice thickness and was expressed as a percentage of the left ventricle volume.

Chemicals and Drug Administration. Antibodies (Santa Cruz Biotechnological Co. Santa Cruz, CA), DNA extraction kit (QIAGEN, Hilden, Germany), polymerase chain reaction (PCR) reagents (Amersham Pharmacia Biotech, Piscataway, NJ), ^{32}P-dCTP (NEM, Boston, MA), Bradford system (Bio-Rad USA, Hercules, CA), TRIZOL (Gibco-BRL, Grand Island, NY), and primers (Bionics, Seoul, Korea) were used. All other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO). An antioxidant, N-acetylcysteine (NAC; 150 mg/kg); a NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC, 120 mg/kg); protein kinase C (PKC) inhibitors, chelerythrine (8 mg/kg) and Gö 6983 (2 mg/kg); a transcription inhibitor, actinomycin D (1.5 mg/kg); and a translation inhibitor, cycloheximide (1.0 mg/kg), were intraperitoneally injected into rats. These agents were administered 30 mins before HOP, as previously described (13).

Identification of DNA Fragmentation. Apoptotic DNA fragmentation was identified as described by Staley *et al.* (14). Briefly, genomic DNA was isolated from the left ventricle by a standard technique using the tissue DNA extraction kit (QIAGEN). One microgram of the extracted DNA was mixed with 1 nmol each of 24–base pair blunt-end linkers in a ligase buffer. The linkers were annealed by heating to 55°C for 10 mins, and linkers were then ligated by T4 DNA ligase. The reaction mixture (100 μl) containing ligated DNA was preheated to 72°C for 3 mins and then incubated with Taq polymerase at 72°C for 5 mins. Samples were amplified for 20 cycles of 1 min each at 94°C and 3 mins at 72°C and were then analyzed by electrophoresis on 1.2% agarose gel. The gels were stained with ethidium bromide and photographed on a UV transilluminator.

TUNEL Assays. To evaluate apoptotic death of myocytes, the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD) was used. Left ventricles were fixed with formalin and embedded in paraffin. Six-micrometer sections were cut from the paraffin blocks and processed for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. The sections were dewaxed and treated with proteinase K, then incubated with equilibration buffer for 10 mins, followed by incubation with working-strength TdT enzyme solution at 37°C for 2 hrs. The reaction was terminated by incubation in working-strength stop/wash buffer for 30 mins at 37°C. Sections were then incubated with antidigoxigenin peroxidase and then incubated with diaminobenzidine and 0.01% H_2O_2 for 5 mins at room temperature. The sections were lightly counterstained with hematoxylin and examined by light microscopy (×100 magnification). The apoptotic cell frequency was measured by calculating the TUNEL-positive cell number per 2.5 mm^2. We analyzed eight different lesions per heart.

Western Blot Analysis. Left ventricles were homog-

enized in a lysis buffer containing 1 m*M* phenylmethylsulfonyl fluoride; 150 m*M* NaCl; 50 m*M* Tris-HCl, pH 7.4; 5% sodium dodecyl sulfate; and 2% Nonidet P-40. The homogenate was centrifuged at 14,000 *g* for 20 mins and the supernatant was collected. For the immunodetection of cytochrome *c*, the homogenate was separated into mitochondrial and cytoplasmic fractions. Twenty micrograms of protein were separated by 12% or 15% denaturing polyacrylamide gel electrophoresis and detected with antibodies. The primary antibodies used were a mouse monoclonal Bcl-2 antibody (Santa Cruz, 1:1500), a rabbit polyclonal Bcl-xL antibody (Santa Cruz, 1:500), a rabbit polyclonal Bax antibody (Santa Cruz, 1:1000), a rabbit polyclonal cytochrome-*c* antibody (Santa Cruz, 1:1000), and a goat polyclonal β-actin antibody (Santa Cruz, 1:1000).

Electrophoretic Mobility Shift Assay (EMSA) of NF-κB. Left ventricles were homogenized in lysis buffer A (20 m*M* Tris, pH 7.8; 10 m*M* KCl; 0.2 m*M* EDTA; 1.5 m*M* $MgCl_2$; 0.5 m*M* dithiothreitol; 1 m*M* Na_3VO_4; and 0.5 m*M* phenylmethylsulfonyl fluoride) and incubated on ice for 15 mins and then centrifuged at 1200 *g* and 4°C for 10 mins. The isolated nuclear pellets were suspended in ice-cold buffer B (20 m*M* Tris, pH 7.8; 420 m*M* NaCl; 0.1 m*M* EDTA; 1.5 m*M* $MgCl_2$; 20% glycerol; 0.5 m*M* dithiothreitol; 1 m*M* Na_3VO_4; 0.5 m*M* phenylmethylsulfonyl fluoride; and 1/1000 vol of Sigma protease inhibitor cocktail) and incubated for an additional 30 mins. The nuclear proteins were then extracted by collecting the supernatant of a 5000 *g* spin of the nuclear homogenates. Double-stranded synthetic oligonucleotides were labeled at the 5′-end with [γ-^{32}P]ATP. The oligonucleotide sequence used for labeling the probes was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Standard DNA binding reaction was carried out in 15 μl of a mixture containing sonicated poly-dI-dC; 14 m*M* Tris, pH 7.4; 70 m*M* KCl; 1.4 m*M* $MgCl_2$; 0.7 m*M* EDTA; 7 m*M* dithiothreitol; 7% glycerol; and 5 μl of the extracted nuclear protein (1 μg/μl). The DNA probe was added, and then the reaction was conducted over 10 mins. The reaction samples were loaded onto 4% polyacrylamide gels made in a 0.3 × Tris/borate/EDTA (TBE) buffer and electrophoresed at a constant voltage of 140 V. After electrophoresis, the gels were vacuum-dried and autoradiographed using an intensifying screen at −70°C.

Semiquantitative Reverse Transcription–PCR. Total RNA was isolated from left ventricles by TRIZOL (Gibco-BRL) according to the manufacturer's instructions, and its quality was checked on a 1% denaturing agarose gel to ensure the presence of the 28S and 18S ribosomal bands. One microgram of total RNA was added to a 25-μl RT-PCR reaction mixture, which contained 10 μCi of ^{32}P-dCTP, prepared according to the manufacturer's instructions. To quantify the Bcl-2 mRNA, specific Bcl-2 primers (5′-ATGGCGCAAGCCGGGAGA-3′, 5′-TCACTTGTGGCCCAGGTA-3′) were used in a reaction involving one cycle of RT at 48°C for 1 hr and 19 cycles of PCR (94°C–55°C–68°C). Rat β-actin primers (5′-GGTGGGTATGGGTCAGAA-3′, 5′-TGCATCCTGTCAGCGATG-3′) were used as internal standards. The resulting PCR fragments were electrophoresed on 4% polyacrylamide gels at 100 V in 0.3 × TBE buffer at 4°C, and the dried gels obtained were autoradiographed.

Statistical Analysis. Results are expressed as means ± SEM. Differences were compared using the unpaired two-tailed *t* test, and $P < 0.05$ was considered significant.

Results

HOP Prevents Myocardial Infarction. Forty-five minutes of global ischemia followed by 90 mins of reperfusion (I/R) caused a significant myocardial infarction in the left ventricle, as shown in the left panel of Figure 1A. However, noticeable infarct was not observed in the right ventricle. In our retrograde perfusion mode, the right ventricle is not loaded by the hydrostatic perfusion pressure. Moreover, the perfusate entered into the right ventricle can be freely released out through incised pulmonary trunk without pressure development. For these reasons, the right ventricle has a lower rate of oxygen consumption and therefore may not be severely injured by ischemia. Thus, we examined myocardial injury only in the left ventricle. The average infarct size expressed as a percentage of the left ventricle was 30.3% ± 2.8% in the I/R only group (Fig. 1A, right panel). The infarct size was significantly reduced in the hearts excised from rats pretreated with HOP. Four or five cycles of HOP significantly limited the infarct size to 16.7% ± 2.9% to 18.7% ± 2.0%, while three cycles of HOP did not reduce the infarct size (Fig. 1A, right panel). The infarct-limiting effect of HOP was observed in a biphasic pattern (Fig. 1B). The early effect started to appear as early as 30 mins after HOP and lasted more than 6 hrs. The delayed effect reappeared around 48 hrs after HOP.

To examine apoptotic cell death, cytochrome-*c* levels were analyzed in the cytosolic fractions. As cytochrome-*c* release from mitochondria is known to occur before cell death, we measured cytochrome *c* 30 or 60 mins after reperfusion. The cytochrome-*c* release increased in I/R hearts (Fig. 2A). HOP prevented the cytochrome-*c* release and conserved the mitochondrial content in the early as well as in the delayed phase (Fig. 2B). DNA fragmentation was observed 90 mins after reperfusion, which was markedly reduced by HOP in both the early and the delayed phases (Fig. 2C). In addition, the TUNEL-positive myocytes were frequently observed in hearts subjected to 90-min reperfusion, which was also reduced by HOP in both the early and the delayed phases (Fig. 2D, left panel). For statistical analysis, the TUNEL-positive cell numbers were counted at a ×100 magnification and compared in I/R only and HOP I/R groups (Fig. 2D, right panel). The TUNEL-positive cell frequency was significantly reduced by HOP in both the early and the delayed phases.

***De Novo* Protein Synthesis Is Required for the I/R Tolerance.** A transcription inhibitor, actinomycin D, or

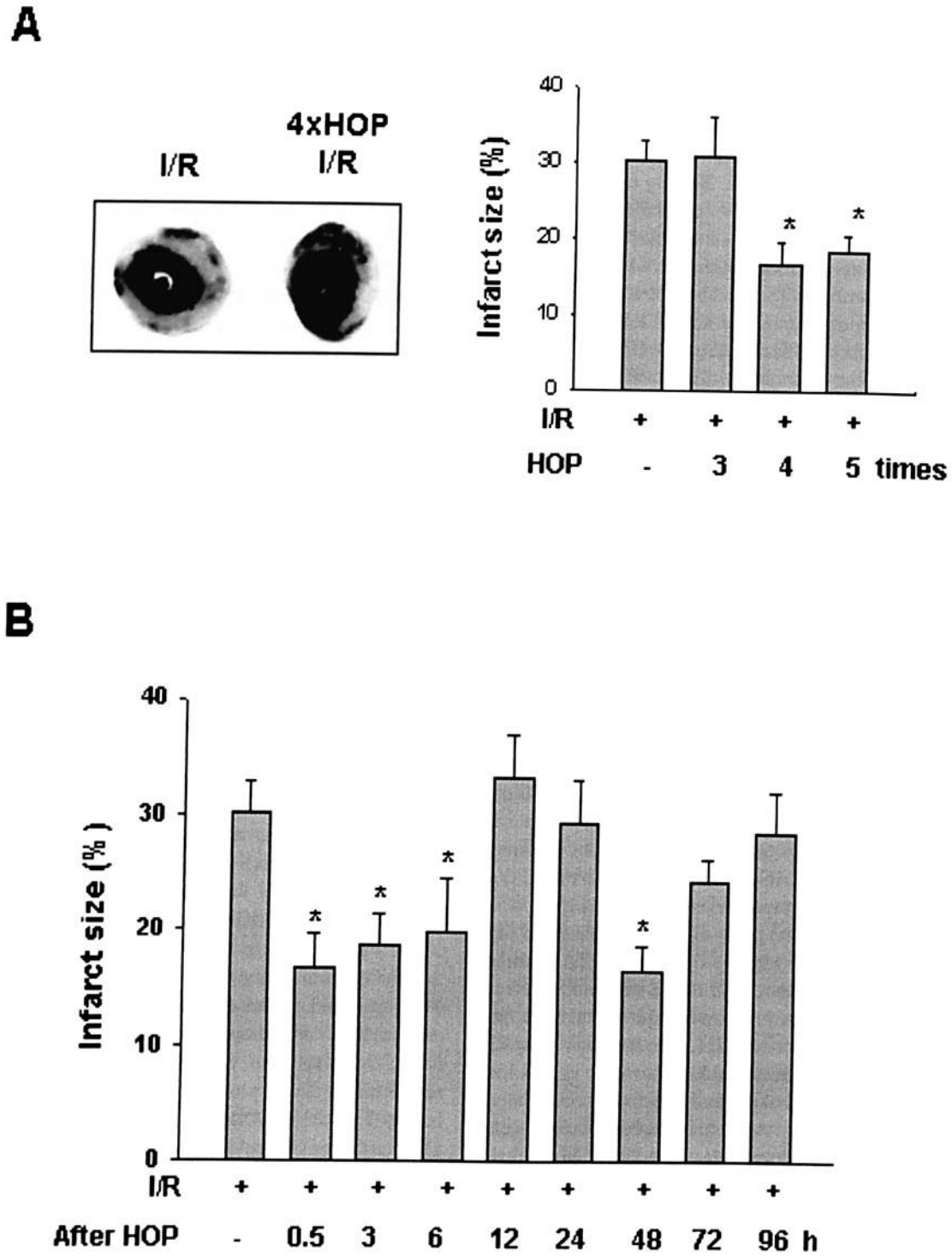

Figure 1. Effect of HOP on myocardial infarction. Hearts excised from the HOP-pretreated rats was subjected to 45 mins ischemia and 90 mins reperfusion (I/R) in a Langendorff apparatus. The infarct size was determined by TTC staining. (A) Left panel: Demonstration of myocardial infarct in heart subjected to I/R only (I/R) or I/R after four times HOP (I/R 4 × HOP). Right panel: The percentages of infarct volumes to the left ventricle volumes. Four and five times repetitions of HOP reduced infarct size. HOP: hyperbaric oxygenation pretreatment, 100% O_2 at 3 atm for 20 mins. HOP was repeated three, four, or five times consecutively, with 20 mins of normoxic intermission. (B) Time course of infarct-limiting effect of HOP. After HOP: time interval between the completion of HOP and the start of I/R. After four cycles of 20-min HOP, rat hearts excised at the indicated time were subjected to I/R. Each bar represents the mean ± SEM of five or more experiments. * $P < 0.05$ versus I/R only.

a translation inhibitor, cycloheximide, abolished the infarct-limiting effects observed in both the early and the delayed phases (Fig. 3), which indicates that the mRNA and protein syntheses were required for the HOP effects.

NF-κB Mediates the HOP Effect. To examine the role of NF-κB in HOP-induced I/R tolerance, the NF-κB activity was measured using EMSA (Fig. 4A). The heart sample treated with lipopolysaccharide was used as a positive

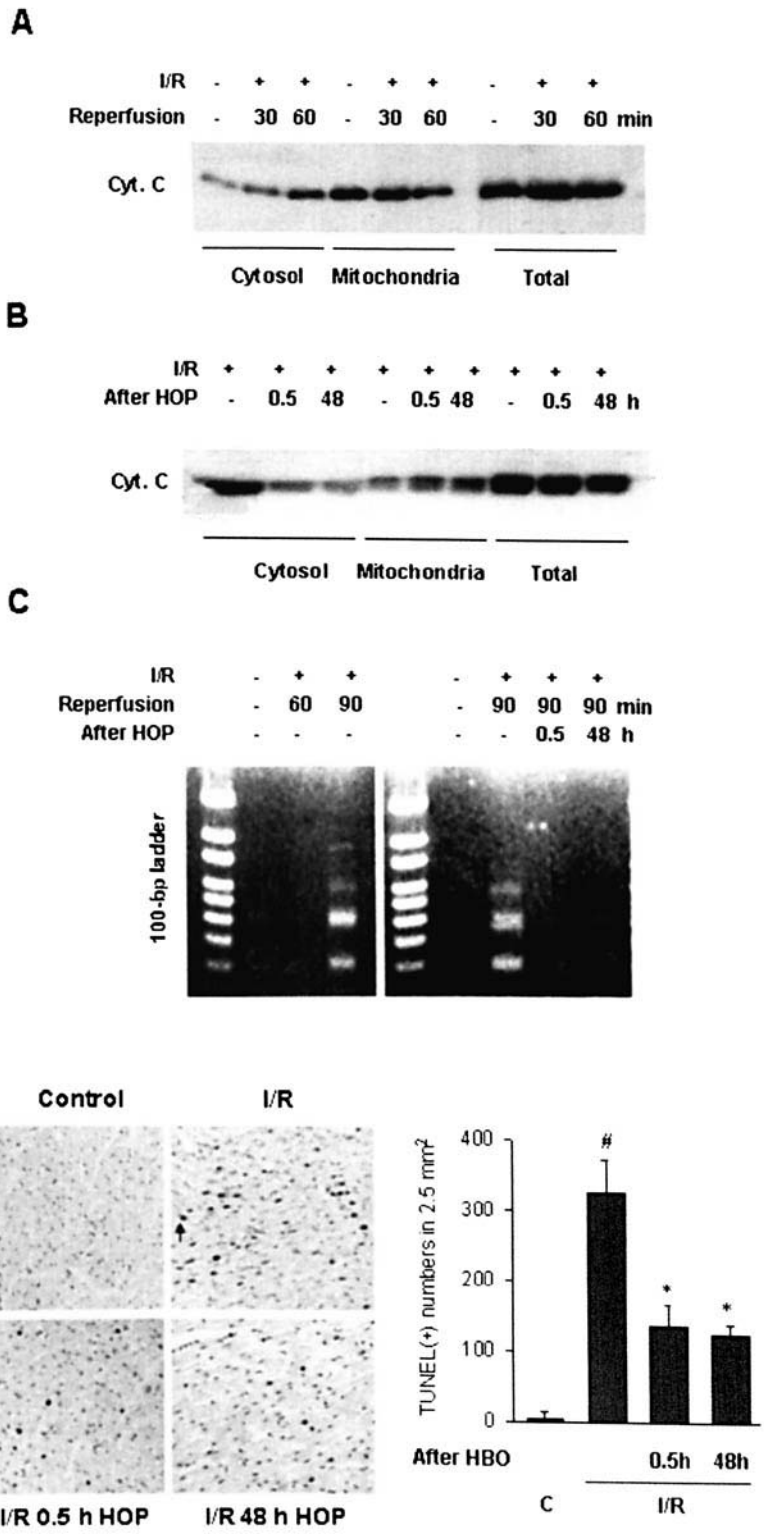

Figure 2. Effect of HOP on myocardial apoptosis. (A) Cytochrome-*c* release from mitochondria after reperfusion of ischemic hearts. Hearts were subjected to 45 mins ischemia followed by 30 or 60 mins reperfusion. Cytochrome-*c* levels in cytosolic, mitochondrial, and total fraction were analyzed by Western blotting. (B) Reduction in cytochrome-*c* release after HOP. Rat hearts were excised 30 mins and 48 hrs after HOP and subjected to 45 mins ischemia and 60 mins reperfusion (I/R). (C) Effect of HOP on DNA fragmentation. DNA ladder was analyzed in LM-PCR product of total DNA extracted from hearts. Hearts excised from rats 30 mins and 48 hrs after HOP were subjected to 45 mins ischemia and 60 or 90 mins reperfusion (I/R). The data presented in each panel are representative of three separate experiments. (D) Effect of HOP on TUNEL staining. Hearts were subjected to 45 mins ischemia and 90 mins reperfusion. Apoptotic cell death was analyzed by TUNEL staining (left panel), and the TUNEL-positive cell numbers per 2.5 mm^2 were plotted (right panel). An arrow indicates the TUNEL-positive cell. Control, non-HOP and non-I/R; I/R, I/R only; I/R 0.5h HOP, I/R 0.5 hrs after 4×HOP; I/R 48 hrs after 4 × HOP. Each bar represents the mean ± SEM of four experiments. # $P < 0.05$ versus control, * $P < 0.05$ versus I/R only.

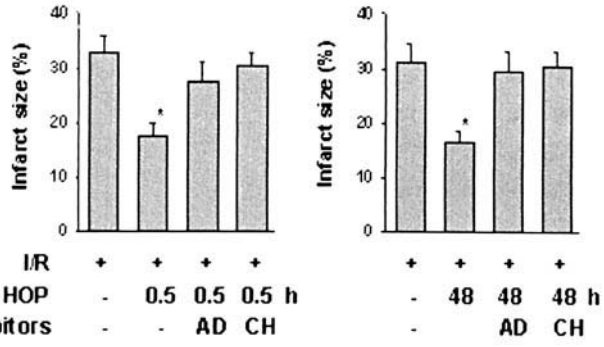

Figure 3. Requirement of *de novo* protein synthesis for the infarct-limiting effect of HOP. Hearts excised from rats 30 mins and 48 hrs after four cycles of HOP were subjected to 45 mins ischemia and 90 mins reperfusion. Myocardial infarct was determined by TTC staining, and the percentages of infarct volumes to the left ventricle volumes were calculated as described in Materials and Methods. AD, actinomycin D (1.5 mg/kg); CH, cycloheximide (1 mg/kg). Inhibitors were injected intraperitoneally 30 mins before HOP. Each bar represents the mean ± SEM of eight experiments. $^*P < 0.05$ versus I/R only.

control to identify NF-κB–DNA binding. NF-κB was activated as early as 5 mins after HOP, while it was not apparent 48 hrs after HOP. A NF-κB inhibitor, PDTC, an antioxidant, NAC, and a PKC inhibitor, chelerythrine, all reduced the NF-κB activation by HOP (Fig. 4A). In addition, the infarct-limiting effects of HOP observed in both phases were completely abolished by these inhibitors and another PKC inhibitor, Gö 6983 (Fig. 4B). These results indicate that NF-κB is activated immediately after HOP *via* ROS and PKC-dependent pathways and in turn provides the I/R tolerance.

HOP Induces Bcl-2 Expression Via NF-κB. Involvement of Bcl-2 family genes in the HOP-induced tolerance against I/R injury was examined. Protein levels of Bcl-2, Bcl-xL, and Bax were analyzed in hearts excised from the HOP-treated rats using Western blotting (Fig. 5). Bcl-2 was induced significantly 30 mins after HOP and lasted more than 6 hrs in an elevated state. Following return to the control level, Bcl-2 expression increased again after 48 hrs. The temporal pattern of Bcl-2 expression matches that of the I/R tolerance demonstrated in Figure 1. In contrast, Bcl-xL and Bax expressions were not changed after HOP. Figure 5B shows Bcl-2 mRNA expression measured using semiquantitative RT-PCR. The Bcl-2 mRNA levels also increased significantly 30 mins after HOP. However, in contrast to the protein expression, the mRNA expression observed at 48 hrs after HOP was not higher than the control level. Both Bcl-2 protein and mRNA inductions were blocked by PDTC, NAC, chelerythrine, or Gö 6983 (Fig. 6A–C). These results, together with the NF-κB–mediated myocardial infarct–limiting effect, as shown in Figure 4, indicate that the gene expression of Bcl-2 induced by activated NF-κB is responsible for the development of myocardial tolerance against I/R injury in the HOP hearts.

Discussion

The present study demonstrates that HOP induces myocardial tolerance against I/R injury in a biphasic pattern, with an early and a delayed effect. HOP enhanced the NF-κB activity and the Bcl-2 expression. All the effects of HOP were abolished by PDTC, NAC, chelerythrine, or Gö 6983. These results indicate that the HOP-induced tolerance against I/R injury is associated with an elevation of Bcl-2 expression, which is mediated by NF-κB activation through ROS and PKC-dependent signaling pathway.

In a previous study we demonstrated for the first time that the long-term, intermittent hyperoxygenation (100% O_2 at 3 atm for 1 hr once a day, for four times) enhances the gene expression of catalase and thereby reduces myocardial infarct size in I/R hearts (12). Our previous study and those of others (15, 16) indicate that the pretreatment with sublethal oxidative stress conditions the hearts to induce myocardial antioxidant enzyme and tolerance to I/R injury produced by subsequent severe oxidative stress of the type occurring in the hearts exposed to prolonged I/R. In the present study, there were no significant changes in the activities of antioxidant enzymes, including catalase (data not shown). In contrast to the long-term hyperoxygenation, short-term hyperoxygenation promotes I/R tolerance *via* some mechanism other than the induction of antioxidant enzymes.

The biphasic pattern of I/R tolerance induced by HOP is comparable to that induced by ischemic preconditioning (IP). In IP hearts, while it is controversial with the early immediate protective effect, *de novo* protein synthesis is required to develop the delayed, second protective effect, which appears 24 to 48 hrs after IP (17, 18). Similarly, actinomycin D and cycloheximide prevented both the early and the delayed infarct-limiting effects of HOP, indicating that *de novo* protein synthesis after HOP is required for developing I/R tolerance. With regard to this mechanism, HOP seems to share a common mechanism of I/R tolerance with IP. Several studies have demonstrated that specific inhibitors of NF-κB activation and nuclear translocation block the cardioprotective effect of IP, indicating that NF-κB plays a crucial role in I/R tolerance in the heart (19, 20). The cellular mechanism by which IP activates NF-κB has been known to involve the formation of ROS and the activation of PKC- and tyrosine kinase–dependent pathways (21). Similarly, HOP activated NF-κB and induced I/R tolerance. A recent study demonstrated the activation of myocardial NF-κB by hyperoxia pretreatment (22). Tahepold *et al.* (22) pretreated rats with normobaric hyperoxygenation for 1 hr and induced I/R injury in isolated hearts. The infarct size was reduced in the hearts of the pretreated rats, and this effect was prevented by the inhibition of NK-κB. In the present study, we also found that NF-κB inhibitors abrogated the infarct-limiting effects shown in both the early and the delayed phase after HOP.

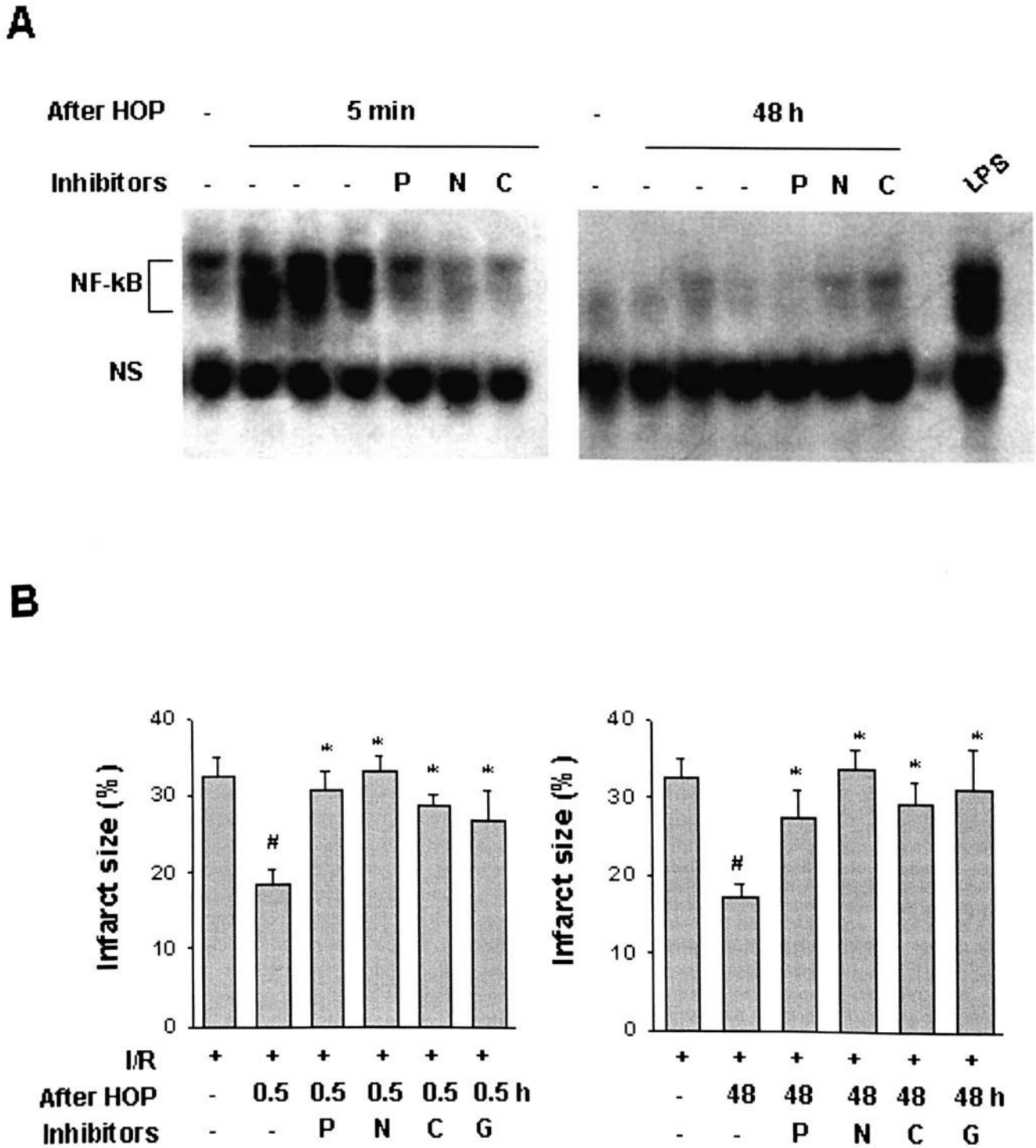

Figure 4. Involvement of NF-κB in HOP-induced I/R tolerance. (A) NF-κB activation by HOP. NF-κB–DNA binding was assessed by EMSA. Hearts were excised from rats 5 mins and 48 hrs after HOP, and the nuclear proteins were extracted. The nuclear protein extracted from LPS-treated (8 mg/kg, iv, for 1 hr) rats was used as a positive control for NF-κB–DNA binding. Pyrrolidine dithiocarbamate (P, 120 mg/kg), N-acetylcysteine (N, 150 mg/kg), or chelerythrine (C, 8 mg/kg) was injected intraperitoneally 30 mins before HOP. NF-κB, the specific binding of NF-κB, and the EMSA probe; NS, the nonspecific protein binding to the probe. The data presented in each panel are representative of three separate experiments. (B) Effects of NF-κB inhibitors on the infarct-limiting effect of HOP. Pyrrolidine dithiocarbamate (P, 120 mg/kg), N-acetylcysteine (N, 150 mg/kg), chelerythrine (C, 8 mg/kg), or Gö 6983 (G, 2 mg/kg) was injected 30 mins before HOP. Hearts excised from rats 30 mins (left panel) and 48 hrs (right panel) after 4 × HOP were subjected to 45 mins ischemia and 90 mins reperfusion, and the infarct size was determined by TTC staining. Each bar represents the mean ± SEM of eight experiments. # $P < 0.05$ versus control, * $P < 0.05$ versus I/R only.

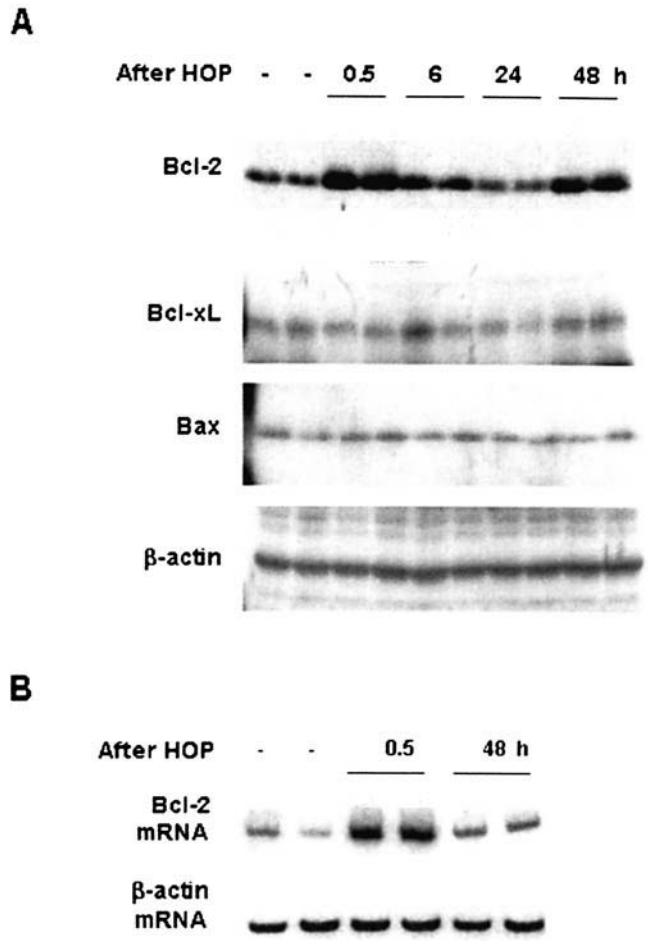

Figure 5. Effect of HOP on Bcl-2 family gene expression. (A) Expression of Bcl-2, Bcl-xL, and Bax proteins was analyzed by Western blotting. ß-Actin was immunodetected with a monoclonal antibody as an internal control. The expression levels were measured in the hearts excised from the HOP rats at the indicated times. (B) Expression of Bcl-2 mRNA was analyzed by semi-quantitative RT-PCR and 4% polyacrylamide gel electrophoresis. ß-Actin mRNA was used as an internal control. The mRNA levels were measured in the hearts isolated from rats 30 mins and 48 hrs after HOP. The data presented in each panel are representative of three separate experiments.

NF-κB may be a common factor to mediate I/R tolerance in both ischemic and hyperoxic preconditioning.

I/R results in apoptosis as well as necrosis of cardiac myocytes. Apoptosis is regulated by a number of factors induced by diverse apoptotic signals, for instance, the pro- and antiapoptotic proteins of Bcl-2 family. Antiapoptotic BCL-2 members include Bcl-2, Bcl-xL, and Bcl-w, and proapoptotic members include Bax, Bad, and Bak. In particular, the balance of Bcl-2 and Bax expression is an important factor to determine the extent of apoptosis in I/R hearts. Indeed, upregulation of Bcl-2 (23, 24) or down-regulation of Bax (25) has been found to attenuate apoptotic cell death and prevent I/R injury. In the present study, the Bcl-2 expression was found to increase evidently after HOP, while there was no significant change in Bax, resulting in an increased ratio of Bcl-2 to Bax. So, then, how is Bcl-2 induced by HOP? NF-κB activated by HOP may target the promoter of the Bcl-2 gene directly. The putative NF-κB binding site in the Bcl-2 promoter was previously identified (26), and this possibility was supported by a report demonstrating that Bcl-2 expression is inhibited by expression of a mutated IκB (27).

With regard to our results, the unexplainable finding is that the early activation of NF-κB is involved in Bcl-2 induction in the delayed phase. The NF-κB activation was evident as early as 5 mins after HOP, and soon after was followed by the early phase induction of Bcl-2 mRNA and protein expression. This supports the possibility that NF-κB activated immediately after HOP directly mediates the early induction of Bcl-2 expression, although the early activation of NF-κB is not likely to induce Bcl-2 in the delayed phase because of a 48-hr time gap between the two events. Moreover, no significant increase in the transcript of Bcl-2 is found in the delayed phase. These results indicate that Bcl-2 in the delayed phase is upregulated at the post-translational step. This possibility is supported by two reports (28, 29), the authors of which demonstrated that Bcl-2 was ubiquitinated and subsequently degraded by the 26S proteasome. However, when Ser87 residue of Bcl-2 was phosphorylated by MAP kinase, Bcl-2 was stabilized. According to this regulatory mechanism, we can speculate that Bcl-2 is phosphorylated and stabilized in the delayed phase of HOP. Since the delayed induction of Bcl-2 was also suppressed by NF-κB inhibitors, it is possible that the Bcl-2 stabilization process is regulated by NF-κB. This topic is worth investigating in a future study.

How does Bcl-2 protect myocardial cells from I/R injury? Although the exact protective mechanism is not well understood in this study, recent findings provide possible answers. First, Bcl-2 overexpression has been found to attenuate the generation of ROS *in vivo* and *in vitro* (30, 31). The antioxidant effect of Bcl-2 may contribute to the prevention of oxidative cellular damage in I/R hearts. Second, the co-localization of Bcl-2 with calcium channels on mitochondria, endoplasmic reticulum, and nuclear membranes is believed to promote calcium homeostasis in these compartments (32, 33). Although we did not investigate this issue, the calcium imbalance by I/R could be ameliorated by Bcl-2 induced by HOP. Third, Bcl-2 inhibits the formation of mitochondrial permeability transition (PT) pore, which is known to play a critical role in control of apoptosis and necrosis (34, 35). The mitochondrial PT pore initiates many injury signals, such as inhibition of mitochondrial oxidative phosphorylation, depletion of ATP, and release of cytochrome *c*. These changes result in dysfunction of cellular energy metabolism, caspase activation, and DNA fragmentation. In particular, mitochondrial PT pore has been found to play a crucial role in I/R injury of hearts. In I/R hearts, transient PT pore opening and ATP maintenance of the mitochondria lead to apoptosis, whereas excessive pore opening and ATP depletion result in necrosis (34, 36). In this respect, HOP

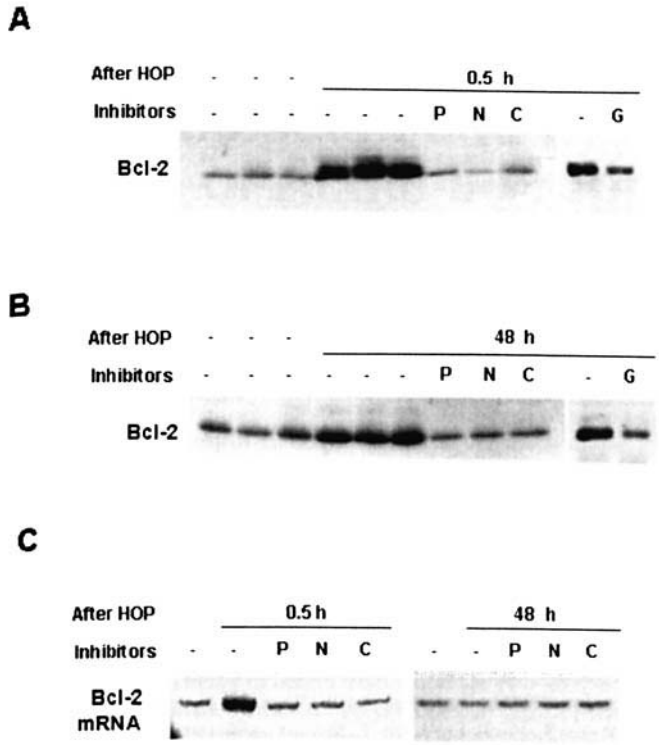

Figure 6. Involvement of NF-κB in Bcl-2 induction by HOP. Rats were treated with pyrrolidine dithiocarbamate (P, 120 mg/kg), N-acetylcysteine (N, 150 mg/kg), chelerythrine (C, 8 mg/kg), or Gö 6983 (G, 2 mg/kg) 30 mins before HOP and subjected to 4 × HOP. The hearts were excised 30 mins (A) and 48 hrs (B) after HOP, and the expression of Bcl-2 protein was analyzed by Western blotting. (C) The expression levels of Bcl-2 mRNA prepared from the hearts excised 30 mins and 48 hrs after HOP were analyzed by RT-PCR. The data presented in each panel are representative of three separate experiments.

may reduce myocardial necrosis as well as apoptosis by inhibiting the PT pore formation in association with the upregulation of Bcl-2 expression. This possibility is supported by recent studies (37–40) that found that Bcl-2 overexpression limits both necrosis and apoptosis in I/R injury of hearts and livers.

In conclusion, the short-term HOP induces myocardial tolerance against I/R injury in association with an elevation of Bcl-2 gene expression through NF-κB activation that is mediated *via* ROS formation and PKC-dependent signaling pathway. HOP, when applied with a standard protocol, is a safe and clinically applicable method for providing a sublethal oxidative stress *in vivo*. Thus, pretreatment of whole animals with the HOP may influence systemically various tissues and organs and perhaps induces a similar protective effect against I/R injury in other organs as well as in hearts. Future studies should be required for evaluation of its beneficial protective effect in many clinical settings related to I/R injury.

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