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Parthenolide, an Inhibitor of the Nuclear Factor-κB Pathway, Ameliorates Cardiovascular Derangement and Outcome in Endotoxic Shock in Rodents

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ABSTRACT

Parthenolide is a sesquiterpene lactone used in folk medicine for its anti-inflammatory activity. Recent in vitro studies have shown that this compound inhibits the nuclear factor (NF)-κB pathway. This study examines the effect of parthenolide in endotoxic shock in rodents. Endotoxic shock was induced by administration of *Escherichia coli* endotoxin in rats. Three groups of rats received parthenolide (0.25, 0.5, or 1 mg/kg) 15 min before endotoxin; another group received parthenolide (1 mg/kg) 3 h after endotoxin. In vehicle-treated rats, administration of endotoxin caused severe hypotension, which was associated with a marked hyporeactivity to norepinephrine in ex vivo thoracic aortas. Immunohistochemistry showed positive staining for nitrotyrosine, poly(ADP-ribose) synthetase (PARS) and apoptosis, whereas Northern blot analysis showed increased mRNA expression of inducible nitric-oxide synthase (iNOS) in thoracic aortas. Elevated levels of plasma nitrate/nitrite were also found. Elevated lung levels of myeloperoxidase activity were indicative of infiltration of neutrophils. These inflammatory events were preceded by cytosolic degradation of inhibitor κBα (IκBα) and activation of nuclear NF-κB in the lung. In vivo pretreatment and post-treatment with parthenolide improved the hemodynamic profile and reduced plasma nitrate/nitrite and lung neutrophil infiltration in a dose-dependent fashion. Vascular hyporeactivity of ex vivo aortas was ameliorated. Treatment with parthenolide also abolished nitrotyrosine formation, PARS expression, and apoptosis and reduced iNOS mRNA content in thoracic aortas. DNA binding of NF-κB was inhibited by parthenolide in the lung, whereas degradation of IκBα was unchanged. In a separate set of experiments, pretreatment or post-treatment with parthenolide significantly improved survival in mice challenged with endotoxin. We conclude that parthenolide exerts beneficial effects during endotoxic shock through inhibition of NF-κB.

Septic shock resulting from Gram-negative bacterial infection is one of the most common causes of death in intensive care units (Parrillo, 1993). The mechanism by which bacterial infection triggers the inflammatory process involves the activation of the transcription factor nuclear factor-κB (NF-κB). Under physiological conditions, NF-κB is sequestered in an inactive form in the cytosol through noncovalent interactions with inhibitor proteins such as IκBα. However, during septic shock, NF-κB has been shown to be activated by bacterial lipopolysaccharide and inflammatory cytokines such as interleukin-6 and tumor necrosis factor. Once activated, NF-κB dissociates from its inhibitors and translocates to the nucleus where it leads to the activation of various proinflammatory and chemotactic agents, cytokines, inducible nitric oxide synthase (iNOS), and adhesion molecules, thus, creating an inflammatory self-maintaining cycle (Baeuerle, 1998; Karin and Delhase, 2000).

Because of the complexity of the pathophysiology of cardiovascular shock, major efforts have recently been focused on identifying novel anti-inflammatory drugs, which can prevent the proinflammatory process at the very early stage of gene expression. Sesquiterpene lactones are derived from *Asteraceae* species plants and have been used as folk remedies for various inflammatory conditions such as rheumatoid arthritis, asthma, psoriasis, and migraine (Hall et al., 1980; Heinrich et al., 1998; Schinella et al., 1998). Despite their popular use as alternative medicines, only a few in vivo experimental studies have been performed with these compounds. Treatment with sesquiterpene lactones has been reported to provide therapeutic efficacy in animal models of paw and ear edema (Hall et al., 1980; Schinella et al., 1998),

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ABBREVIATIONS: NF-κB, nuclear factor-κB; iNOS, inducible nitric-oxide synthase; IκBα, inhibitor κBα; IKK, IκB kinase complex; NO, nitric oxide; PARS, poly(ADP-ribose) synthetase; LPS, lipopolysaccharide; TdT, terminal deoxynucleotidyl transferase; PMSF, phenylmethylsulfonyl fluoride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

chronic arthritis, gastritis, and colitis (Giordano et al., 1992; Wendel et al., 1999). However, their in vivo molecular mechanism of action has not been fully investigated. Several in vitro experimental studies have proposed that the anti-inflammatory property of sesquiterpene lactones is due to their ability to inhibit NF-κB activation (Bork et al., 1997; Hehner et al., 1998, 1999; Heinrich et al., 1998).

In the present study, we investigated the biological effects and the mechanisms of action of parthenolide in in vivo rodent models of endotoxic shock. We observed that parthenolide ameliorated the hypotension and subsequent demise induced by endotoxin and that its therapeutic efficacy was associated with prevention of NF-κB activation.

Experimental Procedures

Hemodynamic Changes. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by U.S. National Institutes of Health (National Institutes of Health Publication 85-23, revised 1996) and with the approval of the Institutional Animal Care and Use Committee. Male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 200 to 275 g were anesthetized with intraperitoneal (i.p.) injection of thiopentone sodium (70 mg/kg). The trachea was cannulated to facilitate respiration. The jugular vein was cannulated for the administration of endotoxin. The carotid artery was cannulated to measure mean arterial blood pressure and heart rate by a pressure transducer connected to a Maclab A/D converter (ADInstruments, Milford, MA). Endotoxic shock was induced by intravenous administration of bacterial lipopolysaccharide from *Escherichia coli* (LPS, 15 mg/kg) (Zingarelli et al., 1996b). Six groups of rats were used in the experiment. The first group (n = 10) received an equal volume of vehicle (0.05% Tween 80) instead of parthenolide, 15 min before endotoxin injection (vehicle + LPS group). The second, third, and fourth groups (n = 10–12) received parthenolide at a dosage of 0.25, 0.5, or 1 mg/kg, (PAR 0.25 + LPS, PAR 0.5 + LPS, and PAR 1.0 + LPS groups). To assess the efficacy of parthenolide as post-treatment, a fifth group (n = 6, LPS + PAR 1.0) received 1 mg/kg parthenolide and a sixth group (n = 6, LPS + vehicle) received the vehicle as post-treatment 3 h after administration of endotoxin. In this latter experiment, the rats that received parthenolide were randomly chosen independently to their initial hemodynamic response to endotoxin. A seventh group of animals underwent the same surgical procedures without administration of endotoxin and served as controls (sham group). Mean arterial blood pressure was monitored for 5 h after endotoxin administration. Animals that died before the end of the experiment were excluded from the study. In another set of experiments, groups of animals (n = 3–5) were sacrificed at different time points after endotoxin administration (15, 30, 60, 120, 180, and 300 min). Plasma samples, lungs, and aortas were collected for biochemical studies and immunohistochemistry.

Measurement of ex Vivo Contractility. In a separate set of experiments, a group of rats, pretreated with either vehicle or 1 mg/kg parthenolide, were sacrificed at 3 h after endotoxin administration, and thoracic aortas were harvested for ex vivo contractility study (Zingarelli et al., 1996b). Aortas were cleared of adhering periadventitial fat and cut into rings 3 to 4 mm wide. Endothelium was removed by gently rubbing the intimal surface with a thin wooden stick. The rings were mounted in organ baths (5 ml) filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs' solution, pH 7.4, consisting of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.7 mM glucose, in the presence of 10 μM indomethacin. Isometric force was measured with isometric transducers (Kent Scientific Corp., Litchfield, CT), digitized by a Maclab A/D converter (ADInstruments), and stored and displayed on a Macintosh personal computer. A tension of 1 g was applied, and the rings were equilibrated for 1 h. Indomethacin was added to prevent the production of cyclooxygenase metabolites. After incubation and washouts, the vessels were precontracted with a medium concentration of norepinepherine (100 nM), and the effect of acetylcholine (10 nM–10 μM) was tested. The lack of a detectable acetylcholine-induced relaxation was taken as evidence that endothelial cells had been removed. Concentration-response curves to norepinepherine (1 nM–10 μM) were then obtained in these endothelium-denuded aortic rings taken from either control rats or rats injected with LPS (with or without pretreatment with 1 mg/kg parthenolide).

Myeloperoxidase Activity. Myeloperoxidase activity was determined as an index of neutrophil accumulation as described previously (Mullane et al., 1985). Lung tissues were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer, pH 7, and centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was allowed to react with a solution of 1.6 mM tetra-methyl-benzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured by spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of hydrogen peroxide/min at 37°C and expressed in units per 100 mg of tissue.

Northern Blot Analysis. Thoracic aortas were homogenized and total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). RNA samples were further enriched for RNA by column spin using the Qiagen protocol (Qiagen, Valencia, CA). One-half of the eluted volume of RNA was electrophoresed on 1% formaldehyde agarose gel. For Northern blot analysis, the RNA was transferred to Magnacharge nylon membrane (Osmonic, Westborough, MA) in 20× standard saline citrate overnight by capillary action, and cross-linked to the membrane with short-wave ultraviolet cross linker (Stratagene, La Jolla, CA). Transferred RNA was visualized by methylene blue staining. Membranes were prehybridized for 2 h at 42°C in NorthernMax solution (Ambion, Austin, TX), and hybridized overnight at 42°C with a murine iNOS cDNA probe (106 cpm/ml) labeled with [^{32}P]dCTP. The specificity of this probe in rat tissues has been previously established (Wong and Menendez, 1999). The blots were then serially washed at 42°C using 2× sodium citrate, sodium chloride-0.1% SDS for 30 min, 1× sodium citrate, sodium chloride-0.1% SDS for 30 min, and at 55°C with 0.2× sodium citrate, sodium chloride-0.1% SDS for 30 min. After probing for iNOS, membranes were stripped with boiling 5 mM EDTA and rehybridized with a ^{32}P-radiolabeled oligonucleotide probe for 18S ribosomal RNA as a house-keeping gene. The relative amount of mRNAs was evaluated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Expression of iNOS was normalized to 18S ribosomal RNA for comparative purposes. Densitometric analysis was performed using ImageQuant (Molecular Dynamics).

Measurement of Nitrite/Nitrate Production. Nitrite/nitrate production, an indicator of nitric oxide synthesis, was measured in plasma samples as described previously (Zingarelli et al., 1996a). Nitrate in the plasma was reduced to nitrite by incubation for 3 h with nitrate reductase (670 mU/ml) and NADPH (160 mM) at room temperature. Nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated H_3PO_4; volume 1:1) to 100-μl samples. The optical density at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Menlo Park, CA). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

Immunohistochemistry for Nitrotyrosine and Poly(ADP-Ribose) Synthetase (PARS). Tyrosine nitration, a marker of nitrosative damage, and PARS activation were evaluated in sections of rat aortas by immunohistochemistry. Paraffin-embedded sections (5 μm thick) were deparaffinated and incubated for 2 h with a blocking solution (0.1 M phosphate-buffered saline containing 0.1% Triton

X-100 and 2% normal goat serum), to minimize nonspecific adsorbtion. Sections were then incubated overnight with primary anti-nitrotyrosine or anti-PARS antibody, or with control solutions. Controls included buffer alone or nonspecific purified rabbit IgG. Specific labeling was detected by incubating for 30 min with a biotin conjugated goat anti-rabbit IgG and amplified with avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) after removing endogenous peroxidase with 0.3% H_2O_2 in 100% methanol for 15 min. Diaminobenzidine was used as a chromogen.

Determination of Apoptosis. Cell death by apoptosis in rat aortas was evaluated by measurement of oligonucleosomal DNA fragments by a histochemical terminal deoxynucleotidyl transferase (TdT) TUNEL-like staining (TdT-FragEL kit; Oncogene Research Products, Cambridge, MA). In brief, after deparaffination, paraffin-embedded sections were permeabilized with protease K (2 mg/ml) in 10 mM Tris, pH 8, at room temperature for 20 min. Endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 5 min. Sections were incubated with a reaction buffer composed by biotin-dCTP and unlabeled dCTP and TdT enzyme in a humidified chamber at 37°C. In this assay, TdT binds to exposed 3′OH ends of DNA fragments and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Byotinilated nucleotides were then detected using a streptavidin-horseradish peroxidase conjugate and diaminobenzidine (Gavrieli et al., 1992).

Protein Extraction. Tissue samples from lungs were homogenized with a Polytron homogenizer in a buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN3, 10 mM β-mercaptoethanol, 20 μM leupeptin, 0.15 μM pepstatin A, 0.2 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, and 0.4 nM microcystin. The homogenates were centrifuged (1,000*g* for 10 min), the supernatants were collected (cytosol extract), and the pellets were solubilized in Triton buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 20 μM leupeptin A, and 0.2 mM PMSF). The lysates were centrifuged (15,000*g* for 30 min, 4°C), and the supernatant (nuclear extract) was collected.

Western Blot Analysis. Cytosol degradation of IκBα was determined by immunoblot analyses. Cytosol extracts were boiled in equal volumes of loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) and 50 μg of protein was loaded per lane on an 8 to 16% Tris-glycine gradient gel. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 5% nonfat dried milk in Tris-buffered saline for 1 h and then incubated with primary antibodies against IκBα for 1 h. The membranes were washed in Tris-buffered saline with 0.1% Tween 20 and incubated with secondary peroxidase-conjugated antibody. Detection was enhanced by chemiluminescence and exposed to photographic film. Densitometric analysis of blots was performed using ImageQuant (Molecular Dynamics).

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays were performed as described previously (Zingarelli et al., 2002). An oligonucleotide probe corresponding to NF-κB consensus sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified in Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). Ten micrograms of nuclear protein was preincubated with electrophoretic mobility shift assay buffer (12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 50 ng/ml poly[d(I-C)], 12% glycerol, v/v, and 0.2 mM PMSF) on ice for 10 min before addition of the radiolabeled oligonucleotide for an additional 10 min. Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide:bisacrylamide) and run in 0.5× Tris borate-EDTA (45 mM Tris-HCl, 45 mM boric acid, and 1 mM EDTA) for 1 h at constant current (30 mA). Gels were transferred to 3M paper (Whatman, Clifton, NJ), dried under a vacuum at 80°C for 1 h, and exposed to photographic film at −70°C with an intensifying screen. Densitometric analysis was performed using ImageQuant (Molecular Dynamics).

Determination of Effect of Parthenolide on NF-κB/DNA Binding in Vitro. The effect of parthenolide on the ability of NF-κB to bind DNA was assessed in in vitro comparative experiments. Nuclear extracts were obtained from lungs derived from control rats or from rats treated in vivo with endotoxin for 15 min as described above. The nuclear extracts were then incubated with vehicle (5 μl of Tris borate-EDTA buffer) or parthenolide (30 nM–10 μM) at room temperature for 30 min. After incubation, electrophoretic mobility shift assays for NF-κB were performed as described above.

Survival Study in Mice. Swiss albino mice (25–30 g, Charles River Laboratories) were injected with endotoxin (60 mg/kg, i.p.). Four groups of mice (n = 10–12 for each group) were used in the experiment. The first group of mice received an equal volume of vehicle (0.05% Tween 80, i.p.) instead of parthenolide 15 min before endotoxin injection (vehicle + LPS group). The second and third groups received parthenolide at a dosage of 0.25 or 0.5 mg/kg, (PAR 0.25 + LPS and PAR 0.5 + LPS). A fourth group received parthenolide at a dosage of 0.5 mg/kg i.p. as post-treatment 3 h after administration of endotoxin (LPS + PAR 0.5 group).

Materials. Primary anti-nitrotyrosine antibody was obtained from Upstate Biotechnology (Saranac Lake, NY). Primary anti-PARS antibody was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). The antibody against IκBα and the oligonucleotide probe for NF-κB consensus were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Reagents, secondary, and nonspecific IgG antibodies for immunohistochemical analyses were from Vector Laboratories (Burlingame, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Data Analysis. All values in the figures and text are expressed as mean ± S.E.M. of n observations, where n represents the number of animals (n = 6–12 animals for each group). The results were examined by analysis of variance followed by the Bonferroni's correction post hoc t test. Statistical analysis of mortality study was performed using the χ^2 test. A p value less than 0.05 was considered significant.

Results

Effect of Parthenolide on Endotoxin-Induced Hypotension. In vehicle-treated rats, administration of endotoxin resulted in a profound decrease in blood pressure, which was characterized by an early phase of hypotension within 5 to 10 min and a delayed phase starting at 2 h thereafter. Pretreatment with 0.5 and 1 mg/kg parthenolide resulted in a significant improvement in blood pressure in a dose-dependent manner, ameliorating both the early and the delayed stages of hypotension (Fig. 1, B and C). In contrast, pretreatment with parthenolide at the low dosage of 0.25 mg/kg did not affect the hemodynamic profile (Fig. 1A). The amelioration of the delayed phase of hypotension was also seen in the endotoxemic rats that received parthenolide (1 mg/kg) as a post-treatment at 3 h after endotoxin administration (Fig. 1D).

Effect of Parthenolide on ex Vivo Vascular Reactivity. Endotoxin also caused a significant depression of the contractile ability of the thoracic aortas to norepinephrine (1 nM–10 μM) ex vivo. However, in vivo pretreatment with 1 mg/kg parthenolide partially restored the vascular reactivity to the vasoconstrictor agent (Fig. 2).

Effect of Parthenolide on Nitric Oxide (NO) Production. The overwhelming release of NO from iNOS during endotoxic shock has been suggested to contribute significantly to cardiovascular dysfunction (Rubanyi, 1998). Therefore, we next determined the effect of in vivo treatment with parthenolide on expression of iNOS in thoracic aortas and

plasma levels of NO stable metabolites, nitrate and nitrite. As determined by Northern blot analysis, expression of iNOS mRNA increased in a time-dependent fashion after endotoxin administration in vehicle-treated rats (Fig. 3). Pretreatment with 0.5 and 1 mg/kg parthenolide reduced expression of iNOS in the aortas. In vehicle-treated rats, the induction of iNOS correlated with increased formation of NO, as plasma levels of nitrate and nitrite were 63.5 ± 5.2 and 77.2 ± 6.8 μM, respectively, at 3 and 5 h after endotoxin administration. Treatment with parthenolide as either a pretreatment or a post-treatment reduced the formation of NO at 5 h after endotoxin administration (Fig. 4).

Effect of Parthenolide on Tyrosine Nitration in Rat Aortas. The increase in NO production correlated with the appearance of a positive immunohistochemical staining for nitrotyrosine, which was scarce and confined at the endothelium at 3 h and widely spread throughout the smooth muscle layer later at 5 h after endotoxin administration. In contrast, nitrotyrosine staining was significantly reduced in a dose-dependent manner by pretreatment with parthenolide. A reduction in nitrotyrosine staining was also seen in the rats that received 1 mg/kg parthenolide as a post-treatment 3 h after endotoxin administration (Fig. 5).

Effect of Parthenolide on PARS Activation in Rat Aortas. To further elucidate the effect of parthenolide on vascular function, we determined the expression of PARS, a nuclear enzyme that is activated after oxidant-induced DNA damage and is proposed to mediate vascular injury (Zingarelli et al., 1996a; Szabó and Dawson, 1998). Thoracic aortas obtained from vehicle-treated rats at 3 and 5 h after endotoxin administration demonstrated a positive immunostaining for PARS compared with the aortas from control sham rats, thus, suggesting the occurrence of PARS activation after endotoxic shock. The positive staining for PARS was mainly localized in the smooth muscle layer and in the endothelium. In contrast, thoracic aortas obtained from parthenolide-treated rats exhibited a marked reduction in PARS expression (Fig. 6).

Effect of Parthenolide on Apoptosis in Rat Aortas. To test whether vascular dysfunction was associated with cell death by apoptosis, we measured oligonucleosomal DNA fragmentation in thoracic aortas. Tissues, obtained from ve-

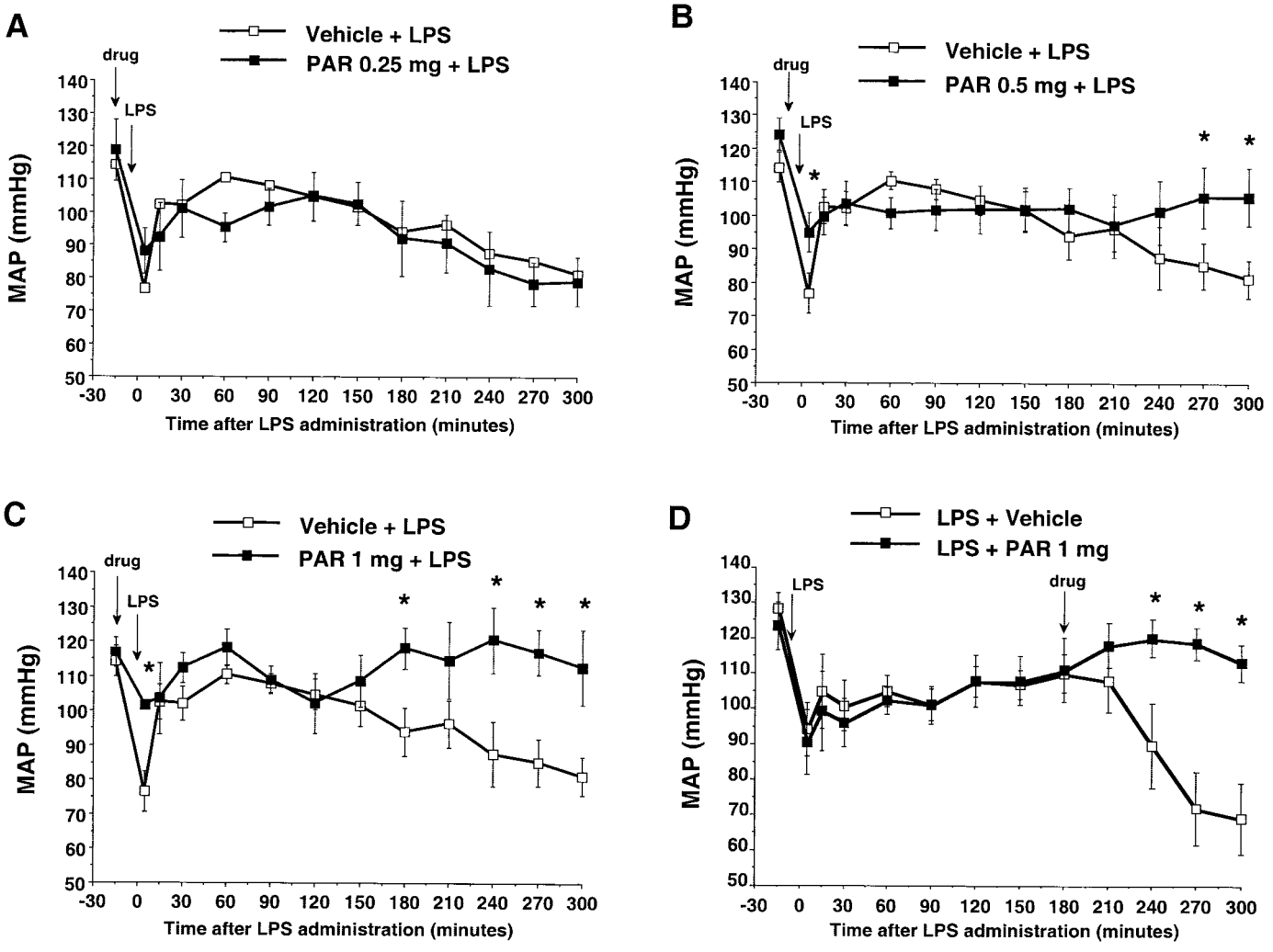

Fig. 1. Effect of in vivo treatment with parthenolide on mean arterial blood pressure (MAP) in rats subjected to endotoxic shock (LPS, 15 mg/kg). Each data point represents the mean ± S.E.M. of 6–15 animals for each group. *, $p < 0.05$ versus vehicle-treated rats subjected to endotoxic shock. A, parthenolide (0.25 mg/kg) was administered 15 min before endotoxin injection (PAR 0.25 mg + LPS). B, parthenolide (0.5 mg/kg) was administered 15 min before endotoxin injection (PAR 0.5 mg + LPS). C, parthenolide (1.0 mg/kg) was administered 15 min before endotoxin injection (PAR 1 mg + LPS). D, parthenolide (1.0 mg/kg) was administered 3 h after endotoxin injection (LPS + PAR 1 mg). Arrows indicate time of administration of vehicle and parthenolide (both indicated as drug) or LPS.

hicle-treated rats at 3 and 5 h after endotoxin administration demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments scattered throughout the endothelial and smooth muscle layers. In contrast, staining for apoptosis was virtually abolished in rats treated with parthenolide (Fig. 7).

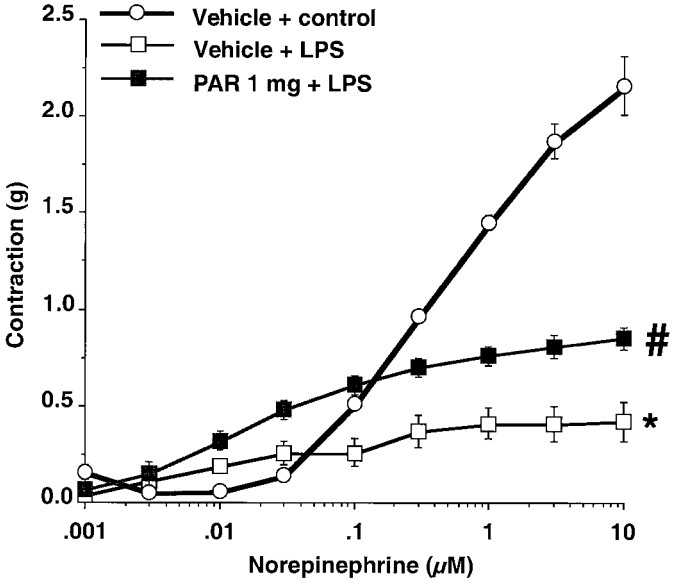

Fig. 2. Effect of in vivo treatment with parthenolide on ex vivo vascular contractility to norepinephrine in endothelium-denuded aortic rings. Each data point represents the mean ± S.E.M. of 6 to 10 rings. *, $p < 0.05$ versus vehicle-treated sham rats (vehicle + control); #, $p < 0.05$ versus vehicle-treated rats subjected to endotoxic shock (vehicle + LPS). Parthenolide (1.0 mg/kg) was administered 15 min before endotoxin injection (PAR 1 mg + LPS).

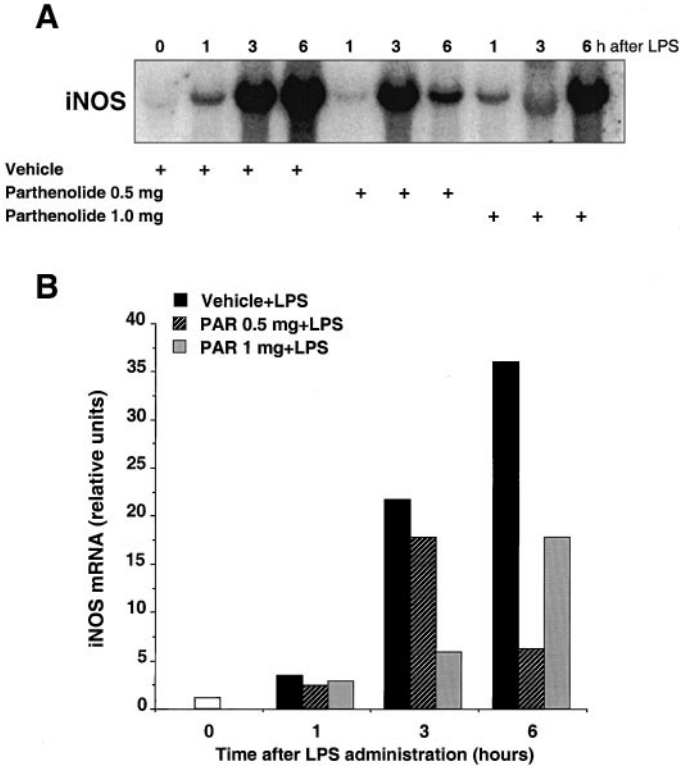

Fig. 3. Effect of in vivo treatment with parthenolide on expression of iNOS mRNA in thoracic aortas. A, representative Northern blot analysis of iNOS mRNA at different time points after endotoxin administration (1, 3 and 6 h). B, densitometric analysis of expression of iNOS mRNA was normalized to 18S ribosomal RNA used as a control. Parthenolide (0.5 or 1.0 mg/kg) was administered 15 min before endotoxin injection.

Effect of Parthenolide on Neutrophil Infiltration in the Lung. Another serious consequence of endotoxic shock is the occurrence of multiorgan failure, which is preceded by accumulation of neutrophils in major vital organs (Balk, 2000). Thus, we next evaluated neutrophil infiltration in the lung by measurement of the activity of myeloperoxidase, an enzyme specific to granulocyte lysosomes and, therefore, directly correlated to the number of neutrophils. Myeloperoxidase activity was similarly elevated at 3 and 5 h (5.57 ± 0.91 and 6.67 ± 1.21 U/100 mg of tissue, respectively) after endotoxin administration in vehicle-treated rats compared with low control values (2.72 ± 0.72 U/100 mg of tissue). Treatment with parthenolide reduced myeloperoxidase activity at 5 h after endotoxin administration, thus, suggesting a reduction in neutrophil infiltration (Fig. 8).

Effect of Parthenolide on Degradation of IκBα and Activation of NF-κB in the Lung. To investigate the cellular mechanisms by which treatment with parthenolide may attenuate endotoxin-induced injury, we evaluated the degradation of IκBα and the subsequent activation of NF-κB in the lung. In a time course study, we found that in vehicle-treated rats endotoxin administration was associated with an early partial reduction of IκBα, as evaluated by immunoblotting (Fig. 9). This event was paralleled by nuclear activation of NF-κB, as early as 15 min after endotoxin administration (Fig. 10). Pretreatment with parthenolide at 0.5 mg/kg reduced activation of NF-κB without affecting the cytosolic disappearance of IκBα.

Effect of Parthenolide on NF-κB/DNA Binding in Vitro. Because in vivo pretreatment with parthenolide reduced activation of NF-κB without affecting degradation of IκBα, we further investigated whether parthenolide may inhibit NF-κB activation by directly altering the ability of NF-κB to bind DNA. To address this issue, in a comparative in vitro experiment we added parthenolide (30 nM –10 μM) or vehicle (5 μl) for 30 min directly to the nuclear extracts of lungs of endotoxin-treated rats. When vehicle was added to

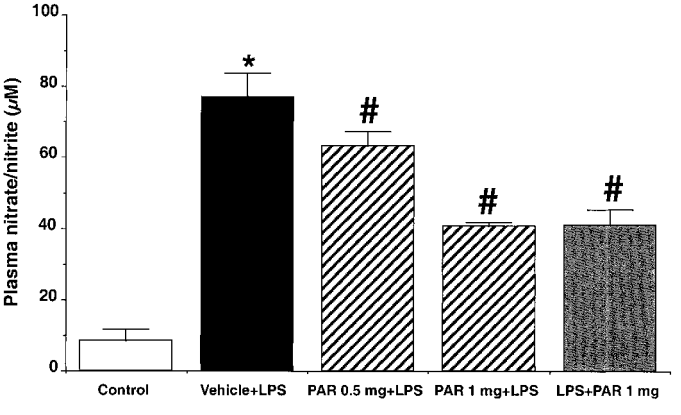

Fig. 4. Effect of in vivo treatment with parthenolide on plasma levels of nitrite/nitrate in rats at 5 h after endotoxin administration. Each data point represents the mean ± S.E.M. of 6 to 10 animals for each group. *, $p < 0.05$ versus vehicle-treated sham rats (control); #, $p < 0.05$ versus vehicle-treated rats subjected to endotoxic shock (vehicle + LPS). In the pretreatment protocol, parthenolide (0.5 or 1.0 mg/kg) was administered 15 min before endotoxin injection (PAR 0.5 mg + LPS and PAR 1 mg + LPS). In the post-treatment protocol, parthenolide (1.0 mg/kg) was administered 3 h after endotoxin injection (LPS + PAR 1 mg).

the nuclear extracts, a remarkable signal for NF-κB/DNA binding was detected (Fig. 11). In contrast, when the nuclear extracts were treated in vitro with parthenolide, the NF-κB/DNA complex was substantially reduced in a concentration-dependent manner (Fig. 11).

Effect of Parthenolide on Endotoxin-Induced Mortality. In a separate set of experiments, administration of endotoxin to Swiss Albino mice resulted in approximately 95% mortality within 24 to 48 h. Parthenolide, given as pretreatment at a concentration of 0.5 mg/kg, significantly improved survival rate, and 50% of animals were still alive at 72 h after endotoxin administration. A significant improvement in survival rate was also seen in endotoxemic mice that received parthenolide (0.5 mg/kg) as a post-treatment 3 h after endotoxin administration, and 60% of animals were still alive at the end of the experimental period (Fig. 12).

Discussion

In the present study, we demonstrated that parthenolide, a sesquiterpene lactone, significantly improved hypotension, vascular hyporeactivity to norepinephrine and survival rate in in vivo models of endotoxic shock. We observed that parthenolide also decreased lung sequestration of neutrophils and plasma levels of NO metabolites and markedly reduced gene expression of iNOS, nitrotyrosine formation, PARS activation, and cell apoptosis in thoracic aortas. These protective effects were associated with a direct inhibition of NF-κB activation in the inflamed lung.

A hallmark of the pathophysiology of endotoxic shock is that endotoxin triggers release of multiple proinflammatory cytokines and reactive toxic species, expression of adhesion molecules, and infiltration of neutrophils in inflamed organs and tissues. This overwhelming inflammatory response to endotoxin then results in cardiovascular derangement with decreased peripheral vascular resistance, multiorgan failure, and eventually death (Parrillo, 1993; Balk, 2000). Considering the pathophysiologic complexity of endotoxic shock, therapeutic strategies are aimed at inhibiting the release of these multiple inflammatory mediators. In vitro and in vivo studies have shown that many of the genes (i.e., cellular adhesion molecules, cytokines, and iNOS) that have been implicated in

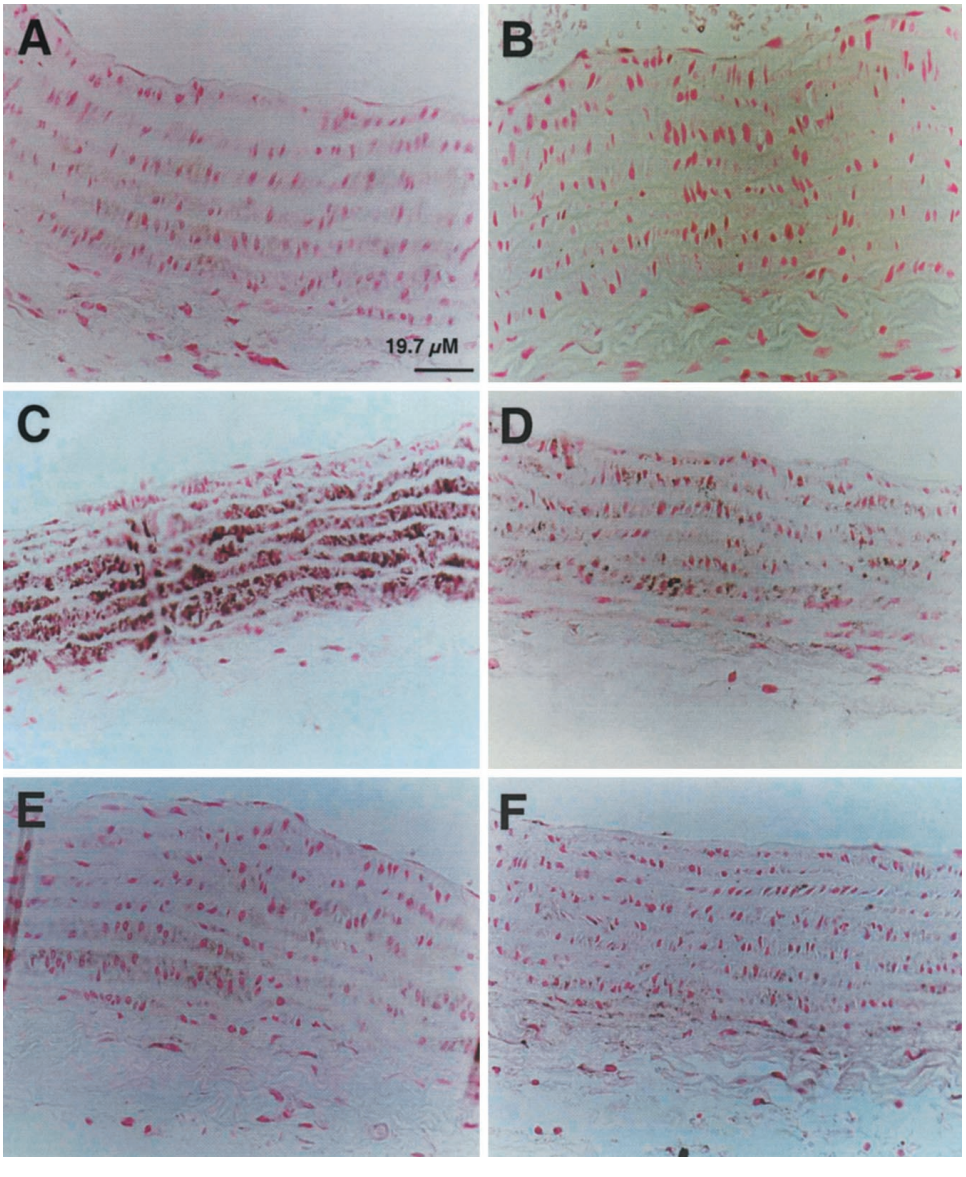

Fig. 5. Representative immunostaining of nitrotyrosine in thoracic aortas. Immunohistochemical staining of nitrotyrosine was considered as a marker of nitrosative stress. Staining was absent in the thoracic aortas from a sham control rat (A). At 3 h after endotoxin administration nitrotyrosine staining was scarcely confined to some endothelial cells (B). At 5 h after endotoxin administration a massive dark staining was localized in the smooth muscle layer of vessels from vehicle-treated rats (C). Immunostaining for nitrotyrosine was reduced in parthenolide-treated rats. D, parthenolide (0.5 mg/kg) was administered 15 min before endotoxin injection (PAR 0.5 mg + LPS). E, parthenolide (1.0 mg/kg) was administered 15 min before endotoxin injection (PAR 1 mg + LPS). F, parthenolide (1.0 mg/kg) was administered 3 h after endotoxin injection (LPS + PAR 1 mg). Magnification 400×; 1 cm = 19.7 μm. A similar pattern was seen in n = 5–6 different tissue sections in each experimental group.

endotoxic shock contain NF-κB binding sites in the promoter/enhancer region (Baeuerle, 1998; Karin and Delhase, 2000). With particular clinical relevance, NF-κB binding activity has been found to be increased in patients with acute inflammation and sepsis and to be correlated with clinical severity and mortality (Bohrer et al., 1997; Arnalich et al., 2000; Paterson et al., 2000). Under the experimental conditions used in our laboratory, we found that activation of the NF-κB pathway is a very early event, because IκBα is degraded and NF-κB is activated already at 15 min after endotoxin administration. However, it is difficult to translate our findings on time course to the human kinetics of NF-κB activation. The time course in humans with which endotoxin induces injury is, in fact, a function of many variables, including the severity of bacteremia and metabolic demands of the organs. Nevertheless, our study demonstrates for the first time how a sesquiterpene lactone, a compound widely used in herbal preparations, can protect from endotoxic shock in vivo. Our data support and extend previous findings demonstrating the therapeutic effect of NF-κB inhibition in experimental models of sepsis. Previous reports have described that in vivo administration of pyrrolidine dithiocarbamate, which inhibits induction of NF-κB through an oxygen radical scavenging mechanism, reduced the extent of microvascular injury, systemic hypotension, and multiple organ failure in rats (Liu et al., 1997, 1999). A major finding of clinical relevance in our study is that parthenolide also exerted beneficial effect when given as post-treatment 3 h after endotoxin challenge (i.e., when most of the adverse hemodynamic and histological effects of endotoxemia occurred or started to occur). In this context, it is interesting to note that binding of NF-κB in mobility shift assays seems to persist longer in nonsurviving than surviving patients with acute sepsis (Bohrer et al., 1997). Therefore, our data clearly indicate that interruption of the NF-κB pathway, even transiently, may be of clinical benefit in sepsis.

In vitro reports have suggested that a target gene for NF-κB is the gene of iNOS (Xie et al., 1994). Enhanced production of NO has been shown to contribute to the hypotension and vascular hyporeactivity to various constrictor agents in septic shock (Rubanyi, 1998). NO, directly or indirectly through formation of peroxynitrite, produces cellular injury and death via several mechanisms including peroxidation of membrane lipids, protein nitration and nitrosylation, and DNA damage (Zingarelli et al., 1996a; Eiserich et al., 1998). The occurrence of DNA breaks has been shown to activate the nuclear enzyme PARS, resulting in the depletion of the cellular energy substrates NAD and ATP. This process, termed "PARS suicide," has been proposed to play an important role in inflammation and shock (Zingarelli et al., 1996a, 1996b; Szabó and Dawson, 1998). In the present study, the beneficial hemodynamic effects of parthenolide in endotoxemic animals seems to be associated with inhibition of the release of NO products in the plasma, and inhibition of for-

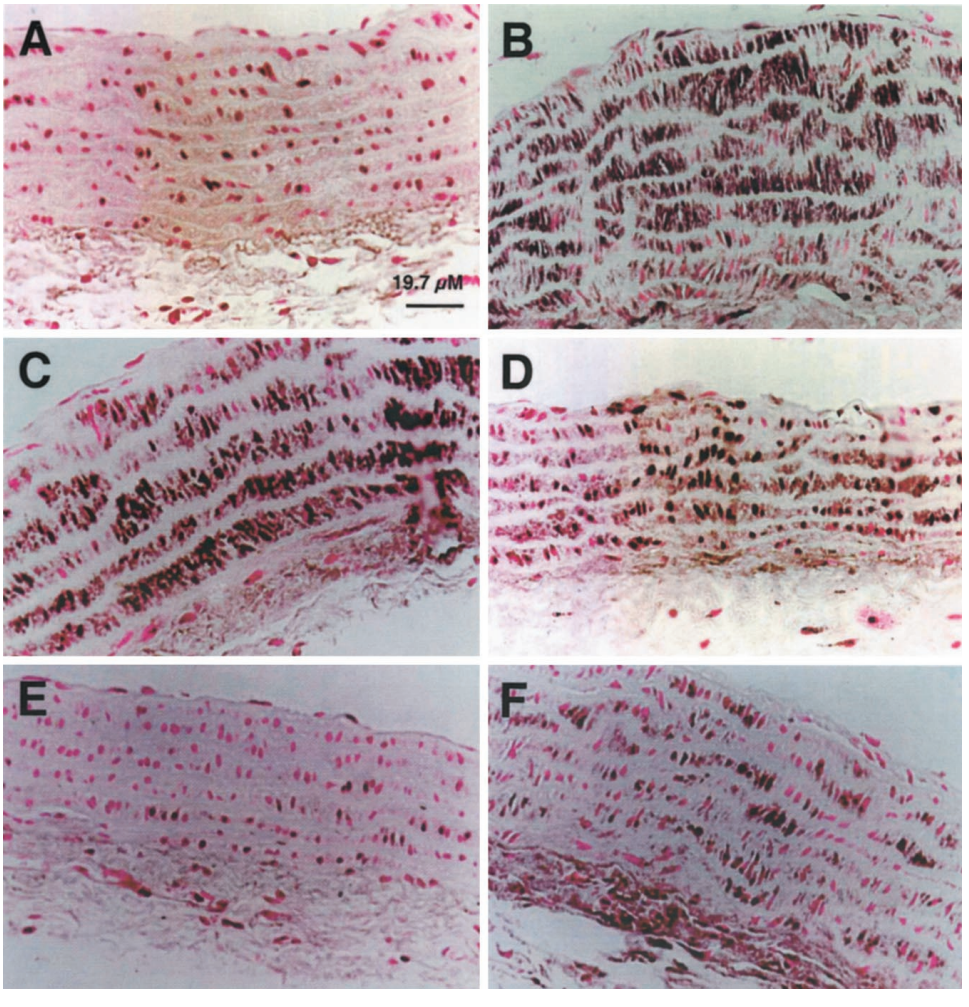

Fig. 6. Representative immunostaining of PARS in thoracic aortas. Staining was absent in the thoracic aortas from a sham control rat (A). A massive dark staining was localized in the smooth muscle layer of vessels from vehicle-treated rats at 3 (B) and 5 h (C) after endotoxin administration. Immunostaining for PARS was reduced in parthenolide-treated rats. D, parthenolide (0.5 mg/kg) was administered 15 min before endotoxin injection (PAR 0.5 mg + LPS). E, parthenolide (1.0 mg/kg) was administered 15 min before endotoxin injection (PAR 1 mg + LPS). F, parthenolide (1.0 mg/kg) was administered 3 h after endotoxin injection (LPS + PAR 1 mg). Magnification, 400×; 1 cm = 19.7 μm. A similar pattern was seen in $n = 5$ to 6 different tissue sections in each experimental group.

mation of nitrotyrosine and expression of PARS in aortas in a dose-dependent fashion. According to our data, these anti-inflammatory effects of parthenolide are secondary to inhibition of iNOS at the genetic level, because parthenolide reduced mRNA expression of the enzyme, thus, preventing the subsequent nitrosative stress and activation of PARS. Similar to our in vivo findings, it has been demonstrated that mRNA expression of iNOS is inhibited by parthenolide in in vitro immunostimulated rat smooth muscle cells (Wong and Menendez, 1999).

The amelioration of vascular contractility to norepinephrine in thoracic aortas observed in parthenolide-treated rats was associated with abolition of apoptotic death of smooth muscle and endothelial cells. These data are in contrast with several in vitro studies suggesting that NF-κB plays a role as a survival factor, responsible in part for "turning on" genes that could block cell death by apoptosis (Li et al., 1999). However, our reports suggest that inhibition of NF-κB activity may also abolish cell death at the early event of transcription of genes mediating the process of oxidative-induced injury. In support of our findings, several reports document that inhibition of NF-κB DNA binding activity can be cytoprotective by preventing cytokine- and oxidant-induced apoptosis (Wrighton et al., 1996; DeMeester et al., 1997).

Although it is difficult to establish the precise mechanism of action of parthenolide in vivo, we propose that the protection afforded by the drug may be secondary to a selective inhibition of the transcription mediated by the NF-κB pathway. Several lines of evidence support our hypothesis. Several common inhibitors of NF-κB such as *N*-acetyl-L-cysteine (Mihm et al., 1991; Schreck et al., 1992a), pyrrolidine dithiocarbamate (Schreck et al., 1992b), acetylsalicylic acid (Frantz and O'Neill, 1995), or curcumin (Singh and Aggarwal, 1995) exert their inhibitory effects by scavenging free radicals. In vitro studies have proven that parthenolide does not interfere with the generation of oxygen radicals (Hehner et al., 1998), whereas it specifically inhibits activation of the NF-κB pathway by targeting the IκB kinase complex (IKK) (Hehner et al., 1999) and/or preventing the degradation of IκBα and IκBβ (Hehner et al., 1998). This last inhibitor effect also accounts for the inhibition of proinflammatory mediator genes, such as the gene for iNOS after endotoxin stimulation in rat smooth muscle cells (Wong and Menendez, 1999) and the gene for interleukin-8 in immunostimulated human respiratory epithelial cells (Mazor et al., 2000). Furthermore, we have recently demonstrated that parthenolide protects against myocardial ischemia and reperfusion injury in the rats by a selective inhibition of IKK activation and IκBα

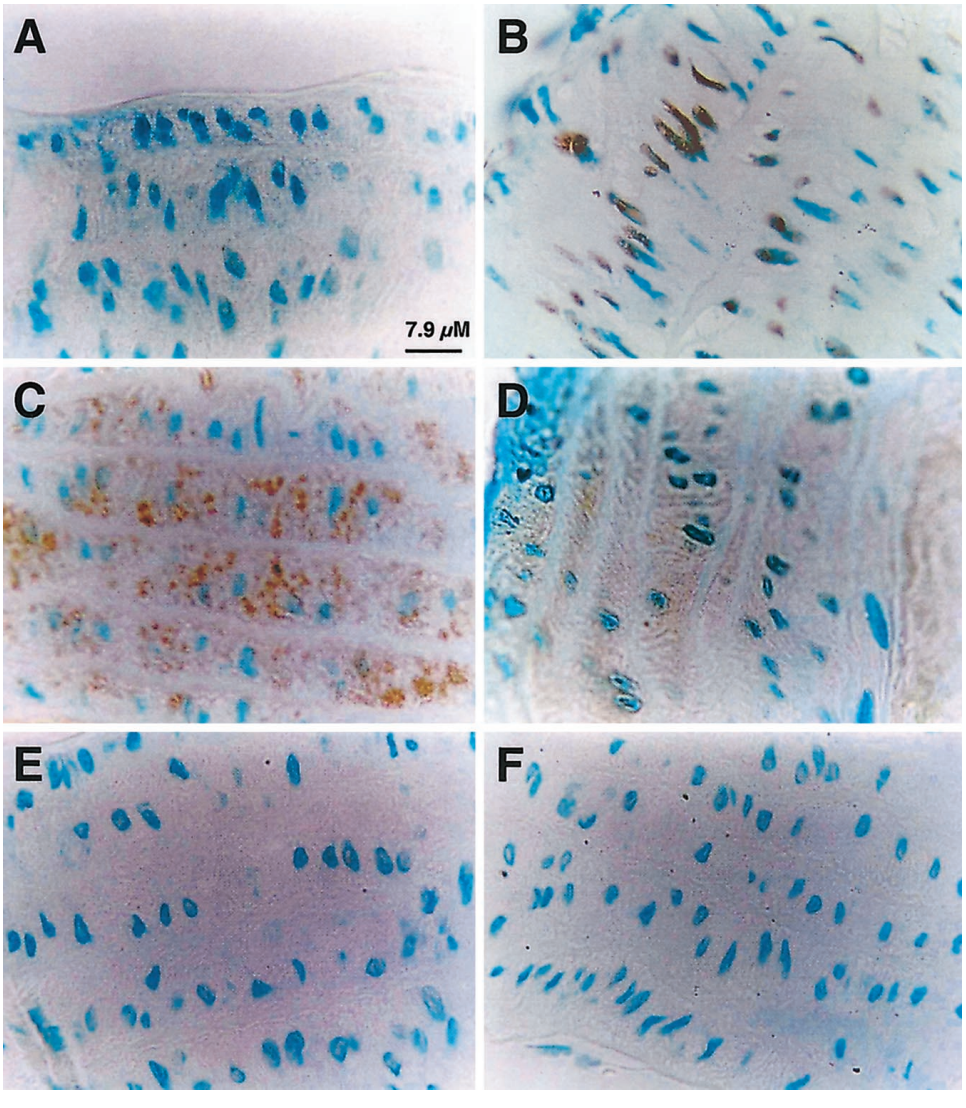

Fig. 7. Representative photomicrographs of in situ TUNEL method of apoptosis in thoracic aortas. Aortic sections from a sham control rat (A) showed negative staining. A dark staining revealed the presence of apoptotic nuclei and intercellular apoptotic fragments in aortic sections from vehicle-treated rats at 3 (B) and 5 h (C) after endotoxin administration. TUNEL staining for apoptosis was reduced in parthenolide-treated rats. D, parthenolide (0.5 mg/kg) was administered 15 min before endotoxin injection (PAR 0.5 mg + LPS). E, parthenolide (1.0 mg/kg) was administered 15 min before endotoxin injection (PAR 1 mg + LPS). F, parthenolide (1.0 mg/kg) was administered 3 h after endotoxin injection (LPS + PAR 1 mg). Magnification, 1000×; 1 cm = 7.9 μm. A similar pattern was seen in $n = 5–6$ different tissue sections in each experimental group.

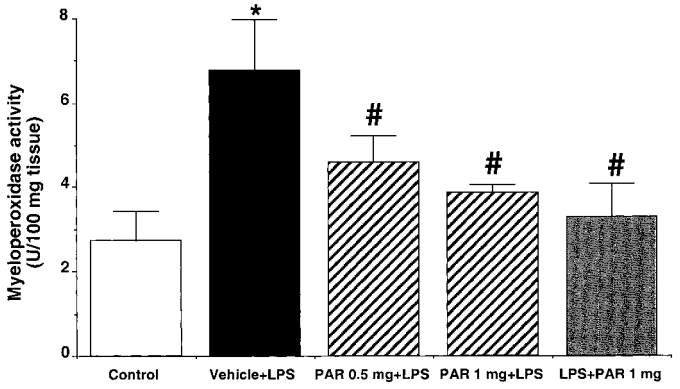

Fig. 8. Effect of in vivo treatment with parthenolide on myeloperoxidase activity in the lung at 5 h after endotoxin administration. Tissue myeloperoxidase activity was enhanced in vehicle-treated rats subjected to endotoxic shock. In rats treated with parthenolide levels of myeloperoxidase were significantly reduced. Each data point is the mean ± S.E.M. of 6 animals for each group. *, $p < 0.05$ versus vehicle-treated sham rats (control); #, $p < 0.05$ versus vehicle-treated rats subjected to endotoxic shock (vehicle + LPS). In the pretreatment protocol, parthenolide (0.5 or 1.0 mg/kg) was administered 15 min before endotoxin injection (PAR 0.5 mg + LPS and PAR 1 mg + LPS). In the post-treatment protocol, parthenolide (1.0 mg/kg) was administered 3 h after endotoxin injection (LPS + PAR 1 mg).

degradation (Zingarelli et al., 2002). In the present study we found that, although in vivo treatment with parthenolide significantly reduced the DNA binding activity of NF-κB, IκBα degradation was not affected. Furthermore, we found that in vitro coincubation of endotoxin-activated nuclear extracts with increasing concentrations of parthenolide inhibited the DNA binding of NF-κB. Taken together, these in vivo and in vitro results provide evidence that parthenolide exerts a direct interference with the DNA binding activity of NF-κB. Sesquiterpene lactones are composed of an isoprenoide ring system and a lactone ring, which together form a reactive Michael system and confer activity through covalent modification of proteins (Bork et al., 1997). Sequiterpene lactones can, in fact, cause irreversible alkylations of thiol groups, for example on cysteine residues (Picman et al., 1979). In vitro studies have demonstrated that a similar compound, helena-

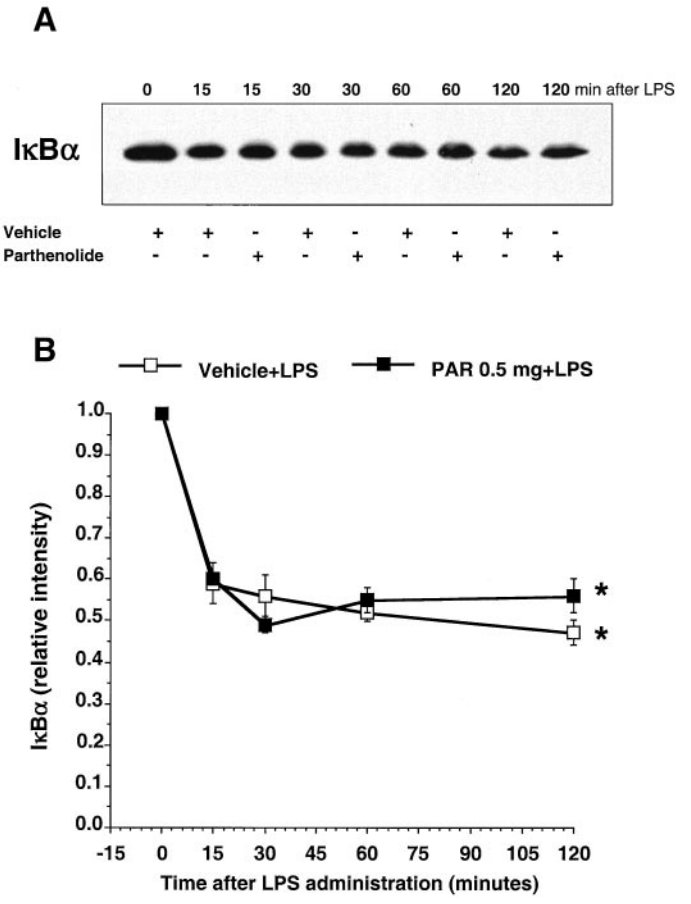

Fig. 9. Effect of in vivo treatment with parthenolide on IκBα degradation in the lung. A, representative autoradiograph of immunoblotting for IκBα. B, image analysis of degradation of IκBα determined by densitometry from the autoradiograph. Results are representative of 3 separate time course experiments. *, $p < 0.05$ of all time points versus respective sham value (time 0). Fold increase was calculated versus respective sham value (time 0) set to 1.0. Parthenolide (0.5 mg/kg) was administered 15 min before endotoxin administration.

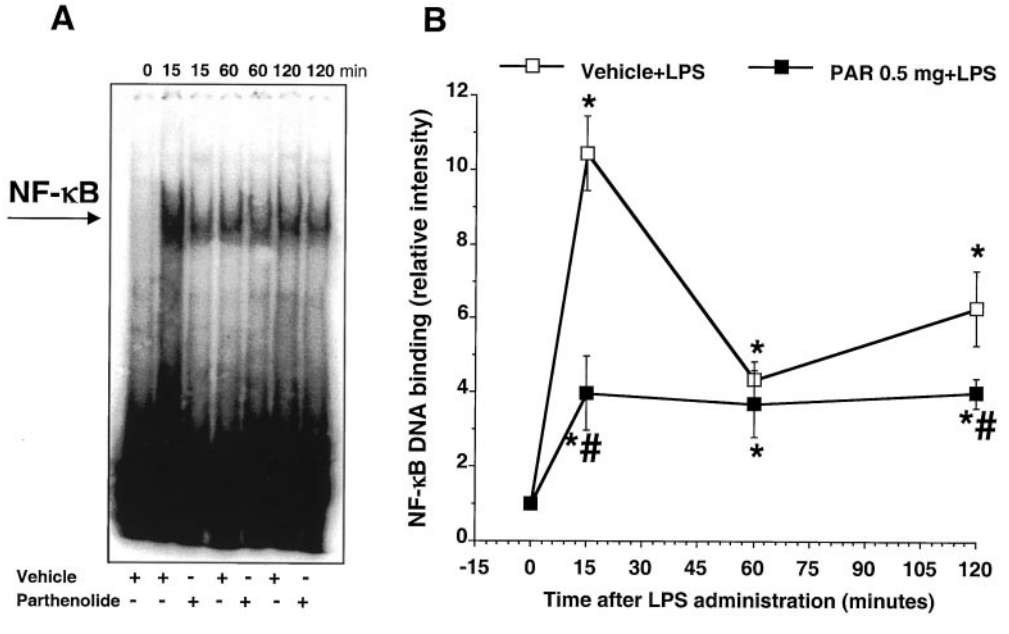

Fig. 10. Effect of in vivo treatment with parthenolide on the activation of NF-κB in the lung. A, representative autoradiograph of electrophoretic mobility shift assay for NF-κB. B, image analysis of activation of NF-κB determined by densitometry from the autoradiograph. Results are representative of three separate time course experiments. *, $p < 0.05$ versus respective sham value (time 0); #, $p < 0.05$ versus vehicle-treated rats subjected to endotoxic shock (vehicle + LPS) at same time point. Fold increase was calculated versus respective sham value (time 0) set to 1.0. Parthenolide (0.5 mg/kg) was administered 15 min before endotoxin administration.

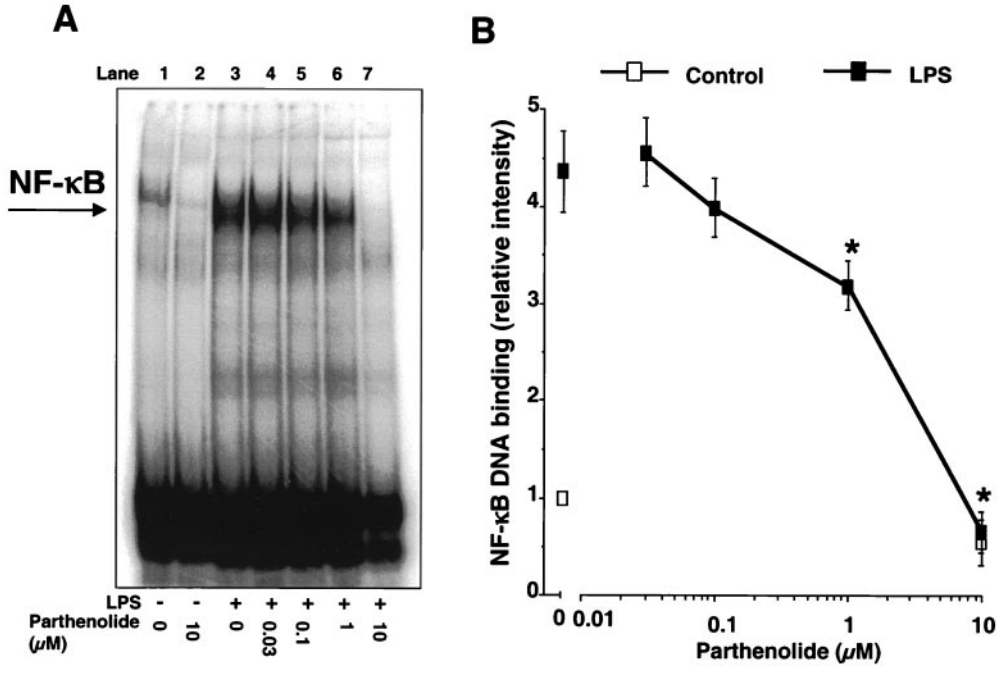

Fig. 11. In vitro effect of parthenolide on NF-κB DNA binding. A, representative autoradiograph of electrophoretic mobility shift assay for NF-κB. Lung nuclear extracts of a control rat were incubated in vitro with vehicle (lane 1) or parthenolide (10 μM, lane 2) for 30 min. Lung nuclear extracts of endotoxin (LPS)-treated rats were incubated in vitro with vehicle (lane 3) or parthenolide at 30 nM (lane 4), 100 nM (lane 5), 1 μM (lane 6) and 10 μM (lane 7) for 30 min. B, image analysis of activation of NF-κB determined by densitometry from the autoradiograph. Results are representative of 3 separate experiments. *, $p < 0.05$ versus respective LPS-activated NF-κB in the absence of parthenolide. Fold increase was calculated versus control lung nuclear extracts set to 1.0.

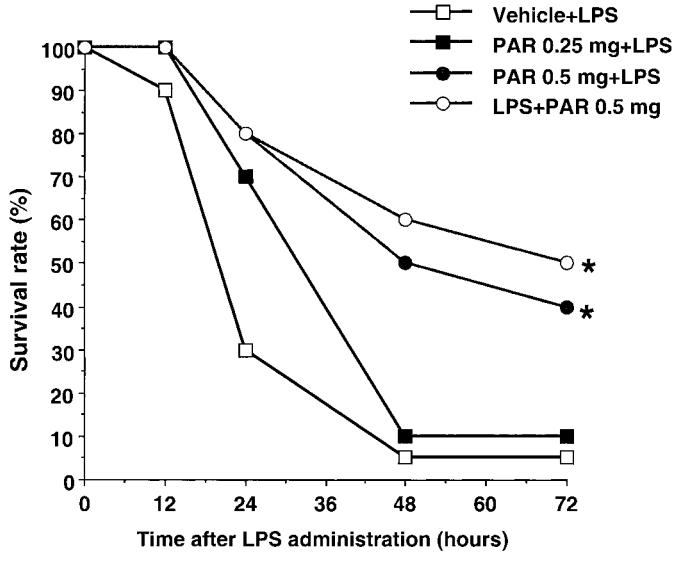

Fig. 12. Effect of in vivo treatment with parthenolide on survival rate in mice subjected to endotoxic shock (LPS, 60 mg/kg). Data are expressed as percentage of initial survival rate (100%). Ten to twelve animals were used for each group. *, $p < 0.05$ versus vehicle-treated mice subjected to endotoxic shock (vehicle + LPS). In the pretreatment protocol, parthenolide (0.25 or 0.5 mg/kg) was administered 15 min before endotoxin injection (PAR 0.25 mg + LPS and PAR 0.5 mg + LPS). In the post-treatment protocol, parthenolide (0.5 mg/kg) was administered 3 h after endotoxin injection (LPS + PAR 0.5 mg).

lin, does not prevent IκBα degradation; but it selectively modifies the *p*-65 subunit of NF-κB at the nuclear level, therefore, inhibiting its DNA binding (Lyβ et al., 1998). Garcia-Pineres et al. (2001) have also shown recently that parthenolide inhibits NF-κB most probably by alkylating cysteine 38 of the *p*-65 subunit. Nevertheless, we cannot exclude the possibility that parthenolide may have several cellular targets. For example, in vitro studies have shown that parthenolide binds directly and inhibits IKKβ (Kwok et al., 2001). Therefore, the discrepancies of the in vivo effects of parthenolide on IκBα degradation between the model of ischemia and reperfusion (Zingarelli et al., 2002) and endotoxin shock (present study) may be due to different experimental conditions, which may affect the molecular reactivity and specificity of parthenolide. These variables may include differences in the rate, dosage and timing of parthenolide treatment, the extension of tissue damage and, therefore, the cellular environment of the target sulfhydryl groups. Furthermore, the pharmacokinetics of these compounds is not known. Therefore, the bioavailability and the contribution of other metabolites cannot be ruled out.

In conclusion, we propose that parthenolide can protect against endotoxic shock in vivo by a specific inhibition of the NF-κB pathway. Because sepsis is a common cause of death and drug resistance is becoming a major medical problem, our findings may provide further information for the development of more potent and specific medications. Furthermore, herbal remedies have become increasingly popular in recent years and many patients prefer plant products to synthetically derived drugs. At the present time, the pharmacokinetics of sesquiterpene lactones is not known. Few in vitro toxicity studies have reported that high concentration of parthenolide and other extracts of *Tanacetum parthenium* may have deleterious effects on smooth muscle cells (Hay et al., 1994). It is, therefore, of great importance to determine the molecular mechanism of action and the biological efficacy and safety of this class of products.

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