Antithrombin Reduces Inflammation and Microcirculatory Perfusion Failure in Closed Soft-Tissue Injury and Endotoxemia

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Background: Closed soft-tissue trauma leads to activation of the coagulation cascade and is often complicated by systemic inflammation and infection. Previous investigations have shown potent anti-inflammatory properties of antithrombin. We herein report on the action of antithrombin on skeletal muscle injury in experimental endotoxemia.

Materials and Methods: By using a pneumatically driven computer-controlled impact device, closed soft-tissue trauma was applied on the left hind limb of pentobarbital-anesthetized rats. Six hours later, endotoxemia was induced by intraperitoneal injection of *Escherichia coli* lipopolysaccharide. An equivalent volume of physiological saline was given in controls. At the same time point, treatment of animals was started by intravenous injection of antithrombin (250 IU/kg body weight) or vehicle solution. Twenty-four hours after trauma, the extensor digitorum longus muscle was microsurgically exposed and analyzed by means of high-resolution multifluorescence microscopy.

Results: Traumatic soft-tissue injury with additional endotoxemia was characterized by nutritive perfusion failure (functional capillary density: 379 ± 20 cm/cm^2), tissue hypoxia (nicotinamide adenine dinucleotide autofluorescence: 77 ± 4 aU), and enhanced leukocyte-endothelial cell interaction (773 ± 35 cells/mm^2). Therapeutic intervention with antithrombin 6 hrs after trauma restored nutritive perfusion and tissue oxygenation (functional capillary density: 469 ± 22 cm/cm^2; nicotinamide adenine dinucleotide autofluorescence: 61 ± 5 aU [$p < 0.05$]) and reduced inflammatory leukocyte adherence (237 ± 20 cells/mm^2 [$p < 0.05$]) toward values found in nontraumatized controls (functional capillary density: 573 ± 13 cm/cm^2; nicotinamide adenine dinucleotide autofluorescence: 56 ± 2 aU; leukocyte adherence: 204 ± 20 cells/mm^2).

Conclusion: Antithrombin ameliorates microcirculatory dysfunction and tissue injury in traumatized animals during endotoxemia. Furthermore, a reduced inflammatory cell response helps to prevent leukocyte-dependent secondary tissue injury. (*Crit Care Med* 2013; 41:867–873)

Key Words: antithrombin; endotoxemia; lipopolysaccharide; microcirculation; nicotinamide adenine dinucleotide fluorescence; trauma.

Among the factors influencing the outcome of complex injuries to the extremities, the severity of the associated soft-tissue trauma is still one of the most decisive prognostic determinants. Due to the alarming frequency of high-velocity/high-energy traffic victims, the prevalence of severe soft-tissue trauma still continues to increase. Extensive trauma leads to activation of the coagulation cascade and is often complicated by systemic inflammation and infection leading to multiple organ dysfunction syndrome (MODS). Sepsis-induced MODS represents the leading cause of death in postoperative and post-traumatic ICUs (1). Modulation of the coagulatory response following trauma and sepsis allows one to affect key features of the pathogenesis of septic shock (2, 3). Antithrombin III, a plasma-derived serine protease inhibitor, controls the activity of thrombin and other proteases of the coagulation cascade (4, 5). Furthermore, antithrombin has previously shown a large number of anti-inflammatory actions, independent of its anticoagulatory effects (6–8). It is supposed that antithrombin interacts with endothelial cells, thus inhibiting leukocyte-endothelial cell interaction. On the other hand, a direct action of antithrombin on leukocyte activation with inhibition of the release of proinflammatory mediators is discussed (5).

To date, however, it has not been investigated whether administration of antithrombin is indeed of therapeutic benefit for traumatized soft tissue. Thus, the aim of our study

was to assess the effects of antithrombin on microvascular nutritive perfusion and local inflammatory cell response in skeletal muscle in a model of closed soft-tissue injury and endotoxemia.

MATERIALS AND METHODS

Animal Model

The experimental protocol was approved by the local animal rights protection authorities and followed the National Institutes of Health guidelines for the care and use of laboratory animals. A total of 35 male Sprague-Dawley rats (body weight [bw]: 250–300 g) were anesthetized with 6% pentobarbital sodium (55 mg/kg bw IP; Narcoren; Merial, Hallbergmoos, Germany). By means of a pneumatically driven and computer-controlled impact device, a standardized soft-tissue trauma was induced in 28 animals on the lateral compartment of the left hind limb, simulating high-velocity trauma of the lower extremity (9). The controlled-impact technique was initially developed as a model of experimental traumatic brain injury in rats, reproducing the pathophysiologic and morphologic responses of severe closed head injury as found in humans (10, 11). The controlled-impact device consists of a compressed nitrogen gas source, an adjustable impactor, a displacement transducer, and a personal computer-assisted interface for data transmission and analysis of time/displacement parameter of the impact. The impact parameters were selected to an impact velocity of 7 m/s, a deformation depth of 11 mm, and an impact duration of 100 msec with an impactor diameter of 10 mm. The left hind limb was placed in a plastic mold (Technovit; Kulzer, Wertheim, Germany) shaped like the hind limb to guarantee optimal energy transmission to the tissue by avoiding hind limb movement during the impact.

Six hours after trauma induction, animals underwent a second hit by intraperitoneal injection of *Escherichia coli* lipopolysaccharide (10 mg/kg bw IP; serotype 128:B12, Sigma, Taufkirchen, Germany) (*trauma/lipopolysaccharide*; n = 7) or the equal volume of saline for control (*trauma*; n = 7). Subsequent to lipopolysaccharide or saline exposure, animals were treated with antithrombin (250 IU/kg bw [Atenative, Octapharma, Langenfeld, Germany]; *therapy/lipopolysaccharide*; n = 7 and *therapy*; n = 7). Following the identical protocol, nontraumatized animals served as controls (*sham*; n = 7). Twenty-four hours after trauma induction, all animals were anesthetized again and placed on a heating pad for maintenance of body temperature at 37°C. Following tracheotomy, animals were mechanically ventilated (tidal volume: 1 mL/100 g bw; 50 breaths/min). Catheters (PE-50; Portex, Hythe, Kent, UK) were placed in the right carotid artery and left jugular vein for continuous monitoring of central hemodynamics (Sirecust; Siemens, Germany).

The left extensor digitorum longus (EDL) muscle was microsurgically prepared to allow direct access for in vivo high-resolution multifluorescence microscopy. The preparation technique was first described by Tyml and Budreau (12) and modified for in vivo microscopy by our group (9). During preparation, tissues were superfused with 37°C warm physiological saline solution to prevent drying. After final exposure of the EDL muscle, tissue was covered with a cover glass.

After baseline recordings (inclusion criteria—mean arterial blood pressure: 100–110 mm Hg, hematocrit: 45%–50%, pCO_2: 35–40 mm Hg, and pH: 7.35–7.45) and a 20-min stabilization period after completion of surgery, in vivo microscopy of the EDL muscle was performed. At the end of the experiments, animals were killed by exsanguination. Muscle tissue was sampled for subsequent histology and immunohistochemistry.

In Vivo Fluorescence Microscopy. For in vivo analysis of the muscle tissue, the surface of the EDL muscle was sequentially scanned from the distal to the proximal part. To reliably assess the microcirculation in the muscle, microscopic images from ten observation areas in each muscle were recorded (9). After intravenous injection of fluorescein isothiocyanate–labeled dextran (15 mg/kg bw; Sigma, Deisenhofen, Germany) and rhodamine 6G (0.15 mg/kg bw; Sigma), in vivo microscopy was performed using a Nikon microscope (E600-FN, Nikon, Tokyo, Japan) equipped with a 100 W mercury lamp and filter sets for blue (excitation/emission: 465–495 nm or > 505 nm), green (510–560 nm or > 575 nm), and ultraviolet (340–380 nm or > 400 nm) epi-illumination. By use of water-immersion objectives (×20/0.75 W and ×40/0.80W, Nikon), final magnifications of ×306 and ×630 were achieved. Images were recorded by means of a charge-coupled device video camera (FK 6990-IQ-S, Pieper, Schwerte, Germany) and transferred to an S-VHS video system for subsequent offline analysis. For analysis of apoptotic cell death, in vivo staining of myocyte nuclei was achieved by intravenous injection of bisbenzimide (10 µmol/kg bw; Hoechst 33342, Sigma).

Microcirculatory Analysis. For quantitative offline analysis, a computer-assisted microcirculation image analysis system was used (CapImage v7.4, Zeintl, Heidelberg, Germany). As previously described (9, 13, 14), functional capillary density was defined as the total length of red blood cell–perfused capillaries per observation area and given in cm/cm². With use of densitometric techniques, macromolecular leakage was assessed in several fields per image and expressed as the ratio of fluorescence intensity selected from the perivascular area to the corresponding intravascular area (plasma gaps in the microvessels between erythrocytes [15]). For assessment of leukocyte-endothelial cell interaction in postcapillary venules, flow behavior of leukocytes was analyzed with respect to free floating, rolling, and adherent leukocytes. Rolling leukocytes were defined as those cells moving along the vessel wall at a velocity less than 40% of that of leukocytes at the centerline and were expressed as a percentage of the total leukocyte flux. Venular leukocyte adherence was defined as the number of leukocytes not moving or detaching from the endothelial lining of the venular vessel wall during an observation period of 20 s. Assuming cylindrical microvessel geometry, leukocyte adherence was expressed as nonmoving cells per endothelial surface (n/mm²), calculated from the diameter and length of the vessel segment analyzed. In postcapillary venules, centerline red blood cell velocity was determined using the line shift method

(CapImage, Dr. Zeintl Software, Heidelberg, Germany). To minimize bias in selection of venules with respect to the local leukocyte-endothelial cell interaction, postcapillary venules were chosen during visualization in blue light epi-illumination, which does not allow visualizing leukocyte flow behavior. Thereafter, filter set was changed to green light epi-illumination for assessment of inflammatory leukocyte response. Reduced nicotinamide adenine dinucleotide (NADH) fluorescence of skeletal muscle tissue was densitometrically assessed after 2 s of ultraviolet epi-illumination by computer-assisted gray level determination (16). To avoid interference of gray levels with microvascular structures, analysis was strictly limited to the intercapillary space. Within ten observation fields of skeletal muscle, apoptotic cell death was assessed by counting the number of bisbenzimide-stained cells that showed apoptosis-associated condensation, fragmentation, and crescent-shaped formation of their nuclear chromatin (17, 18).

Laboratory Analysis

Arterial blood samples were withdrawn for analysis of blood gases (Rapidlab 348, Bayer Vital, Fernwald, Germany) and blood cell count using a Coulter counter (AcTdiff, Coulter, Hamburg, Germany).

Histology and Immunohistochemistry. At the end of each experiment, EDL muscle tissue was fixed in 4% phosphate-buffered formalin for 2–3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 4-μm sections were cut and exposed to an apoptosis-specific staining kit (indirect in situ DNA nick-end labeling [terminal deoxynucleotidyl transferase dUTP nick-end labeling, TUNEL] assay, ApopTag; Chemicon International Inc., Temecula, CA) according to the manufacturer's instructions. Quantitative analysis was performed by counting the number of TUNEL positive tissue–confined cells in 50 consecutive high-power fields (HPF; ×400 magnification). For quantitative assessment of leukocyte tissue infiltration, tissue specimens were stained by the AS-D chloroacetate esterase technique. Leukocytes were identified by positive staining and morphology in 50 HPF in a blinded fashion using a light microscopy (×40 objective) and recorded by the number of cells/HPF.

Statistical Analysis. Results were given as mean ± SEM. After proving the assumption of normality (Kolmogorov-Smirnov), comparisons between the experimental groups were performed by a univariate analysis of variance, followed by the Bonferroni post hoc multiple comparison procedure (SigmaStat, Jandel, San Rafael, CA). To assess the correlation between different microcirculatory parameters, linear regression analysis was used. Statistical significance was set at $p < 0.05$.

RESULTS

Systemic Hemodynamics

Animals that underwent local soft-tissue trauma and endotoxemia revealed increased heart rate and pronounced thrombocytopenia when compared with non-lipopolysaccharide-exposed and sham-operated animals. No changes were observed for hemoglobin, leukocyte count, electrolytes, and blood clotting parameters (**Table 1**).

TABLE 1. Systemic Hemodynamics and Blood Cell Count and Clotting Parameters

	Sham	Trauma	Trauma/ Lipopolysaccharides	Therapy	Therapy/ Lipopolysaccharides
Hemodynamics					
Mean arterial blood pressure (mm Hg)	109±7	111±10	111±7	107±4	99±7
Heart rate (beats/min)	364±8	377±17	420±11[a]	382±17	417±8[a]
Blood parameters					
Hemoglobin (mmol/L)	8.8±0.3	8.3±0.2	8.6±0.2	8.0±0.3	8.4±0.1
Hematocrit (%)	43±4	42±1	42±1	41±4	40±1
Thrombocytes (10^9/L)	752±61	776±70	160±66[a–c]	694±118	157±53[a–c]
Leukocytes (10^9/L)	8.5±0.7	8.3±0.9	11.1±2.2	11.4±1.8	10.3±1.3
Na^+ (mmol/L)	139±3	139±1	140±1	140±1	139±1
K^+ (mmol/L)	3.9±0.4	3.9±0.1	4.0±0.3	4.1±0.1	3.8±1.6
Thromboplastin time (%)	149±1	141±5	135±6	135±1	135±0
Partial thromboplastin time (s)	26.0±3.4	22.3±0.9	30.2±3.7	29.4±1.3	29.4±1.3

Values are given as mean ± SEM. Blood samples were withdrawn at 24 h after soft-tissue trauma.
[a] $p < 0.05$ vs. sham.
[b] $p < 0.05$ vs. trauma.
[c] $p < 0.05$ vs. therapy.

Microvascular Perfusion

Closed soft-tissue trauma and trauma with subsequent endotoxemia caused a tremendous impairment of functional capillary density with values less than 400 cm/cm². Treatment with antithrombin restored nutritive capillary perfusion in endotoxemic animals (therapy/lipopolysaccharide: 469 ± 22 cm/cm²) and nonendotoxemic animals (therapy: 438 ± 29 cm/cm²), however, not reaching physiologic baseline values (**Fig. 1A**). In line with nutritive perfusion failure, significantly increased NADH autofluorescence of skeletal muscle tissue was observed after closed soft-tissue injury (trauma: 80 ± 2 aU) and after subsequent endotoxemia (trauma/lipopolysaccharide: 77 ± 4 aU), indicating pronounced tissue hypoxia. In contrast, animals treated with antithrombin exhibited significant reduction of tissue NADH (therapy: 57 ± 3 aU; therapy/lipopolysaccharide: 61 ± 5 aU), further underlining the concomitant improvement of capillary perfusion (Fig. 1B). Regression analysis revealed a significant inverse correlation between functional capillary density and NADH autofluorescence of skeletal muscle tissue with a regression coefficient of $r = -0.51$ ($p < 0.05$). Macromolecular leakage increased upon trauma and endotoxemia. Therapeutic intervention with antithrombin was accompanied by a significantly reduced macromolecular leakage, as found in nontraumatized animals (**Fig. 2**). Capillary diameters and red blood cell velocity did not significantly differ among the groups, indicating comparable microhemodynamic conditions.

Inflammatory Cell Response

Soft-tissue trauma alone as well as in combination with endotoxemia provoked a marked inflammatory cell response with leukocytes rolling along and firmly attaching (**Table 2**) to the venular endothelium. Antithrombin effectively limited the inflammatory response with reduced numbers of leukocytes interacting with the postcapillary venular endothelium after both trauma and trauma with the subsequent endotoxic hit (Table 2). In line with in vivo fluorescence microscopic analysis of leukocyte-endothelial cell interaction, histology of traumatized muscle tissue revealed an inflammatory cell infiltration,
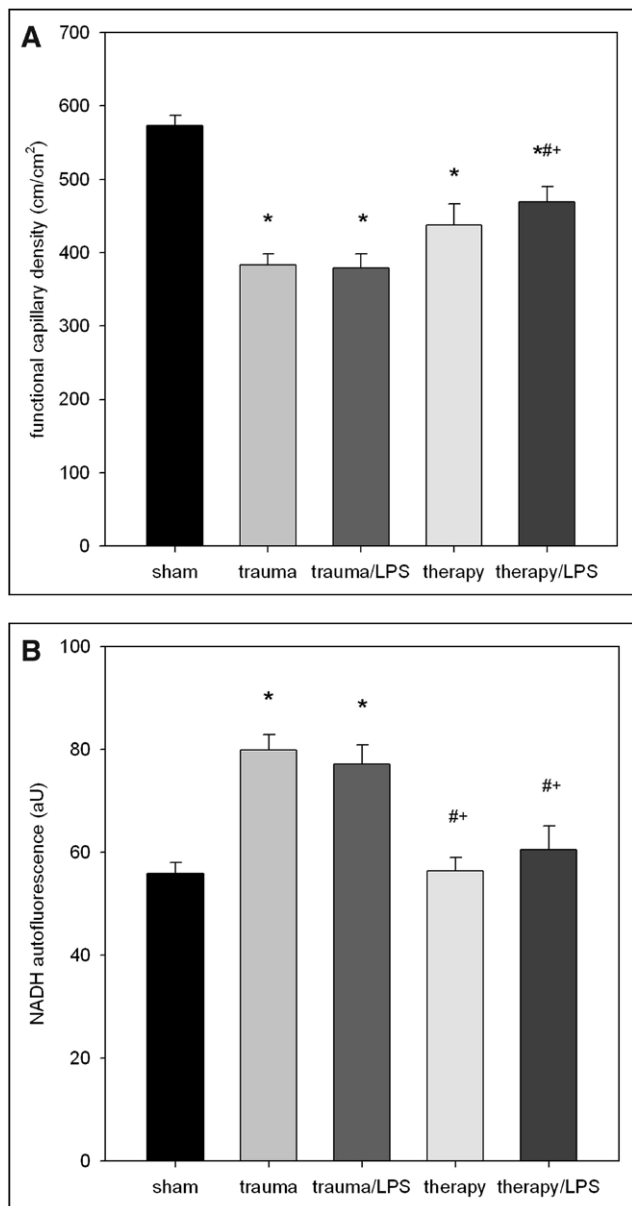

Figure 1. Functional capillary density (**A**) and nicotinamide adenine dinucleotide (NADH) autofluorescence (**B**) of skeletal muscle tissue at 24 hrs after soft-tissue trauma and subsequent exposure to lipopolysaccharide (LPS). Animals were treated either with antithrombin (therapy; therapy/LPS) or vehicle solution (trauma; trauma/LPS). Sham-operated animals without trauma and endotoxemia (sham) served as controls. Values are given as mean ± SEM. *$p < 0.05$ vs. sham; #$p < 0.05$ vs. trauma, +$p < 0.05$ vs. trauma/LPS.
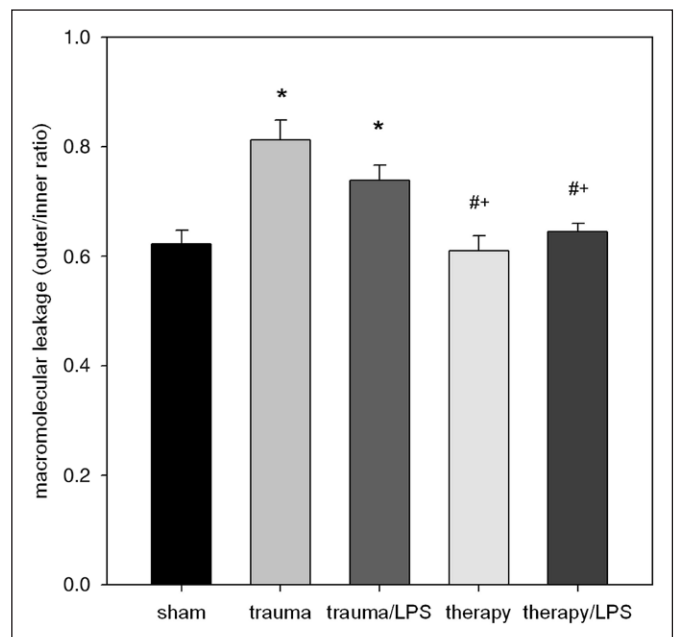

Figure 2 . Macromolecular leakage of fluorescein isothiocyanate dextran (ratio) within skeletal muscle tissue at 24 hrs after soft-tissue trauma and subsequent exposure to lipopolysaccharide (LPS). Animals were treated either with antithrombin (therapy; therapy/LPS) or vehicle solution (trauma; trauma/LPS). Sham-operated animals without trauma and endotoxemia (sham) served as controls. Values are given as mean ± SEM; *$p < 0.05$ vs. sham; #$p < 0.05$ vs. trauma, and +$p < 0.05$ vs. trauma/LPS.

TABLE 2. Leukocyte-Endothelial Cell Interaction

	Sham	Trauma	Trauma/Lipopoly saccharides	Therapy	Therapy/Lipopoly saccharides
Leukocyte rolling (%)	30±2	41±5	48±3[a]	43±2	48±3[a]
Leukocyte adherence (n/mm^2)	204±20	686±37[a]	773±35[a]	176±38[b,c]	237±20[b,c]

Values are given as mean ± SEM. Blood samples were withdrawn at 24 h after soft-tissue trauma.

[a] $p < 0.05$ vs. sham.
[b] $p < 0.05$ vs. trauma.
[c] $p < 0.05$ vs. trauma/lipopolysaccharides.

which was reduced in antithrombin-treated animals (data not shown).

Tissue Cell Death

Upon trauma, nontreated animals revealed enhanced numbers of apoptotic muscle cell nuclei, both in vivo and by indirect in situ DNA nick-end labeling (TUNEL) assay. Treatment with antithrombin reduced apoptotic cell death in both methods (**Fig. 3***A* and ***B***).

DISCUSSION

In this study, we could show that antithrombin prevents leukocyte adherence, microvascular perfusion failure, and microvascular leakage in traumatized skeletal muscle tissue under conditions of systemic endotoxemia. With respect to the fact that the severity of soft-tissue trauma is a critical prognostic factor for morbidity and mortality following complex injuries of the extremities (13), the findings allow one to conclude that treatment with antithrombin might reduce secondary tissue damage.

Antithrombin III has been shown to reduce mortality rate and to prevent organ dysfunction in different animal models (19, 20). Furthermore, antithrombin was capable in delaying microvascular thrombus formation in a murine model (21, 22). Beside its anticoagulatory actions, antithrombin reveals anti-inflammatory activities in a variety of experimental models, which are independent of its direct influence on the coagulatory cascade (7, 23). Beneficial effects of antithrombin could also be shown in patients with severe sepsis (24), which, however, have not been confirmed in the multinational Kyber-Sept trial (25). Despite the fact that antithrombin application is currently not recommended as an adjunctive therapy by the surviving sepsis campaign guidelines (26), there is evidence for antithrombin to effectively reduce mortality rate in a subgroup of septic patients not receiving heparin (27). To our knowledge, the benefit of antithrombin application on traumatized skeletal muscle tissue in an experimental set-up comparable to the present one has not been investigated yet.

The animal model used in this study was formerly described by our group and allows the intravital fluorescence microscopy of traumatized skeletal muscle for quantitative analysis of microvascular perfusion and inflammatory cell interaction under endotoxemic conditions (9, 28). Soft-tissue trauma alone leads to an increase in leukocyte-endothelial cell interaction and a marked reduction in capillary perfusion. In line with nutritive perfusion failure, high-NADH autofluorescence was observed, indicating local tissue hypoxia (25). In general, interruption of oxidative phosphorylation caused by inadequate oxygen supply is reflected by an increase of NADH levels (29). Thus, in vivo NADH-fluorescence microscopy allows noninvasive investigation of organ metabolism as a result of alterations in oxidative phosphorylation (17). In line with this, we could demonstrate a strong negative regression between NADH autofluorescence and microvascular perfusion within the traumatized skeletal muscle tissue. Microcirculatory disorders were even more pronounced by the additional lipopolysaccharide challenge 6 hrs after trauma induction, representing an endotoxemic second hit, which probably leads to thrombotic occlusion of microvessels secondary to the perfusion failure caused by capillary microdamage by the soft-tissue trauma itself. Treatment with antithrombin was capable in modulating these trauma- and endotoxemia-induced microvascular sequelae.

It is known that the glycocalyx, a layer of glucosaminoglycans on the endothelial surface, is impaired in sepsis, thus enabling interaction of white blood cells and platelets with the endothelial surface (30, 31). Massive leukocyte rolling and adherence to the endothelial surface in both endotoxemic animals and animals with isolated soft-tissue trauma could be demonstrated. Furthermore, we observed a significant decrease in platelet counts in endotoxemic animals, indicating a septic consumption coagulopathy caused by increased interaction of platelets with the endothelial surface (30). These consumed thrombocytes cannot be restored by antithrombin but have to be reproduced, which takes longer than the observation time is. Thrombocytopenia was not induced by soft-tissue trauma alone. This issue might be due to the fact that trauma alone leads to a circumscribed endothelial damage with only local increase of cell-cell interaction, in contrast to a systemic impairment of the endothelial surface in sepsis.

Antithrombin was highly effective in preventing leukocyte adherence to the venular endothelium. Antithrombin-treated animals revealed values of leukocyte adherence comparable to those in sham controls. The mechanism of action remains controversial. While some authors credit the effect of antithrombin on leukocyte-endothelial cell interaction with its anticoagulant activity as the primary mechanism of action (32), others assume the mechanism being independent on the coagulatory pathway (8, 30). Further studies are needed to fully characterize the
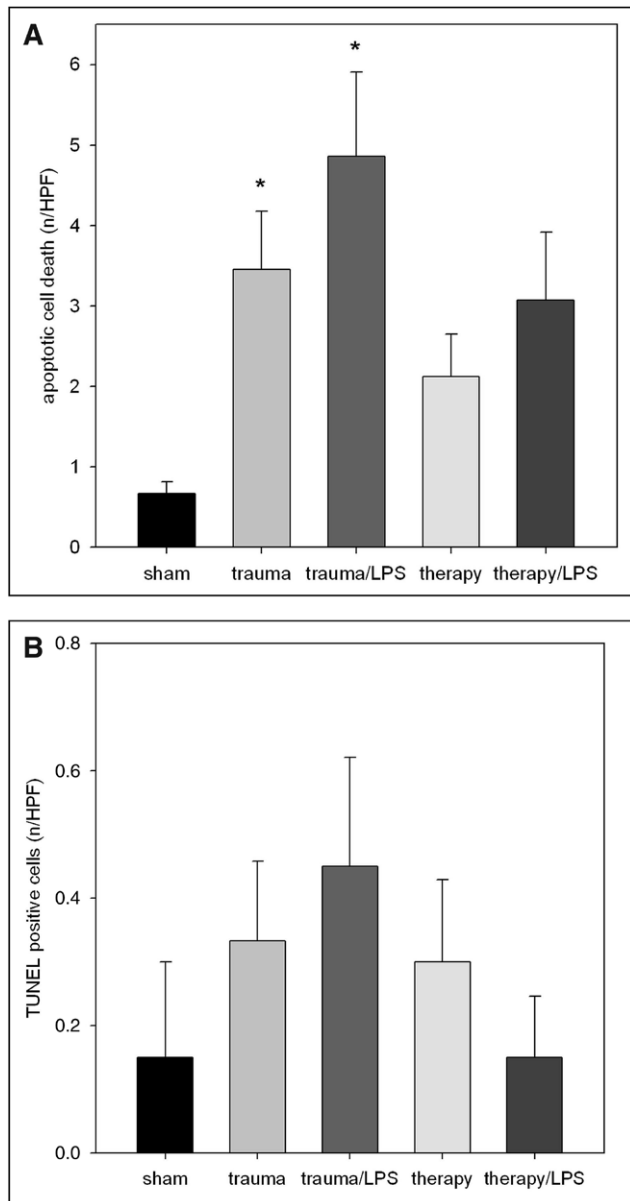
Figure 3. Quantitative analysis of cell apoptosis, as assessed by in vivo staining with bisbenzimide (**A**) or by indirect in situ DNA nick-end labeling (terminal deoxynucleotidyl transferase dUTP nick-end labeling [TUNEL]) assay (**B**) of skeletal muscle tissue in animals at 24 hrs after soft-tissue trauma and subsequent exposure to lipopolysaccharide (LPS). Animals were treated either with antithrombin (therapy; therapy/LPS) or vehicle solution (trauma; trauma/LPS). Sham-operated animals without trauma and endotoxemia (sham) served as controls. Values are given as mean ± SEM. *$p < 0.05$ vs. *sham*. HPF = high-power fields.

mechanisms of antithrombin-mediated protection intraumatized patients with sepsis.

In summary, the protective effect of antithrombin on traumatized skeletal muscle tissue implies that antithrombin reveals potent anti-inflammatory properties to prevent skeletal muscle from secondary tissue damage in order to hasten healing and to improve performance following closed soft-tissue injury. Furthermore, we observed effective amelioration of tissue perfusion and thus adequate tissue oxygen supply. Despite its well-known anticoagulatory properties, we did not observe adverse

side effects, like bleeding complications in traumatized tissue. Our investigation shows that the anticoagulant pathway of antithrombin may represent one key mechanism in preventing traumatized muscle tissue from microvascular dysfunction in a double-hit model.

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