ANTIOXIDANTS & REDOX SIGNALING
Volume 4, Number 5, 2002

Forum Original Research Communication

Hepatic Redox Regulation of Transcription Factors Activator Protein-1 and Nuclear Factor-κB After Hemorrhagic Shock *In Vivo*

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ABSTRACT

Ischemia and reperfusion result in a hepatocellular stress gene response, characterized by a zonal heterogeneity with pericentral hepatocytes being the primary target. In the present study, we assessed cell type-specific and zonal pattern of activation of redox-sensitive transcription factors nuclear factor-κB (NFκB) and activator protein-1 (AP-1) in a graded model of hemorrhage and their modulation by the antioxidants trolox and tempol. Hemorrhagic hypotension (35–40 mm Hg) up to 3 h without subsequent resuscitation led to an only moderate activation of NFκB and AP-1. In contrast, fluid resuscitation after 1 or 2 h of hemorrhage induced a profound activation of AP-1 within the first hour of reperfusion. Consistent with a regulation by oxygen free radicals, activation of AP-1 was substantially attenuated by antioxidants. The faint activation of NFκB with various intervals of hemorrhage was unaffected by antioxidants and did not exceed activation with sham operation. Immunohistochemistry for the AP-1 subunit c-Jun revealed a predominant expression in nuclei of pericentral and midzonal hepatocytes. These data suggest activation of AP-1 in hepatocytes most susceptible to injury and reprogramming of gene expression in low-flow ischemia. Whereas activation of NFκB is weak in this model and is not modulated by either reperfusion or antioxidants, regulation of AP-1 after hemorrhage and subsequent resuscitation seems to depend on oxygen free radical formation because it requires reperfusion and is inhibitable by antioxidants. *Antioxid. Redox Signal.* 4: 711–720.

INTRODUCTION

HEPATOCELLULAR DYSFUNCTION frequently occurs after hemorrhagic shock and trauma despite adequate restoration of systemic and regional hemodynamics. Both ischemia and reperfusion associated with hemorrhagic shock and resuscitation contribute to the biphasic injury primarily affecting the pericentral region of the acinus (5). Prolonged ischemia seems to promote acute pericentral necrosis, whereas reperfusion initiates a delayed inflammatory reaction, also termed "ischemic hepatitis" (3). Although progress has been made with respect to early resuscitation, depression or disturbances of cellular functions associated with the delayed inflammatory response propagate the development of multiple organ failure and sepsis, the leading cause of late death in the critically ill (15). Severe disturbances of hepatocellular homeostasis resulting from hemorrhagic hypotension induce a variety of stress response genes, including cytokines and adhesion molecules, but also the acute phase and the heat shock response. As hemorrhagic shock and resuscitation produce characteristic changes in the redox state of the cell, the so-called redox-sensitive transcription factors nuclear factor-κB (NFκB) and activator protein-1 (AP-1) may play a significant role for reprogramming of hepatocellular gene expression under conditions associated with resuscitation from hemorrhage (4, 17). Although oxygen free radicals (OFR), in particular hydrogen peroxide have been proposed as regulators of NFκB activation based on experiments in cultured

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Jurkat T cells, this concept of universal regulation of NFκB by OFR has since been questioned (2). In addition, little is known about the regulation of these transcripton factors by oxidants and antioxidants *in vivo*.

Therefore, in the present study the activation patterns of NFκB and AP-1 were characterized in a graded model of hemorrhage associated with low-flow ischemia of the liver with or without resuscitation/reperfusion, as well as their modulation by the antioxidants trolox and tempol.

MATERIALS AND METHODS

All chemicals used were purchased from Sigma (München, Germany) if not specified otherwise. All chemicals were of the highest purity commercially available.

Animals

Male Sprague-Dawley rats [220–280 g body weight (b.w.)] were obtained from Charles River (Sulzfeld, Germany). Pellet food was withheld overnight before preparative surgery, while animals had free access to water. All experiments were performed in accordance with the German legislation on protection of animals and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* [DHEW Publication No. (NIH) 86–23, revised 1985].

Experimental design

Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg b.w.). Subsequently, a tracheotomy was performed to facilitate spontaneous breathing. The right jugular vein was cannulated for drug administration and fluid resuscitation. A continuous infusion of Ringer's solution (10 ml/kg/h) was supplied to compensate for losses during preparative surgery. The left carotid artery was cannulated to allow blood withdrawal and measurement of systemic arterial blood pressure with a standard pressure transducer (Medex Medical, Ratingen, Germany).

Animals (n = 6/group) were subjected to hemorrhagic shock with a mean arterial blood pressure (MAP) of 35–40 mm Hg for 1, 2 or 3 h in a modified Wiggers model followed by fluid resuscitation. In the first group, MAP was lowered to 35–40 mm Hg for 1 h followed by 2 h of resuscitation. In the second group, hemorrhagic hypotension was maintained for 2 h followed by 1 h of fluid resuscitation, whereas in the third group MAP was lowered for 3 h without resuscitation. This model was used to produce a graded ischemic liver injury.

Shed blood was collected in syringes containing citrate–phosphate–dextrose solution (0.14 ml/ml of shed blood). In animals receiving resuscitation fluids, 60% of the shed blood was returned during the first 10 min of resuscitation. In addition, twice the shed blood volume was infused in the form of Ringer's solution during the first hour of resuscitation. The infusion rate of Ringer's solution was lowered to a volume equaling the maximal bleedout volume for the second hour of resuscitation if appropriate.

As activation of AP-1 did require resuscitation/reperfusion (see below), either the radical scavenger tempol (30 mg/kg b.w. as bolus injection followed by a continuous infusion of 30 mg/kg b.w./h), the vitamin E analogue trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid; 6 mg/kg b.w.), or an equal volume of 0.9% NaCl as vehicle was administered along with resuscitation fluids to elucidate the role of formation of reactive oxygen intermediates. In addition to the studies addressing the influence of different grades of ischemic injury, in these experiments the time course of AP-1 activation was further characterized. Rats were subjected to hemorrhagic hypotension (MAP 35–40 mm Hg) for 1 h. Nuclear protein was prepared immediately after hypotension or after resuscitation intervals of 2 or 5 h.

Time-matched sham-operated controls underwent preparative surgery, but did not undergo hemorrhage and received a constant infusion of 10 ml/kg/h Ringer's solution over the whole period of the experiment.

As a positive control experiment for NFκB activation, some animals (n = 4) were injected with endotoxin (ETX) [lipopolysaccharide (LPS) serotype O26:B6; 1 mg/kg b.w. i.p.]. Six hours after injection, livers were harvested and nuclear protein was prepared.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously (17). Approximately 150 mg of frozen liver tissue was homogenized in ice-cold buffer A [1:10 (wt/vol) 10 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* dithiothreitol (DTT), 1 m*M* phenylmethylsulfonyl fluoride (PMSF)], incubated on ice for 10 min, and centrifuged. The pellet was resuspended in ice-cold buffer B (0.1% Triton X-100 in buffer A), incubated on ice for 10 min, and centrifuged at 850 *g* for 10 min. The crude nuclear pellet was carefully rinsed with buffer A, resuspended in 50 µl of buffer C [20 m*M* HEPES, pH 7.9, 25% glycerol (vol/vol), 0.42 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, 1 m*M* PMSF], incubated on ice for 30 min, and centrifuged at 20,000 *g* for 15 min at 4°C. Nuclear protein concentrations were assessed by a Bradford assay (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard. Gel shift reagents were used according to the manufacturer's protocol (Promega Corp., Madison, WI, U.S.A.). Antibodies cross-reactive to AP-1 subunits (c-Jun, JunD, c-Fos, JunB, FosB, Fra1, and Fra2) or to NFκB subunits [p50 (NFκB1), p52 (NFκB2), p65 (rel A), p68 (rel B), and p75 (c-Rel)] were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). The double-stranded AP-1 or NFκB consensus oligonucleotide probes (Promega) were end-labeled with 10 µCi of [γ-^{32}P]ATP at 222 TBq/mmol (Amersham, Freiburg, Germany). Binding reactions, containing 35 fmol of oligonucleotide and 5 µg of nuclear protein, were conducted at room temperature for 20 min in a total volume of 10 µl binding buffer [10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.5 m*M* DTT, 4% glycerol (vol/vol), and 0.5 µg of poly dI:dC]. For competition reactions, a one-fold or 50-fold excess of unlabeled oligonucleotide was added 5 min prior to the addition of the labeled probe. For supershift analysis, 1 µg of each antibody was added to the nuclear protein 5 min prior to the addition of the radiolabeled

probe. Following binding reactions, samples were subjected to a nondenaturing 5% polyacrylamide gel electrophoresis in low ionic strength buffer (45 m*M* Tris-borate, 1 m*M* EDTA) at 180 V/22 mA for 2 h. Gels were vacuum-dried and exposed to x-ray film (X-Omat XAR-5, Eastman Kodak, Rochester, NY, U.S.A.) at −70°C. Signals were analyzed densitometrically (Bio-Rad GS 700 Imaging Densitometer).

Immunohistochemical staining for c-Jun

Formalin-fixed, paraffin-embedded, dewaxed liver sections were used to assess the spatial and cell type-specific expression pattern of c-Jun. Sections were subjected to an antigen retrieval using microwave irradiation. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide/methanol. After subsequent treatment with normal horse serum, slides were incubated at 37°C for 1 h with a monoclonal mouse anti-rat c-Jun primary antibody (1:200; Santa Cruz Biotechnology). As secondary antibody, a biotinylated horse anti-mouse antibody was used for streptavidin-biotin-complex peroxidase staining. 3,3´-Diaminobenzidine and 3% $CoCl_2$ were used as chromogens, and slides were counterstained with hematoxylin.

Statistical analysis

Data are presented as means ± SEM. Differences were evaluated by ANOVA followed by post-hoc multiple comparison according to the Student–Newman–Keuls method using the SigmaStat software package (Jandel Scientific, San Rafael, CA, U.S.A.). A $p < 0.05$ was considered significant.

RESULTS

Macrohemodynamic response to hemorrhage and resuscitation

Hemorrhagic shock was reversible in all animals subjected to hemorrhagic hypotension for 1 or 2 h as reflected by recovery of MAP, heart rate (HR), and base excess (BE) upon resuscitation (data not shown).

In the group with 3 h of hemorrhage (no resuscitation), all animals required several intravenous injections of 0.5 ml Ringer's after 130 ± 9 min to maintain the blood pressure at 35–40 mm Hg, indicating a decompensated hemorrhagic shock.

In those groups receiving resuscitation fluids with or without antioxidants, similar shed blood volumes were required to achieve and maintain a MAP of 35–40 mm Hg (shock vehicle, 36.6 ± 1.5 ml/kg b.w.; shock tempol, 38.2 ± 2.1 ml/kg b.w.; shock trolox, 34.9 ± 1.6 ml/kg b.w.). Base deficit as an indicator of ischemic injury was comparable at the end of hemorrhagic shock in all groups subjected to hemorrhagic hypotension for 1 h followed by resuscitation (shock vehicle, −16.1 ± 1.6 mmol/L; shock tempol, −18.2 ± 1.0 mmol/L; shock trolox, −14.9 ± 0.9 mmol/L). In the shock groups treated with trolox or tempol, no significant differences were observed in MAP, HR, BE, and body temperature irrespective of the treatment as compared with vehicle animals.

Sham-operated time-matched controls maintained normal MAP, HR, body temperature, and BE throughout the experiments (data not shown).

Activation pattern of transcription factor NFκB after hemorrhagic shock

Nuclear protein of liver specimens of unmanipulated controls and time-matched sham-operated controls, as well as rats subjected to hemorrhagic shock for 1 h (2 h of resuscitation), 2 h (1 h of resuscitation), or 3 h (no resuscitation), was obtained and corresponding samples were subjected to EMSA for NFκB. Although activation of NFκB was observed after 1, 2, or 3 h of hemorrhagic hypotension, it did not exceed the levels observed in sham-operated time-matched controls (Fig. 1, lanes 3–10). NFκB binding activity was almost completely inhibited by a onefold excess (Fig. 1. lane 13) and completely inhibited by a 50-fold excess (Fig. 1, lane 14) of unlabeled NFκB oligonucleotide, but was unaffected by unlabeled AP-1 oligonucleotide (Fig. 1, lane 15) confirming the specifity of the binding reaction. Using nuclear extracts of

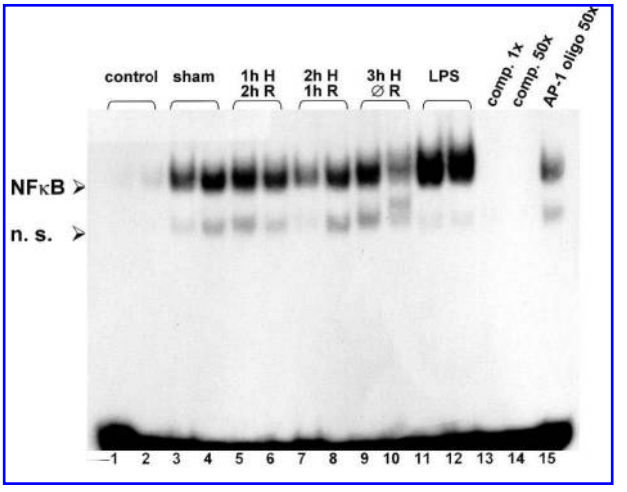

FIG. 1. Activation pattern of NFκB after hemorrhagic hypotension and resuscitation. The activation of NFκB observed after 1, 2, or 3 h of hemorrhagic hypotension did not exceed the levels observed in sham-operated time-matched controls. The panel shows a representative EMSA of nuclear extracts from livers of unmanipulated controls (control, lanes 1 and 2), sham-operated time-matched controls (sham; lanes 3 and 4), 1 h of hemorrhagic shock/2 h of resuscitation (1h H/2h R; lanes 5 and 6), 2 h of hemorrhagic shock/1 h of resuscitation (2h H/1h R; lanes 7 and 8), and 3 h of hemorrhagic shock without resuscitation (3h H/Ø R; lanes 9 and 10). As positive controls, a profound activation of NFκB was observed after endotoxemia (LPS = ETX, lanes 11–12). Each lane represents nuclear protein of an individual animal. For competition reactions, a onefold (1x) or 50-fold (50x) excess of unlabeled NFκB oligonucleotide was added, leading to a concentration-dependent attenuation of binding activity (lanes 13 and 14). The unlabeled oligonucleotide for AP-1 was used as a nonspecific competitor (lane 15). Sample used in lanes 13 and 14 corresponds to lane 3. n.s., not specific (unspecific binding of the oligonucleotide).

animals 6 h after endotoxemia (1 mg/kg b.w.; LPS O26:B6) as positive controls, a profound activation of NFκB was observed compared with hemorrhagic shock or sham operation (Fig. 1, lanes 11 and 12). Each lane represents the analysis of a nuclear extract of an individual animal.

To confirm the identity of the activated protein–DNA complex, a supershift analysis was performed. A supershift was observed in the presence of antibodies directed against p50 (NFκB1; Fig. 2, lane 3) and p65 (rel A; Fig. 2, lane 5) in the nuclear protein obtained after 1 h of hemorrhagic hypotension and 2 h of resuscitation. No supershift was detected in the presence of p52 (NFκB2), p68 (rel B), and p75 (c-Rel; Fig. 2, lanes 4, 6, and 7). These results suggest that the NFκB complex is composed of the subunits p50 (NFκB1) and p65 (rel A).

Treatment with the antioxidant tempol at the time point of resuscitation did not significantly affect the activation of NFκB 2 h or 5 h after beginning of fluid resuscitation (Fig. 3, lanes 9–12) compared with animals treated with vehicle (Fig. 3, lanes 5–8). Furthermore, activation of NFκB observed after sham operation did not differ from activation observed after hemorrhagic shock (Fig. 3, lanes 3 and 4). Unmanipulated controls showed no activation of NFκB (Fig. 3, lanes 1 and 2). Using nuclear extracts of animals 6 h after endotoxemia (1 mg/kg b.w.; LPS O26:B6) as positive controls, a profound and significantly increased activation of NFκB was observed compared with hemorrhagic shock and resuscitation (Fig. 3, lanes 13 and 14). Similar results were obtained by administration of the chemically different antioxidant trolox after 1 h of hemorrhagic hypotension followed by 5 h of fluid resuscitation. No differences in activation pattern of NFκB were observed between sham-operated controls and animals after hemorrhagic shock and resuscitation with or without administration of trolox (arbitrary densitometric units: control, 0.4 ± 0.02; sham, 3.3 ± 1.4; shock vehicle, 2.0 ± 0.8; shock trolox, 3.9 ± 2.4; ETX, 80.3 ± 18.7*#; *$p < 0.05$, compared with sham-operated controls; #$p < 0.05$, compared with shock vehicle).

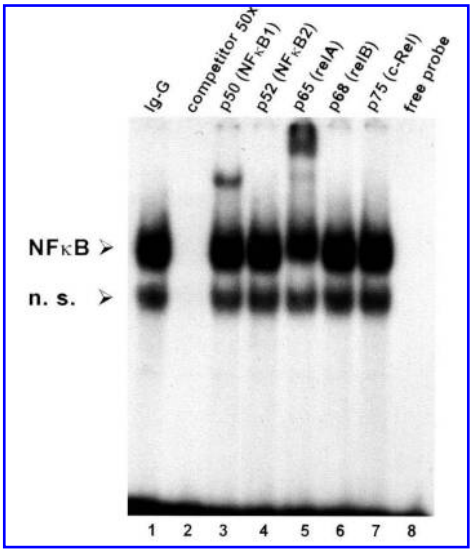

FIG. 2. Supershift analysis of protein subunits binding to the NFκB oligonucleotide. Nuclear extracts from a liver after hemorrhagic hypotension and fluid resuscitation were preincubated with antibodies to rabbit IgG, p50 (NFκB1), p52 (NFκB2), p65 (rel A), p68 (rel B), or p75 (c-Rel) as described in Materials and Methods. Supershift analysis indicates the presence of the NFκB subunits p50 (NFκB1, lane 3) and p65 (rel A, lane 5) in the oligonucleotide/protein complex in a representative sample after 1 h of hemorrhagic hypotension followed by 2 h of resuscitation. For competition reaction, a 50-fold excess of unlabeled NFκB oligonucleotide was added to the binding reaction, almost completely abolishing the binding activity (lane 2). Lane 8 shows free probe without addition of nuclear extract. n.s., not specific (unspecific binding of the oligonucleotide).

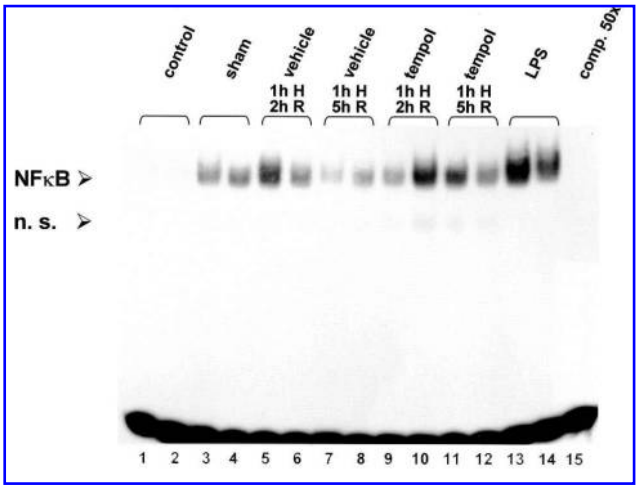

FIG. 3. Activation pattern of NFκB after hemorrhagic shock and resuscitation with or without administration of tempol. Administration of the antioxidant tempol at the time point of resuscitation after 1 h of hemorrhagic hypotension did not significantly affect the activation of NFκB 2 h or 5 h after beginning of fluid resuscitation (lanes 9–12) compared with animals treated with vehicle (lanes 5–8). The activation of NFκB observed after sham operation did not differ from activation observed after hemorrhagic shock (lanes 3 and 4). Unmanipulated controls showed almost no activation of NFκB (lanes 1 and 2). As positive controls, a profound activation of NFκB was observed after endotoxemia (LPS = ETX, lanes 13 and 14). For competition reactions, a 50-fold (50x) excess of unlabeled NFκB oligonucleotide was added, almost completely abolishing the binding activity (lane 15). n.s., not specific (unspecific binding of the oligonucleotide).

Activation pattern of transcription factor AP-1 after hemorrhagic shock

In contrast to NFκB, a profound activation of AP-1 was observed in nuclear extracts of animals subjected to hemorrhagic hypotension for 1 h (2 h of resuscitation) (Fig. 4, lanes 3 and 4) or 2 h (1 h of resuscitation) (Fig. 4, lanes 5 and 6). No activation of AP-1 was observed in sham-operated time-matched controls (Fig. 4, lanes 1 and 2), whereas 3 h of hemorrhagic shock without resuscitation resulted in a moderate activation of AP-1 (Fig. 4, lanes 7 and 8). AP-1 binding activity was partially inhibited by a onefold excess and al-

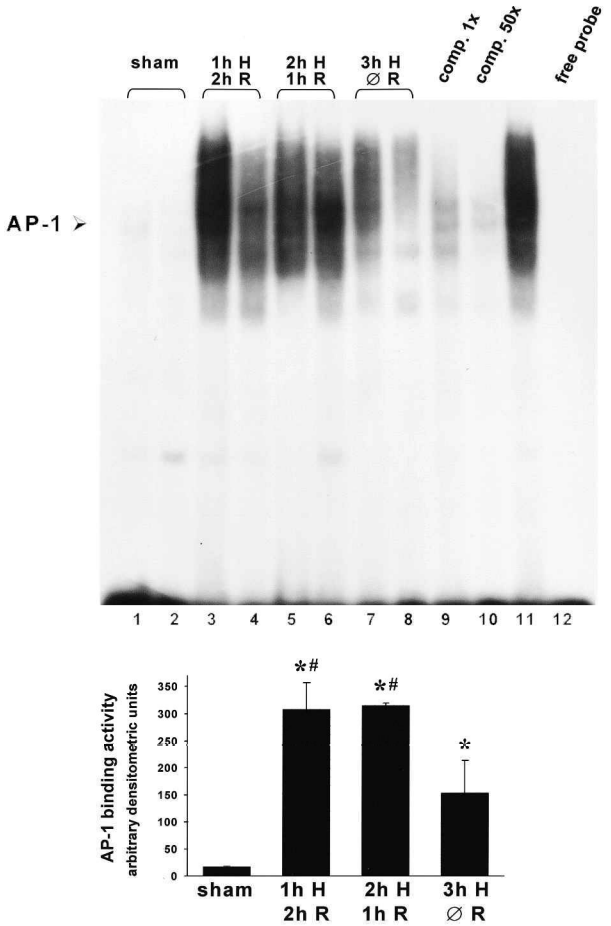

FIG. 4. Activation pattern of AP-1 after hemorrhagic hypotension and resuscitation After hemorrhagic shock for 1 or 2 h followed by fluid resuscitation for 2 or 1 h, respectively, a profound activation of AP-1 was observed, whereas 3 h of hemorrhagic hypotension without resuscitation resulted in a significantly weaker activation of AP-1. The upper panel represents an EMSA of nuclear extracts from livers of sham-operated time-matched controls (sham; lanes 1 and 2), 1 h of hemorrhagic shock/2 h of resuscitation (1h H/2h R; lanes 3 and 4), 2 h of hemorrhagic shock/1 h of resuscitation (2h H/1h R; lanes 5 and 6), and 3 h of hemorrhagic shock without resuscitation (3h H/Ø R; lanes 7 and 8). Each lane represents nuclear protein of an individual animal. For competition reactions, a onefold (1x) or 50-fold (50x) excess of unlabeled AP-1 oligonucleotide was added, leading to a concentration-dependent attenuation of binding activity (lanes 9 and 10). The unlabeled oligonucleotide for NFκB was used as a nonspecific competitor for AP-1 shifts (lane 11). Lane 12 shows the free probe. The lower panel represents the densitometric data from $n = 4$ animals. *$p < 0.05$ as compared with sham-operated controls; #$p < 0.05$ vs. 3h H/Ø R.

most completely inhibited by a 50-fold excess of unlabeled AP-1 oligonucleotide (Fig. 4, lanes 9 and 10). The unlabeled oligonucleotide for NFκB was used as a nonspecific competitor and did not inhibit binding activity (Fig. 4, lane 11).

To confirm the identity of the activated protein–DNA complex, a supershift analysis was performed. A supershift was observed in the presence of antibodies directed against c-Jun, JunD, and c-Fos in nuclear protein from livers obtained after 1 h of hemorrhage and 2 h of resuscitation (Fig. 5, lanes 3, 5, and 6). No supershift was detected in the presence of the antibodies directed against JunB, FosB, Fra-1, and Fra-2 (Fig. 5, lanes 4, 7, 8, and 9). Competition reactions with a 50-fold excess of unlabeled AP-1 oligonucleotide completely abolished the binding activity (Fig. 5, lane 2).

These results suggest that the AP-1 complex is composed of the subunits c-Jun, JunD, and c-Fos.

No AP-1 activation was observed after 1 h of hemorrhagic hypotension without resuscitation (Fig. 6, lanes 3 and 4). The observed profound activation of AP-1 after 1 h of hemorrhagic hypotension followed by 2 or 5 h of resuscitation (Fig. 6, lanes 5–8) was significantly attenuated by treatment with the antioxidant tempol (30 mg/kg b.w. as bolus injection followed by a continuous infusion of 30 mg/kg b.w./h) given at the onset of fluid resuscitation (Fig. 6, lanes 9–12). No activation of AP-1 was observed in sham-operated animals (Fig. 6, lanes 1 and 2). Administration of the chemically different antioxidant trolox (6 mg/kg b.w.) significantly attenuated the observed activation of AP-1 after 1 h of hemorrhagic hypotension followed by 5 h of resuscitation (arbitrary densitometric units: sham, 6.8 ± 0.9; shock vehicle, 96.8 ± 17.3*;

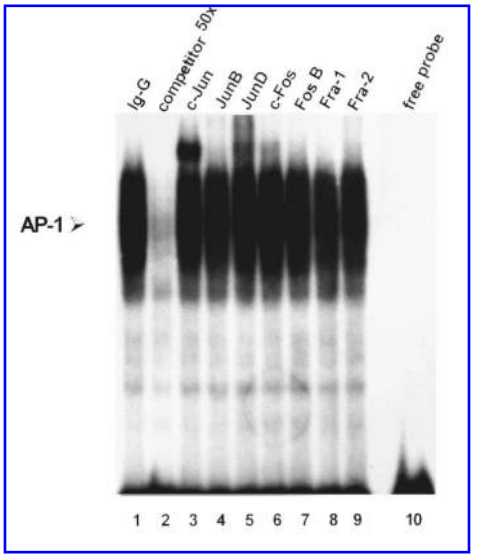

FIG. 5. Supershift analysis of protein subunits binding to the AP-1 oligonucleotide. Supershift analysis indicates the presence of the AP-1 subunits c-Jun (lane 3), JunD (lane 5), and c-Fos (lane 6) in the oligonucleotide/protein complex in a representative sample after 1 h of hemorrhagic hypotension followed by 2 h of resuscitation. Therefore, the AP-1 complex seems to be composed of homo- and heterodimers of c-Jun, JunD, and c-Fos. Nuclear extracts from a liver after hemorrhagic hypotension and fluid resuscitation were preincubated with antibodies to rabbit IgG, c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, or Fra-2 as described in Materials and Methods. For competition reaction, a 50-fold excess of unlabeled AP-1 oligonucleotide was added to the binding reaction, almost completely abolishing the binding activity (lane 2). Lane 10 shows free probe without addition of nuclear extract.

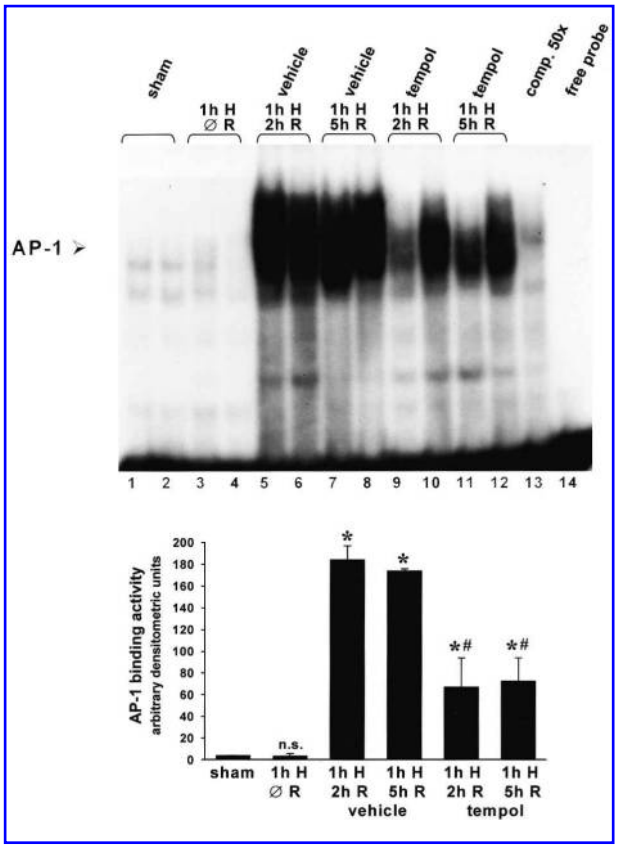

FIG. 6. Activation pattern of AP-1 after hemorrhagic shock and resuscitation with or without administration of tempol. After hemorrhagic hypotension for 1 h followed by 2 or 5 h of fluid resuscitation, a profound activation of AP-1 was observed, which was markedly decreased after treatment with the antioxidant tempol. The panel represents an EMSA of nuclear extracts from livers of sham-operated time-matched controls (lanes 1 and 2), 1 h of hemorrhagic shock without reperfusion (lanes 3 and 4), 1 h of hemorrhagic shock/2 or 5 h of resuscitation with administration of saline as vehicle (lanes 5–8), and 1 h of hemorrhagic shock/2 or 5 h of resuscitation after treatment with tempol (lanes 9–12). Each lane represents nuclear protein of an individual animal. For competition reactions, a 50-fold (50x) excess of unlabeled AP-1 oligonucleotide was added, leading to an attenuation of binding activity (lane 13). Lane 14 shows free probe without addition of nuclear extract. The lower panel represents the densitometric data from $n = 4$ animals. *$p <$ 0.05 vs. sham; #$p < 0.05$ compared with respective vehicle control. n.s., not significant as compared with sham.

shock trolox, 41.9 ± 12.9#; *$p < 0.05$ vs. sham-operated controls, #$p < 0.05$ compared with shock vehicle).

Immunohistochemistry of AP-1 subunit c-Jun

To investigate the cell type and spatial expression pattern of the AP-1 subunit c-Jun *in vivo,* immunohistochemistry for c-Jun was performed on liver sections. No c-Jun immunoreactive protein was observed in liver sections of sham-operated controls (Fig. 7A). A substantial *de novo* expression of c-Jun immunoreactive protein was observed in nuclei of parenchymal cells of the pericentral and midzonal regions after 1 h of hemorrhagic hypotension followed by 2 h of resuscitation (Fig. 7B), whereas 2 h of hemorrhage followed by 1 h of resuscitation resulted in the presence of c-Jun immunoreactive protein in virtually all nuclei of the acinus (Fig. 7C).

In contrast, *de novo* expression of c-Jun after 3 h of hemorrhagic shock without resuscitation was only detected in individual hepatocytes scattered over the acinus (Fig. 7D).

Administration of the antioxidant tempol at the onset of resuscitation attenuated substantially the expression of c-Jun immunoreactive protein in hepatocytes in pericentral and midzonal regions after 2 h (Fig. 8C), as well as after 5 h (Fig. 8D) of resuscitation, consistent with the results obtained by EMSA.

DISCUSSION

In the present study, we have characterized the hepatic activation pattern of the putatively "redox-sensitive" transcription factors NFκB and AP-1 in a model of low-flow ischemia with or without subsequent resuscitation *in vivo.* A profound activation of AP-1 was observed after resuscitation/reperfusion that was inhibited by antioxidants, suggesting a regulatory role of OFR for AP-1 activation. Low-flow ischemia alone failed to activate AP-1 (after 1 h of hemorrhagic hypotension) or led to a moderate activation of AP-1 (after 3 h of hemorrhagic hypotension). In contrast, activation of NFκB after low-flow ischemia was weak compared with activation after endotoxemia and was not affected by antioxidants, nor did activation upon hemorrhagic shock and resuscitation exceed levels observed in sham-operated controls. Thus, AP-1 may fulfill criteria of a transcription factor that is regulated along with changes in the cellular redox state *in vivo* in the rat liver.

Transcription factors bind to specific consensus sequences in promoter regions of downstream effector genes and activate or depress effector gene expression. We have previously shown that expression of effector genes regulated by AP-1 (like heme oxygenase-1 after hemorrhagic shock) or NFκB (like inducible nitric oxide synthase after endotoxemia) contributes to maintenance of organ blood flow and hepatocellular integrity (16, 17), indicating a functional significance of the discriminate activation pattern of AP-1 and NFκB upon different stress events in the liver.

Based on data obtained in cultured Jurkat T cells, activation of NFκB by OFR has been suggested and contributed to the development of the concept of redox regulation of transcription factors (19). However, recent evidence suggests that in cell lines other than Jurkat T cells, activation of NFκB is not mediated by OFR, and implicates that the reactive oxygen-mediated activation of NFκB may be restricted to certain cell types *in vitro* (2). In addition, an OFR-independent cell type-specific activation of NFκB by cytokines was demonstrated (2), whereas little is known about OFR-dependent regulation of NFκB *in vivo.* With respect to endotoxemia, administration of the antioxidant dimethyl sulfoxide inhibited NFκB activation, which correlated with suppression of tumor necrosis factor-α formation, reduced ICAM-1 gene transcription, and protection against ETX-induced liver injury (6).

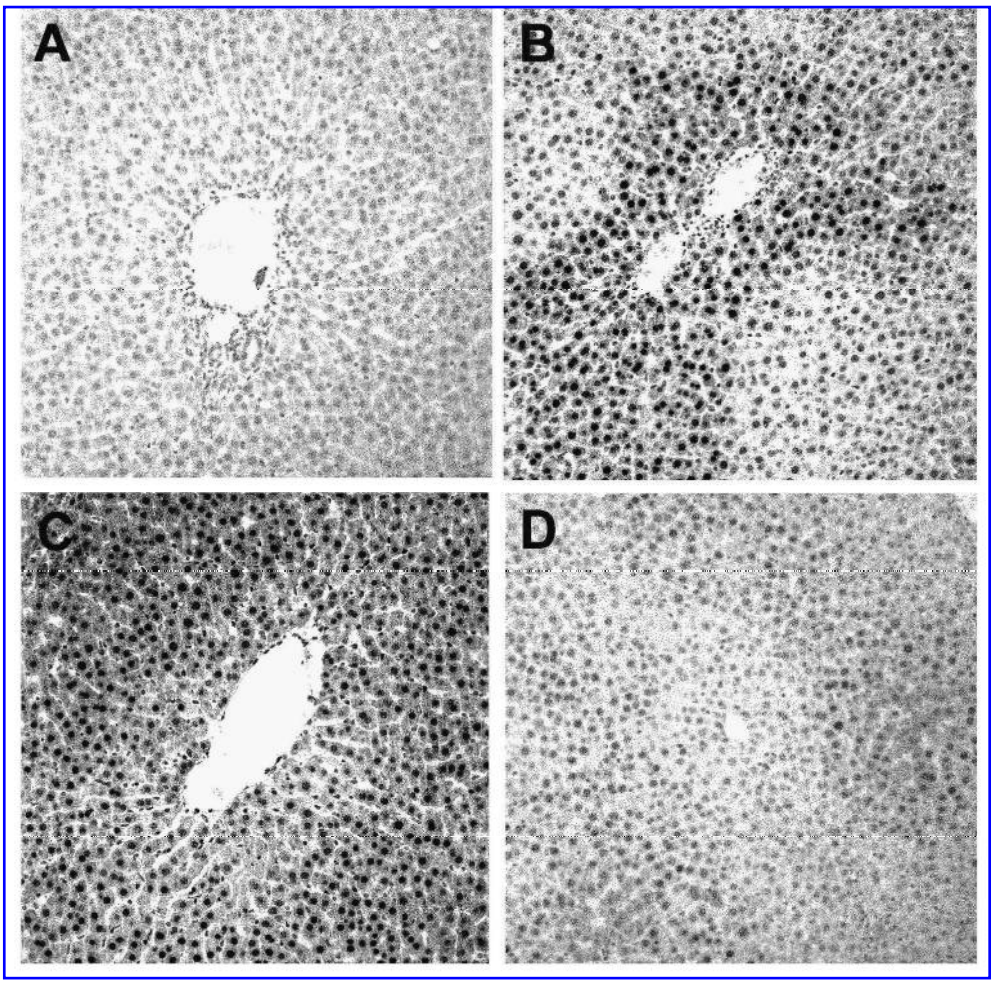

FIG. 7. Cell type-specific and acinar expression pattern of c-Jun immunoreactive protein. Immunohistochemical detection of AP-1 subunit c-Jun was performed in paraffin-embedded dewaxed liver sections using a monoclonal mouse anti-rat c-Jun primary antibody. Samples were obtained from sham animals and from animals subjected to 1, 2, or 3 h of hemorrhagic hypotension with 2 or 1 h of resuscitation or without resuscitation, respectively. No c-Jun immunoreactive protein was detected in sham operated animals (**A**). One hour of hemorrhagic shock/2 h of reperfusion resulted in a profound *de novo* expression of c-Jun immunoreactive protein in nuclei of hepatocytes of the pericentral and midzonal region (**B**), whereas 2 h of hemorrhagic shock/1 h of reperfusion resulted in a *de novo* expression of c-Jun immunoreactive protein in nuclei over the whole acinus (**C**). After 3 h of hemorrhagic hypotension without resuscitation, there was only a weak *de novo* expression of c-Jun in some hepatocytes scattered over the whole acinus (**D**).

Although NFκB is activated in the intact rat liver in the present study by hemorrhage and resuscitation, this activation does not exceed the activation observed after sham operation. Furthermore, the observed activation is weak compared with the activation of NFκB after endotoxemia. Administration of the antioxidants trolox or tempol did not alter the activation of NFκB after hemorrhage and resuscitation. Therefore, the mechanisms of activation of NFκB and its role for the hepatocellular response to hemorrhage and resuscitation are not clear. In the present model, activation is not modulated by reperfusion or antioxidants; therefore, production of reactive oxygen species as a mechanism of activation may be unlikely. In contrast, recent evidence suggests an important functional role of OFR-dependent NFκB activation for survival, tumor necrosis factor-α cytokine response, and vascular reactivity to phenylephrine in a severe model of decompensated hemorrhagic shock. These contradictory results may in part be dependent on the severity of the hemorrhagic shock model, which may also reflect an increased OFR formation after prolonged and severe decompensated hemorrhagic shock (1).

In contrast, activation of AP-1 was observed after 1 or 2 h of hemorrhagic hypotension when hypotension was followed by even short periods of resuscitation. The AP-1 complex seems to be composed of c-Jun, JunD, and c-Fos as assessed by supershift analysis. Consistent with these results on the whole organ level, a profound accumulation of c-Jun immunoreactive protein was observed in nuclei of midzonal and pericentral hepatocytes *in vivo*. Administration of two chemically different antioxidants (trolox or tempol) attenuated activation of AP-1 on the whole organ level and reduced c-Jun

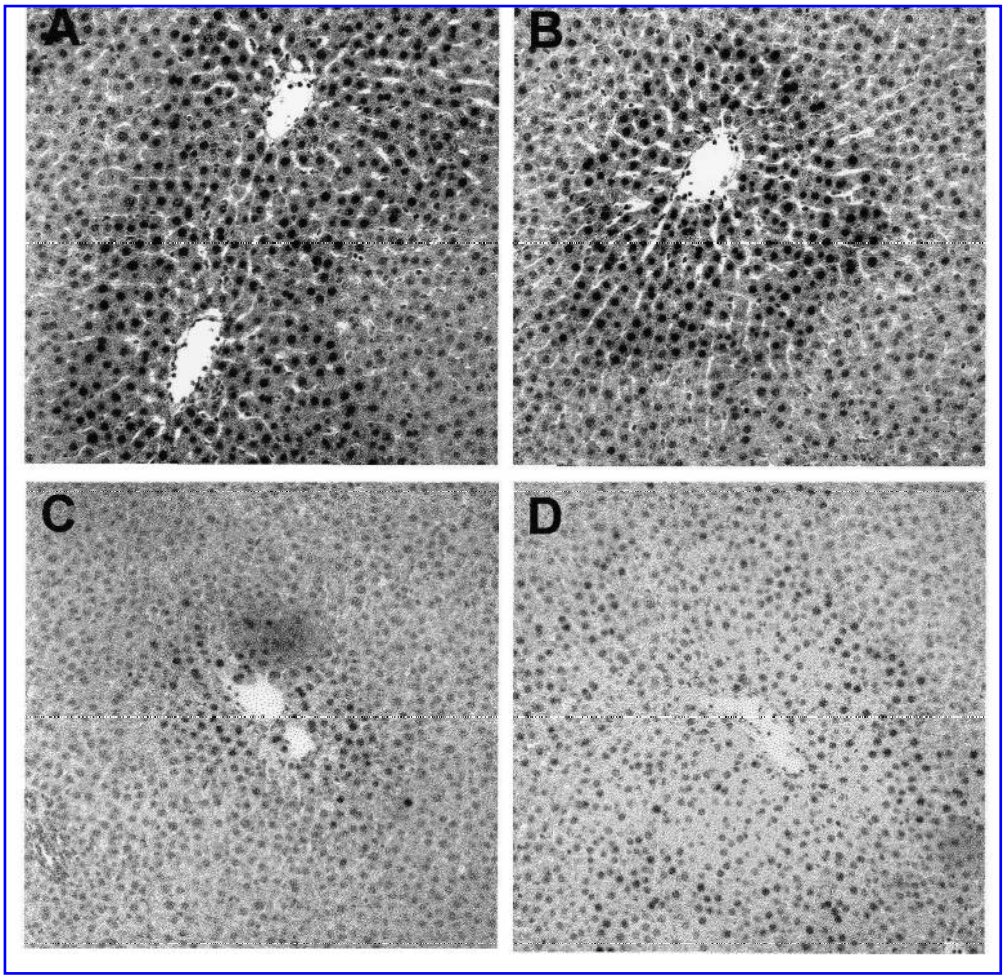

FIG. 8 Expression pattern of c-Jun immunoreactive protein after hemorrhagic shock and resuscitation with or without administration of the antioxidant tempol. The immunohistochemical detection of c-Jun was performed in liver samples obtained in another series of experiments from rats subjected to hemorrhage for 1 h followed by resuscitation for 2 (**A** and **C**) or 5 (**B** and **D**) h. The antioxidant tempol (30 mg/kg b.w. as bolus injection followed by a continuous infusion of 30 mg/kg b.w./h) was administered along with resuscitation fluids at the beginning of the reperfusion period. The profound *de novo* induction of c-Jun in the nuclei of the pericentral and midzonal hepatocytes observed after 1 h of hemorrhage and 2 (**A**) or 5 (**B**) h of reperfusion was markedly attenuated by treatment with tempol (**C** and **D**).

immunoreactive protein after hemorrhage and resuscitation. Hemorrhagic shock without resuscitation did not lead to a detectable AP-1 activity after 1 h, whereas a weak activation of AP-1 and a weak *de novo* synthesis of c-Jun in individual hepatocytes of the whole acinus were observed after 3 h of hemorrhagic hypotension. These data suggest that activation of AP-1 seems to depend on the formation of OFR during reperfusion, leading to a spatial activation pattern in nuclei of midzonal and pericentral hepatocytes, which correlates with the localization of OFR formation. A similar expression pattern has been documented in midzonal and pericentral regions of the acinus after low-flow ischemia in the perfused rat liver (20).

Early results by Schenk *et al.* (18) and Meyer *et al.* (13) suggest that AP-1 is a secondary antioxidant responsive factor. In contrast, recent studies measuring OFR production by electron paramagnetic resonance spectroscopy in human hepatoma cells (HepG2) showed that *tert*-butylhydroquinone leads to a prooxidant activation of AP-1, not supporting the notion that the induction of AP-1 is an antioxidant response (14).

Our results *in vivo* show a significant reduction of the observed activation of AP-1 after hemorrhagic hypotension and resuscitation by the antioxidants tempol and trolox. No AP-1 activation is observed after 1 h of hemorrhage without resuscitation and only a weak activation is observed after 3 h of hemorrhage. Therefore, resuscitation/reperfusion seems to be a critical determinant for AP-1 activity *in vivo,* suggesting that OFR formation may be dependent on reperfusion *in vivo*.

This is in line with a large body of evidence in models of global liver ischemia suggesting intravascular oxidant stress in the liver upon reperfusion (7). In the early phase of reperfusion after ischemia, Kupffer cells were identified as a major source of early postischemic oxidant stress in the liver *in vivo* (9, 11). In addition, accumulation and induction of neu-

trophils within 5–6 h contribute to formation of OFR and liver injury (10, 12).

Hemorrhagic shock leads to a profound decrease in cardiac output and reduction in organ blood flow. This low-flow state results in a decrease in oxygen tension and hypoxia. Especially in the liver, the oxygen partial pressure is subject to a periportal–pericentral oxygen gradient in liver sinusoids already under physiological conditions. Therefore, low-flow ischemia associated with hemorrhagic hypotension results primarily in hypoxia of pericentral localized hepatocytes. This may lead to a decrease in the normally very high antioxidative capacity of hepatocytes (8), rendering the hepatocytes of the midzonal and pericentral region most susceptible to OFR. In line with this concept, the immunohistochemical detection of c-Jun indicated a substantial *de novo* expression of c-Jun immunoreactive protein in parenchymal cells of pericentral and midzonal regions of the liver acinus after hemorrhagic shock and resuscitation, which was attenuated by administration of antioxidants. However, the expression pattern of c-Jun in the liver seems to depend on the duration of low-flow ischemia as well. Short periods of hemorrhagic hypotension (1 h followed by 2 h of resuscitation) induced c-Jun *de novo* synthesis primarily in pericentral and midzonal regions of the acinus, whereas longer periods of hemorrhage (2 h followed by 1 h of resuscitation) resulted in a *de novo* synthesis of c-Jun immunoreactive protein over the whole liver acinus. This would be consistent either with a further shift to the periportal region of depletion of antioxidative properties of hepatocytes or with an increase and shift to the periportal region in intravascular OFR formation after prolonged ischemia and reperfusion. Ischemia alone failed to show a spatial expression pattern of c-Jun, suggesting an important contribution of reperfusion and reoxygenation for the generation of intravascular OFR and supporting the concept of a zonal activation pattern of redox-sensitive transcription factors and reprogramming of gene expression in the liver acinus due to reperfusion and reoxygenation.

In summary, in the present study we assessed the hepatic activation patterns of NFκB and AP-1 in a graded model of low-flow ischemia. Whereas the observed activation of NFκB is weak and not modulated by antioxidants, activation of AP-1 may depend on reactive oxygen species and shows a spatial expression pattern dependent on the extent of ischemia and reperfusion. This activation of AP-1 is inhibitable by antioxidants, requires reperfusion, and shows a spatial expression pattern that may correlate with OFR formation, which has been documented in midzonal and pericentral regions of the acinus in low-flow ischemia, and may lead to a zonal reprogramming of gene expression in the liver 718acinus.

ABBREVIATIONS

AP-1, activator protein-1; BE, base excess; b.w., body weight; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ETX, endotoxin; HR, heart rate; LPS, lipopolysaccharide; MAP, mean arterial pressure; NFκB, nuclear factor κB; OFR, oxygen free radicals; PMSF, phenylmethylsulfonyl fluoride.

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Received for publication July 5, 2001; accepted September 14, 2001.

This article has been cited by:

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