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Menadione Cytotoxicity to Hep G2 Cells and Protection by Activation of Nuclear Factor-κB

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SUMMARY

Menadione (vitamin K-3,2-methyl-1,4-naphthoquinone), a redox cycling reagent, generates reactive oxygen intermediates and causes oxidative injury. The addition of menadione to Hep G2 cells produced a time- and concentration-dependent loss of cell viability. Preincubation of Hep G2 cells with low, nontoxic concentrations of menadione increased the viability of the cells against toxic doses of menadione or H_2O_2. Maximum protection was found with menadione concentrations of ~3 μM and preincubation times of ~45 min. This protective effect could be blocked by the protein synthesis inhibitor cycloheximide and by a variety of antioxidants. The transcription factor nuclear factor-κF (NF-κB) is known to be activated by many compounds, including reactive oxygen intermediates. Menadione activated NF-κB as determined by electrophoretic mobility shift assays. This activation was prevented by the same antioxidants that blocked protection against cytotoxicity produced by preincubation with menadione. Anti-p50 IgG prevented the menadione-stimulated binding of NF-κB to the oligonucleotide probe, whereas anti-p65 IgG produced a supershift of the NF-κB/oligonucleotide complex. Salicylate prevented the activation of NF-κB by menadione, and under these conditions, salicylate potentiated the cytotoxicity of menadione or H_2O_2. Transfection with a plasmid containing cDNA encoding mouse IκBβ, an inhibitor of NF-κB, resulted in increased toxicity by menadione. Furthermore, when protein kinase C was down-regulated by prolonged treatment with active phorbol ester (phorbol-12-myristate-13-acetate), the Hep G2 cells became more sensitive to menadione treatment. However, short term treatment with PMA, which activated NF-κB, resulted in protection against menadione cytotoxicity. Menadione cytotoxicity was enhanced when the Hep G2 cells were depleted of GSH. An increased level of GSH was observed after menadione pretreatment; this increase was blocked by salicylate, thereby linking the GSH increase to activation of NF-κB by menadione. The results of the current study suggest that menadione pretreatment protects Hep G2 cells from oxidative injury through an NF-κB-related mechanism, which may involve, in part, increased production of GSH.

Partially reduced intermediates formed from the initial univalent reduction of O_2 are reactive and toxic in biological systems. These intermediates include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) (1). The reduction of molecular oxygen includes four electron transfer steps. Superoxide radical is generated by accepting one electron from a donor; hydrogen peroxide is generated by accepting a second electron; hydroxyl radical is generated by accepting the third electron; and water is the final product after acceptance of all four electrons. In addition to oxygen, a number of exogenous compounds, such as menadione, and a number of other redox cycling agents can be metabolized to reactive species, which may also result in oxidative injury. Menadione (vitamin K-3,2-methyl-1,4-naphthoquinone) could be reduced by NADPH-P450 reductase, NADH dehydrogenase, and other flavoproteins *in vivo* to give the menadione semiquinone radical (2). This radical itself may react directly with other cellular constituents to cause injury. It may also recycle back to menadione through rapid reaction with molecular oxygen to yield superoxide radical. The cytotoxicity of menadione seems to be associated with superoxide generation, protein thiol oxidation, and alteration in the Ca^{2+} homeostasis (3, 4). Menadione may cause toxicity due to depletion of GSH by conjugate formation (e.g., naphthoquinones such as menadione react readily with GSH to form glutathione conjugates; ≤20% of the GSH in hepatocytes may be consumed by its direct reaction with menadione to form 2-methyl-3-glutathionyl-1,4-naphthoquinone) (5, 6).

To defend themselves from oxidative injury, organisms have evolved protective systems to convert the reactive oxy-

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ABBREVIATIONS: GSH, glutathione, reduced form; PKC, protein kinase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, DL-dithiothreitol; EMSA, electrophoretic mobility shift assay; MEM, minimum essential medium; LDH, lactate dehydrogenase; NAC, *N*-acetylcysteine; PMA, phorbol-12-myristate-13-acetate; SOD, superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PDTC, pyrrolidine dithiocarbamate; ROI, reactive oxygen intermediate.

gen intermediates to less reactive components. These systems include enzymes, such as SOD, catalase, glutathione peroxidase, and glutathione reductase, and cellular antioxidants, such as GSH, vitamin C, and vitamin E. SOD acts as a protective protein by converting superoxide to H_2O_2. Catalase usually localized in peroxisomes converts H_2O_2 to water and oxygen. Glutathione peroxidase removes the H_2O_2 by utilizing GSH (which is oxidized to GSSG and reduced back by glutathione reductase).

Active oxygen species, such as $O_2^{\cdot-}$, H_2O_2, and ·OH, produced as normal by-products of cellular metabolism, clearly have effects on multiple cell types and organisms. Bacteria such as *Escherichia coli* can develop resistance to normally lethal concentrations of H_2O_2 through induction of a series of defense and repair enzymes. Genes for such enzymes were induced by pretreatment with H_2O_2 (7). Adaptive responses to the oxidative stress of H_2O_2 in yeast *Saccharomyces cerevisiae* strain RZ53 could increase their viability against a higher dose of H_2O_2 (8). It has been shown that eukaryotic cells are also capable of transient adaptive responses to oxidative stress (9–12). Preincubation of mammalian cell lines, such as Chinese hamster ovary fibroblast cells, embryonic mouse fibroblast C3H 10T½ cells, Chinese hamster lung fibroblast V79 cells, and rat liver epithelial clone 9 cells, with relatively low doses of H_2O_2 was found to increase the resistance of these cells to H_2O_2 (13).

NF-κB, a ubiquitous heterodimeric transcription factor composed of p50 and p65 subunits, was originally identified as an inducible B cell-specific factor able to bind to the κB motif in the intronic κ light chain enhancer (14, 15). IκB binds and modulates the NF-κB activity. Inactive NF-κB is localized in the cytoplasm. A variety of stimuli, including viruses, bacterial lipopolysaccharide, active PMA, cytokines, and antigens, induce the dissociation of IκB from NF-κB dimer, which leads to translocation of NF-κB to the nucleus, in which it binds to the κB site and modulates transcription (for reviews, see Refs. 16 and 17). Induction of NF-κB has also been shown as an early response to oxidative stress. Several genes related to oxidative stress, such as Mn-SOD (18, 19), NAD(P)H:quinone oxidoreductase (DT-diaphorase) (20), inducible nitric oxide synthetase (21, 22), and ferritin H (23), could be regulated by the activation of NF-κB.

The recent observations that 2,3-dimethoxy-1,4-naphthoquinone and menadione, redox cycling quinones, increased the activity of γ-glutamylcysteine synthase in bovine pulmonary artery endothelial cells and Chinese hamster lung fibroblast V79 cells (24, 25) led to the current study to investigate whether menadione pretreatment of Hep G2 cells protected these cells against a toxic concentration of menadione and of H_2O_2, whether menadione activates NF-κB, and whether this activation and elevated GSH levels play a role in any protective action by menadione pretreatment.

Materials and Methods

Hep G2 cell line. The Hep G2 cell line (HB 8065; American Type Culture Collection, Rockville, MD) (26), a human hepatocellular carcinoma cell line, was cultured in MEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere in 5% CO_2 at 37°. Most reagents were from Sigma Chemical (St. Louis, MO).

MTT assay. Cytotoxicity of menadione and H_2O_2 generated by glucose/glucose oxidase on Hep G2 cells was determined by the MTT assay (27). Tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and are therefore useful for the measurement of cytotoxicity. To determine cytotoxicity of menadione or H_2O_2, 5×10^4 cells/ml/well were plated onto a 24-well plate and incubated in 5% CO_2 at 37°. Test reagents were added to the culture medium for a designated incubation time, typically 18 hr. The MTT assay was performed using the Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). Briefly, 15% volume of dye solution was added to each well after the appropriate incubation time. After 1 hr of incubation at 37°, an equal volume of solubilization/stop solution was added to each well for an additional 1-hr incubation. The absorbance of the reaction solution at 570 nm was recorded. The absorbance at 630 nm was used as reference. The net $A_{570nm} - A_{630nm}$ was taken as the index of cell viability. The reading taken from the wells with cells cultured with control medium was used as 100% viability value. The percent viability was calculated by the formula $(A_{570nm} - A_{630nm})_{sample}/(A_{570nm} - A_{630nm})_{control} \times 100$.

LDH release assay. LDH activity was measured as another index of cytotoxicity; $1–2 \times 10^6$ cells/2 ml/well were plated onto a six-well plate, and test reagents were added and incubated at 37° for 18 hr. The supernatant was collected to measure LDH activity as LDH_{out}. Cells were harvested by scraping, washed with PBS, suspended in 1 ml of PBS, and sonicated for 10 sec. The Lactate Dehydrogenase Assay Kit (Sigma Chemical, St. Louis, MO.) was used for the quantitative kinetic determination of LDH activity. The reagent contains 50 mM lactate plus 7 mM NAD^+ in a pH 8.9 buffer system. To determine the LDH activity, 50–200-μl aliquots were added to the LDH assay system, and the increase in absorbance at 340 nm due to NADH formation was recorded for kinetic calculation. The LDH activity of the cell suspension was measured as LDH_{in}. The cytotoxicity index was expressed as the ratio of LDH_{out} to LDH_{in}.

EMSA. Nuclear extracts were isolated according to a modification of the method of Dignam *et al.* (28). Briefly, cells were pretreated with various reagents for 45 min. Then, 10^7 cells were harvested and washed once with PBS and twice with buffer A (consisting of 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT). The cell pellet was suspended in 200 μl of buffer A plus 0.1% Nonidet P-40 and incubated on ice for 15 min with brief mixing. After centrifugation for 10 min at 4°, the supernatant was removed, and the nuclear pellet was suspended in 15 μl of buffer C (consisting of 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT), incubated for 15 min on ice, mixed briefly, and centrifuged for 10 min at 4°. The supernatant was diluted with 75 μl of modified buffer D (consisting of 20 mM HEPES, pH 7.9, 20% glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT) and stored at −70°. Protein concentration was determined by the BioRad (Hercules, CA) DC Protein Assay. A double-stranded oligonucleotide containing a tandem repeat of the consensus sequence of the NF-κB DNA binding site, -GGGGACTTTCC- (underlined below), was used as a probe. To determine the sequence specificity of the DNA/protein interaction, 20-fold additional nonradioactive oligonucleotides and a oligonucleotide containing mutations in the NF-κB consensus sequence were added to compete with the NF-κB probe. The sequences of the oligonucleotides were 5′-GATCCAAGGGGACTTTCCATGGATCCAAGGGGACTTTCCATG-3′, 3′-GTTCCCCTGAAAGGTACCTAGGTTCCCCTGAAAGGTACCTAG-5′ (wild type), and 5′-GATCCAAGCTCACTTTCCATGGATCCAAGCTCACTTTCCATG-3′, 3′GT-TCGAGT-GAAAGGTACCTAGGTTCGAGTGAAAGGTACCTAG-5′ (mutated).

Probes were end-labeled by T4 polynucleotide kinase (Life Technologies, Gaithersburg, MD) with [γ-^{32}P]ATP (DuPont-New England Nuclear, Boston, MA). Briefly, in a 1.5-ml microcentrifuge tube, 5 μl of 5× polynucleotide kinase buffer (5× = 300 mM Tris, pH 7.5, 50 mM $MgCl_2$, 75 mM β-mercaptoethanol, 1.65 μM ATP), 5 ng of oligonucleotide, 100 μCi of [γ-^{32}P]ATP, 5 units of T4 kinase, and H_2O were mixed in a 25-μl reaction volume and incubated for 45 min at 37°.

The reaction was terminated by the addition of 50 μl of 50 mM Tris·HCl, pH 7.5. The labeled oligonucleotide was purified on a Pharmacia ProbeQuant G-50 MicroColumn. An EMSA was performed at room temperature for 20 min in a total 25-μl reaction volume containing 5 μl of 5× incubation buffer (5× = 50 mM Tris·HCl, pH 7.5, 500 mM NaCl, 5 mM DTT, 5 mM EDTA, 20% glycerol, 0.4 mg/ml sonicated salmon sperm DNA), 8–12 μg of nuclear extract, and 5×10^4 cpm of labeled oligonucleotide, followed by polyacrylamide gel electrophoresis for DNA. The dried gels were analyzed after autoradiography with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

GSH assay. We subcultured overnight 3×10^6 cells onto a 10-mm Petri dish. Culture medium was replaced by fresh medium or medium containing 3 μM menadione. After incubation at 37° in a 5% CO_2 incubator for 45 min, the cells were rinsed twice, and fresh medium was added. After additional incubations, the cells were harvested by scraping. Cells were washed with PBS, suspended in PBS, and sonicated for 10 sec. The mixture was used to measure the content of intracellular GSH by the GSH-400 colorimetric assay (Bioxytech S.A., France). Briefly, an initial sample volume of 200 μl was reacted with 50 μl of reagent R1 (solution of 1.2×10^{-2} M; a patented chromogenic reagent in 0.2 N HCl) and mixed thoroughly. Then, 50 μl of solution R2 was added and thoroughly mixed, followed by incubation for 30 min at 37°. The final absorbance at 400 nm was measured. Reduced GSH was used to prepare a standard curve. The intracellular GSH value was standardized against the protein concentration of the mixture.

Transduction of Hep G2 cells with IκBβ cDNA plasmid. The full-length mouse IκBβ cDNA (29), excised from pBSK-IκBβ plasmid (kindly provided by Dr. Sankar Ghosh, Yale University School of Medicine, New Haven, CT). was inserted into the *Not*I restriction site of pCI-neo expression vector (Promega, Madison, WI) in the sense orientation to form pCI- IκBβ. Transfection of Hep G2 cells was carried out by using the LipofectAmine reagent (Life Technologies, Gaithersburg, MD). Hep G2 cells were grown to 80–90% confluence and harvested by trypsinization, and 1.5×10^6 cells were seeded onto a 100-mm culture dish and grown until 50–70% confluency. Cells were rinsed with serum-free MEM before transfection. Solution A (15 μg of the appropriate plasmid DNA in 800 μl of serum-free MEM) and solution B (100 μl of LipofectAmine reagent in 800 μl of serum-free MEM) were mixed gently and incubated at room temperature for 30 min to form a DNA/liposome complex. The complex was diluted with 6.4 ml of MEM, added to the Petri dish containing the Hep G2 cells, and followed by incubation for 5 hr at 37° in a CO_2 incubator. Then, 8 ml of MEM with 20% fetal calf serum was added to each culture dish. After 18 hr of incubation, fresh MEM was added, and the cells were incubated for an additional 4 days. The cells were collected by trypsinization and used for Western blot analysis and menadione toxicity studies.

Statistics. Results refer to mean ± standard deviation and are average values from three to five values per experiment, with experiments repeated at least three times.

Results

Menadione cytotoxicity to Hep G2 cells. Oxidative stress generated by menadione semiquinone, superoxide, H_2O_2, ·OH, and the elimination of GSH was considered to be a primary event involved in menadione cytotoxicity. Hep G2 cells (5×10^4 monolayers) in each 24-well plate were used for menadione cytotoxicity assays, as measured with the MTT assay. The data were expressed as average values obtained from three to five wells. Under these conditions, most cells were killed after an overnight incubation in the presence of 25–50 μM menadione (Fig. 1A). For most subsequent experiments, incubation times of 18 hr and menadione concentrations of 15–20 μM were chosen as routine incubation conditions (the LD_{50} value of an 18-hr incubation was ~18–19 μM). The cytotoxicity by menadione was also dependent on the cell number (data not shown). Menadione cytotoxicity was validated by assays of LDH leakage and morphology. The cytotoxicity by menadione was prevented by the addition of NAC (Fig. 1B) or by iron chelators such as α,α-dipyridyl (data not shown).

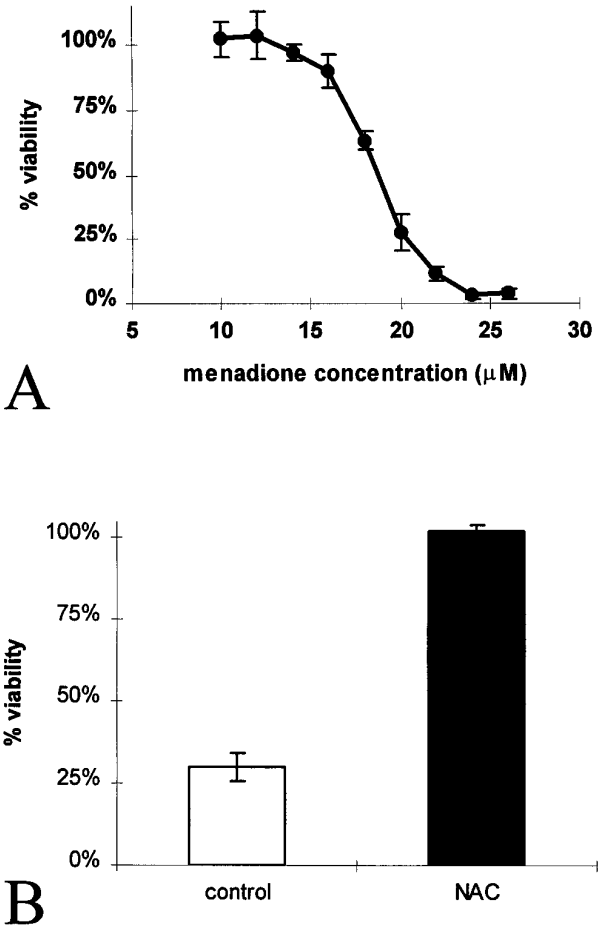

Fig. 1. Menadione cytotoxicity to Hep G2 cells; 5×10^4 cells/ml/well Hep G2 cells were plated onto a 24-well plate. A, Different concentrations of menadione were added to the culture medium for 18 hr. B, 5 mM NAC or control medium was added to the culture medium 15 min before 18 μM menadione. The percentage of viability was determined as described in Materials and Methods.

Preincubation of Hep G2 cells with menadione protects against cytotoxicity of H_2O_2 or menadione. Glucose oxidase/glucose was used to generate H_2O_2 in the culture medium (which contains 1 mg/ml glucose) to avoid the rapid decomposition of H_2O_2, which occurs in the complete medium, and to avoid the addition of high bolus concentrations of the oxidant. Glucose plus glucose oxidase was also toxic to the Hep G2 cells in a dose- and time-dependent manner (data not shown). To study whether menadione was able to activate cell defensive systems, Hep G2 cells were preincubated with lower doses of menadione (1–5 μM), which by themselves were not toxic. Preincubation of Hep G2 cells with these lower doses of menadione increased the viability of Hep G2 cells and protected them against a toxic dose of H_2O_2 (generated from glucose oxidase/glucose system) compared with cells incubated with culture medium lacking menadione (Fig. 2A). The protective effect of preincubation with

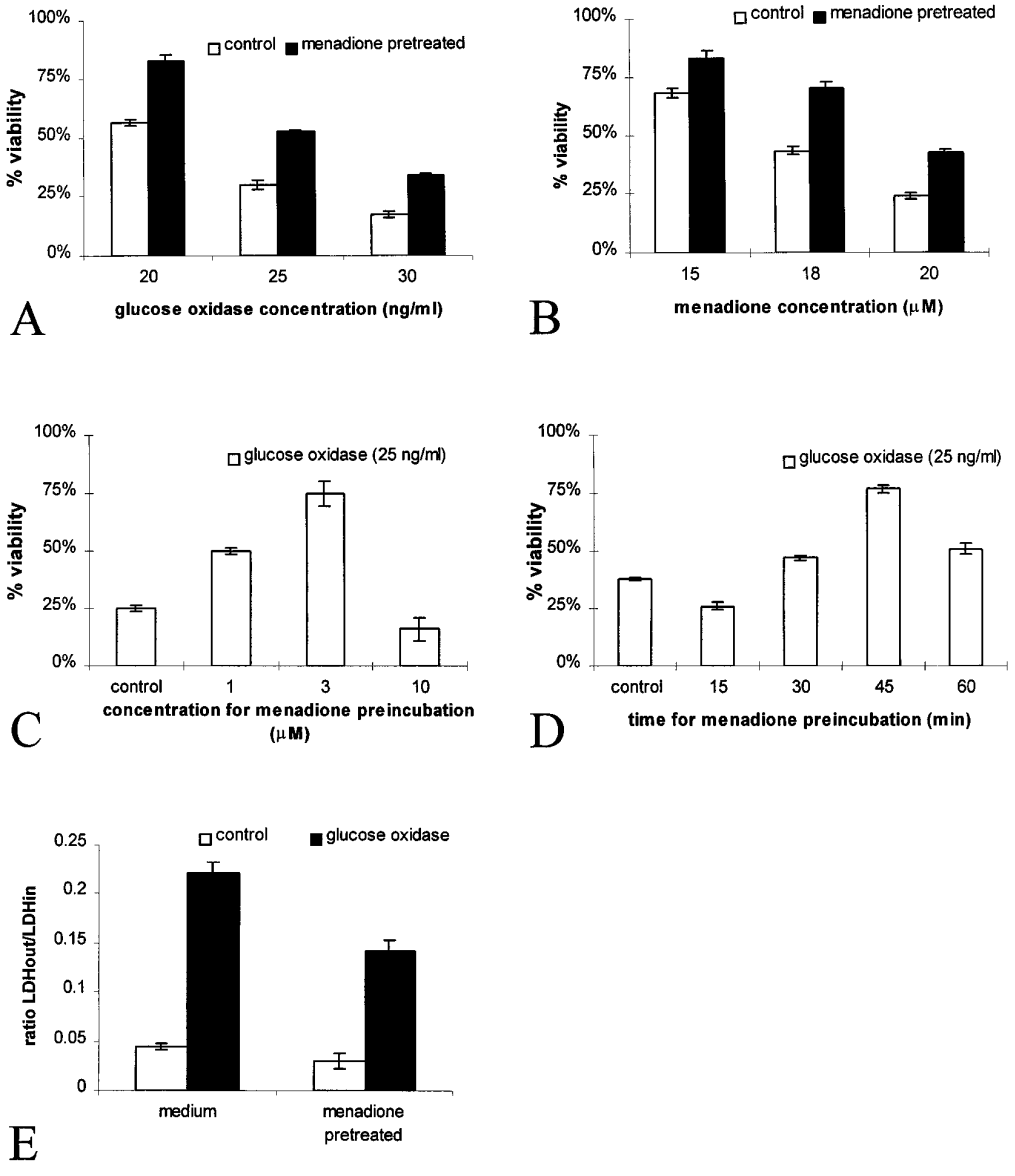

Fig. 2. Preincubation of Hep G2 cells with menadione protects against cytotoxicity of H_2O_2 or menadione. A, Cells were pretreated with control medium or 3 μM menadione for 45 min and then were exposed to H_2O_2 generated by 20, 25, or 30 ng/ml glucose oxidase plus 1 mg/ml glucose for 18 hr, followed by MTT assay. B, The cells were pretreated with control medium or 3 μM menadione for 45 min and then exposed to 15, 18, or 20 μM menadione for 18 hr. C, Dose-dependence of menadione preincubation. Hep G2 cells were pretreated with control medium and different concentrations of menadione for 45 min and then exposed to 25 ng/ml glucose oxidase (and 1 mg/ml glucose) for 18 hr. D, Time course of menadione preincubation. Hep G2 cells were pretreated with 3 μM menadione for different times and then exposed to 25 ng/ml glucose oxidase (and 1 mg/ml glucose) for 18 hr. The percent viability was determined as described in Materials and Methods. E, Cells were pretreated with control medium or 3 μM menadione for 45 min and then exposed to H_2O_2 generated by 25 ng/ml glucose oxidase plus 1 mg/ml glucose and followed by LDH release assay.

menadione was related to the concentration of menadione, with maximum protection occurring at 3–5 μM menadione (Fig. 2C), and was dependent on the length of preincubation time with menadione, becoming maximal at 45 min (Fig. 2D). In addition to protection against toxicity of H_2O_2, the pretreatment with menadione resulted in protection against menadione toxicity (Fig. 2B). Protection by pretreatment with menadione was also observed by using the LDH release assay instead of the MTT assay to determine cytotoxicity (Fig. 2E).

Menadione activation of the transcription factor NF-κB in Hep G2 cells. Oxidative stress generated by H_2O_2 can activate the transcription factor NF-κB (30–32). Because H_2O_2 is generated during the metabolism of menadione, it seemed reasonable to determine whether NF-κB was activated during or after the preincubation with menadione. Activation of NF-κB was measured with an EMSA. Pretreatment of Hep G2 cells with 3 μM menadione led to the activation of NF-κB (Fig. 3A, compare *lane 3* with *lane 1*; *lane 2* is from the nuclear extract prepared from Hep G2 cells incubated with 50 ng of PMA, a known activator of NF-κB) (33). When 20-fold unlabeled oligonucleotide containing the consensus sequence of NF-κB binding sites was added to the EMSA reaction mixtures, the DNA binding and mobility shift ability of nuclear extracts from PMA- or menadione-activated Hep G2 cells were inhibited (Fig. 3A, *lanes 5 and 6*). However, 20-fold unlabeled oligonucleotide containing mutations in the NF-κB binding site did not affect the EMSA assay (Fig. 3A, *lanes 8 and 9*). The activation of NF-κB by menadione was time dependent and could be observed as early as 10 min after menadione addition (Fig. 3B). This time period (10 min) was earlier than the time in which maximal protection against H_2O_2 toxicity by menadione pretreatment occurs (45 min). NF-κB contains p50 and p65 subunits; treatment of nuclear extracts with antibodies against p50 and p65 can result in either prevention of binding to the probe or a "supershift" of the NF-κB/oligonucleotide complex. Incubation of the nuclear extract from menadione-treated Hep G2 cells with anti-p50 IgG (Santa Cruz Biochemicals, Santa Cruz, CA) prevented binding to the oligonucleotide probe, whereas treatment with anti-p65 IgG (Upstate Biotechnology, Lake Placid, NY) resulted in a supershift of the complex (Fig. 4).

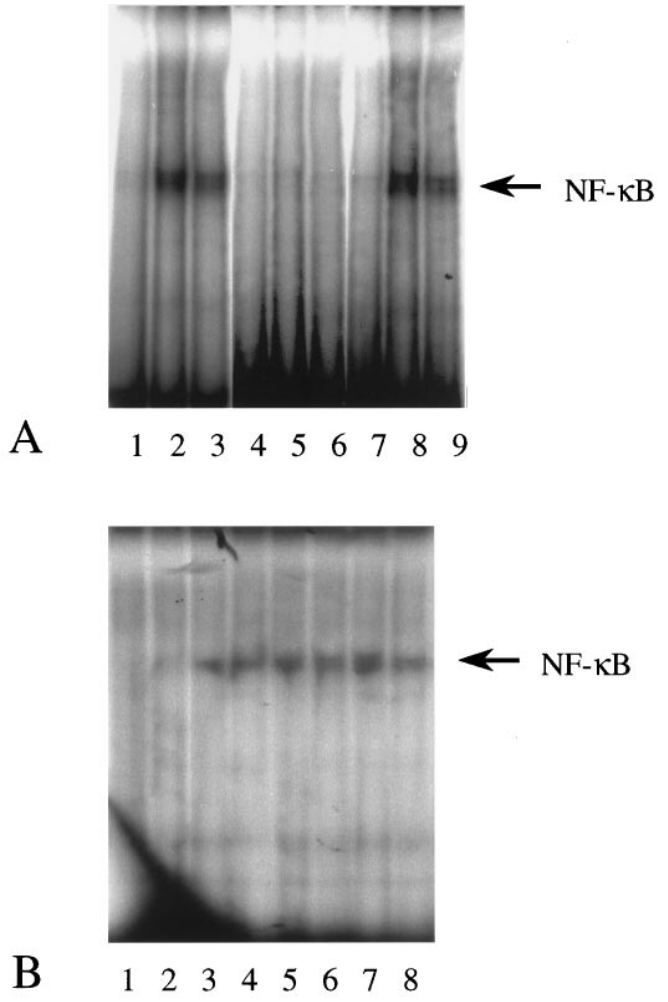

Fig. 3. Activation of NF-κB in Hep G2 cells. A, Nuclear extracts were prepared from the Hep G2 cells treated with control medium (*lanes 1, 4, and 7*), 50 ng/ml PMA (*lanes 2, 5, and 8*), or 3 μM menadione (*lanes 3, 6, and 9*) for 45 min and incubated with labeled oligonucleotide containing consensus sequence of NF-κB binding sites (*lanes 1–3*) for EMSA (see Material and Methods). Twenty-fold more unlabeled oligonucleotides containing NF-κB sequence (*lanes 4–6*) and unlabeled oligonucleotide containing mutant NF-κB sites (*lanes 7–9*) were present in the incubation period for competition. B, EMSA was performed by using nuclear extracts prepared from Hep G2 cells incubated with medium alone (*lane 1*) or 3 μM menadione for 5, 10, 15, 30, 45, 60, and 120 min (*lanes 2–8*, respectively).

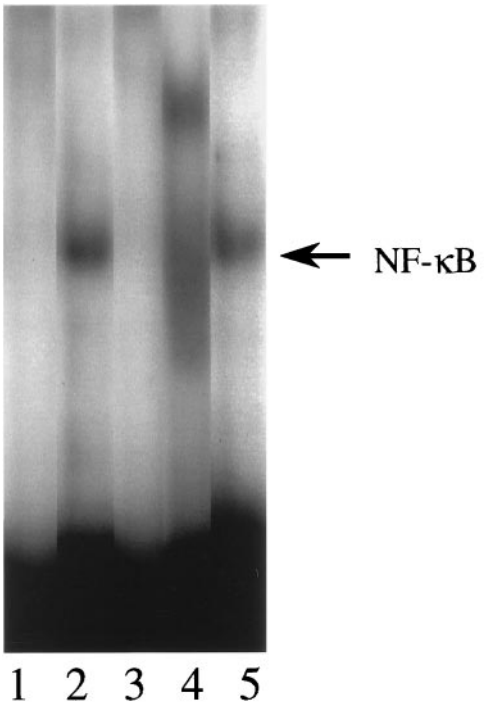

Fig. 4. Effect of anti-p50 and anti-p65 antibodies on NF-κB binding to NF-κB consensus sequence oligonucleotide probe. EMSA was carried out as described in Materials and Methods with nuclear extracts prepared from the Hep G2 cells treated with control medium (*lane 1*) or 3 μM menadione for 45 min. Nuclear extract from the cells stimulated with menadione was incubated in the binding buffer in the absence of antibody (*lane 2*) or in the presence of 2 μg of anti-p50 IgG (*lane 3*), 2 μg of anti-p65 IgG (*lane 4*), or 2 of μg preimmune IgG (*lane 5*) overnight at 4°. Labeled oligonucleotide containing the consensus sequence of NF-κB binding site was then added to each reaction mixture.

Preimmune IgG had no effect. Salicylate inhibited the activation of NF-κB in Jurkat cells (34). We therefore studied whether salicylate could inhibit the activation of NF-κB by menadione in the Hep G2 cells. Indeed, when 10 mM salicylate was added with menadione to the Hep G2 cells, the activation of NF-κB was inhibited (Fig. 5, compare *lane 2* with *lane 1*).

Salicylate potentiation of menadione cytotoxicity. As shown in Fig. 5, salicylate prevented activation of NF-κB by menadione in Hep G2 cells. If activation of NF-κB played an important role in the mechanism by which menadione protected Hep G2 cells against H_2O_2 or menadione cytotoxicity, it would be anticipated that salicylate would prevent the protection by menadione and perhaps even potentiate the cytotoxicity. Indeed, this proved to be the case; results (Fig. 6A) show that in the absence of salicylate, menadione at a concentration of 10 μM was not toxic to Hep G2 cells. However, in the presence of salicylate (2.5–20 mM), menadione toxicity was clearly observed. A menadione dose-dependent curve of cytotoxicity is shown in Fig. 6B; concentrations of 2.5–7.5 μM menadione were not toxic to the Hep G2 cells in the absence of salicylate, but striking toxicity was found in the presence of salicylate. Salicylate not only increased the toxicity of menadione but also potentiated the toxicity of H_2O_2. A time course for the potentiation of 10 μM menadione and 100 μM H_2O_2 toxicity by salicylate is shown (Fig. 6C). In other experiments, we observed that aspirin (acetylsalicylic acid) had the same actions as salicylate (data not shown). Salicylate, in the absence of menadione, was not toxic to the Hep G2 cells. Although salicylate may have other actions, such as antioxidant properties, such properties would be expected to decrease, not enhance, the toxicity of menadione.

Effect of antioxidants on NF-κB activation by menadione, and protection against cytotoxicity by menadione preincubation. It has been suggested that ROIs act as an intermediate during the activation of NF-κB (30, 35, 36). The effects of several antioxidants on NF-κB activation by menadione were evaluated. The antioxidants NAC, PDTC, thiourea, or uric acid were added during the pretreatment period with menadione and subsequently removed by washing the cells before the addition of a toxic concentration of menadione. As shown in Fig. 5, preincubation of Hep G2 cells with 3 μM menadione for 45 min in the presence of NAC, PDTC, thiourea, or uric acid did not lead to activation of

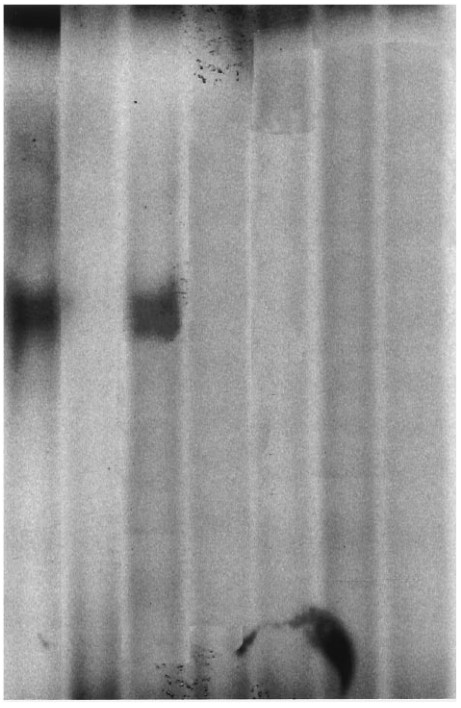

Fig. 5. Effects of salicylate, cycloheximide, and antioxidants on NF-κB activation by menadione. EMSA was carried as described in Materials and Methods with nuclear extracts prepared from Hep G2 cells treated with 3 μM menadione in the absence of any additions (*lane 1*) or in the presence of 10 mM sodium salicylate (*lane 2*), 20 μg/ml cycloheximide (*lane 3*), 10 mM NAC (*lane 4*), 50 μM PDTC (*lane 5*), 5 mM thiourea (*lane 6*), or 0.5 mM uric acid (*lane 7*) for 45 min

NF-κB (Fig. 5, compare *lanes 4–7* with *lane 1*). When Hep G2 cells were preincubated with 3 μM menadione in the presence of these antioxidants, the protective effect of menadione produced by this preincubation was no longer observed (Table 1). Although antioxidants may have nonspecific effects on cellular metabolism and viability, the results with four different antioxidants are suggestive that ROIs derived from menadione metabolism play a role in the activation of NF-κB by menadione and that when NF-κB activation is prevented by these antioxidants, there is loss of the protective effect produced by menadione pretreatment.

Depletion of PKC by PMA eliminates protective effect of menadione preincubation. Prolonged treatment of cells with the active phorbol ester PMA is known to down-regulate PKC (37) and inhibit PKC-dependent NF-κB activation (38). Hep G2 cells were treated with medium or 100 ng/ml PMA for 24 hr, followed by a 45-min preincubation with 3 μM menadione or medium and then exposure to 18 μM menadione. After treatment with PMA, preincubation with 3 μM menadione (a concentration that enables the cells to become more resistant to menadione and H_2O_2 cytotoxicity) did not increase the resistance of Hep G2 cells to the higher dose of menadione (Fig. 7A). Analogous to the results with salicylate, after 24 hr of PMA treatment, Hep G2 cells became more sensitive to menadione (Fig. 7B). It is interesting to speculate that down-regulation of PKC eventually suppresses the activation of NF-κB by ROIs generated from

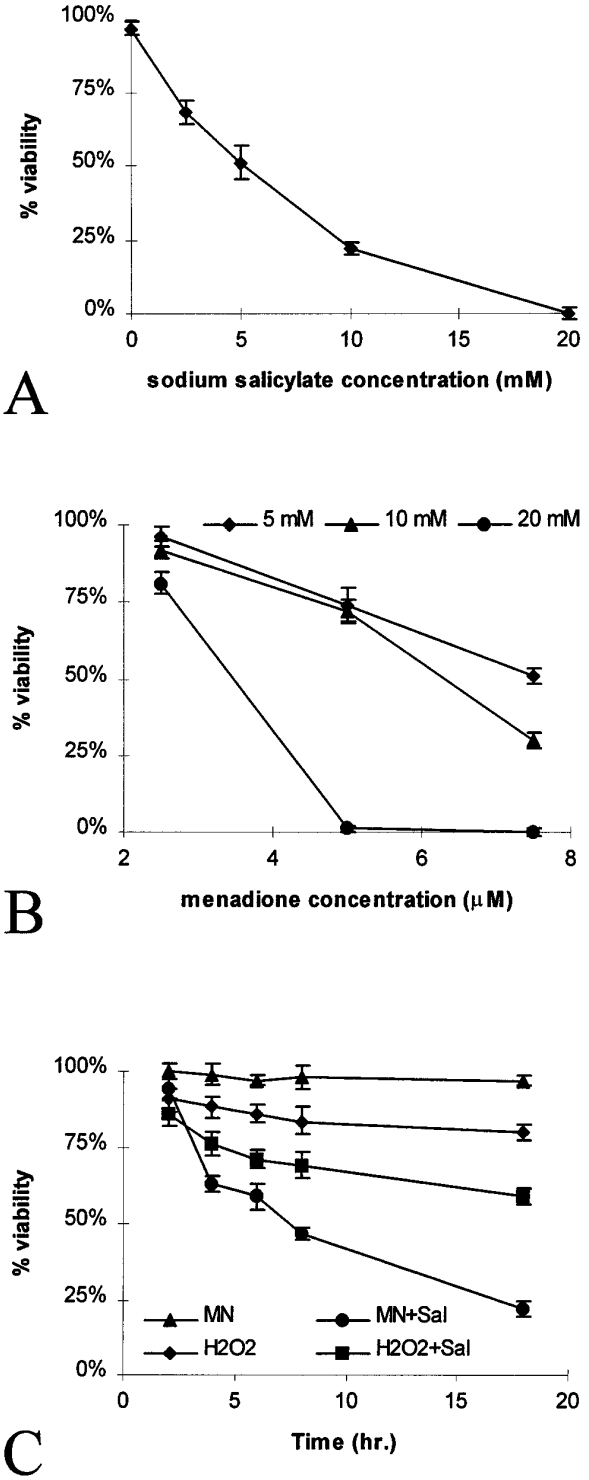

Fig. 6. Salicylate potentiation of menadione or H_2O_2 cytotoxicity to Hep G2 cells. Hep G2 cells were placed onto 24-well plates for assays, and viability was determined by the MTT assay, with results expressed as the percent viability of cells exposed to menadione or H_2O_2 plus salicylate compared with cells exposed to salicylate alone. At these concentrations and for a 6-hr incubation, salicylate alone had no toxic effect. A, Cells were incubated with 10 μM menadione in the presence of different concentrations of sodium salicylate for 6 hr. B, Dose-dependence curves were made by incubation of Hep G2 cells with the combination of various concentrations of menadione and sodium salicylate for 18 hr. C, Time course was carried out by incubating Hep G2 cells with 10 μM menadione (*MN*) or 50 μM H_2O_2 with or without 10 mM sodium salicylate (*Sal*) for the indicated time points.

TABLE 1

Effect of antioxidants on protective effect of menadione preincubation

Hep G2 cells were pretreated with control MEM or 3 μM menadione for 45 min in the presence of the indicated additions. Final concentrations of the additions were 20 μg/ml cycloheximide, 10 mM NAC, 50 μM pyrrolidine dithiocarbamate, 5 mM thiourea, and 0.5 mM uric acid. The cells were then exposed to 18 μM menadione for 18 hr, and viability was determined as described in Materials and Methods. Results are mean ± standard deviation of three to five wells per experiment from three separate experiments.

Addition	Viable cells	
	Control	Menadione
	%	
Experiment 1		
Control	54 ± 3.8	85 ± 3.6
Cycloheximide	44 ± 3.3	49 ± 4.5
NAC	55 ± 3.1	61 ± 4.5
Experiment 2		
Control	28 ± 2.0	46 ± 2.7
PDTC	24 ± 2.0	28 ± 0.8
Thiourea	26 ± 5.6	27 ± 5.1
Uric acid	31 ± 6.6	26 ± 6.7

menadione metabolism and thereby eliminates the possible protective effect that results from NF-κB activation. Short term treatment with PMA activates NF-κB (Fig. 3, *lane 2*); this could lead to protection of the Hep G2 cells against a toxic concentration of menadione, analogous to menadione pretreatment. This proved to be the case; treatment of the cells with 25 ng/ml PMA for 25 min increased the resistance to menadione (Fig. 7C).

Effect of cycloheximide, a protein synthesis inhibitor, on protection of menadione cytotoxicity by menadione preincubation. Activation of NF-κB, a transcription factor, should result in activation of target genes, followed by synthesis of enzymes or factors that may play a role in protection against toxicity of H_2O_2 or menadione. We therefore evaluated whether protein synthesis was necessary for the protection against menadione cytotoxicity produced by the menadione pretreatment. When the protein synthesis inhibitor cycloheximide was present during the preincubation with menadione, a protective effect against menadione cytotoxicity was not observed (Table 1). Because the immediate activation of NF-κB is not a protein synthesis-dependent event (14, 15), the presence of a protein synthesis inhibitor should not alter the activation of NF-κB. In the presence of menadione plus cycloheximide, NF-κB activation was comparable to that in the presence of menadione alone (Fig. 5, compare *lane 3* with *lane 1*), probably because cycloheximide itself is an activator of NF-κB in certain cell lines, including the Hep G2 cells. Because cycloheximide blocks the protective effect of menadione preincubation but does not prevent NF-κB activation, these results suggest that certain proteins or factors may be synthesized during or after the preincubation with menadione that produce the actual protection.

Effect of IκBβ on menadione toxicity. IκB binds to NF-κB, preventing its translocation into the nucleus and thereby preventing NF-κB modulation of transcription. To further implicate a role for NF-κB in the protection afforded by preincubation with low, nontoxic concentrations of menadione, the Hep G2 cells were transfected with an expression vector containing mouse IκBβ cDNA. The level of IκBβ overexpression in Hep G2 cells is shown in the Western blot in Fig. 8A. Menadione toxicity was increased by the transfection

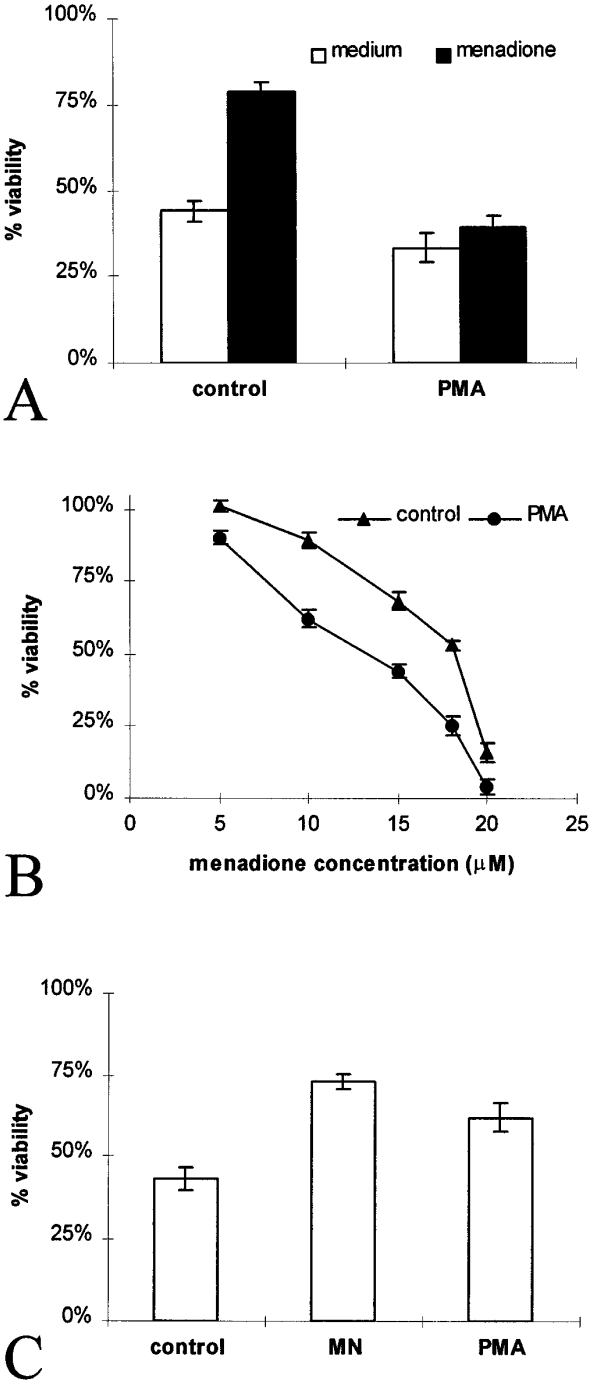

Fig. 7. Effects of PMA treatment on the protection by menadione preincubation against menadione cytotoxicity. A, Hep G2 cells were treated with 100 ng/ml PMA or medium for 24 hr and then preincubated with normal medium or 3 μM menadione for 45 min, followed by incubation with 18 μM menadione and subsequent cytotoxicity assay. B, After prolonged treatment with PMA or medium for 24 hr, Hep G2 cells were exposed to a series of different menadione concentrations for cytotoxicity assay. C, Hep G2 cells were preincubated with medium (*control*), 3 μM menadione (*MN*), or 25 ng/ml PMA for 25 min and then exposed to 18 μM menadione for cytotoxicity assay. The percent viability was determined as described in Materials and Methods.

with IκBβ ~2.5-fold compared with control transfection with pCI plasmid (Fig. 8B).

Intracellular GSH level after menadione preincubation. GSH is a tripeptide with nucleophilic and reducing

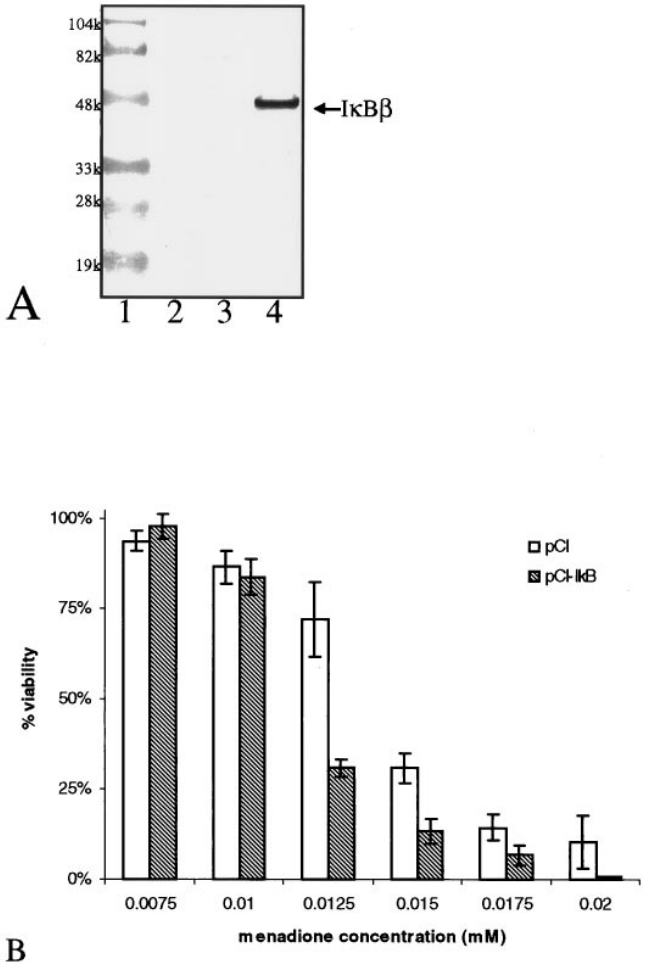

Fig. 8. Effect of overexpression of IκBβ on menadione cytotoxicity to Hep G2 cells. Transfection of the Hep G2 cells with control plasmid pCI or pCI-IκBβ was carried out as described in Materials and Methods. A, Western blot analysis was carried out on cell extracts using anti-IκBβ IgG (Santa Cruz Biochemicals) as primary antibody and goat anti-rabbit IgG-alkaline phosphatase as secondary antibody. *Lane 1*, molecular mass marker. *Lane 2*, Hep G2 cell. *Lane 3*, pCI transfectant. *Lane 4*, pCI-IκBβ transfectant. B, Cytotoxicity of the indicated concentrations of menadione in the pCI- and IκBβ-transfected Hep G2 cells was determined after an 18-hr incubation period.

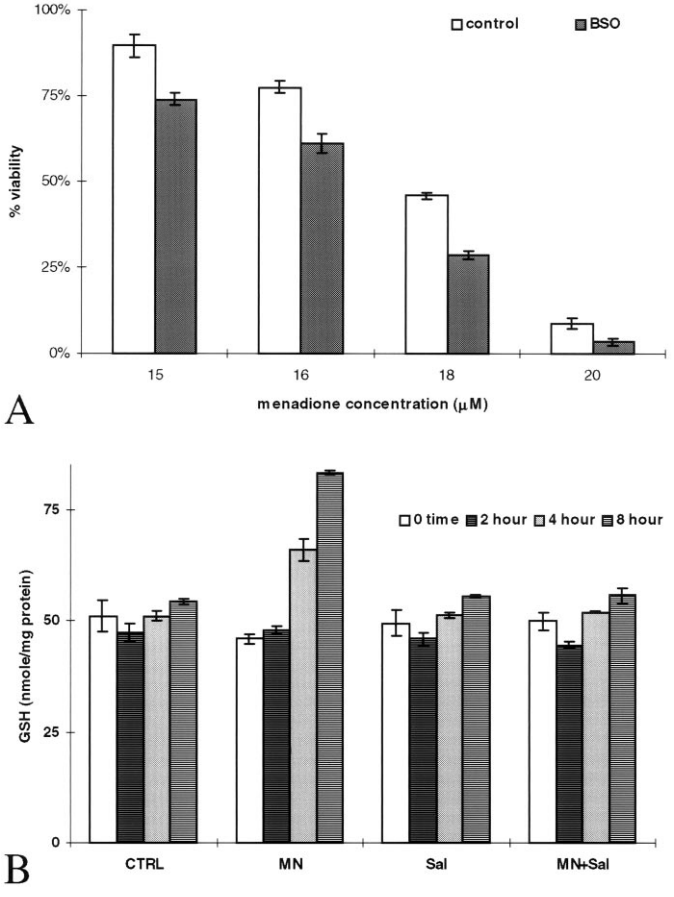

Fig. 9. A, Hep G2 cells were incubated with or without 0.1 mM buthionine sulfoximine (*BSO*) for 2 hr, followed by incubation in the presence of a series of different menadione concentrations for 18 hr. The cytotoxicity was determined as described in Materials and Methods. B, Intracellular GSH level after menadione preincubation. Hep G2 cells were preincubated with control medium (*CTRL*), 3 μM menadione (*MN*), 10 mM sodium salicylate (*Sal*), or 3 μM menadione plus 10 mM sodium salicylate (*MN+Sal*) for 45 min (salicylate was added 15 min before menadione) and then rinsed twice with medium, followed by additional incubation for 2, 4, or 8 hr with medium. Cells were harvested at the indicated time for GSH assay as described in Materials and Methods.

properties that play a central role in metabolic pathways as well as in the antioxidant system of the most aerobic cells. Because NAC protected Hep G2 cells from menadione cytotoxicity (Fig. 1B), GSH may play an important role in protection against this cytotoxicity. Depletion of GSH could therefore enhance the menadione cytotoxicity. Treatment of the Hep G2 cells with 0.1 mM buthionine sulfoximine overnight, a condition that depletes >90% of the cellular GSH, resulted in an increase in the menadione cytotoxicity (Fig. 9A). To evaluate possible factors responsible for the protective effect induced by menadione preincubation, the Hep G2 cells were treated with 3 μM menadione or medium for 45 min followed by removal of the menadione and continued incubation in normal medium. As shown in Fig. 9B, the GSH level of the cells that were pretreated with normal medium was unchanged for 8 hr after the medium change. However, the Hep G2 cells pretreated with 3 μM menadione showed an increased intracellular GSH level during incubation in the absence of menadione. When 10 mM sodium salicylate (which by itself had no effect on the GSH level) was present with 3 μM menadione during the preincubation, an increased GSH level was not observed. This links the increased GSH level to a salicylate-sensitive reaction, suggesting a possible role for NF-κB activation in the pathway leading to the elevated levels of GSH and to protection against menadione cytotoxicity.

Discussion

Adaptive responses to the oxidative stress of H_2O_2 in yeast *S. cerevisiae* strain RZ53 could increase the viability against a higher dose of H_2O_2 (8). It has been shown previously that preincubation of mammalian cells with low doses of H_2O_2 can protect these cells from the cytotoxicity of higher doses of H_2O_2 (13). However, the mechanism for this protection is still unknown. In the current study, we evaluated a possible protective effect of preincubation of lower, nontoxic doses of menadione on oxidative injury produced by toxic doses of menadione and H_2O_2. A major goal was to investigate the

possible role of NF-κB activation in the protection mechanism produced by menadione preincubation.

Preincubation of Hep G2 cells with a low dose of menadione (3 μM) was found to protect these cells from oxidative injury caused by the subsequent addition of a higher toxic dose of menadione or H_2O_2 generated by the glucose oxidase/glucose system. When antioxidants, such as NAC, PDTC, thiourea, or uric acid, were present with menadione during the preincubation period, no protective effect by menadione was observed. This could reflect the production of ROIs (generated by the metabolism of menadione), which are responsible for the eventual protective effect. NAC, a nucleophile, reacts with a variety of reactive species and helps to maintain cellular GSH levels. Thiourea and uric acid react with hydroxyl radical-like species. PDTC, a metal chelator, prevents iron-catalyzed formation of potent oxidizing species.

With the EMSA, menadione was shown to activate NF-κB in Hep G2 cells. The NF-κB specific binding in EMSA was confirmed by the addition of excess unlabeled NF-κB oligonucleotide, which blocked the specific binding, and of excess mutated NF-κB oligonucleotide, which did not block the specific binding, to the EMSA reactions, and by the ability of anti-p50 IgG to block binding to the oligonucleotide probe, while anti-p65 IgG produced a supershift of NF-κB/probe complex. These results suggest the presence of p50 and p65 subunits in the NF-κB complex activated by menadione. The activation of NF-κB by menadione may be related to the oxidative stress generated from the metabolism of menadione because the same antioxidants that blocked the protective effect produced by preincubation with menadione (Table 1) also inhibited the activation of NF-κB by menadione. Concentrations of menadione that activated NF-κB were also effective concentrations for protection against cytotoxicity of H_2O_2 or menadione. Nevertheless, the activation of NF-κB by menadione was a relatively rapid response, evident as early as 10 min after treatment. The time needed to activate NF-κB by menadione was shorter than the menadione preincubation time when maximum protection was observed. This suggests that formation of some proteins or factors must first be induced before the cells achieve the ability to resist oxidative stress.

Sodium salicylate has been shown to inhibit the NF-κB activation in some cell lines (34), and it inhibited NF-κB activation by menadione in Hep G2 cells. Associated with this prevention of NF-κB activation was a potentiation of the cytotoxicity of menadione and H_2O_2. Concentrations of menadione or H_2O_2 that were not toxic to the cells in the absence of salicylate were toxic in the presence of salicylate. This raises the possibility that by inhibiting NF-κB activation, salicylate eliminated the protective effect generated by menadione-induced low level oxidative stress. In a similar manner, overexpression of IκBβ in Hep G2 cells increases the toxicity of menadione. Active phorbol esters such as PMA are known for their ability to activate PKC, which phosphorylates IκB and then activates NF-κB. Prolonged culture in PMA-containing medium down-regulates PKC activity (37), which results in inhibition of PKC-dependent NF-κB activation. When Hep G2 cells were maintained in culture medium containing 100 ng/ml of PMA for 24 hr, those cells became more sensitive to menadione than the control cells, which were maintained in normal medium. Menadione preincubation failed to increase the viability of PMA-treated cells in the menadione cytotoxicity assay. However, short term treatment with PMA, which activates PKC and, subsequently, NF-κB, protected against menadione cytotoxicity. The results with salicylate, IκBβ, and PMA suggest a role for NF-κB in the protection by menadione preincubation.

NF-κB activation, which includes the release of IκB and translocation of NF-κB into the nucleus, is not sufficient to cause the protection observed after menadione pretreatment. Activation of NF-κB regulates the expression of certain genes that contain NF-κB binding sequences in their upstream regulation regions (39, 40). It has been suggested that genes involved with oxidative stress might be induced after activation of NF-κB. Cycloheximide could also activate NF-κB in Hep G2 cells, but it did not increase the viability of these cells in response to menadione. When cycloheximide was present with menadione during preincubation, there was no protective effect against menadione cytotoxicity. These data indicate that some proteins must be synthesized in response to the activation of NF-κB caused by the pretreatment with menadione, and it is likely that such proteins are responsible for protecting the cells from oxidative injury.

Menadione preincubation was found to increase significantly the intracellular GSH level of Hep G2 cells. This increase may play an important role in the mechanism of the protective effect of menadione preincubation, especially because menadione cytotoxicity was elevated when GSH was depleted after treatment of the cells with buthionine sulfoximine. Shi *et al.* (24) found an increased GSH concentration in bovine pulmonary artery endothelial cells after the cells were treated with 2,3-dimethoxy-1,4-naphthoquinone and menadione, whereas Ochi (25) showed that menadione increased GSH levels in Chinese hamster V79 cells. The increase in GSH levels in Hep G2 cells by menadione pretreatment may be one consequence of the activation of NF-κB by menadione; salicylate inhibits the GSH increase induced by menadione treatment under conditions in which salicylate potentiates menadione cytotoxicity in the Hep G2 cells. Menadione is metabolized to ROIs, which are toxic to the cells when produced in high amounts that overwhelm cellular defensive mechanisms. At lower concentrations, menadione may induce cellular defense, partially by increasing GSH levels. This protective effect would be eliminated in the presence of salicylate if the protection is an NF-κB-dependent event. The salicylate potentiation of menadione cytotoxicity is consistent with the suggestion that menadione protects the Hep G2 cells against higher menadione cytotoxicity through an NF-κB dependent pathway.

The NF-κB family is activated by a variety of stimuli, such as cytokines, viruses, and UV light, as well as oxidative stress (for reviews, see Refs. 16 and 17). Based on the observation that induction of NF-κB is an early response to oxidative stress, the NF-κB signaling pathway seems to be a natural protective mechanism against injury. Certain proteins related to oxidative stress, such as Mn-SOD (18, 19), DT-diaphorase (20), and inducible nitric oxide (21, 22), and ferritin H (23), have been shown to be induced after NF-κB activation. Results in the current study show that ROIs generated from menadione metabolism can induce a protective mechanism against oxidative stress in Hep G2 cells. This protection mechanism seems to involve an NF-κB activation and may be due in part to elevation of cellular GSH levels. Further studies are under way to identify the ultimate en-

zymes or factors induced after NF-κB activation that provided the protection against menadione or H_2O_2 cytotoxicity.

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