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The angiotensin-converting enzyme inhibitor captopril inhibits poly(ADP-ribose)polymerase activation and exerts beneficial effects in an ovine model of burn and smoke injury

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Abstract

We investigated the effect of the angiotensin converting enzyme (ACE) inhibitor captopril in a clinically relevant ovine model of smoke and burn injury, with special reference to oxidative stress, activation of poly(ADP-ribose) polymerase in the lung and in circulating leukocytes. Female, adult sheep (28–40 kg) were divided into 3 groups. After tracheostomy and under deep anesthesia both vehicle-control (n=5) and captopril (20 mg/kg/d, iv., starting 0.5 hour before the injury) treated (n=5) groups were subjected to 2×20%, third degree burn injury and were insufflated with 48 breaths of cotton smoke. A sham group not receiving burn/smoke was also studied (n=5). Animals were mechanically ventilated and fluid resuscitated for 24 h in the awake state. Burn and smoke injury resulted in an upregulation of ACE in the lung, evidenced by immunohistochemical determination and Western blotting. Burn and smoke injury resulted in pulmonary dysfunction, as well as systemic hemodynamic alterations. Captopril treatment of burn and smoke animals improved PaO₂/FiO₂ ratio and pulmonary shunt fraction and reduced the degree of lung edema. There was a marked increase in PAR levels in circulating leukocytes after burn/smoke injury, which was significantly decreased by captopril. The pulmonary level of ACE and the elevated pulmonary levels of TGF-β in response to burn and smoke injury were significantly decreased by captopril treatment. Our results suggest that the ACE inhibitor captopril exerts beneficial effects on the pulmonary function in burn/smoke injury. The effects of the ACE inhibitor may be related to the prevention of ROS-induced PARP over-activation. ACE inhibition may also exert additional beneficial effects by inhibiting the expression of the pro-fibrotic mediator TGF-β.

Keywords

smoke injury; burn injury; ARDS; oxidative stress; nitrosative stress; angiotensin; vascular dysfunction; cell death; nitric oxide; lung

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Introduction

Thermal injury remains a significant health problem (1). After smoke inhalation, there is a rapid onset of hyperemia in the upper airway of humans and sheep, followed by an increase in microvascular permeability to proteins in the pulmonary and bronchial circulation resulting fluid loss and a complex sequence of pathophysiological events, in which free radicals and oxidants are known to play a significant pathogenetic role (2–4).

During various forms of critical illness DNA damage, resulting from oxidative and nitrosative stress activates the nuclear enzyme poly(ADP-ribose)polymerase-1 (PARP) (reviewed in 5,6). Upon binding to broken DNA, PARP becomes activated and cleaves NAD⁺ into nicotinamide and ADP-ribose. It then polymerizes ADP-ribose on nuclear acceptor proteins including histones, transcription factors, and PARP itself. Excessive PARP activation induced by oxidative stress leads to the depletion of cellular NAD⁺ and ATP pools, and results in cell death, cell dysfunction and organ injury. In addition, PARP activation promotes pro-inflammatory mediator production in various forms of critical illness (5,6). The pathogenic role of PARP in burn injury has been previously demonstrated in rodent as well as large animal models of burn injury, burn/smoke injury or acute lung injury: pharmacological inhibitors of PARP improve hemodynamics, pulmonary function and survival (7–9). The majority of studies published on the role of PARP and critical illness have focused on solid organs such as heart, lung, liver or kidney. However, our recent studies have also implicated the role of PARP activation in circulating leukocytes in various forms of critical illness, including an ovine model of combined burn and smoke injury (10–14).

The classical roles of the renin-angiotensin-aldosterone-system (RAAS) and the vasoconstrictor hormone angiotensin II relate to the regulation of the cardiovascular system. Angiotensinogen, a 452 amino acid alpha2-globulin, produced by the liver, is cleaved by renin to form angiotensin I, which is a decapeptide with 10 amino acids. Angiotensin I is not biologically active and needs to be further converted to the octapeptide angiotensin II by the angiotensin-converting-enzyme (ACE), which is primarily expressed in the endothelial cells of multiple tissues. The classical physiological functions of angiotensin II include stimulation of aldosterone production in the adrenal cortex, leading to increased blood volume as a result of water and sodium retention in the kidney. In addition, angiotensin II has direct vasoconstrictor effects (acting directly as a vasoconstrictor on vascular smooth muscle cells) and indirect vasoconstrictor effects (by enhancing the peripheral synaptic transmission, by an increase of the central sympathetic tone) (15).

Traber and colleagues have demonstrated in 1985 that there is an increase in angiotensin-converting enzyme (ACE) activity in sheep subjected to smoke injury (16). However, at the time angiotensin II was viewed exclusively as a vasoconstrictor hormone. Consequently, the active pathogenic role of the angiotensin pathways in the pathogenesis of this disease has not yet been explored. Over the last decade, multiple lines of studies have indicated that angiotensin II can exert direct cytotoxic effects via generation of intracellular oxidants, through activation of NAD(P)H oxidase (17–19). These effects induce endothelial cell damage by intracellular generation of free radicals and oxidants, which is associated with nuclear and mitochondrial dysfunction. Several groups, including ours, have established that one of the downstream cellular signaling pathways of angiotensin II involves DNA strand breakage and PARP activation (20,21). Therefore, in the current study we investigated the potential active role of angiotensin II in smoke and burn injury, with special reference to oxidative stress and PARP activation in the lung and in circulating leukocytes.

Materials and Methods

Animal model

Adult female Merino sheep (30–40 kg) were cared for in the Investigate Intensive Care Unit at our institution, a facility accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The experimental procedures for burn and smoke injury were conducted similar to previous studies (9,14). The protocol was approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX). The National Institutes of Health and American Physiological Society guidelines for animal care were strictly followed.

Surgical preparation

Fifteen female Merino sheep, weighing 31.8 ± 1.0 kg, were anesthetized with ketamine (Bioniche Pharma, Lake Forest, IL, 15–20 mg/kg) followed by isoflurane (Terrell™, RxElite, Meridian, I.D., 1.4 – 1.8 vol %, 50% O₂ mixture), endotracheally intubated (10G) and, while under general anesthesia, chronically instrumented for hemodynamic monitoring with a 7-French Swan-Ganz thermodilution catheter (Edwards Lifesciences LLC, Irvine, CA) placed into the pulmonary artery via the common jugular vein, a 16 G femoral artery catheter (BD Intracath, Beckton Dickinson Infusion Therapy Systems Inc. Sandy, UT) placed in the arteria femoralis dextra. Through a left thoracotomy at the level of the 5th intercostal space, a silastic catheter was positioned in the left atrium. Postoperative analgesia was performed with 0.9 mg buprenorphin over 24h. The animals were given post surgery a five-day recovery period with free access to food and water before the start of the experiment. On the day of study the sheep were re-anesthetized, and a tracheotomy tube (Shiley 10 SCT, Tyco Healthcare, Pleasanton CA) was positioned just below the larynx. A urinary retention catheter (Foley 14 C.R., Bard Inc. Covington, GA) was placed into the urinary bladder via the ureter.

Experimental protocol and measurements

Animals were randomized into three groups, with 5 animals in each group: an uninjured and untreated group (Sham), a burn and smoke injury with vehicle treatment (Burn&Smoke), and a burn and smoke injury with captopril treatment (Burn&Smoke +Captopril). The Burn&Smoke and the Burn&Smoke+Captopril groups of sheep received a 3rd-degree flame burn of 20% was applied to each flank (total area 40%) using a Bunsen burner until the skin was thoroughly contracted. It was previously determined this degree of injury to be a full-thickness burn, i.e., including both epidermis and dermis, in which the nerve endings are destroyed by heat. Inhalation injury was induced while sheep were in the prone position. A modified bee smoker was filled with 50 burning cotton toweling and connected to the tracheostomy tube. The sheep was insufflated with a total 48 breaths of cotton smoke. The temperature of the smoke did not exceed 40 °C. The sheep was recovering from anesthesia after injury but were placed on ventilation (volume control) for 24 h in awake state. Fluid resuscitation during the experiment was performed with Ringer's lactate solution following the Parkland formula ($4 \text{ ml \% burned surface area}^{-1} \text{ kg body wt}^{-1}$ for the first 24 h). The sheep were studied, fluid resuscitated and mechanically ventilated in awake state with free access to food but not to water. Measurements of cardiopulmonary data and urinary output were collected and blood samples were taken for 24 hours at baseline and every 3 to 6 hours. In the Burn&Smoke+Captopril group, continuous intravenous infusion of captopril started 30 minutes prior to the injury with a dose of 20 mg/kg/day. The corresponding control Burn&Smoke animals received equivalent volume of vehicle. After 24 hours the sheep were euthanized by removing the animals' heart while they were under deep anesthesia with 1,500 mg ketamine and 100 mg xylazine. Lungs were collected for analysis of wet-to-dry

ratio, for histological analysis and for immunohistochemical analysis for the detection of angiotensin II.

Immunohistochemical analysis

Paraffin sections of 5 μ m thickness were prepared from sheep lung blocks. Sections were dried in a slide oven at 60 degrees for 30 minutes to ensure that the sections adhered to the slides, and are completely dry. Sections were deparaffinized in four changes of xylene for five minutes each and then rehydrated through a series of graded alcohols with a final rinse in distilled water. Endogenous peroxides were quenched by soaking sections in two changes of 3% Methanol hydrogen peroxide for 5 minutes each. Prior to immunohistochemistry, antigen retrieval was performed using citrate at pH 6.0. Briefly, slides were incubated for 20 minutes in Target Retrieval Solution (Dako Corporation, Carpinteria, CA) preheated to 99°C, then allowed to cool down for 20 minutes. The slides were then rinsed in three changes of distilled water and placed into a container of Tris Buffered Saline with tween 20 (Signet Pathology Systems, Inc., Dedham, MA) for five minutes to decrease surface tension and facilitate coating. Slides were processed at room temperature in a Dako horizontal auto-stainer, using the biotin-streptavidin method. Both avidin and biotin were obtained from Vector Laboratories, as part of the AB blocking kit, and diluted 1:5 using Dako antibody diluent. Tris buffered saline was used to rinse slides between each of the consecutive processing steps. The ACE primary antibody (rabbit polyclonal, Santa Cruz Biotechnology, CA) was diluted in the biotin solution at 1:200, and applied for 1 hour. Sections were incubated with diluted avidin for 7 minutes, rinsed, and incubated with the primary antibody (ACE) biotin solution for 1 hour. Afterwards, slides were incubated in universal secondary antibody LSAB2 (Dako) for 15 minutes, followed by LSAB2 labeling agent (Dako) for 15 minutes, and then diaminobenzidine (DAB, Dako) for 5 minutes. Slides were rinsed in distilled water, counterstained with hematoxylin for 1 minute, rinsed in distilled water first, 0.25% ammonia water, and distilled water as final step. Slides were then dehydrated through graded series of alcohols, four changes of xylene, and finally coverslipped with synthetic glass and permount mounting media.

Cardiopulmonary data collection

Cardiopulmonary data were collected by continuous measurement of systemic arterial, left atrial and pulmonary arterial pressure catheters were connected to pressure transducers (Baxter-Edwards Critical Care, Irvine, CA) and the Swan-Ganz thermodilution catheter on a clinical monitor (Model V24C, Philips Medicine System, Bollinger Germany). Measurements for heart rate, mean arterial blood pressure, mean pulmonary artery pressure, mean wedge pressure, blood temperature were collected directly. Measurement of cardiac output was performed by rapid injection of 10 ml of normal Saline with a temperature of 0–4°C into the proximal port of the Swan-Ganz catheter.

Blood analysis

Measurement of hematocrit was performed by spinning two capillaries of arterial blood for 3 minutes at 3,000 rpm (IEC Centrifuge, Damon group GMI-IEC Corp., Ramsey, MI) and analyzed manually with a hematocrit reading chart (Arthur H. Thomas Co. Philadelphia, PA). Clinical blood gas machines (682 CO-Oxymeter and GEM Premier 3000, both from Instrumentation Laboratory Comp., Bedford, MA) were used to analyze pO₂, pCO₂, pH, BE, Hb, SO₂, COHb, sodium, potassium, calcium, lactate and glucose in femoral arterial and pulmonary arterial samples (1 ml, heparinized). Cell blood count was analyzed from arterial blood using a HemaVet HV 950FS (Drew Scientific Inc., Dallas, TX).

Mechanical ventilation

Sheep tolerate mechanical ventilation in awake state after tracheostomy. Mechanical ventilation was applied with Siemens Servo 300 ventilators (Siemens AG Medical Solutions, Erlangen, Germany) in pressure control mode, starting with 20 breath per minute. Breath rate was adjusted according to PaCO₂ levels measured during the blood gas analyses. Maximum pressures were set to 40 cmH₂O. FiO₂ was adjusted according to arterial PaO₂ levels. Respiration rate, inspirational peak and pause pressures were recorded.

Leukocyte isolation

Six ml arterial and 6 ml venous blood were drawn from the femoral artery and the pulmonary artery at baseline, immediately after the 2nd burn and at 1, 3, 6 and 24 hours after the injury. Blood samples were drawn into Lavender Vacutainer tube containing EDTA. Leukocytes were isolated as described (14) by Histopaque method (Histopaque-1077, Sigma Aldrich, St. Louis, MO). 6 ml of blood samples were carefully layered on 6 ml of Histopaque-1077, which was previously heated to room temperature. After centrifugation (400 g, 30 min), the leukocytes between the layer of the Histopaque and plasma were collected. Leukocytes were isolated according to the manufacturer's instruction. Freshly isolated leukocytes were used as Western blot samples or were incubated.

Western blot and Oxyblot analysis

Freshly isolated leukocytes were homogenized in RIPA Buffer with EDTA (Boston BioProducts, Ashland, MA) containing protease inhibitor cocktail (1:100, Sigma Aldrich, St. Louis, MO). After homogenization samples were harvested in SDS-polyacrylamide gel electrophoresis sample buffer (NuPage, Invitrogen, Carlsbad, CA). Proteins were separated on 4–12% SDS-polyacrylamide gels. After blocking (5% non-fat dry milk in phosphate-buffered saline), membranes were probed overnight at 4°C with recognizing the following antigens: anti-PAR (1:1000, rabbit, polyclonal) (Trevigen, Gaithersburg, MD), anti-actin (1:10,000, rabbit, polyclonal) (Santa Cruz Biotechnology Inc, Santa Cruz, CA). On the next day membranes were washed 3 times for 15 min in phosphate buffered saline containing 0.5% Tween before addition of anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1000, Southern Biotech, Birmingham, AL). The antibody-antigen complexes were visualized by means of enhanced chemiluminescence by Syngene gel documentation system (Syngene, Frederick, MD) and the results were quantified by GeneTools from Syngene.

To investigate the amount of ROS western blot samples were analyzed by OxyBlot Protein Oxidation Detection Kit (Millipore, Billerica, MA). Samples were prepared as the manufacturer suggested. Results were visualized by means of enhanced chemiluminescence by Syngene gel documentation system results were quantified by GeneTools from Syngene.

In case of lung samples, 100 milligrams of lung samples were homogenized in ice-cold 50 mM Tris-buffer, pH 8.0 (containing protease inhibitor 1:100 and 50 mM sodium metavanadate, Sigma Aldrich, St. Louis, MO, USA) and harvested in 2x concentrated SDS polyacrilamide gel electrophoresis buffer. For Western blot analysis, TGF-β antibody was purchased from Abcam (1:500, rabbit, polyclonal) and angiotensin II antibody (H-170) was bought from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Western blotting for TGF-β and angiotensin II were also conducted using a TGF-β antibody (Abcam, 1:500, rabbit, polyclonal) and an angiotensin II antibody (H-170, Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Statistical analysis

ANOVA with Bonferroni post hoc comparison was used for data analysis. Data are expressed as mean $\pm$ S.E.M. Significance was set as $p<0.05$.

Results

Effect of captopril on the cardiovascular and pulmonary alterations

There was a similar degree of tachycardia after burn and smoke injury in both the Burn&Smoke and the Burn&Smoke+Captopril group (Fig. 1a). The mean arterial pressure remained unchanged in the Sham and the Burn&Smoke group, while in the Burn&Smoke+Captopril group a statistically significant fall in blood pressure was seen at the 24h time point (sham 24h: 99.8 $\pm$ 3 mmHg, Burn&Smoke 24h: 107.0 $\pm$ 3 mmHg and Burn&Smoke+Captopril 24h: 88.4 $\pm$ 5 mmHg) (Fig. 1b). There were no statistically significant differences in systemic vascular resistance index (SVRI) between the three groups studied (Fig. 1c). There were no differences in the mean pulmonary artery pressure and the left atrial pressure between any of the animal groups (Table 1). The mean pulmonary wedge pressure of the Burn&Smoke+Captopril group was significantly higher (15.6 $\pm$ 1 mmHg) than of the Sham group at 6 h (11.0 $\pm$ 1 mmHg). In addition, we noted a statistically significant drop of the central venous pressure in the Burn&Smoke group (5.2 $\pm$ 1 mmHg) 3 h after injury compared to the Sham (7.8 $\pm$ 1 mmHg) and the Burn&Smoke+Captopril group (8.6 $\pm$ 1 mmHg) (Table 1).

At 24h we measured a significant higher pulmonary shunt fraction Q_s/Q_t in the Burn&Smoke group (26 $\pm$ 4) than in the Sham (15 $\pm$ 1) and the Burn&Smoke+Captopril groups (18 $\pm$ 3) (Fig. 2a). In addition, we discovered that the PaO_2/FiO_2 ratio in the Burn&Smoke+Captopril group (24h:425 $\pm$ 21 mmHg) remained at normal baseline levels throughout the study, while the control group (24h: 318 $\pm$ 55 mmHg) progressively decreased over time, reaching, at 24h, 300 mmHg, which is the definition for acute lung injury in this experimental model (Fig. 2b). There was an increase in the bronchial obstruction score values in the Burn&Smoke animals, as compared to the Sham, while there was no difference between the scores of the Sham and Burn&Smoke+Captopril groups (Fig. 3a). Furthermore, Burn&Smoke+Captopril treatment tended to decrease wet/dry lung weight ratios during smoke/burn injury, although the difference did not reach statistical significance ($P<0.1$) (Fig. 3b). Captopril treatment during smoke and burn injury reduced ventilatory pressures at 24h (pause and peak pressure) (Table 1).

The total fluid balance was significantly higher after burn and smoke injury in both Burn&Smoke and Burn&Smoke+Captopril groups than in the Sham group (Table 1). Blood plasma protein levels exhibited a significant drop in both injured groups compared to the Sham group. Values for cardiac output, hematocrit, hemoglobin, pulmonary artery pressure and left atrial pressure showed no statistical difference between the three groups (Table 1).

Effect of captopril on pulmonary histopathology and on the pulmonary expression of ACE

There was a 2-fold increase in the expression of ACE in the lung, as quantified by Western blotting in the Burn&Smoke group as compared to Sham animals (Fig. 4). This increase was significantly reduced in the Burn&Smoke+Captopril group (Fig. 4). Immunohistochemical staining for ACE of paraffin sections collected from lung of normal sheep (sham) and of sheep exposed to smoke/burn at 24 hours both showed positive immunostaining in the bronchial epithelium and vascular endothelial cells. However, in the lung sections collected from sheep exposed to smoke inhalation/burn injury, additional ACE positive staining was observed also within infiltrating inflammatory cells. In these sections positive staining in bronchiolar epithelium appeared more intense than in sections from Sham animals (Fig. 5).

There was an increase in oxidized protein levels in the lung in response to Burn&Smoke, as detected by the Oxyblot assay, which was unaffected by Captopril (Fig. 6). TGF- β levels were increased by burn and smoke injury, and captopril treatment significantly decreased this elevation (Fig. 7). The antibody used in the current study recognizes the C terminus of TGF- β (both the latent and the activated form).

Effect of captopril treatment on PARP activation in circulating leukocytes

We have recently demonstrated that smoke/burn injury, in addition to inducing local pulmonary damage, induces oxidative damage in circulating leukocytes and activates the nuclear enzyme PARP. In the current study, the level of PAR (the product of PARP) was increased significantly (almost 5–6 fold, $p < 0.05$) immediately after burn and smoke injury both in the arterial and venous samples. This elevation was decreased significantly ($p < 0.05$) in the Captopril-treated animals for the entire duration of the study (Figs. 8, 9).

Discussion

Smoke inhalation injury can result in severe respiratory distress, which is subsequently followed by the development of pulmonary fibrosis (2). The renin-angiotensin system (RAS) plays a key role in maintaining blood pressure homeostasis, fluid and salt balance. Angiotensin-converting enzyme (ACE) plays a central role in generating angiotensin II from angiotensin I, and capillary blood vessels in the lung are one of the major sites of ACE expression and angiotensin II production (15–18). Both angiotensin II and transforming growth factor β (TGF- β) activity are associated with both local fibrotic conditions such as hypertrophic scarring and systemic conditions seen in large burn and sepsis. Angiotensin II and TGF β both activate the Smad protein system, which leads to the expression of genes related to fibrosis and excessive extracellular matrix formation (ECM). In fibrotic conditions, angiotensin II acts both independently and synergistically with TGF- β (22,23).

In the present study we examined the effect of ACE inhibitor captopril administration on a variety of physiological and biochemical parameters in a well-established, clinically relevant third degree burn and smoke injury. The dose of captopril used in the current study was associated with significant hemodynamic effects: at 24h a decrease in SVR and mean arterial blood pressure occurred, consistently with the well-known role of angiotensin II in the regulation of blood pressure.

Previous work has already demonstrated that oxidative stress plays a major role in the pathophysiological process induced by thermal injury (see: Introduction). However, the source of these oxidants and free radicals are incompletely understood. Because high levels of angiotensin II are produced in various forms of acute lung injury (24–26), we hypothesized that angiotensin II and downstream pathways of reactive oxidants contribute to the pathogenesis of burn/smoke-induced lung injury. In humans, the ACE activity and angiotensin II has been shown to be elevated in bronchial lavage exposed to burn and smoke trauma with the highest values present in the first day after the injury (24). In accordance with these findings, the ACE levels were markedly elevated by burn/smoke trauma in the current model, and were localized to endothelial cells and tissue-infiltrating inflammatory cells. Interestingly, the ACE inhibitor captopril reduced pulmonary ACE levels. This finding that is somewhat surprising, as captopril is an inhibitor of the activity, and not the expression of ACE. We hypothesize that ACE inhibition maintains endothelial function, improves vascular patency, reduces tissue-infiltrating leukocyte accumulation and thereby interrupts positive feedback cycles of ACE upregulation. Effects of captopril on these pathways may explain the improved P/F ratio and improved histological status of the captopril-treated animals. However, captopril treatment of the animals did not affect oxidative protein content in the lungs. We believe that the main portion of the oxidative protein modification occurs

as a direct result to smoke/burn injury, and is not related to secondary activation of ACE, production of angiotensin II and the generation of secondary oxidants. Nevertheless, it is possible (and remains to be determined) whether captopril inhibits reactive oxidant formation in a localized fashion (in pulmonary endothelial cells or in tissue-infiltrating mononuclear cells). The results demonstrating that captopril-treated animals have a lower PARP activation in their circulating leukocytes (see below) may be consistent with such a localized action.

Angiotensin II upregulates the expression of the profibrotic cytokine TGF- β , which is involved in both the conversion of fibroblast and accumulation of collagen (21,22). The current results demonstrated that burn and smoke trauma induces an elevation in pulmonary TGF- β levels, which was significantly diminished by captopril administration. We hypothesize that such a suppression may be beneficial in inhibiting the development of lung fibrosis in the later stage of the disease. However, additional studies, using a subchronic model of burn/smoke injury are required to directly address this question.

Reactive nitrogen species (RNS) and ROS are involved in the development of lung injury in response to smoke inhalation (27,28). RNS and ROS induce DNA damage and consequently activate the nuclear enzyme PARP enzyme (5,6). We have demonstrated a marked increase in PARP activation in circulating leukocytes, an effect that was drastically suppressed by captopril treatment of the animals. We hypothesize that captopril, by preventing angiotensin-II induced NADPH oxidase activation, suppresses intracellular ROS production, which leads to a lower degree of DNA injury and PARP activation. PARP plays several roles in the cell: activation of PARP can lead to cell death via energy depletion and necrosis; PARP activation can also regulate the translation and expression of multiple pro-inflammatory mediators (5,6). By preventing PARP activation, we hypothesize that captopril may affect the fate/activation state of immune cells and consequently the immune/inflammatory response during burns. In specific, based on previously published evidence in other models (5,6,10–13) our working hypothesis is that (a) circulating leukocytes in which PARP is activated produce higher amounts of pro-inflammatory cytokines; (b) these cells upregulate their adhesion receptors and develop a propensity for adhesion/tissue infiltration and (c) thereby PARP in circulating cells contributes to systemic inflammatory response and remote organ injury. We further hypothesize that (d) these responses are attenuated by captopril, because a reduced oxidative stress response suppresses DNA strand breakage (the obligatory trigger of PARP enzymatic activation) in leukocytes. This hypothesis needs to be directly addressed in future studies.

In summary, our results demonstrate that the ACE inhibitor captopril exerts significant protective effects in the early stage of burn and smoke injury. We hypothesize that the mode of the beneficial effect is inhibition of intracellular ROS generation, inhibition of PARP activation and inhibition of TGF- β upregulation. We must point out that the current design utilized a pre-treatment protocol; in order to be clinically effective, obviously the compound must be also tested in a post-treatment paradigm. Additionally, the effect of captopril on survival needs to be evaluated. Furthermore, we must point out that the current dose of captopril used exerted hemodynamic effects. Because of the well-known hemodynamic instability of burn patients, it would be essential to determine, in future studies, whether a dose of captopril can be identified that is devoid of significant systemic hemodynamic effects, without losing its protective effect against pulmonary dysfunction. Finally, we most point out that the current study only utilized captopril, a single (although widely used) member of the ACE inhibitor class; further work needs to be conducted to test whether the current findings are also applicable to ACE inhibitors of different structural classes. The results of such additional studies will help to test the potential future practical utility of the current approach.

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Figure 1. Hemodynamic changes after smoke inhalation injury: (A) HR, heart rate, beats/min., (B) MAP, mean arterial pressure, mmHg and (C) SVRI, systemic vascular resistance index, dyn*sec/cm⁵/m² in the Sham, Burn&Smoke and Burn&Smoke+Captopril groups of animals. Data are expressed as mean $\pm$ SEM of 5 animals per group. Significance was expressed as *p<0.05 vs. Sham, #p<0.05 vs. Burn&Smoke.

Figure 2.

Pulmonary functionality changes after smoke inhalation injury: (A) Q_s/Q_t , pulmonary shunt fraction and (B) PaO_2/FiO_2 ratio, mmHg in the Sham, Burn&Smoke and Burn&Smoke +Captopril groups of animals.. Data are expressed as mean $\pm$ SEM of 5 animals per group. Significance was expressed as * $p < 0.05$ vs. Sham, # $p < 0.05$ vs. Burn&Smoke.

Figure 3. Histological values after smoke inhalation injury: (A) Bronchial obstruction score, percent of obstruction and (B) Wet/Dry weight ratio in the Sham, Burn&Smoke and Burn&Smoke +Captopril groups of animals. Data are expressed as mean ± SEM of 5 animals per group. Significance was expressed as * $p < 0.05$ vs. Sham, # $p < 0.05$ vs. Burn&Smoke.

Figure 4.

a.) Representative Western blot analysis of ACE in sheep lung 24 hours after combined smoke and burn injury (2×20%, third degree burn and 48 smoke inhalation). Actin blot is showing as loading control. **b.)** Densitometric evaluation of ACE/actin in sheep lung after combined (2×20%, third degree) and smoke (inhalations) injury. Data represent mean±SEM of n=5 animals for the Sham, Burn&Smoke and Burn&Smoke+Captopril groups of animals. *p<0.05 represents a significant elevation in ACE/actin signal in Burn&Smoke group, compared to the Sham group.

Figure 5. Immunohistochemical staining for ACE in lung sections of sheep from the sham group (A, B) and Smoke&Burn group at 24h (C, D). Original magnification: 20x (A, B, C), 40x (D).

Figure 6.

a.) Representative Oxyblot analysis of sheep lung at 24 hours in the Sham, Burn&Smoke and Burn&Smoke+Captopril groups of animals. Actin blot is showing as loading control. **b.)** Densitometric evaluation of Oxyblot/actin level in sheep lung after combined burn (2×20%, third degree) and smoke (48 inhalations) injury. Data represent mean±SEM of n=5 animals for each group.

Figure 7.

a.) Representative TGFβ (latent and activated form) analysis of sheep lung at 24h in the Sham, Burn&Smoke and Burn&Smoke+Captopril groups of animals. Actin blot is showing as loading control. **b.)** Densitometric evaluation of TGFβ/actin levels. Data represent mean $\pm$ SEM of n=5 animals for each group. *p<0.05 represents a significant increase in TGFβ/actin signal in Burn&Smoke+Captopril group compared to the Sham group, #p<0.05 represents a significant attenuation in TGFβ/actin signal in Burn&Smoke+Captopril group, compared to either the Burn&Smoke group or to the Sham group.

Figure 8.

Representative PAR Western blot analysis in circulating leukocytes isolated from arterial and venous blood at baseline conditions and at various time points (up to 24h) in the Sham, Burn&Smoke and Burn&Smoke+Captopril groups of animals. Actin blots are shown as loading control.

Figure 9.

Densitometric evaluation of the time course change the level of PAR/actin in leukocytes in circulating leukocytes isolated from arterial blood at baseline conditions and at various time points (up to 24h) in the Sham, Burn&Smoke and Burn&Smoke+Captopril groups of animals. Data represent mean $\pm$ SEM of n=5 animals for each group. *p<0.01 represents a significant increase in PAR/actin signal in the Burn&Smoke group compared to baseline values; group, compared to the Burn&Smoke group; #p<0.05 represents a significant inhibition of PAR/actin signal in Burn&Smoke+Captopril group compared to the Burn&Smoke group.

Table 1

Summary of cardiopulmonary hemodynamics.

Variable	Group	BL	3h	6h	12h	18h	24h
CO	S	4.98±0.26	5.07±0.37	4.92±0.31	4.31±0.31	4.48±0.21	4.35±0.15
	B/S	4.93±0.27	5.18±0.24	5.21±0.34	5.31±0.65	5.02±0.48	5.38±0.42
	B/S+C	5.16±0.15	4.83±0.45	5.35±0.38	4.95±0.47	5.03±0.36	5.57±0.53
CI	S	4.68±0.96	4.77±1.02	4.63±0.96	4.05±0.86	4.23±0.88	4.10±0.84
	B/S	4.81±0.97	5.06±1.02	5.11±1.06	5.18±1.18	4.95±1.12	5.28±1.14
	B/S+C	5.47±1.09	5.12±1.11	5.67±1.18	5.25±1.15	5.34±1.13	5.90±1.28
LAP	S	6.40±0.51	9.40±1.03	8.80±0.86	8.80±0.80	8.80±0.80	8.60±1.03
	B/S	5.25±0.85	5.00±0.71	7.00±1.30	5.20±0.58	5.20±0.80	6.20±0.66
	B/S+C	6.80±0.66	9.20±0.80	9.80±0.80	10.20±1.24	9.40±1.63	9.00±0.84
CVP	S	4.60±0.68	8.20±0.97	7.80±0.49	7.00±0.84	7.80±0.73	7.60±0.51
	B/S	3.80±0.20	5.40±0.98	7.00±1.30	5.20±0.58	5.20±0.80	6.20±0.66
	B/S+C	4.80±0.58	7.20±0.97	8.00±1.38	8.40±0.75	8.60±1.25	7.80±1.02
Plasma-Protein	S	6.44±0.17	6.38±0.23	6.48±0.17	6.46±0.19	6.56±0.19	6.40±0.19
	B/S	6.52±0.53	5.48±0.43	5.18±0.39	5.02±0.43	4.86±0.41	4.82±0.38
	B/S+C	6.44±0.26	5.36±0.39	5.18±0.32	5.14±0.22	5.02±0.25	5.00±0.21
FiO ₂	S	0.21±0.0	1.00±0.0	0.21±0.0	0.21±0.0	0.21±0.0	0.21±0.0
	B/S	0.21±0.0	1.00±0.0	0.33±0.06	0.21±0.0	0.26±0.04	0.32±0.05
	B/S+C	0.21±0.0	1.00±0.0	0.21±0.0	0.21±0.0	0.21±0.0	0.21±0.0
PaO ₂	S	106.6±3.71	564.0±25.2	138.6±5.6	131.8±4.0	129.8±2.2	131.0±2.8
	B/S	101.0±3.26	440.8±51.7	182.2±25.2	103.0±7.5	104.6±8.7	95.6±15.0
	B/S+C	112.8±4.33	563.6±15.3	122.0±3.0	120.4±5.5	113.4±4.2	107.2±4.4
PaCO ₂	S	42.0±0.71	22.4±1.03	22.2±2.31	24.4±2.23	27.8±2.33	28.0±2.55
	B/S	39.0±0.71	31.2±3.67	30.0±1.70	28.6±1.17	27.4±1.36	32.0±1.05
	B/S+C	39.6±1.94	31.4±2.36	29.8±1.24	28.4±1.81	26.4±1.54	27.8±0.73
Art pH	S	7.46±0.04	7.64±0.02	7.61±0.03	7.56±0.01	7.50±0.03	7.48±0.02
	B/S	7.48±0.01	7.62±0.03	7.59±0.01	7.52±0.02	7.55±0.01	7.52±0.02
	B/S+C	7.48±0.02	7.60±0.04	7.62±0.02	7.57±0.05	7.59±0.03	7.54±0.02
aBE	S	5.28±2.39	3.68±1.81	1.78±1.95	0.30±1.22	-1.04±1.12	-1.80±1.05

Variable	Group	BL	3h	6h	12h	18h	24h
SaO ₂	B/S	5.36±0.83	8.36±1.85	6.80±1.50	1.14±1.10	1.68±0.98	2.64±0.33
	B/S+C	5.88±1.52	7.00±1.66	8.38±0.68	4.06±2.41	3.36±0.69	2.80±1.06
	S	91.54±0.86	94.38±0.13	93.50±0.08	93.14±0.19	93.00±0.20	93.12±0.15
DO ₂	B/S	90.98±0.47	93.96±0.29	93.26±0.44	90.92±0.90	91.18±1.11	87.68±3.49
	B/S+C	91.66±0.77	94.62±0.35	92.68±0.34	92.56±0.63	92.58±0.54	91.14±0.92
	S	654.9±40	737.2±70	627.2±43	567.0±52	564.3±47	544.3±42
PvO ₂	B/S	641.4±46	746.9±58	663.3±27	652.8±66	616.9±27	647.6±27
	B/S+C	691.7±52	800.2±83	759.0±48	728.1±86	711.1±57	744.8±65
	S	41.60±2.79	47.80±3.51	39.80±2.42	38.00±2.21	39.20±2.48	38.40±3.19
Temp	B/S	42.40±2.01	40.60±2.38	35.20±1.66	36.00±1.26	38.20±1.88	38.40±2.52
	B/S+C	46.40±1.36	42.40±2.14	37.80±0.97	35.40±1.89	32.40±1.63	37.40±1.03
	S	39.20±0.14	39.38±0.20	39.36±0.22	39.30±0.23	39.30±0.26	39.48±0.35
Hb	C	39.01±0.14	39.16±0.15	39.18±0.12	39.24±0.15	39.68±0.20	39.82±0.27
	B/S+C	39.16±0.11	38.84±0.16	38.36±0.24	38.58±0.12	38.80±0.14	39.06±0.14
	S	9.10±0.20	8.58±0.25	8.66±0.20	8.78±0.47	8.54±0.40±	8.38±0.34
Hct	C	8.76±0.68	8.62±0.74	8.22±0.59	8.36±0.62	8.50±0.92	8.66±0.95
	B/S+C	8.20±0.69	8.82±0.56	8.62±0.58	8.82±0.37	8.54±0.33	8.28±0.51
	S	26.4±10.8	25.4±1.29	25.8±0.86	25.0±1.41	25.6±1.57	25.2±1.11
	B/S	25.2±1.66	23.8±2.03	23.4±1.21	23.8±1.46	23.6±2.20	25.2±3.01
	B/S+C	24.2±1.85	24.8±1.59	24.8±1.77	25.0±1.00	23.8±1.16	23.8±1.11

S:Sham, B/S:Burn&Smoke, B/S+C:Burn&Smoke+Captopril