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Tissue Factor Expression in Vital Organs during Murine Traumatic Shock

Role of Transcription Factors AP-1 and NF-κB

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Background: **Tissue factor (TF) is a cell-surface glycoprotein responsible for initiating the extrinsic pathway of coagulation that has been shown to have a role in the pathophysiology of sepsis and reperfusion injury. The purpose of this study was to investigate TF expression in vital organs and to determine possible regulatory mechanisms of TF expression in the lung during traumatic shock in rats.**

Methods: **Noble-Collip drum trauma was induced in anesthetized Sprague-Dawley rats. Anesthetized rats without trauma served as controls. TF activity was measured in plasma and lung tissue. TF messenger RNA (mRNA) was measured in the lung, liver, and small intestine using ribonuclease protection assays. Electromobility shift assays were used to quantify binding of nuclear extracts from lung to TF-specific consensus domains for transcription factors NF-κB and AP-1.**

Results: **TF activity in plasma increased up to 14-fold and +232% in the lung ($P < 0.001$ for plasma and lung) 2 h after trauma. TF mRNA level was significantly increased in the lungs ($P < 0.01$), small intestine ($P < 0.01$), and liver ($P < 0.05$) 1 h after trauma compared to sham-operated control rats. TF mRNA expression continued to increase in the lungs and the liver (both, $P < 0.001$) 2 h after trauma. TF sequence-specific complex binding to AP-1 and NF-κB domains was enhanced in the lungs of trauma rats (+395%, $P < 0.001$ and +168%, $P < 0.001$, respectively).**

Conclusions: **These results suggest that TF may play an important role in the pathophysiology of severe trauma and that regulatory elements AP-1 and NF-κB may be involved in the regulation of TF mRNA expression in traumatic shock. (Key words: Nuclear regulatory factors; procoagulant; trauma.)**

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TISSUE factor (TF) is a cell-surface glycoprotein responsible for initiating the extrinsic pathway of coagulation.[1–4] Although TF is expressed constitutively in nonvascular cells, monocytes and endothelial cells within the vascular compartment do not normally express TF constitutively.[5]

During stimulation by endotoxin (lipopolysaccharide), tumor necrosis factor, and other factors, cells initiate transcription of TF.[6–8] Transcriptional regulation in response to lipopolysaccharide has been localized to a 56–base pair (bp) element in the 5′-region of the human TF gene and requires the transcription factors AP-1 (c-Fos/c-Jun heterodimers) and NF-κB (p65/c-Rel heterodimers).[9,10] Lipopolysaccharide-induced TF mRNA levels were reduced by interleukin 4 (IL-4) and cyclic adenosine monophosphate (cAMP) at the transcriptional level.[11,12]

The expression of TF is also dependent on P-selectin. Purified P-selectin has induced TF expression on monocytes in a concentration-dependent manner by 50- to 100-fold.[5,13] During tissue injury, P-selectin expression on platelets and the vascular endothelium promotes remodeling of the injured vessels.[5,14] Moreover, binding of P-selectin to monocytes in the area of vascular injury may initiate thrombosis by induction of TF expression.[13,15] However, in situations such as trauma, TF is probably induced by stimuli and involves TF generation from cells that do not normally express significant amounts of TF.[16] A naturally occurring inhibitor of TF (TF pathway inhibitor, TFPI) inhibits thromboplastin-induced coagulation, which could potentially inhibit thrombogenesis, thereby diminishing tissue injury.[17–19]

Noble-Collip drum trauma in anesthetized rats is a well-described model of traumatic shock that is characterized by marked microcirculatory perturbation and endothelial dysfunction. The dysfunctional endothelium loses its ability to release nitric oxide, resulting in increased surface expression of endothelial cell adhesion molecules.[20–23] TF activity is increased in the blood and

liver in hepatic ischemia–reperfusion injury in rats.[24] Moreover, elevated plasma TF occurs in trauma and sepsis, leading to intravascular coagulation.[25,26] However, TF gene expression has not been studied in complex cardiovascular disease states, such as traumatic shock, in which multiple organ dysfunction occurs.

The first aim of the current study was to investigate plasma and lung alterations in TF activity and mRNA expression in the lung, small intestine, and liver during traumatic shock in rats. Secondly, using gel-shift assays, we measured the binding activity of AP-1 and NF-κB transcription factors in the lung during murine traumatic shock, which have been shown *in vitro* to play an important role in the regulation of TF promoter activity.

Materials and Methods

Experimental Protocol

Male Sprague-Dawley rats weighing 175–225 g were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) before any experimental procedures. The studies were carried out in accordance with the National Institute of Health's Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Traumatic shock was induced in pentobarbital-anesthetized rats by whole-body trauma administered in a Noble-Collip drum apparatus. Anesthetized rats were subjected to a total of 550 revolutions at a rate of 60 rpm. A carotid artery was cannulated for the determination of arterial blood pressure to confirm the occurrence of traumatic shock, according to established procedures.[22] All trauma rats underwent autopsy to confirm the presence of gross evidence of traumatic injury (*i.e.*, bowel ischemia, serosanguinous ascites, splanchnic vascular engorgement) to the splanchnic viscera. Rats were excluded from the study if these findings were not observed to a significant degree. Less than 10% of rats (*i.e.*, 2 of 24) did not meet these criteria. Noble-Collip drum trauma results in a mortality rate of 90% within 5 h.[22] After 30, 60, and 120 min, the lungs, small intestine, and liver were removed for immediate total RNA extraction. Animals were randomly divided into experimental groups consisting of rats subjected either to trauma or to a sham-trauma protocol. Sham-trauma rats were anesthetized, cannulated, and monitored at the same time as traumatized rats.

RNA Preparation

Total RNA was extracted from the lungs, small intestine, and liver using the acid guanidium-phenol-chloroform extraction method described by Chomczynski and Sacchi.[27] The lungs, small intestine, and liver (200–400 mg pieces) were minced on ice and homogenized in a Polytron tissue homogenizer with 4 ml guanidine isothiocyanate solution (Ultra Pure; Gibco/BRL, Gaithersburg, MD). RNA was precipitated with equal volumes of 2-propanol. RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water.

Quantification of Tissue Factor mRNA by Ribonuclease Protection Assays

We prefer nuclease protection over methods such as Northern blot analysis for a number of reasons.[28] The ribonuclease protection assay is superior for the detection of low-abundance RNAs contained in total RNA, as described previously. The sensitivity of the ribonuclease protection assay comes from the use of a complementary, *in vitro* radiolabeled transcript probe specific for the target mRNA. The probe and total RNA are hybridized in solution, after which the mixture is treated with ribonuclease to degrade all remaining single-stranded (unhybridized) RNA. The hybridized portion of the probe will be protected from digestion and can be visualized after electrophoresis by autoradiography of the mixture in a gel. The plasmid for synthesis of the rat TF probe for ribonuclease protection assays was created by synthesis of a complementary DNA (cDNA) using rat lung total RNA and oligodeoxythymidine followed by cloning. Rat TF cDNA was amplified using forward primer (5′-CTACACTGTTCAGATAAGCGATAG-3′) and reverse primer (5′-GTCCACCCAAATCCCACTTTC-CTG-3′). The 1,077-bp polymerase chain reaction (PCR) fragment of rat TF, containing nucleotides 243–1319 (GenBank accession No. U07619; Bethesda, MD) was cloned using a polymerase chain reaction 2.1-TOPO Cloning Kit (gift from Invitrogen Corp., Carlsbad, CA). For ribonuclease protection assays an *Eco*RI–*Hind*III fragment of rat TF was subcloned in the pBluescript II KS^+ vector (Stratagene, La Jolla, CA). The plasmid, containing fragment of TF cDNA, was digested with Xba I and used as a template for *in vitro* transcription of a 559-base radiolabeled antisense probe that contained a 487-base protected fragment using T3 RNA polymerase (Boehringer Mannheim, Indianapolis, IN) in the presence of ^{32}P-UTP (Amersham Corp., Arlington Heights, IL). All constructs, used in this investigation, were verified by sequencing the insert in the plasmid. The rat TF sequence was found to be 100% identical to the published sequences (GenBank accession No. U07619).

For ribonuclease protection assay, 2 μg total RNA was used for detection of β-actin mRNA, 10 μg was used for detection of TF mRNA in the lung, and 50 μg was used for detection of TF mRNA in the liver and small intestine according to previously described procedures.[29] Expression of mRNA was quantified using storage phosphor technology (Molecular Dynamics, Sunnyvale, CA). Intensity of each TF mRNA band was normalized for β-actin mRNA level (β-actin plasmid obtained from Ambion, Austin, TX).

Tissue Factor Activity in Lung Cytoplasm and Plasma

Tissue factor activity was measured in rat plasma and lung cytoplasm (postmitochondrial supernatant) using a TF activity assay kit (America Diagnostica, Greenwich, CT). This assay is designed for detection of human TF that activates a chromogenic enzyme cleaved from *de novo* factor VIIa/TF (personal oral communication with Dr. R. Hart, America Diagnostica, August 1999, and package insert). This assay is specific for TF; however, rat TF is capable of activating human plasma.[30,31]

Nuclear Extracts and Gel Mobility Shift Assay

Nuclear extracts were prepared from lungs of sham-operated control or trauma rats as described by Andrews and Faller.[32] Binding reactions for electrophoretic mobility shift assays (EMSA) of the transcription factors AP-1, SP-1, and NF-κB were carried out according to previously described procedures.[21] A 30-bp fragment (−226 to −197) of 5′-region of the rat TF gene was used to analyze AP-1 binding.[31,33] This fragment of the TF gene (5′-TGGTGTTGAATCACGGTTGAGTCACCCCTT-3′) contains two (distal and proximal) AP-1 sites [AP-1 (D+P)]. It was created by annealing synthetic oligonucleotides: forward (5′-TGGTGTTGAATCACGGTTGAG-3′) and reverse (3′-GCCAACTCAGTGGGGAA-5′). Three mutant forms of this fragment were used also for analysis of AP-1 binding: mutant 1, containing native proximal and mutated (TGA**CGAC**) distal AP-1 site [AP-1 (P)]; mutant 2, containing native distal and mutated (TGAG**GGC**) proximal site [AP-1 (D)]; and mutant 3, containing mutated AP-1 sites (both). The mutated nucleotides are shown in bold. The NF-κB 22-bp probe (5′-GGGTCTCGGAGTTTCCTACGGG-3′; from −191 to −170 of mouse TF gene) was created by annealing forward (5′-GGGTCTCGGAGTT-3′) and reverse (3′-GAGCCTCAAAGGATGCCC-5′) oligonucleotides. For creation of a mutant form of NF-κB, the same forward oligonucleotide and a mutated reverse oligonucleotide (3′-GAGCCTCAA**TCT**ATGCCC-5′) were used. The SP-1 probe (5′-GGGGCCC**ATT**GGGGCGGGGAGTCGCGA-3′; from −107 to −81 of rat TF gene) was created by annealing of forward (5′-GGGGCCC**ATT**GGGGCGG-3′) and reverse (3′-CCCCCGCCCCTCAGCGCT-5′) oligonucleotides. This probe, with mutations (C**ATT**GGGGCG) in the Egr-1 site was used for analysis of SP-1 binding. A fragment containing mutations in Egr-1 and SP-1 sites was used as a control for nonspecific binding. The radiolabel was introduced at both termini with α[^{32}P]-deoxycytidine triphosphate in the presence of three other cold deoxynucleotide triphosphates. To show the specificity for binding to AP-1, NF-κB, and SP-1 elements, we used mutant probes and antibodies against cRel (C)-G, and NF-κB p65 (C-20) X (both antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA). Nuclear extracts from lungs of control and trauma rats reacted with SP-1 oligonucleotide as further control.

Statistical Analysis

All values are represented as the mean ± SEM. Data comparing all organ TF gene expression percent changes were subjected to analysis of variance followed by the Dunnett method for evaluation of differences between groups. Comparison of individual organ trauma to control TF mRNA was subjected to the Student *t* test for paired observations. Values of $P < 0.05$ were considered statistically significant.

Results

Traumatized rats exhibited mean arterial blood pressures of 56.3 ± 2.8 mmHg 2 h after trauma, whereas the mean arterial blood pressures in sham-trauma rats were 148.8 ± 9.0 mmHg at this time. To verify that the anesthetized rats experienced traumatic shock, intestinal myeloperoxidase (an enzymatic marker for infiltrated leukocytes) and plasma-free amino nitrogen (a marker for plasma proteolysis) activities were determined 30, 60, and 120 min after trauma. All rats subjected to Noble-Collip drum trauma developed signs of shock. Gross evidence of traumatic injury (*i.e.*, bowel ischemia, serosanguinous ascites, splanchnic vascular engorgement) to the splanchnic viscera was found in all rats used in this investigation of trauma rats.

Tissue factor concentration and its TF mRNA expression at the transcriptional level were investigated in trauma and sham-operated control rats. TF activity was measured in plasma and lung extracts 30, 60, and 120 min after trauma. Plasma TF activity was very low in control rats and increased up to 14-fold at 30 and 60 min

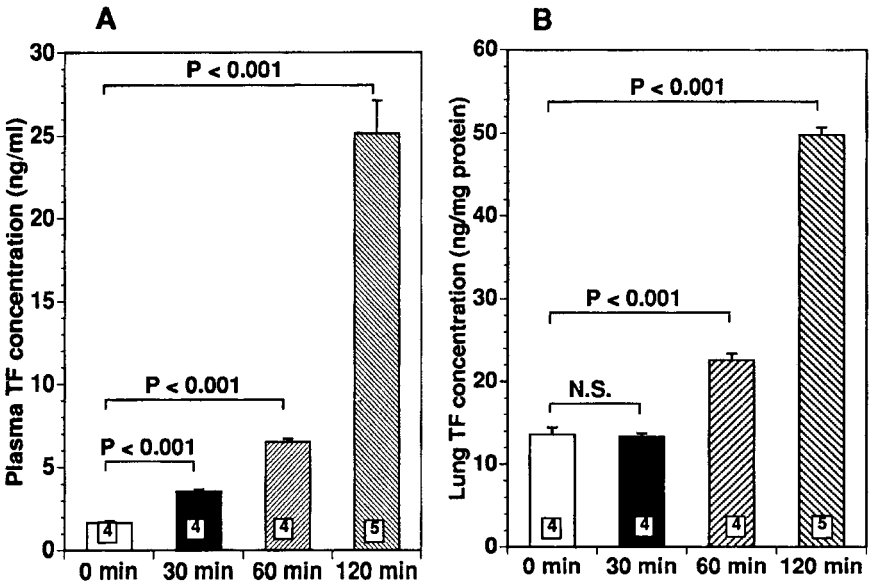

Fig. 1. Tissue factor concentration in plasma (*A*) and lung cytoplasm (*B*) from sham-operated control rats (0 min) and anesthetized rats subjected to Noble-Collip drum trauma 30, 60, and 120 min after trauma. Bar heights represent the mean and brackets indicate ± SEM. Numbers at the bottom of each bar represent the number of rats studied in each group.

and 2 h ($P < 0.001$) after trauma (fig. 1A). In the lung, TF activity did not change at 30 min but increased at 1 and 2 h after trauma compared with sham-operated control rats (fig. 1B).

Tissue factor mRNA expression was also assessed in trauma and sham-operated control rats. There was a marked increase in TF mRNA signal in the lungs and liver 2 h after Noble-Collip drum trauma, compared with the same organs from sham-operated control rats (fig. 2). Control experiments measuring the levels of β-actin transcripts indicate similar mRNA levels in these organs of sham-operated control animals and trauma rats (fig. 2). Quantification of the effect of trauma on TF mRNA expression in the lungs, liver, and small intestine 30, 60, and 120 min after Noble-Collip drum trauma is shown in figures 3 A–C. These data show that TF mRNA slightly increased in the lungs (+34%; $P < 0.01$), small intestine (+40%; $P < 0.01$), and liver (+24%; $P < 0.05$) 1 h after trauma and significantly increased in the lungs and liver (+134 and +139%, $P < 0.001$, respectively) 2 h after Noble-Collip drum trauma, compared with sham-operated anesthetized rats. Much smaller increases in TF mRNA expression were observed in the small intestine 2 h after trauma (+47%; $P < 0.001$, fig. 3C). Although there was an increase in mRNA in the small intestine, there were no significant changes in TF gene expression in the lung and liver 30 min after trauma, compared with control sham-operated animals (figs. 3A and B).

To investigate the possible molecular mechanisms of the regulation of TF gene expression in the lung during traumatic shock, we performed electrophoretic mobility

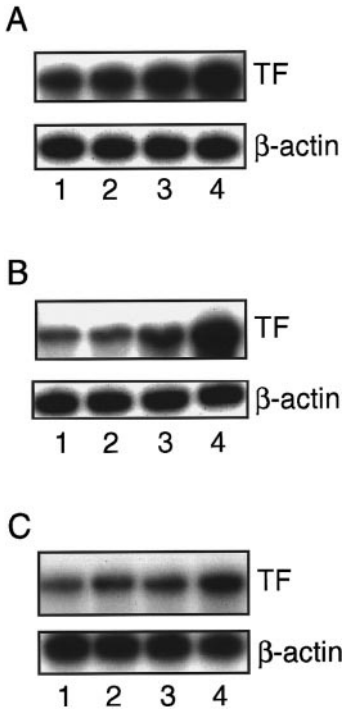

Fig. 2. Representative polyacrylamide gel autoradiograph used in a typical ribonuclease protection assay of tissue factor (TF) and β-actin mRNA in the lung (*A*), liver (*B*), and small intestine (*C*) of (1) sham-operated control and post-trauma rats at (2) 30 min, (3) 60 min, and (4) 2 h. Compared with control values, there is a marked increase in TF mRNA levels in the lung and liver isolated from rats subjected to Noble-Collip drum trauma. Intensities of the TF mRNA bands were normalized to those of β-actin mRNA (lower row of each figure).

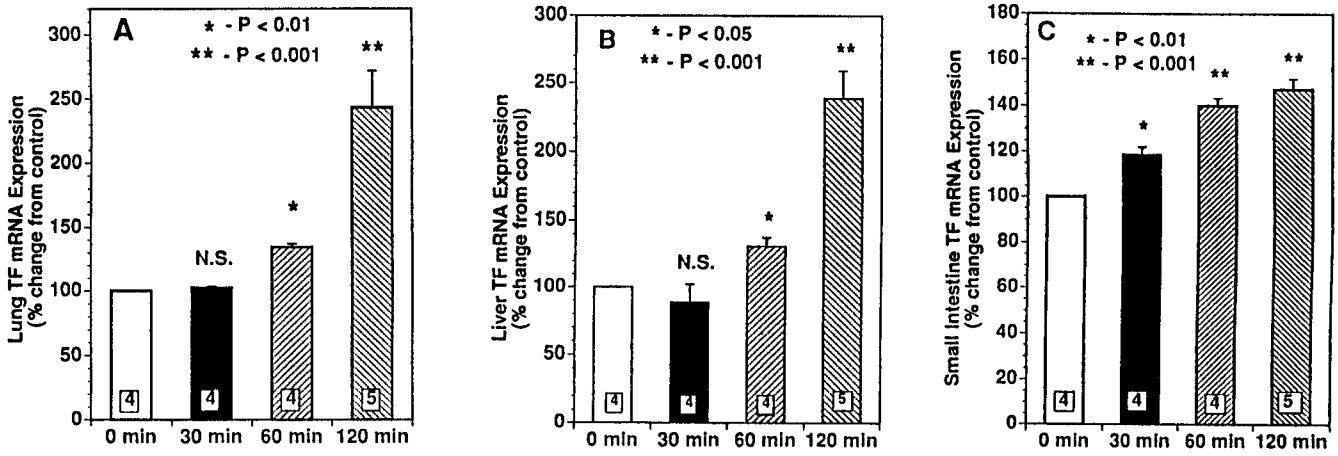

Fig. 3. Quantification of the effect of trauma on tissue factor mRNA in the rat lung (*A*), liver (*B*), and small intestine (*C*) 30, 60, and 120 min after Noble-Collip drum trauma. Control values are shown as 0 min (bars). Total RNA from these rat organs was analyzed by ribonuclease protection assay and quantified using storage phosphor technology. Data were normalized to β-actin mRNA. Bar heights are the mean values obtained from a series of four to five experiments ± SEM. Number of rats studied are indicated at the bottom of the bars.

shift assays using lung nuclear extracts and probes specific for the regulatory region of the rat TF gene. Because the 5′-region of rat TF gene contains two, closely spaced AP-1 elements, an NF-κB (cRel/p65)–binding motif and an SP-1 sequence, nuclear proteins were incubated with AP-1 (D+P), AP-1 (D), AP-1 (P), NF-κB, and SP-1 probes. As shown in figure 4, lung nuclear proteins were bound significantly with the SP-1 probe, but this binding did not change with trauma. In the presence of a 100-fold excess of the unlabeled SP-1 probe, binding of the labeled SP-1 probe was inhibited by competition (data not shown). Sequence-specific binding with a 30-bp AP-1 (D+P) probe containing distal (D) and proximal (P) AP-1 sites was strongly increased in lung nuclear extract from rats subjected to Noble-Collip drum trauma. Binding to this probe was inhibited by competition in the presence of a 100-fold excess of the unlabeled probe. Reasoning that distal or proximal AP-1 sequence elements may be responsible for induction of binding, we used two mutant AP-1 probes for gel-shift analysis. One probe, AP-1 (P), containing mutated distal and native proximal AP-1 sequences, was not bound by nuclear proteins of lung extracts from either control or trauma rats (data not shown).

The AP-1 (D) probe, containing mutated proximal and native distal AP-1 binding motif, bound nuclear proteins from the lung in control and trauma rats (fig. 5). These results showed an increase in binding of nuclear proteins with the AP-1 (D) probe in trauma rats compared with sham-operated control rats when equivalent amounts of protein extracts were used. AP-1 binding was completely inhibited by competition of a 100-fold excess of the unlabeled AP-1 probe (fig. 5). Sequence-specific complex binding to AP-1 (D) domains were enhanced (+395%; $P < 0.001$) in the lung 2 h after trauma (fig. 6). A double mutated AP-1 probe containing mutations in both AP-1 binding motifs was not bound to nuclear proteins from the lung of sham-operated control rats and rats subjected to Noble-Collip drum trauma (data not shown).

The TF-specific NF-κB probe was bound to nuclear proteins from rat lungs of sham-operated control and trauma rats (fig. 7). This NF-κB (cRel/p65) probe exhibits a relatively lower level of rat-lung nuclear protein binding than do p50/p52 (P-selectin specific) or p50/p65 probes (data not shown). Binding of lung nuclear proteins from rats subjected to Noble-Collip drum trauma to the NF-κB probe was increased when equivalent amounts of protein extracts were used (binding with SP-1 probe; fig. 7). Quantification of the effect of trauma on TF-specific NF-κB binding of lung nuclear proteins from control and trauma rats 2 h after Noble-Collip drum trauma showed an increase in binding in trauma compared with sham-operated control (+168%; $P < 0.001$; fig. 6). Specificity of NF-κB regulatory protein binding to the TF-specific NF-κB motif was studied using a probe containing a mutated NF-κB regulatory element and by competitive binding. The labeled mutant NF-κB probe was not bound by lung nuclear proteins from control or trauma rats (fig. 7). The unlabeled NF-κB probe in 100-fold excess was completely inhibited from binding to lung nuclear proteins from control and trauma rats. Bind-

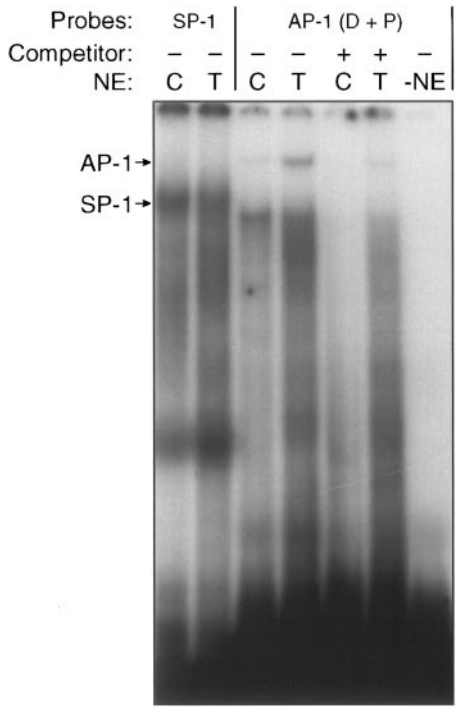

Fig. 4. Specificity of AP-1 binding to the tissue factor AP-1 regulatory element in rat lung nuclear extract (NE) from sham-operated control (C) rats and rats 2 h after they were subjected to Noble-Collip drum trauma (T). Binding reactions were carried out with AP-1 (D + P) probe containing distal (D) and proximal (P) AP-1 sites. Nuclear extracts were incubated during the following conditions from left to right: labeled SP-1 probe, in which binding was not changed in trauma; labeled AP-1 probe, which showed an increase in its binding post-trauma compared with sham-operated controls; unlabeled AP-1 probe in 100-fold excess plus labeled AP-1 probe, in which binding was inhibited by competition; and control lane with no nuclear extract (−NE).

ing of lung nuclear proteins from control and trauma rats with a labeled NF-κB probe was inhibited by an antibody against cRel (data not shown).

Discussion

Traumatic shock is a multifactorial disorder that generates various molecular and cellular mediators.[34] Among these are biologically active lipids, cytokines, endotoxins, and oxygen-derived free radicals. This results in a generalized inflammatory event that is similar to the "systemic inflammatory response syndrome."[35] The primary insult, however, is not caused by sepsis.[22,35] Recently, attention has been focused on leukocyte endothelial interaction and the factors that regulate leukocyte adhesion.[22] Significant nonbacterial inflammatory events occur immediately after Noble-Collip drum trauma that directly affect leukocyte–endothelium cell interaction and lead to endothelial dysfunction. In our traumatic shock model, the time course of endothelial dysfunction occurred within 15–30 min and exhibited reduced endothelium-derived nitric oxide, translocation of P-selectin from the endothelium to the cell surface, and increased leukocyte rolling and adherence.[21,22] One humoral substance that may contribute to increased leukocyte–endothelium interaction is TF. The rapid translocation of P-selectin protein from the Weibel-Palade bodies of vascular endothelial cells and from the α granules of activated platelets may help to explain the in-

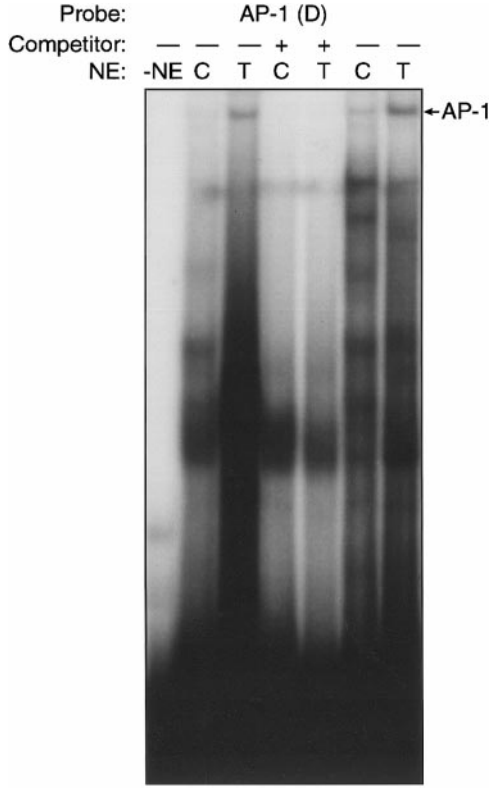

Fig. 5. Specificity of AP-1 binding to the tissue factor AP-1 (distal [D]) regulatory element in rat lung nuclear extract isolated from sham-operated control (C) rats and rats 2 h after Noble-Collip drum trauma (T). Binding reactions were carried out with AP-1 (D) probe containing native distal (D) and mutated (inactive) proximal AP-1 sites. Nuclear extracts were incubated during the following conditions from left to right: Control lane with no nuclear extract (−NE); labeled AP-1 (D) probe, which showed an increase in its binding post-trauma compared with sham-operated controls. Lanes 4 and 5 show complete inhibition by competitor (unlabeled AP-1 probe) in 100-fold excess plus labeled AP-1 probe). Lanes 6 and 7 show labeled AP-1 (D) probe with nuclear extract from other control and trauma rats.

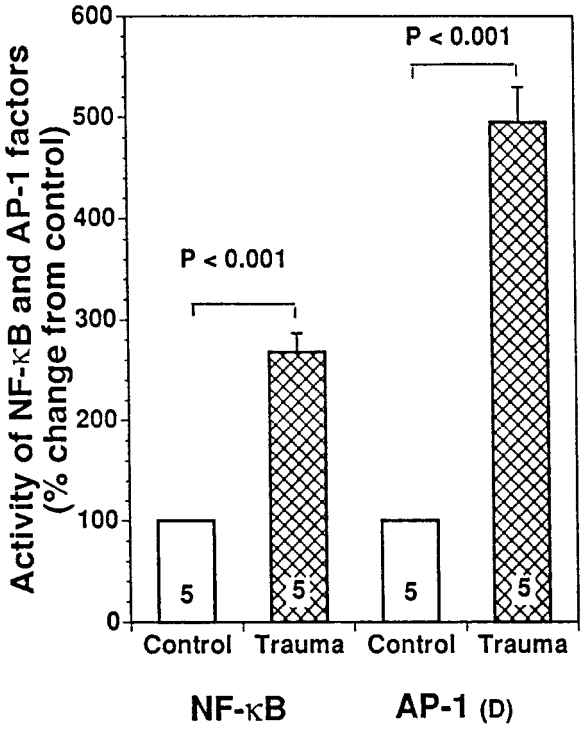

Fig. 6. Quantification of the effect of trauma on tissue factor (TF)–specific NF-κB and AP-1, binding in rat lung nuclear extract 2 h after sham trauma (control). Nuclear extracts from rat lungs were analyzed by electrophoretic mobility shift assays to TF-specific AP-1 (D) and NF-κB and were quantified using storage phosphor technology. Data were normalized to binding of SP-1. Bar heights represent the mean ± SEM. Numbers at the bottom of bars represent the number of rats studied.

crease in TF we observed within the circulation and in various tissues.

Soft tissue trauma, such as that occurring in Noble-Collip drum trauma, results in significantly increased plasma and lung TF activity and up-regulation of TF mRNA expression at the transcriptional level in the lung, the liver, and, to a lesser extent, the small intestine. Plasma TF activity was very low in sham-operated control rats but increased dramatically in trauma rats. Baseline lung TF activity was higher in control rats relative to plasma and increased after trauma to a lesser extent relative to plasma. Similar results in TF activity were observed in hepatic ischemic–reperfusion injury of the rat.[24]

Recently, we showed that P-selectin mRNA expression is significantly increased in the lung during trauma and that this rapid increase is caused by increased binding of P-selectin–specific NF-κB heterodimers (p50/p52) to the P-selectin promoter.[21] A parallel increase in TF and P-selectin mRNA levels in Noble-Collip drum trauma rats suggests that there is a positive correlation between P-selectin and TF gene expression. These results, are consistent with the data of Celi *et al.*,[13] which show that the expression of TF is, at least in part, P-selectin dependent. During tissue injury, P-selectin expression on platelets and stimulated endothelium mediates the binding of leukocytes to the vessel wall and the accumulation of leukocytes within a thrombus.[5] Furthermore, blockade of P-selectin by specific anti–P-selectin antibodies, nitric oxide, or ligand competitors, inhibits the accumulation of leukocytes and the deposition of fibrin within a thrombus.[5,36–39] Purified P-selectin induced TF expression on mononuclear leukocytes in a dose-dependent manner by 50- to 100-fold.[5,13] P-selectin also increased TF mRNA in monocytes and the expression of TF antigen on the monocyte surface. TF expression and procoagulant activity of mononuclear cells were inhibited if monoclonal P-selectin antibody was added to cells be-

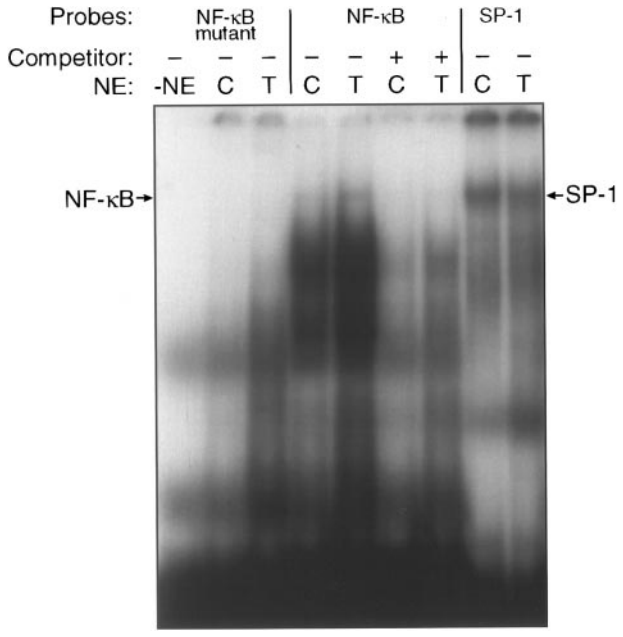

Fig. 7. Specificity of NF-κB binding to the tissue factor NF-κB regulatory element in rat lung nuclear extracts from sham-operated control (C) rats and rats 2 h after Noble-Collip drum trauma (T). Nuclear extracts were incubated during the following conditions from left to right: Control lane with no nuclear extract (−NE); labeled mutated (inactive) NF-κB probe, which did not bind to lung nuclear extract from either trauma or sham-operated control rats. Lanes 4 and 5 show labeled NF-κB probe, which developed an increase in its binding post-trauma compared with sham-operated controls. Lanes 6 and 7 indicate unlabeled NF-κB probe in 100-fold excess plus labeled NF-κB probe in which binding was completely inhibited by competition. Lanes 8 and 9 show results from labeled SP-1 probe in which binding was not changed in trauma rats.

fore P-selectin exposure.[13] The binding of P-selectin to monocytes in the area of vascular injury may initiate thrombosis through induction of TF expression.[13]

In this study, we also analyzed binding of lung nuclear proteins from sham-operated control and trauma rats with AP-1, Egr-1, and NF-κB probes specific for the regulatory region of the rat TF gene using electrophoretic mobility shift assays. Sequence-specific complex binding to AP-1 and NF-κB domains was shown to be significantly enhanced in the lung 2 h after trauma. Because the 5′-region of the rat TF gene contains two, closely spaced AP-1 elements, we incubated the nuclear proteins with different AP-1 probes. Both AP-1 sites include core sequence elements, TGAATCA (distal) and TGAGTCA (proximal), that resemble the consensus binding site of the AP-1 family of transcription factors. Oeth *et al.*[10] demonstrated that the two AP-1 sites bind c-Fos/c-Jun heterodimers in unstimulated and lipopolysaccharide-stimulated human monocytic cells. However, Groupp and Donovan-Peluso[40] demonstrated that lipopolysaccharide induces a change in the AP-1 binding from JunD/Fos in control THP-1 monocytes to c-Jun/Fos and JunD/Fos. Furthermore, Bierhaus *et al.*[16] showed that the distal AP-1 site of the human TF gene was inducible by Jun homodimers, but the proximal AP-1 site was inducible by Jun/Fos heterodimers during tumor necrosis factor α–mediated induction in bovine aortic endothelial cells. It is possible that the molecular mechanism of induction of TF expression may be dependent on the specific inducer and cell type. Reasoning that the distal or the proximal AP-1 sequence element may be responsible for induction of binding, we used two AP-1 probes. One, AP-1 (P), containing mutated distal and native proximal AP-1 sequence, was not bound to nuclear proteins from the lung in control and trauma rats. The AP-1 (D) probe, containing mutated proximal and native distal AP-1 binding motif, was bound by nuclear proteins from the lung of sham-operated control rats and rats subjected to Noble-Collip drum trauma. These results clearly showed that the distal AP-1 site is responsible for induction of nuclear protein binding and, possibly, induction of TF gene expression in the lung in trauma. Bierhaus *et al.*[16] reported that the proximal site participates in tumor necrosis factor α–mediated induction of endothelial TF gene expression. At the distal AP-1 site, only minor induction of binding activity was observed during tumor necrosis factor α–mediated induction. However, there are data that maximal lipopolysaccharide induction of the TF promoter in human monocytic cells necessitated both the AP-1 site and the κB site within the lipopolysaccharide response element.[10,41] Therefore, it appears that the incipient stimulus is important in determining how the two AP-1 TF binding sites are activated and bound.

Activation of TF gene expression by lipopolysaccharide may also be mediated by IκBα because agents that block the nuclear translocation of c-Rel/p65 heterodimers by preventing IκBα phosphorylation or proteolytic degradation inhibits lipopolysaccharide-induced TF gene transcription in monocytic cells.[41]

The demonstration of increased AP-1 and NF-κB binding offers a potential explanation for the increase in TF mRNA expression in trauma. We found significant increases in the binding of the transcription factors, AP-1 and NF-κB, from lung nuclear extracts of trauma rats consistent with this possibility.

In conclusion, our study provides strong evidence that TF mRNA expression and TF activity are increased in the lung, liver, and small intestine during traumatic shock in rats. This enhanced TF activity and mRNA expression, occurring within 1 to 2 h, was accompanied by induction of P-selectin gene expression at the transcriptional level and may play a significant pathophysiologic role in the early thrombosis of severe trauma. We also demonstrated by electrophoretic mobility shift assays using lung nuclear extracts that the AP-1 and NF-κB binding to the TF specific promoter is induced after trauma and may be important for TF gene expression in trauma conditions. Further investigations of the mechanisms by which trauma affects TF expression and the biologic significance of trauma-induced alteration in TF expression are needed. Specifically, the relation among TF, leukocyte–endothelium cell interaction, and modulators of cell adhesion related to shock warrants further study.

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References

1. Mackman N: Regulation of the tissue factor gene. FASEB J 1995; 9:883–9

2. Edgington TS, Mackman N, Brand K, Ruf W: The structural biology of expression and function of tissue factor. Thromb Haemost 1991; 66:67–79

3. Østerud B: Tissue factor: A complex biological role. Thromb Haemost 1997; 78:755–8

4. Ruf W, Edgington TS: Structural biology of tissue factor, the initiator of thrombogenesis in vivo. FASEB J 1994; 8:385–90

5. Furie B, Furie BC: P-selectin induction of tissue factor biosynthesis and expression. Haemostasis 1996; 26(suppl 1):60–5

6. Ahern SM, Miyata T, Sadler JE: Regulation of human tissue factor expression by mRNA turnover. J Biol Chem 1993; 268:2154–9

7. Gregory SA, Morrissey JH, Edgington TS: Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin. Mol Biol Cell 1989; 9:2752–5

8. Yamamoto K, Loskutoff DJ: Fibrin deposition in tissues from endotoxin-treated mice correlates with decreases in the expression of urokinase-type but not tissue-type plaminogen activator. J Clin Invest 1996; 97:2440–51

9. Mackman N, Brand K, Edgington TS: Lipopolysaccharide-mediated transcriptional activation of the tissue factor gene in THP-1 monocytes requires both activator protein 1 and nuclear factor kappa B binding site. J Exp Med 1991; 174:1517–22

10. Oeth P, Parry GCN, Mackman N: Regulation of the tissue factor gene in human monocytic cells: Role of AP-1, NF-kappaB/Rel, and SP-1 proteins in uninduced and lipopolysaccharide-induced expression. Arterioscler Thromb Vasc Biol 1997; 17:365–74

11. Ollivier V, Houssaye S, Ternisien C, Elbim C, Leon A, de Verneuil H, Mackman N, Edgington TS, de Prost D: Endotoxin-induced tissue factor mRNA in human monocytes is negatively regulated by a cyclic AMP-dependent mechanism. Blood 1993; 81:973–9

12. Ramani M, Ollivier V, Ternisien C, Vu T, Elbim C, Hakim J, de Prost D: Interleukin 4 prevents the induction of tissue factor mRNA in human monocytes in response to LPS or PMA stimulation. Br J Haematol 1993; 85:462–8

13. Celi A, Pellegrini G, Lorenzet R, De Blasi A, Ready N, Furie BC, Furie, B: P-selectin induces the expression of tissue factor on monocytes. Proc Natl Acad Sci U S A 1994; 91:8767–71

14. Lefer AM: Selectins: Vital vasculotropic vectors involved in vascular remodeling. Circulation 1997; 96:3258–60

15. Weyrich AS, Elstad MR, McEver RP, McIntyre TM, Moore KL, Morrissey JH, Prescott SM, Zimmerman GA: Activated platelets signal chemokine synthesis by human monocytes. J Clin Invest 1996; 97: 1525–34

16. Bierhaus A, Zhang Y, Deng Y, Mackman N, Quehenberger P, Haase M, Luther T, Mueller M, Boehrer H, Greten J, Martin E, Baeuerle PA, Waldherr R, Kisiel W, Ziegler R, Stern DM, Nawroth PP: Mechanism of the tumor necrosis factor alpha-mediated induction of endothelial tissue factor. J Biol Chem 1995; 270:26419–32

17. Asada Y, Marutsuka K, Hatakeyama K, Sato Y, Hara S, Kisanuki A, Sumiyoshi A: The role of tissue factor in the pathogenesis of thrombosis and atherosclerosis. J Atherosclr Thromb 1998; 4:135–9

18. Broze GJ Jr: Tissue factor pathway inhibitor and the current concept of blood coagulation. Blood Coagul Fibrinolysis 1995; 6(suppl 1):S7–13

19. Koudsi B, Yu CD, Ferguson EW Jr, Miller GA, Merkel KD, Wun TC, Kraemer, BA. Prevention of spinal cord injury after transient aortic clamping with tissue factor pathway inhibitor. Surgery 1996; 119: 269–74

20. Lefer AM, Lefer DJ: Pharmacology of the endothelium in ischemia-reperfusion and circulatory shock. Annu Rev Pharmacol Toxicol 1993; 33:71–90

21. Armstead VE, Minchenko AG, Campbell B, Lefer AM: P-selectin is upregulated in vital organs during murine traumatic shock. FASEB J 1997; 11:1271–9

22. Scalia R, Pearlman S, Campbell B, Lefer AM: Time course of endothelial dysfunction and neutrophil adherence and infiltration during murine traumatic shock. Shock 1996; 6:177–82

23. Rockey DC, Chung JJ: Reduced nitric oxide production by endothelial cells in cirrhotic rat liver: Endothelial dysfunction in portal hypertension. Gastroenterology 1998; 114:344–51

24. Kobayashi Y, Yoshimura N, Nakamura K, Yamagishi H, Takahiro O: Expression of tissue factor in hepatic ischemic-reperfusion injury of the rat. Transplantation 1998; 66:708–16

25. Gando S, Kameue T, Nanzaki S, Hayakawa T, Nakanishi Y: Participation of tissue factor and thrombin in posttraumatic systemic inflammatory syndrome. Crit Care Med 1997; 25:1820–6

26. Nakagawa K, Zhang Y, Tsuji H, Yoshizumi M, Kasahara T, Nishimura H, Nawroth PP, Nakagawa T: The angiogenic effect of tissue factor on tumors andn wounds. Semin Thromb Hemost 1998; 24:207–10

27. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–9

28. Smith CP: Northern analysis versus nuclease-protection mapping, Methods for mapping transcribed DNA sequences, Essential Molecular Biology: A Practical Approach, 1st edition. Edited by Brown TA, Rickwood D, Hames BD. New York. Oxford University Press, 1993, pp 238–41

29. Minchenko AG, Bauer T, Salceda S, Caro J: Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. Lab Invest 1994; 71:374–9

30. Mackman N, Fowler BJ, Edgington TS, Morrissey JH: Functional analysis of the human tissue factor promoter and induction by serum. Proc Natl Acad Sci U S A 1990; 87:2254–8

31. Taby O, Rosenfield CL, Bogdanov V, Nemerson Y, Taubman MB: Cloning of the rat tissue factor cDNA and promoter: Identification of a serum-response region. Thromb Haemost 1996; 76:697–702

32. Andrews NC, Faller DV: A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 1991; 19:2499

33. Mackman N: Regulation of tissue factor gene. Thromb Haemost 1997; 78:747–54

34. Galvin MJ, Sollott SJ, Lefer AM: Mechanism of the protective effect of conditioning in rats subjected to Noble-Collip drum trauma. Circ Shock 1979; 6:23–9

35. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ: Definition for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest 1992; 101:1644–55

36. Yago K, Zenita K, Ginya H, Sawada M, Ohmori K, Okuma M, Kannagi R, Lowe JB: Expression of alpha-(1,3)-fucosyltransferases which synthesize sialyl Le(x) and sialyl Le(a), the carbohydrate ligands for E-and P-selectins, in human malignant cell lines. Cancer Res 1993; 53:5559–65

37. Gauthier TW, Scalia R, Murohara T, Guo JP, Lefer AM: Nitric oxide protects against leukocyte-endothelium interactions in the early stages of hypercholesterolemia. Arterioscler Thromb Vasc Biol 1995; 15:1652–9

38. Skurk C, Nuss C, Lefer AM: Beneficial effecfts of oligotide, a novel oligodeoxyribonucleotide, in murine traumatic shock. Shock 1995; 3:13–20

39. Skurk C, Buerke M, Guo JP, Paulson J, Lefer AM: Sialyl Lewis^x-containing oligosaccharide exerts beneficial effect in murine traumatic shock. Am J Physiol 1994; 267:H2124–31

40. Groupp ER, Donovan-Peluso M: Lipopolysaccharide induction of THP-1 cells activates binding of c-Jun, Ets, and Egr-1 to the tissue factor promoter. J Biol Chem 1996; 271:12423-30

41. Oeth P, Yao Y, Fan ST, Mackman N: Retinoic acid selectively inhibits lipopolysaccharide induction of tissue factor gene expression in human monocytes. Blood 1998; 91:2857–65