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Nuclear Factor-κB as a target of cyclosporin in acute hypovolemic hemorrhagic shock

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Abstract

Background: Cyclosporin is an immunosuppressive drug that blocks Nuclear Factor κB (NF-κB) activation. We investigated the role of NF-κB in acute hypovolemic hemorrhagic (Hem) shock and the effects of cyclosporin in this model of experimental shock. **Methods:** Hem shock was induced in male anesthetized rats by intermittently withdrawing blood from an iliac catheter over a period of 20 min (bleeding period) until mean arterial blood pressure (MAP) fell and stabilized within the range of 20–30 mmHg. Two minutes after bleeding cessation, animals received intravenously cyclosporin (1 mg kg^{-1}) or its vehicle. Survival rate and survival time were evaluated for 120 min after bleeding was discontinued. Plasma TNF-α levels were investigated at different time points after bleeding cessation. Moreover we investigated levels of TNF-α mRNA in the liver, vascular reactivity, liver NF-κB binding activity and levels of the inhibitory protein IκBα in the cytoplasm. **Results:** Hemorrhagic shocked rats died in 27±6 min following the cessation of bleeding, experienced a marked hypotension (mean arterial blood pressure=20–30 mmHg) and had enhanced plasma levels of Tumor Necrosis Factor-α (208±22 pg ml^{-1}, 20 min after the end of bleeding). Furthermore, aortas taken 20 min after bleeding from hemorrhagic shocked rats showed a marked hypo-reactivity to phenylephrine (PE: 1 nM–10 μM) compared with aortas harvested from sham shocked rats. Hem shocked rats also had increased levels of TNF-α mRNA in the liver (15–20 min after the end of bleeding). Electrophoretic mobility shift assay showed that liver NF-κB binding activity increased in the nucleus 10 min after the end of hemorrhage and remained elevated until the death of animals. Western blot analysis suggested that the levels of inhibitory protein IκBα in the cytoplasm decreased at 5 min after the end of bleeding. Cyclosporin inhibited the loss of IκBα protein from the cytoplasm and prevented NF-κB binding activity in the nucleus. Furthermore, cyclosporin increased survival time (118±7 min; $P<0.01$) and survival rate (vehicle=0% and cyclosporin=80%, at 120 min after the end of bleeding), reverted the marked hypotension, decreased liver mRNA for TNF-α, reduced plasma TNF-α (28±7 pg ml^{-1}), and restored to control values the hypo-reactivity to PE. **Conclusions:** Our results suggest that acute blood loss (50% of the estimated total blood volume over a period of 20 min) causes early activation of NF-κB which triggers an inflammatory cascade leading to a fatal outcome. Cyclosporin blocks NF-κB activation and protects against hypovolemic hemorrhagic shock. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Blood pressure; Cytokines; Infection/inflammation; Shock

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1. Introduction

Acute hypovolemic hemorrhagic shock is a very lethal experimental model of circulatory shock [1]. In this model, mean arterial blood pressure decreases to 20–30 mmHg and all animals die within 25–30 min after the end of bleeding [2]. Furthermore, a rapid systemic release of TNF-α occurs [3] which seems to be responsible for the loss of vascular reactivity after stimulation of the vasculature with vasoconstrictor stimuli: indeed a passive immunization with specific monoclonal antibodies raised against the inflammatory cytokine improves survival, increases mean arterial blood pressure and restores the hypo-responsiveness to vasoconstriction stimuli [4].

However the mechanism by which the systemic release of TNF-α is triggered during acute hypovolemic hemorrhagic shock in the rat still remains to be elucidated. Nuclear Factor kappa B (NF-κB) is a ubiquitous rapid response transcription factor which modulates gene expression in various situations that require rapid and sensitive immune and inflammatory response and that causes the expression of several cytokines and adhesion molecules such as E-selectin, Vascular cell adhesion molecule 1 (VCAM-1) and Intercellular Adhesion Molecule 1 (ICAM-1) [5].

Cyclosporin (CsA) is an immunosuppressive drug that blocks T-cell proliferation in response to legation of the T-cell receptor [6,7]. It binds with affinity to a family of cytoplasmatic immunosuppressive binding proteins called immunophilins. The drug exerts its effect principally through impairment of gene expression in target cells. The immunophilin–drug complex inhibits calcineurin phosphatase [8] and therefore the drug blocks calcium-dependent events, such as cytokine gene expression, nitric oxide synthase activation, cell degranulation and apoptosis [9–11]. It has also been suggested that cyclosporin prevents NF-κB activation by inhibiting the action of calcineurin which indirectly induces the degradation of IκBα, the inhibitory protein of NF-κB [12]. Finally, cyclosporin has been shown to protect against splanchnic artery occlusion shock [13]. However the effects of this drug on hemorrhagic shock have not yet been investigated.

The aim of our study was to investigate the effect of cyclosporin in this experimental model and the involvement of NF-κB in acute hypovolemic shock in the rat.

2. Methods

2.1. Acute hypovolemic hemorrhagic shock protocol

Male Sprague–Dawley rats (230–250 g body wt.) were used with food in pellets and tap water available ad libitum. Housing conditions and experimental procedures were in strict accordance with European Community regulations on the use and care of animals for scientific purposes (CEE Council 89/609; Italian D.L.: 22-1-92 No. 116).

The present model of volume-controlled hemorrhagic shock has been repeatedly described in detail elsewhere [14–16]. This experimental model has neither volume replacement procedure nor resuscitation maneuvers. In brief under general anesthesia (urethane, 1.25 g kg^{-1} i.p.) and after heparinization (heparin sodium, 100 IU kg^{-1}), rats were instrumented with indwelling polyethylene catheters in the left common carotid artery, to record arterial blood pressure and into the right jugular vein to inject drugs.

The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure was displayed on a polygraph. Arterial blood pressure is reported as mean arterial pressure (MAP).

Another cannula was implanted into the iliac artery. Acute hypovolemic hemorrhagic shock was induced by a graded withdrawal of blood (2–2.5 ml^{-1} 100 g body wt.) until the MAP fell and stabilized at levels of 20–25 mmHg. The withdrawn blood approximates 50% of the estimated total blood volume.

Sham shocked rats underwent all surgical procedures experienced by the hemorrhagic shocked animals, but they were not bled.

2.2. Survival evaluation

Two minutes after bleeding cessation, treated rats received intravenously cyclosporin (1 mg kg^{-1}) or its vehicle. Survival rate and survival time were evaluated for 120 min after the bleeding was discontinued.

2.3. Plasma TNF-α levels

Plasma was drawn at different time points after bleeding cessation. TNF-α concentrations were determined by an ELISA kit (Genzyme).

2.4. Isolation of nuclear and cytoplasmatic proteins

Briefly, 70 mg of pulverized liver samples (obtained at different time points) were homogenized in 0.8 ml ice cold hypotonic buffer [10 mM Hepes pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DDT); protease inhibitors: 0.5 mM phenyl methylsulfonyl fluoride, aprotinin, pepstatin, leupeptin (10 μg ml^{-1} each); and phosphatase inhibitors: 50 mM NAF, 30 mM β-glycerophosphate, 1 mM Na_3VO_4 and 20 mM ρ-nitrophenyl phosphate]. The homogenates were centrifuged for

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30 s at 2000 rev./min at 4°C to eliminate any unbroken tissues. The supernatants were incubated on ice for 20 min, vortexed for 30 s after addition of 50 μl of 10% Nonidet P-40 and then centrifuged for 1 min at 4°C in an Eppendorf centrifuge. Supernatants containing cytoplasmatic protein were collected and stored at −80°C. After a single wash with the hypotonic buffer without Nonidet P-40, the pellets were suspended in an ice-cold hypertonic salt buffer (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors, and phosphatase inhibitors), incubated on ice for 30 min, mixed frequently, and centrifuged for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C. The concentration of total proteins in the samples was determined by a commercially available protein assay reagent (MICROBCA, protein assay kit, Pierce, Rockford, IL, USA). To estimate possible contamination of the nuclear extracts with the cytoplasmatic extracts, when preparing the nuclear and cytoplasmatic proteins, lactate dehydrogenase (LDH) activity was determined with a commercially available kit for the quantitative kinetic determination of LDH activity (Sigma, St Louis, MO). Values were expressed as LDH activity units per milligram of protein. To establish that the nuclear extracts contained mainly nuclear proteins, 40 μg of nuclear protein preparations were subjected to Western blot analysis for histone H3, a nuclear protein, with anti-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY).

2.5. Electrophoretic mobility shift assay

NF-κB binding activity was performed in a 15-μl binding reaction mixture containing 1% binding buffer [50 μg/ml of double-stranded poly(dI-dC), 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, and 10% glycerol], 15 μg of nuclear proteins, and 35 fmol (50 000 cpm, Cherenkov counting) of double-stranded NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-8′) which was end-labeled with [γ^{32}P]ATP (3000 Ci $mmol^{-1}$ at 10 mCi ml^{-1}; Amersham Life Sciences, Arlington Heights, IL) using T4 polynucleotide kinase. The binding reaction mixture was incubated at room temperature for 20 min and analyzed by electrophoresis on 5% non-denaturing polyacrylamide gels. After electrophoresis, the gels were dried using a gel-drier and exposed to Kodak X-ray films at −70°C. The binding bands were quantified by scanning densitometry of a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). The results for each time point from each group were expressed as relative integrated intensity compared with the sham shock group liver measured in the same batch because the integrated intensity of group samples from different electrophoretic mobility shift assay (EMSA) batches would be affected by the half-life of the isotope, exposure time, and background levels.

2.6. Western blot analysis of IκBα in cytoplasm

Cytoplasmatic proteins (40 μg) from each sample were mixed with 2×SDS sample buffer [62 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue], heated at 95°C for 5 min, and separated by SDS–polyacrylamide gel electrophoresis. After electrophoresis on 12.5% polyacrylamide gels, the separated proteins were transferred from the gels into Hybond electrochemiluminescence membranes (Amersham) using a Bio-Rad semidry transfer system (Bio-Rad) for 2 h. The membranes were blocked with 5% non-fat dry milk in TBS–0.05% Tween for 1 h at room temperature, washed three times for 10 min each in TBS–0.05 Tween 20, and incubated with a primary IκBα antibody (Santa Cruz Biotechnology) in TBS–0.05% Tween 20 containing 5% non-fat dry milk for 1–2 h at room temperature. After being washed three times for 10 min each in TBS–0.05% Tween 20, the membranes were incubated with a second antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham). The IκBα protein signal was quantified by scanning densitometry using a bio-image analysis system (BIO-PROFIL Celbio, Milan, Italy).

The results from each experimental group were expressed as relative integrated intensity compared with normal livers measured with the same batch.

2.7. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from liver section at several time points. The methods used in the current study have been described elsewhere [17]. In brief, approximately 100 mg of liver was homogenized with 800 μl RNAZOL STAT (Teltest, Firendswood, TX, USA) in a microfuge tube, after which 80 μl chloroform was added. After vortexing and centrifugation, the aqueous phase was transferred to a new microfuge tube containing an equal volume of cold isopropanol and the RNA recovered by precipitation by chilling at −80°C for 15 min. The pellet was washed with cold ethanol 70%, centrifuged, dried in speed vacuum, centrifuged a second time and then dissolved in 20 μl of buffer. A 2-μg portion of total RNA was subjected to first strand cDNA synthesis in a 20-μl reaction mixture containing the AMV reverse transcriptase (Superscript II; BRL USA), each dNTP, the specific primers, Tris–HCl and $MgCl_2$.

After dilution of the product with distilled water, 5 μl were used for each polymerase chain reaction (PCR) which contained the Taq polymerase (Perkin Elmer), the buffer as supplied with the enzyme, each dNTP and the specific primers, designed to cross introns and to avoid confusion between mRNA expression and genomic contamination.

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The following oligonucleotide pairs were used (5′ oligo/3′oligo), each sequence as 5′ to 3′:

TNF-α: CACGCTCTTCTGTCTTACTGA/
GGACTCCGTGATGTCTAAGT

GAPDH: ACCACCATGGAGAAGGTCGG/
CTCAGTGTAGCCCAGGATGGC

The optimal cycle number for TNF-α was 25 and we used a PCR negative and a PCR positive control without cDNA or with a known cDNA, respectively. A portion of the PCR product was electrophoresed and transferred to a nylon membrane which was prehybridized with oligonucleotide probes, radiolabeled with [^{32}P]ATP by a T4 oligonucleotide kinase. After an overnight hybridization at 55°C, filters underwent autoradiography in a dark-room with a fixed camera. The captured image, sent to an image analysis software package (BIO-PROFIL, Celbio, Milan, Italy) was subjected to densitometric analysis.

2.8. Vascular reactivity

Thoracic aortas were removed 20 min after the end of bleeding and placed in cold Krebs' solution of the following composition (nM): NaCl 118.4, KCl 4. 7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.7; then aortas were cleaned of adherent connective and fat tissue and cut into rings of approximately 2 mm in length. In some rings, the vascular endothelium was removed mechanically by gently rubbing the luminal surface with a thin wooden stick. Rings were then placed under 1 g of tension in an organ bath containing 10 ml of Krebs' solution at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μM) in order to exclude the involvement of prostaglandins and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Varese, Italy). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs' solution at 15- to 20-min intervals and basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (PE, 100 nM). When the contraction was stable, the functional integrity of endothelium was assessed by a relaxant response to acetylcholine (ACh, 100 nM). The tissue was then washed occasionally for 30 min. Concentration–response curves were obtained by cumulative concentrations of PE (1 nM–10 μM) in intact or endothelium-denuded aortic rings. The results (mean±S.D.) are expressed as g of tension mg^{-1} tissue.

2.9. Cell culture

Peritoneal rat macrophages were harvested from normal rats and cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (4×10^{-3} M) and 10% fetal calf serum. Each sample contained 1×10^{6} cells/ml. Macrophages were pre-incubated for 120 min at 37°C with cyclosporin (1 μM) followed by H_2O_2 (250 μM) or PMA (25 ng/ml), and then tested for NF-κB as described before.

2.10. Drug

Cyclosporin was obtained from Sandoz S.p.A., Milan, Italy. The compound was administered intravenously, 2 min following the end of bleeding. The substance was prepared fresh daily and administered in a volume of 1 ml kg^{-1}.

2.11. Statistical analysis

Data are expressed as means±S.D. and were analyzed by analysis of variance for multiple comparison of results; Duncan's multiple range test was used to compare group means. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. For survival rate, statistical analysis was done with Fisher's exact probability test.

3. Results

3.1. Activation of Nuclear Factor Kappa B

NF-κB activation in the nuclear extracts of liver was determined by EMSA at different time points. The top of Fig. 1 shows a representative EMSA picture indicating peak activation of NF-κB. The bottom of the figure shows quantitative data obtained at different time points.

NF-κB binding activity was present at very low levels in sham operated animals and under basal conditions. NF-κB rapidly increased 10 min after bleeding and remained elevated throughout the experimental protocol (Fig. 1). The administration of cyclosporin markedly reduced NF-κB binding activity (Fig. 1).

3.2. Loss of IκBα protein in the cytoplasm

Nuclear Factor Kappa B (NF-κB) activation was also indirectly investigated by studying its inhibitory cytoplasmatic protein IκBα in the liver.

The top of Fig. 2 shows representative Western blot analysis indicating peak reduction of IκBα protein in the cytoplasm of liver obtained from sham shocked animals and rats subjected to acute hypovolemic hemorrhagic shock and treated with vehicle or cyclosporin. The bottom of Fig. 2 represents quantitative data at different time points. IκBα levels showed peak reduction at 5 min after the bleeding was discontinued. Thereafter, IκBα levels in cytoplasm slightly increased (Fig. 2).

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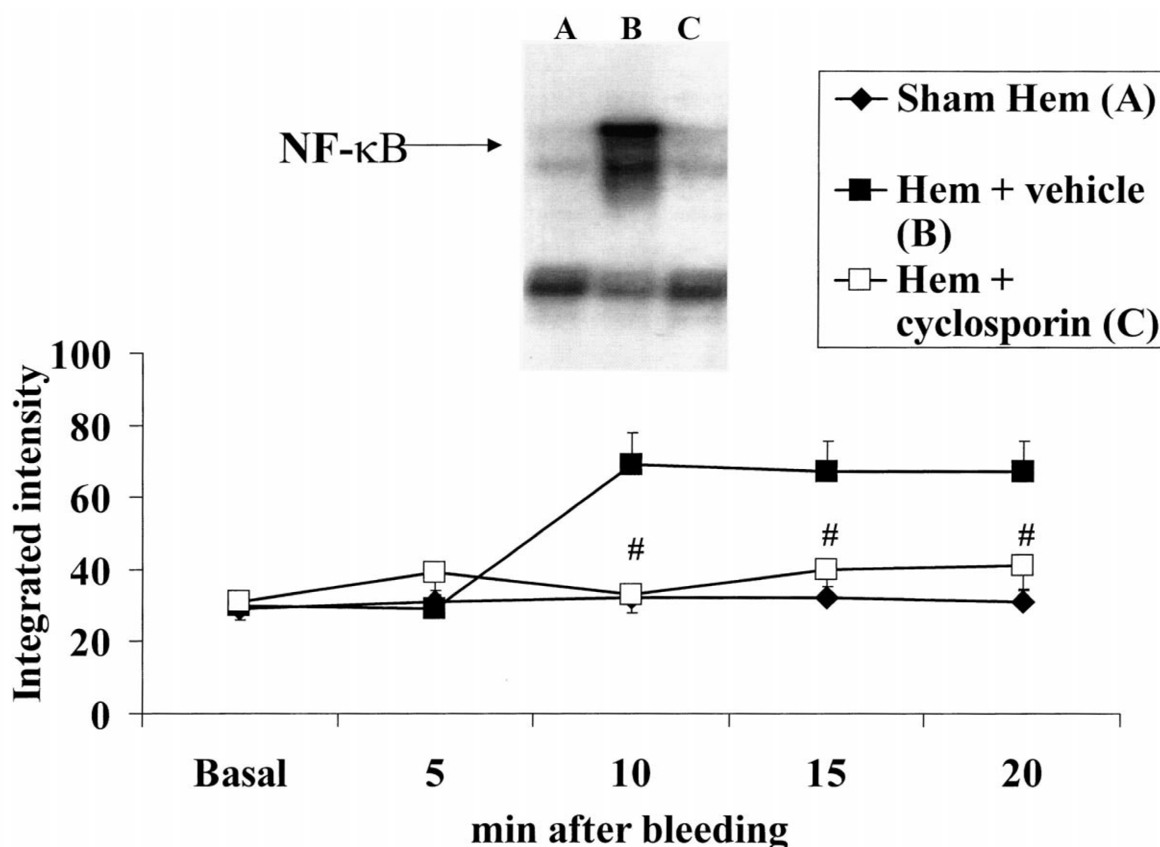

Fig. 1. Electrophoretic mobility shift assay (EMSA) of NF-κB binding activity in rat liver. Samples were normal liver of sham operated rats (Sham Hem) and liver of rats subjected to acute hypovolemic hemorrhagic shock (Hem) and treated with vehicle (1 ml kg^{-1}) or cyclosporin (1 mg kg^{-1}). The top of the figure shows a representative EMSA picture highlighting NF-κB binding activity 10 min after bleeding cessation. The bottom of the figure shows quantitative data and indicates the integrated intensity of NF-κB binding activity at different time points. Each point represents the mean±S.D. of seven experiments. $^{\#}P<0.01$ versus Hem+vehicle.

The administration of cyclosporin blunted the consistent loss of IκBα protein from the cytoplasm (Fig. 2).

3.3. TNF-α mRNA expression

The top of Fig. 3 shows representative autoradiograms highlighting peak mRNA expression for liver TNF-α in rats subjected to acute hypovolemic shock and treated with vehicle or cyclosporin. The bottom of Fig. 3 depicts quantitative data and indicates the relative amount of liver TNF-α mRNA at different time points in hemorrhaged rats treated with vehicle or the immunosuppressive agent.

No significant change in hepatic TNF-α mRNA expression was observed during the first 10 min after the end of bleeding. In contrast, liver mRNA levels for TNF-α became significantly elevated at 15 min (Fig. 3).

Administration of cyclosporin (Fig. 3) blunted hepatic TNF-α mRNA expression.

3.4. Plasma TNF-α levels

Sham shocked rats had very low levels of TNF-α (Fig. 4). In hemorrhaged animals, the plasma levels of the inflammatory cytokine increased 15 min after the bleeding was discontinued and reached its maximum increase at 20 min (Fig. 4). The administration of cyclosporin markedly reduced the plasma levels of TNF-α (Fig. 4).

3.5. Arterial blood pressure and contractile response to phenylephrine

Fig. 5 shows that hemorrhaged rats experienced a profound and long lasting hypotension culminating in the death of animals 25–30 min after bleeding cessation. Animals treated with cyclosporin (injected intravenously 2 min after the end of bleeding) had a marked increase in blood pressure (Fig. 5).

In intact aortic rings prepared from hemorrhaged animals (20 min after the end of bleeding) the contractile response to phenylephrine (PE: 1 nM–10 μM) was significantly reduced. The maximum force of contraction induced by 10 μM PE in aortic rings from sham rats was 1.9±0.8 g mg^{-1} tissue whereas it was 0.7±0.5 g mg^{-1} tissue in rings from hemorrhaged rats. Removal of endothelium did not increase the constrictor response elicited by PE in rat aortic rings obtained from both hemorrhagic

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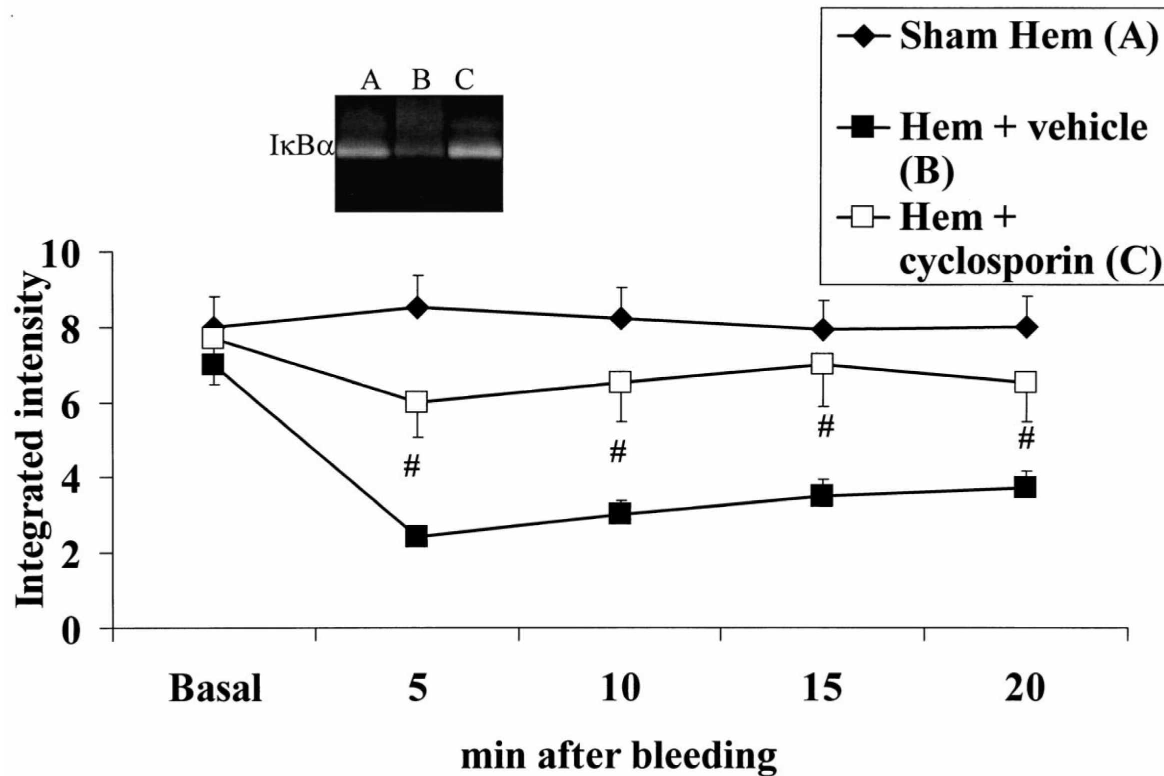

Fig. 2. Western blot analysis of IκBα protein levels in the cytoplasm of rat liver. Samples were normal liver of sham shocked rats (Sham Hem) and liver of rats subjected to acute hypovolemic shock (Hem) and treated with vehicle (1 ml kg^{-1}) or cyclosporin (1 mg kg^{-1}). The top of the figure shows a representative Western Blot picture highlighting IκBα protein levels at 5 min after bleeding cessation. The bottom of the figure shows quantitative data and indicates the integrated intensity of IκBα protein at different time points. Each point represents the mean±S.D. of seven experiments. $^{\#}P<0.01$ versus Hem + vehicle.

shocked rats and sham operated animals (Fig. 6). However, the contractile response to PE in endothelium-denuded aortic rings was also significantly smaller in hemorrhagic shocked rats than in sham operated animals. Administration of cyclosporin improved the impaired contractility response to PE in hemorrhaged rats.

3.6. Survival rate and survival time

Table 1 shows the effect of acute hypovolemic shock on survival rate and survival time. Of 10 rats, none survived for 30 min after bleeding subsequent to vehicle injection. Cyclosporin treatment significantly increased survival rate and time in shocked rats compared with those treated with saline. In those animals, mean survival time was 118±7 min and 2-h survival rate was 80% (Table 1).

3.7. In vitro experiment

To investigate whether cyclosporin directly affects NF-κB, we studied the effect of cyclosporin on activation of the transcription factor induced by several agents (Fig. 7). The results shown in Fig. 7 indicate that cyclosporin completely blocked the activation of NF-κB induced by H_2O_2 and phorbol ester (Fig. 7).

4. Discussion

NF-κB is an early transcription factor which modulates gene expression in various situations that require rapid and sensitive immune and inflammatory response. The prototypic inducible form of NF-κB is a heterodimer composed of NF-κB1 and Rel A, which both belong to the NF-κB/Rel family of proteins. Inactive NF-κB is present in the cytoplasm complexed with the inhibitory protein IκBα. NF-κB is activated by a number of incoming signals from the cell surface [18]. Released from IκBα inhibition, NF-κB translocates into the nucleus and binds to κB motif of the target gene, in turn causing activation of several factors (cell adhesion molecules; cytokines) involved in the inflammatory response [19–21].

Hemorrhagic shock initiates an inflammatory response characterized by the upregulation of cytokine expression [22] and accumulation of neutrophils in a variety of tissues [23]. These changes are prominent in the lungs and liver and are likely to contribute to end organ damage and resultant dysfunction after shock. The mechanism by which hemorrhage triggers this inflammatory response is the object of intensive investigation. Heightened adrenergic activity [24] and systemic release of pro-inflammatory agents from the gut [25,26] have been hypothesized to

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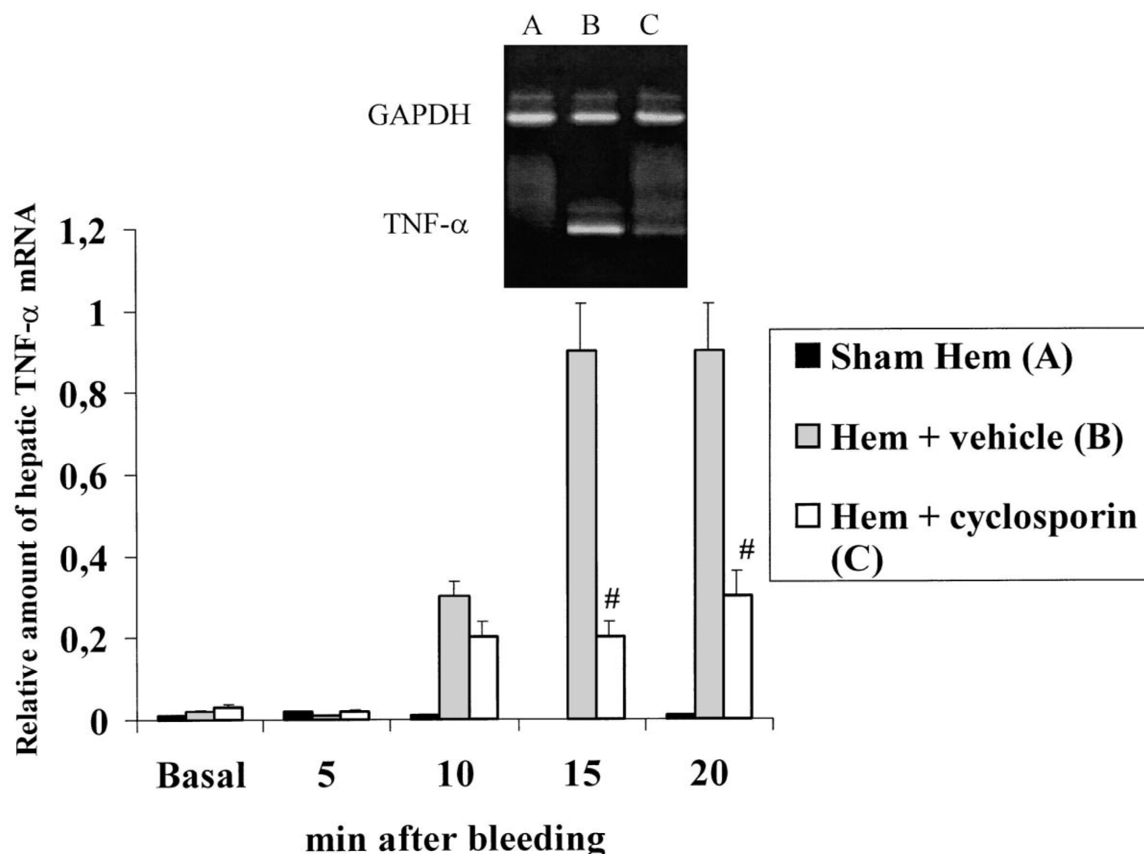

Fig. 3. Liver TNF-α mRNA expression in samples obtained from rats subjected to sham shock (Sham Hen) or acute hypovolemic hemorrhagic shock (Hem) and treated with vehicle (1 ml kg^{-1}) or cyclosporin (1 mg kg^{-1}). The top of the figure shows a representative RT-PCR picture highlighting peak TNF-α expression 15 min after bleeding cessation. The bottom of the figure depicts quantitative data and indicates the relative amount of TNF-α mRNA at different time points. Each point represents the mean±S.D. of seven experiments. $^{\#}P<0.01$ versus Hem+vehicle.

contribute to organ injury after hemorrhage. In addition, reactive oxygen species (ROIs) are produced after resuscitation from hemorrhagic shock and have been implicated in a number of signal transduction pathways [27].

Previous experiments have suggested that NF-κB is activated in lung mononuclear cells during a murine hemorrhagic model of shock consisting in withdrawing 30% of the calculated blood volume over a period of 60 s [28,29]. Furthermore NF-κB activation has been shown in the lungs after resuscitation in a rat model of resuscitated

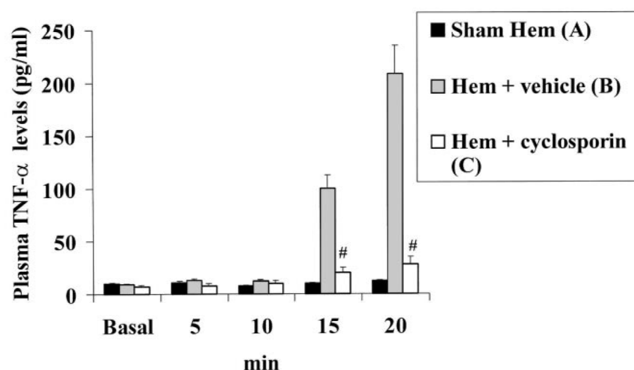

Fig. 4. Plasma levels of TNF-α in rats subjected to sham shock (Sham Hem) or acute hypovolemic hemorrhagic shock (Hem) and treated with vehicle (1 ml kg^{-1}) or cyclosporin (1 mg kg^{-1}). Each point represents the mean±S.D. of seven experiments. $^{\#}P<0.005$ versus Hem+vehicle.

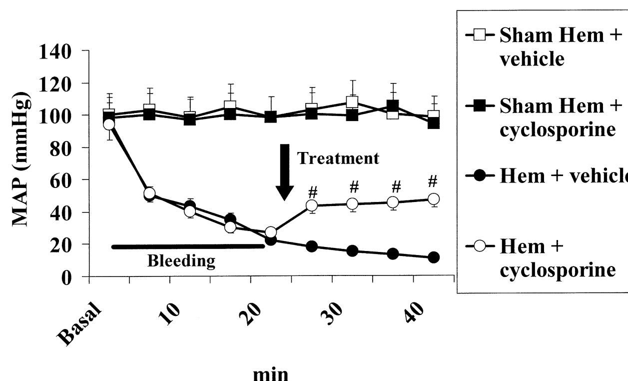

Fig. 5. Mean arterial blood pressure in rats subjected to sham shock (Sham Hem) or acute hypovolemic hemorrhagic shock (Hem) and treated with vehicle (1 ml kg^{-1}) or cyclosporin (1 mg kg^{-1}). Each point represents the mean±S.D. of seven experiments. $^{\#}P<0.005$ versus Hem+vehicle.

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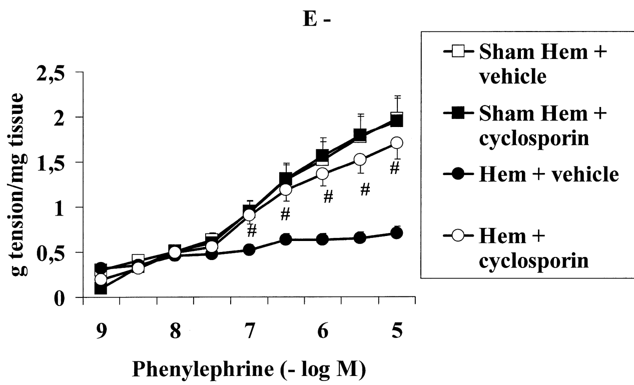

Fig. 6. Contractile response to cumulative doses of phenylephrine (PE) in endothelium-denuded aortic rings prepared from rats subjected to sham shock (Sham Hem) or acute hypovolemic hemorrhagic shock (Hem) and treated with vehicle (1 ml kg^{-1}) or cyclosporin (20 mg kg^{-1}). Each point represents the mean±S.D. of seven experiments. $^{\#}P<0.001$ versus Hem + vehicle.

hemorrhagic shock [30]. In addition, it has been suggested that NF-κB is involved in inflammatory gene activation in lung mononuclear cells during hemorrhage or hemorrhage–resuscitation and that ROIs induce NF-κB translocation [31]. In this model, NF-κB required at least 1 h after the hemorrhage procedure to be activated.

Acute hypovolemic hemorrhagic shock is a lethal type of shock (all animals die within 25–30 min after bleeding cessation) and is characterized by a serious hypotension, increased levels of TNF-α and vascular failure due to the loss of vascular reactivity after stimulation with vasoconstrictor stimuli. The inflammatory cytokine seems to play a pivotal role in the pathogenesis of this experimental model of circulatory shock since administration of anti-TNF-α monoclonal antibodies significantly increases survival, improves hypotension and restores the impairment in vascular reactivity [4]. However the mechanism(s) causing

Table 1
Survival time and survival rate in rats subjected to acute hypovolemic hemorrhagic shock (Hem)

Treatment	Survival time (min)	Survival rate		
		30 min	60 min	120 min
Sham Hem + vehicle (1 ml kg^{-1})	>90	10/10	10/10	10/10
Sham Hem + cyclosporin (1 mg kg^{-1})	>90	10/10	10/10	10/10
Hem + vehicle (1 ml kg^{-1})	27±6	0/10	0/10[a]	0/10[a]
Hem + cyclosporin (1 mg kg^{-1})	118±7[b]	10/10[b]	10/10[b]	8/10[b]

Each point represents the mean±S.D. of 10 experiments.
[b] $P<0.01$ versus Hem + vehicle.
[a] $P<0.01$ versus Sham.

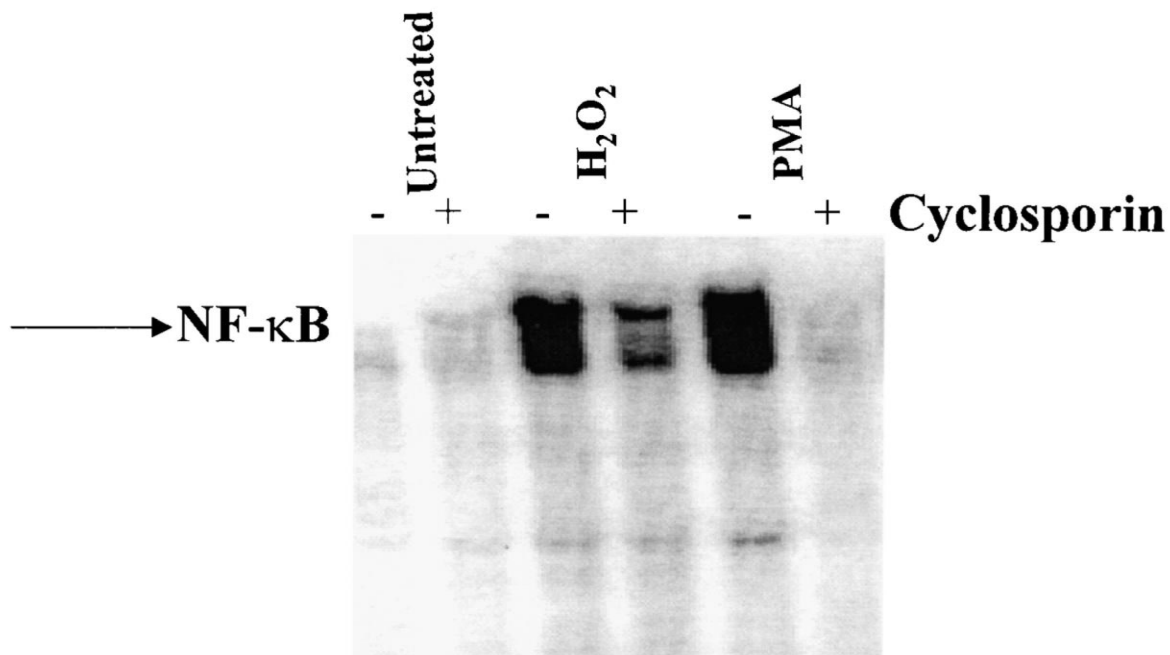

Fig. 7. Effects of cyclosporin on different activators (H_2O_2 and PMA) on NF-κB. Macrophages were pre-incubated for 120 min at 37°C with cyclosporin (1 μM) followed by H_2O_2 (25 μM) and PMA (25 ng/ml).

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systemic production of the inflammatory cytokine in this rapid and short lasting hemorrhagic shock model still remain to be elucidated. It has been suggested that during systemic stress (i.e. circulatory shock), a parasympathetic nervous system activity influences, via the vagus nerve, liver production of TNF-α and the consequent release of the mature protein in the bloodstream [32]. Therefore we decided to study NF-κB activation and TNF-α production in the liver.

We found that peak liver NF-κB activation was achieved 10 min after bleeding cessation. In addition, IκBα protein levels sharply decreased at 5 min after the end of bleeding. This confirms that NFκB represents a rapid and early signal mechanism for controlling gene expression. In keeping with this hypothesis, liver TNF-α mRNA levels increased at 15–20 min after the end of bleeding and the peak message for this inflammatory cytokine was reached 20 min following bleeding cessation. Furthermore, the plasma levels of TNF-α raised later in the bloodstream and were significantly enhanced at 20 min after the end of bleeding, just before the occurrence of animal death. This strongly supports the idea that acute blood loss primes liver NF-κB which in turn activates an inflammatory cascade leading to the expression of TNF-α and finally culminating in the fatal outcome of the animals.

The NF-κB activation process can be inhibited by pharmacological agents at each activation step [33]. The immunosuppressive agent cyclosporin prevents NF-κB activation by inhibiting the action of calcineurin, a phosphatase that indirectly induces IκB degradation. Calcineurin in fact may activate the proteolytic degradation of IκB through protein kinase activation [34]. However cyclosporin could also prevent NF-κB activation by inhibiting proteosomal proteolysis of IκBα [12].

With this background we investigated the ability of cyclosporin to block NF-κB activation. The drug succeeded in blocking NFκB activation, in turn turning off the TNF-α gene and therefore limiting acute vascular failure. The inhibition of NF-κB was a direct effect: as a matter of fact, cyclosporin, under in vitro conditions, blocked the activation of NF-κB induced by H_2O_2 and phorbol ester.

Indeed the reversal of vascular failure (i.e. blunted responsiveness of aortic rings and marked hypotension) is likely due to the reduction in the circulating levels of TNF-α: as a matter of fact, the inflammatory and pleiotropic cytokine has been shown to play a pivotal role in mediating the loss of vascular reactivity after stimulation with vasoconstrictor stimuli [4]. More specifically, TNF-α stimulates inducible nitric oxide synthase (iNOS) activity in the smooth muscle cells, thereby leading to the production of a large amount of the vasoactive substance in the smooth muscle cells. Thus, enhanced production of nitric oxide, due to a TNF-α-induced activation of iNOS, is finally responsible for the impairment in vascular reactivity and in development of vascular failure in acute hemorrhagic shock.

In conclusion, all these data indicate that acute blood loss may activate NF-κB early, which in turn triggers an inflammatory cascade leading to inflammatory cytokine production and to vascular derangement. Furthermore, the present results suggest that cyclosporin may represent a good inhibitor of NF-κB activation with potential antishock effects.

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