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Local Wound p38 MAPK Inhibition Attenuates Burn Induced Cardiac Dysfunction

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Abstract

Background—Topical inhibition of activated p38 MAPK within burn wounds attenuates the local and systemic inflammatory response. In this study we investigated the effects of local activated p38 MAPK inhibition on burn-induced cardiac dysfunction.

Methods—Using a standardized rat model of scald burn injury, rats were given a 30% total body surface area partial thickness burn or sham injury and wounds were treated with an activated p38 MAPK inhibitor (SB) or vehicle. Systemic blood pressure measurements were recorded *in vivo* followed by *in vitro* assessment of sarcomere contraction in single cell suspensions of isolated cardiomyocytes.

Results—Systolic blood pressure or maximum left ventricular pressures *in vivo* and peak cardiomyocyte sarcomere contractility *in vitro* were significantly reduced following burn injury. These functional deficits were abolished 24 hours after burn injury following local p38 MAPK inhibition. *In vitro* incubation of normal cardiomyocytes with homogenate from burned skin or burn serum resulted in a similar pattern of impaired cardiomyocyte contractility. These effects were reversed in normal cardiomyocytes exposed to burn skin homogenates treated topically with a p38 MAPK inhibitor. Western blot analysis showed that cardiac p38 MAPK activation was not affected by dermal blockade of activated p38 MAPK arguing against systemic absorption of the inhibitor and indicating the involvement of systemic cytokine signaling.

Conclusions—Topical activated p38 MAPK inhibition within burned skin attenuates release of pro-inflammatory mediators and prevents burn induced cardiac dysfunction following thermal injury. These results support inhibition of burn-wound inflammatory signaling as a new therapeutic approach to prevent potential post-thermal injury multi-organ dysfunction syndrome.

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Keywords

inflammation; burn injury; mitogen-activated kinase; cardiomyocytes

INTRODUCTION

The United States ranks first among industrialized nations in death and disability from burn injuries ¹. Locally, the burn wound inflammatory response plays an important role in wound healing, ultimate depth of the burn injury, and the risk of infection ^{2–5}. Systemically, a large burn wound inflammatory response may induce broad activation of the immune system (systemic inflammatory response syndrome, SIRS) putting distant organs such as the cardiopulmonary system at increased risk of dysfunction (multi-organ dysfunction syndrome, MODS) ^{5–7}.

It is well established that burn injury leads to impaired cardiac function possibly resulting in end organ hypoperfusion and impaired peripheral microcirculation ^{6, 8, 9}. Several pro-inflammatory mediators and intracellular signaling pathways that potentially contribute to burn-induced cardiac dysfunction have been described ^{6, 10}. Among them, p38 mitogen-activated protein kinase (MAPK) is known to play a central role in the cellular response to external stress such as thermal injury ¹¹. Although the consequences of p38 MAPK activation in the heart are not yet fully understood, p38 MAPK has been reported to play an important role in cardiac hypertrophy, ischemia-reperfusion injury, and cardiomyocyte apoptosis ^{12, 13}. Once activated, p38 MAPK results in downstream activation of pro-apoptotic transcription factors such as p53 and stimulates robust pro-inflammatory cytokine production of IL-6, IL1- β , and TNF- α in cardiomyocytes ^{14–17}. Inhibition of activated p38 MAPK by parenteral administration of a specific p38-inhibitor has been shown to decrease the levels of those systemic mediators thereby attenuating burn-induced myocardial contractile defects ¹⁸.

In previous studies, we were able to show that local blockade of activated p38 MAPK in experimental burn wounds decreased not only the local dermal inflammatory response, but also reduced skin microvascular damage and wound-bacterial growth ^{19, 20}. In addition to inducing local burn wound apoptosis, the burn-induced dermal inflammatory response may act as an ongoing trigger for the systemic inflammatory response syndrome (SIRS) and result in end organ dysfunction. Attenuation of burn wound inflammatory signaling with local blockade of dermal activated p38 MAPK decreased systemic levels of pro-inflammatory cytokines and reduced burn-induced acute lung injury ^{21, 22}. Given that attenuation of local inflammatory signaling reduces SIRS and subsequent systemic complications after burn injury, we hypothesize that local inhibition of the activated p38 MAPK pathway will also attenuate burn-induced myocardial dysfunction.

MATERIAL AND METHODS

Reagents

Unless otherwise indicated, all reagents were purchased from Sigma Aldrich (St. Louis, MO).

Experimental animals

Adult male Sprague-Dawley rats (Harlan Inc., Indianapolis, IN) weighing 300 to 350 g were used in all experiments. Prior to use, the animals were housed in a specific pathogen-free environment and allowed to acclimate to their surroundings for one week. Standard rat chow and water were available to the animals *ad libitum*. All experiments were performed in accordance with the guidelines set forth by the National Institutes of Health for care and use

of animals. The experimental protocol was approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Burn procedure

Under isoflurane anesthesia dorsal hair was closely clipped and residual stubble removed using Nair[®] depilatory cream (Church & Dwight Inc., Princeton, NJ). The rats were then placed in a prefabricated mold device with a rectangular opening which exposed the dorsal skin surface while protecting the remaining skin from burn exposure. The exposed skin surface was immersed in 60°C water for 27 seconds producing a partial-thickness dermal burn over 30% of the total body surface area (TBSA). Sham animals underwent an identical procedure, except that they were immersed in room temperature water (24°C). The rats were immediately dried and resuscitated with lactated Ringer's solution (4 mL/kg/% TBSA burn). One half of the calculated resuscitation volume was given i.p. and the remaining volume was given as divided dose subcutaneous injections immediately post-burn. The animals also received buprenorphine 0.1 mg/kg by subcutaneous injection every 8 hours for the first 24 hours following burn injury. Sham animals received the same resuscitation and analgesia treatment. Rats were sacrificed at 1, 6, 12 or 24 hours after burn injury for *in vivo* and *in vitro* experiments.

Local inhibition of p38 MAPK

Where indicated, burn and sham animals received local treatment with SB202190 (Calbiochem, San Diego, CA), a specific activated p38 MAPK inhibitor^{19, 21}. A solution of acetone/olive oil was employed as the vehicle for SB202190 as it easily dissolves SB202190 and can penetrate the skin. SB202190 was dissolved in 1:4 mixture by volume of acetone/olive oil yielding a concentration of 10⁻⁵M. Vehicle-treated animals received acetone/olive oil application only. 2 mL of either SB202190 inhibitor + vehicle or vehicle alone were applied locally to the burn wound immediately following burn injury and every 8 hours thereafter for a total of 3 treatments.

In vivo measurement of blood pressure

Under isoflurane anesthesia the right carotid artery was exposed, and a 2.5 French microtip catheter (Millar Instruments, Houston, TX) was advanced into the right common carotid artery aortic junction. Correct positioning was verified initially by fluoroscopy and thereafter by appearance of the characteristic systemic blood pressure curve. Mean (MAP), maximum (SBP) and minimum (DBP) blood pressure as well as heart rate (beats per minute) were recorded for 5 minutes using a signal transduction and amplification system connected to a standard Microsoft Windows[®] operating system PC equipped with recording and analysis software (PowerLab[®] 8SP Base, Bridge Amp, Chart[®] 5 Software, ADInstruments, Colorado Springs, CO).

Cardiomyocyte isolation

Rats were anticoagulated with 1000 units i.p. of heparin sodium (Abbott Laboratories, North Chicago, IL) and anesthetized with isoflurane anesthesia at the selected experimental time point. A bilateral sternal flap thoracotomy was created, the heart was rapidly excised and rinsed in ice-cold Krebs-Henseleit buffer, supplemented with 5 mM taurine. The heart perfusion and the cardiomyocyte isolation procedures were performed as described previously¹⁰. Briefly, the distal aortic arch was cannulated and mounted on a Langendorff perfusion system (ADInstruments Inc., Colorado Springs, CO) connected to a PC-based signal transduction/amplification system (PowerLab[®], ADInstruments Inc., Colorado Springs, CO). Retrograde coronary artery perfusion was performed using Krebs-Henseleit buffer. Enzyme digestion was then performed by using calcium-free Krebs-Henseleit perfusion solution containing Collagenase type II 0.5 mg/mL (Worthington Biochemical Corp., Lakewood, NJ) and

Hyaluronidase 0.2 mg/mL (Sigma Aldrich Inc., St. Louis, MO). After gradual increase of calcium concentration the heart was removed from the cannula, gently minced, and the resulting cardiomyocyte pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM, GIBCO Corp., Grand Island, NY). Cardiomyocyte concentration was determined using a Hemacytometer, cell viability was assessed by Trypan blue dye exclusion and cell morphology. Myocytes with a rod-like shape, clearly defined edges and sharp striations were counted as viable cells, whereas cells with membrane blebbing, loss of striation pattern and rounded cells were classified as non-viable. Cell suspensions with a viability of > 75% were used for all subsequent experiments. Purity of the cardiomyocyte suspension and possible contamination with leukocytes (neutrophil granulocytes, lymphocytes, macrophages) was assessed with FACScan Flow Cytometry System (Becton Dickinson, San José, CA) revealing a purity of > 99% cardiomyocytes.

Plating of cardiomyocytes and stimulation with burn skin homogenates or serum

Cardiomyocytes were plated onto sterile 22×22 mm glass coverslips precoated with 40 µg/mL natural mouse laminin (Invitrogen Corp., Carlsbad, CA) at a density of 5×10^4 cells/coverslip/well and incubated in 6-well tissue culture plates (37°C, 5 % CO₂) for 3 hours to allow for cell attachment. DMEM was then carefully pipetted off and replaced with 2 mL/well serum-free M199 medium supplemented with 10 mM Glutathione, 0.2 mg/mL BSA (GIBCO, Grand Island, NY), 15 mM HEPES, 26 mM NaHCO₃. Where indicated, single CM were incubated with burn serum (100 µL) or burn skin homogenates. Full-thickness skin from burn or sham rats was homogenized in 1mg/mL M199, centrifuged and 200 µL of the supernatant was added to the appropriate wells. Culture plates containing single cell suspensions of cardiomyocytes with skin homogenates were placed in an incubator (37°C, 5% CO₂) for additional 18 hours. All media and other reagents used for the cardiomyocyte isolation were certified endotoxin-free by the manufacturers.

Single-cell sarcomere contraction and relaxation analysis

Plated cardiomyocytes that had been incubated for a total of 21 hours underwent single-cell sarcomere contraction and relaxation analysis using a variable rate CCD video camera system (MyoCam®, IonOptix Corp., Milton, MA) equipped with sarcomere length detection software (IonWizard®, IonOptix Corp., Milton, MA). Microscopic imaging was performed using an inverted microscope system (Eclipse® TE-2000S, Nikon Corp., Melville, NY) connected to the CCD video camera system. A coverslip with the plated cardiomyocytes was placed in a prefabricated chamber, which was filled with warm (37°C) M199 and mounted on the microscope system. The chamber system was connected to a Grass stimulator system (Grass Inc., West Warwick, RI). Electrical pacing stimulation was performed using a 100 mV stimulus of 4 ms duration and a frequency of 1 Hz. The measurement of sarcomere contraction at different experimental conditions and the selection of the cardiomyocytes from each coverslip were performed in a randomized fashion. For each measured cardiomyocyte, a rectangle-shaped region of interest was defined and sarcomeres within the focused region were selected for analysis typically including 15–20 sarcomeres. Sarcomere contractions were recorded for 75 seconds. Parameters measured using the IonWizard® (IonOptix Inc., Milton, MA) software included peak sarcomere shortening, contraction velocity, and relaxation velocity.

Limulus amebocyte assay

A chromogenic Limulus amebocyte assay (QCL-1000®, Cambrex Corp., Baltimore, MD) was performed to confirm endotoxin-free conditions in all cardiomyocyte suspensions. Briefly, samples of the cell culture supernatants were mixed with the LAL reagent and chromogenic substrate reagent over a short incubation period (16 minutes), and read on a spectrophotometer at a wavelength of 405 nm. The assay has a sensitivity range of 0.1 EU/mL – 1.0 EU/mL.

Western blot analysis

24 hours after sham or burn injury, whole hearts were harvested and flushed with calcium-free Krebs-Henseleit buffer solution by cannulation of the aorta. Protein from whole ventricular homogenate lysates (40 µg) was separated by electrophoresis in a denaturing 10% polyacrylamide gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. Equal loading was facilitated by protein estimation (BCA Protein Assay, Pierce, Rockford, IL) and confirmed by detection of GAPDH as housekeeping protein. Non-specific binding sites were blocked with TBS-T containing 5% non-fat dry milk for 1 hour at room temperature. The membrane was then incubated with the following antibodies in a 1:10 dilution overnight: mouse anti-p38 MAPK, mouse anti-phospho-p38 MAPK or rabbit anti-MAPK-activated protein kinase-2/3 (MAPKAPK-2/3, Cell Signaling, Danvers, MA). The anti-MAPKAPK antibody detects endogenous levels of MAPKAPK-2 protein phosphorylated at threonine 334 and also cross-reacts with MAPKAPK-3 phosphorylated at threonine 313 (as shown in Figure 4). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were developed by enhanced chemiluminescence technique according to the manufacturer's protocol (Amersham Pharmacia Biotech, Piscataway, NJ). The results were quantified using digital pixel density and image analysis software Kodak ID (Scientific Imaging Systems, New Haven, CT), normalized to GAPDH and expressed as ratios.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 4 (GraphPad, Inc., San Diego, CA). Results are expressed as the mean value $\pm$ standard error of the mean (SEM) unless otherwise noted. Analysis of variance (ANOVA) followed by Tukey's post-hoc tests was used to test for differences among the experimental groups for each of the grouping variables. Statistical significance was defined as a p-value < 0.05 .

RESULTS

Local blockade of p38 MAPK after thermal injury preserves left ventricular function

Prior to isolation of rat cardiomyocytes (CMs), the left carotid artery was catheterized with a 2.5 F microcatheter and systemic blood pressures in the aortic root including maximum left ventricular pressure were recorded. Animals were treated topically with either the activated p38 MAPK inhibitor, SB202190, or vehicle at 0, 8, and 16 hours after burn or sham injury. Systemic blood pressures and heart rate values were recorded 24 hours following burn or sham injury. Mean, systolic, and diastolic blood pressure were all significantly reduced in burn + vehicle compared to sham-injured animals. Local inhibition of activated p38 MAPK with SB202190 resulted in restoration of blood pressures similar to the levels of sham animals (Figure 1A–C). It is important to note that topical activated p38 MAPK inhibition in sham animals had no effect on mean, systolic, or diastolic blood pressure values supporting the premise that topical application of SB202190 does not directly affect these parameters. Instead, topical application of the SB202190 abrogated the activity of local activated p38 MAPK in the skin. It is the activation of local p38 MAPK in the skin that is necessary for cardiac dysfunction to develop after burn injury. Heart rates did not differ significantly in any of the groups (Figure 1D).

Local blockade of p38 MAPK preserves burn-induced sarcomere shortening parameters

We investigated the effects of burn injury and inhibition of local activated p38 MAPK (at 0, 8, and 16 hours after burn or sham injury) on peak sarcomere shortening 24 hours post burn or sham injury. The data demonstrate that blockade of activated p38 MAPK in the skin abolishes burn-induced depression of peak sarcomere shortening. Following burn injury, in

burn + vehicle treated rats, peak sarcomere shortening was markedly reduced (by >50%) (Figure 2A). Peak sarcomere shortening values for cardiomyocytes isolated from burn + SB202190 animals were markedly improved compared to the values of burn + vehicle animals (Figure 2A). Dermal blockade of activated p38 MAPK with SB202190 resulted in normal sarcomere function 24 hours post sham injury.

In addition to evaluation of peak sarcomere shortening, we analyzed sarcomere contraction velocity (Figure 2B) and relaxation velocity (Figure 2C) values at 24 hours post burn or sham injury. Both velocity parameters were significantly reduced in cardiomyocytes isolated from burn animals compared to cells derived from sham animals. Significantly slower contraction and relaxation velocities occurred in CMs isolated from burn + vehicle animals compared to their sham counterparts (Figure 2B–C). However, local blockade of activated p38 MAPK improved the contraction velocity as well as the relaxation velocity. By 24 hours after burn, CMs from rats treated with SB202190 had sarcomere shortening velocities comparable to levels recorded in cardiomyocytes isolated from sham animals. To rule out an intrinsic effect of SB202190 on these measured velocities, sham rats treated with SB202190 were compared to sham rats injected with vehicle; there was no difference in sarcomere shortening velocities between these two treatment groups.

Burn skin or burn serum treated with SB abolishes burn-induced impairment of cardiomyocyte contractility

In order to investigate the effects of burn skin and burn serum on contractility parameters after burn injury, we exposed isolated cardiomyocytes from healthy rats to serum or skin homogenates from either normal rats or burn rats treated with SB202190 or vehicle for 18 hours. As shown in Figure 3A–C, exposure of isolated CMs to normal skin homogenate had no effect on sarcomere contractility when compared to baseline data of sham burn + vehicle rats (Figure 2A–C). In contrast, incubation of isolated CMs with homogenates of burn skin treated with vehicle resulted in significant deterioration in peak sarcomere shortening when compared to CMs exposed to normal skin homogenates. However, peak sarcomere shortening of isolated cardiomyocytes incubated with homogenate from SB202190 treated burn skin was significantly improved compared to CMs exposed to burn skin treated with vehicle homogenate (Figure 3A). A similar pattern was found with regard to contraction and relaxation velocities. CM exposed to homogenates of burn skin treated with SB202190 showed velocity levels in isolated CMs comparable to CM exposed to normal skin (Figure 3B–C). Comparable results were obtained when CMs were exposed to serum of these animals (Figure 3D–F). Peak sarcomere shortening as well as contraction and relaxation velocities were greatly reduced in isolated CMs exposed to serum from burn animals treated with vehicle. In contrast, myocardial contractility was almost unchanged when normal CMs were incubated with serum from SB202190 treated burn animals. These data indicate a direct association between the local inflammatory response (activation of skin p38 MAPK) and the increase in inflammatory mediators within the circulation leading to deterioration of distant organ function in the setting of thermal injury.

Local inhibition of p38 MAPK does not impact expression of cardiac p38 MAPK

In the next set of experiments we addressed the question whether local dermal blockade of activated p38 MAPK will also inhibit the expression of p38 MAPK in the heart. We therefore harvested heart tissue at 24 hours after burn or sham injury. Homogenized ventricular tissue extracts were used for Western blotting to identify expression of baseline p38 MAPK, phosphorylated p38 MAPK as well as phosphorylated MAPK-activated protein kinase-2/3 (MAPKAPK-2/3), a direct downstream target for activated p38 MAPK. As shown in Figure 4, there was no difference in the expression of either p38 MAPK (baseline or phosphorylated) or MAPKAPK-2/3 between rats treated with vehicle or SB202190. Dermal application of

SB202190 did not result in direct inhibition of expression of activated p38 MAPK or MAPKAP-2/3. These results coupled with our prior research suggest that burn wound inhibition of p38 MAPK attenuates the release of local and systemic inflammatory mediators into the circulation. It is the systemic inflammatory mediators that affect end organ dysfunction.

DISCUSSION

Thermal injury resulting in tissue damage instigates a tightly controlled immune response characterized by release of pro-inflammatory cytokines and chemokines, activation of the complement system, and production of other mediators directed at repairing and healing the tissue damage. When the dermal inflammatory response remains controlled and localized to the site of tissue damage it is highly effective in recruiting inflammatory cells and initiating repair mechanisms. However, when the balance between pro- and anti-inflammatory mediators is lost or when the extent of injury exceeds the body's healing capability, the inflammatory response is no longer localized and the systemic inflammatory response syndrome (SIRS) with potential for multi-organ dysfunction ensues. Extensive research has been conducted on these mechanisms especially in the setting of systemic infection and sepsis²³. In contrast, burn injuries usually present with an initial absence of bacterial wound infection but still possess the risk of systemic shock, inadequate tissue perfusion, and progressive end organ dysfunction all of which can result in burn-related mortality^{24–27}. Burn wounds are typically characterized by three separate zones. Centrally there is a region of necrotic cells and no blood flow. Surrounding the necrotic cells is a zone of stasis with injured cells that are still potentially viable. The outermost zone is marked by hyperemia and is the site of the local dermal inflammatory response. It is in the zone of hyperemia where high levels of inflammatory mediators are released²⁸. Therefore, local mechanisms at the burn wound level are attractive research foci in order to elucidate the mechanisms leading to ongoing tissue necrosis and SIRS.

One of the intracellular cascades that is activated in response to cellular stress and injury is p38 MAPK¹¹. Once phosphorylated, p38 induces the synthesis of an array of pro-inflammatory mediators such as IL-1 β , IL-6 and TNF- α , all of which are known to be cardio-depressive agents^{15–17, 29–31}. Skin is a major source of inflammatory agents following thermal injury potentially contributing to SIRS^{32, 33}. Partial thickness burn injury is known to produce a rise in dermal p38 MAPK phosphorylation starting as early as 1 hour after injury and is detectable up to 12 hours post-burn¹⁹. Macrophages isolated from the spleen of burn injured animals 7 days after injury demonstrate maximal activation of p38 MAPK at 30 minutes after stimulation with lipopolysaccharide in tissue culture.³⁴ The macrophages isolated from thermal injured animals had levels of phosphorylated p38 MAPK following LPS exposure that were significantly greater than those from macrophages taken from sham injured animals.

In our experiments, topical burn wound treatment with SB202190 did not produce a difference in local levels of phosphorylated p38 MAPK. Western blot analysis did however confirm that treatment with SB202190 produced downstream target inhibition of p38 MAPK 24 hours after thermal injury with a significant reduction in expression of dermal mitogen activated protein kinase-activated protein kinase-2 (MAPKAP-2). This inhibition of activated p38 MAPK resulted in attenuation of the local pro-inflammatory cytokine response (reduction in dermal levels of IL-1 β , IL-6, and TNF- α compared to vehicle treated controls). Topical modulation of burn wound inflammation also reduced the serum levels of IL-1 β and IL-6 24 hours post-injury²¹. This attenuation of dermal inflammation and systemic pro-inflammatory cytokines by topical SB 202190 following burn injury was also associated with reduced evidence of acute lung injury as measured by pulmonary inflammatory response, pulmonary function, and survival following pneumonia after thermal injury^{19–22}.

In the current study we show that burn-induced cardiac dysfunction can be abrogated by inhibition of activated p38 MAPK within the burn wound. Local application of SB202190 blocked the development of impaired left ventricular function *in vivo* at 24 hours after burn injury. Hemodynamic values for MAP, SBP and DBP remained at normal values after burn injury in SB-treated rats, whereas vehicle treated animals experienced a significant reduction in these physiologic parameters when compared to sham burn rats. In our experiments we attributed these hemodynamic changes to the measured deficits in cardiomyocyte contractility found *in-vitro*. However, the decrease in systemic hemodynamic values could also be attributable to changes in pre- and afterload following thermal injury. To guard against this as a confounding variable, animals in all groups were resuscitated with the same amount of crystalloid fluid to minimize the effect of preload changes after burn injury. With regard to afterload, it is possible that the release of pro-inflammatory mediators could contribute to a lower mean arterial pressure through peripheral arteriole vasodilation³⁵. Blockade of dermal activated p38 MAPK may therefore result in preserved afterload in addition to improved contractility thus offering another potential explanation for the improved hemodynamic values obtained in the SB202190 treated group.

These *in vivo* results prompted us to investigate if similar treatment would have a beneficial impact on *in vitro* contractility parameters in isolated rat cardiomyocytes (CMs). We showed that CMs isolated from burn animals exhibit significantly decreased peak sarcomere shortening as well as reduced contraction and relaxation velocities when compared to CMs from sham animals. Our analysis is sarcomere-specific and independent of cell size, and it provides results similar to the method of cellular edge detection shortening measurement used by other investigators in a prior study³⁶. In concordance with our *in vivo* data, local blockade of activated p38 MAPK in burned rats abolished the burn-induced reduction in sarcomere contractility. Similar to the effects seen for the *in vivo* blood pressure measurements, complete restoration of peak sarcomere shortening to levels comparable to CMs from sham animals was observed at 24 hours after burn injury. This suggests that inhibition of local dermal phosphorylated p38 MAPK in burn injured animals may block the generation of systemic inflammation resulting in decreased cardiac contractility. Our previous studies have shown that burn-induced acute lung injury can be ameliorated by local activated p38 MAPK inhibition^{21, 22}. Therefore, the attenuation of cardiac dysfunction by topical modulation of dermal wound inflammation confirms our hypothesis that the dermal response to thermal injury leads to SIRS and end-organ damage. In addition, these results point to the prominent role of local activated p38 MAPK in eventual myocardial dysfunction following thermal injury.

To further investigate the direct relationship between local burn wound inflammation and subsequent compromise of cardiac function we exposed isolated cardiomyocytes from normal rats to homogenates of burned skin in tissue culture. Exposure to burn skin homogenate directly impaired cardiac contractility parameters in normal cardiomyocytes such as peak sarcomere shortening, contraction velocity, and relaxation velocity. Interestingly, cardiomyocytes exposed to burn skin treated with dermal application of SB showed significantly less deterioration of contractility measurements. Likewise, serum isolated from SB treated burn rats only slightly impaired myocardial contractility of normal CMs while serum from burned animals treated with vehicle significantly decreased contractility. These data coupled with prior investigations from our laboratory help explain a potential pathway leading from local burn injury to remote end-organ failure (cardiac and pulmonary dysfunction)^{19–22}.

Expression and phosphorylation of p38 MAPK in cardiomyocytes is known to take place in a time-dependent manner starting as early as 1 hour after burn injury and lasting for at least 4 hours¹⁸. We found the same pattern for expression of baseline p38 as well for phosphorylated p38 MAPK as indicated by Western blots. Given the rapid phosphorylation of p38 MAPK within 15 – 60 minutes after thermal injury we applied the p38 MAPK inhibitor immediately

following burn injury (and two more times thereafter) to ensure blockade of the phosphorylated and therefore activated p38 MAPK as quickly as possible^{18, 19}. However, we found no difference in cardiac expression of p38 MAPK between rats treated with topical SB202190 or vehicle arguing against absorption and systemic effects of SB. Similarly, MAPKAPK-2, which is a direct target of activated p38MAPK and becomes rapidly phosphorylated and activated, showed no difference between these two treatment groups confirming our results³⁷. We therefore propose that other intermediate pathways and mediators may link the local inflammatory response and systemic end-organ effects. The nature of these mediators remains to be shown, but we hypothesize that pathways becoming activated either concurrently with or downstream of p38 MAPK locally in the burn wound will exert their systemic effects on remote organs thereby contributing to organ failure and potentially MODS. Possible candidate mechanisms include the activation pathways of c-Jun protein kinase (JNK) and stress-activated protein kinase (SAPK). Cellular stress activates ATF-2 by phosphorylation of Thr69 and Thr71. In addition to p38 MAPK, both JNK and SAPK have been shown to phosphorylate ATF-2 at these sites in vitro and in cells transfected with ATF-2^{38–40}.

In summary, activation of downstream targets of p38 MAPK and release of pro-inflammatory mediators from burn skin can be abrogated by local phosphorylated p38 MAPK inhibition. This leads to improved cardiac function following burn injury. Cardiac activation of p38 MAPK is not altered by such local treatment indicating that other concurrent pathways may be involved. We provide evidence that local control of inflammatory response to burn injury is associated with attenuated end-organ dysfunction, which may be used as a novel therapeutic approach in the future.

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Non-standard abbreviations

DPBS

Dulbecco's phosphate buffered saline

CMs

cardiomyocytes

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In-vivo Hemodynamics 24 hours after burn injury

Figure 1.

In vivo measurement of systemic hemodynamics, mean arterial blood pressure (MAP, A), systolic blood pressure (SBP, B), diastolic blood pressure (DBP, C) and heart rate (D) 24 hours after burn or sham injury. Activated p38 MAPK-inhibitor (SB) or vehicle was applied locally at the time 0, 8, and 16 hours after burn or sham injury. * $p < 0.05$ burn SB vs. burn vehicle (ANOVA). $n = 5$ animals in each group.

In-vitro sarcomere contractility 24 hours after burn injury

Figure 2.

In vitro measurement of peak sarcomere shortening values (A), contraction velocity (B) and relaxation velocity (C). Activated p38 MAPK-inhibitor (SB) or vehicle was applied locally at the time 0, 8, and 16 hours after burn or sham injury. Cardiomyocytes were harvested 24 hours after burn or sham injury. Using IonWizard® Software and the IonOptix® Acquisition System, 10–15 sarcomeres were included in a rectangle-shaped region of interest (ROI) and contractions were recorded for 75 s, averaged and analyzed. The total number of analyzed cells per group and time point was n=45. * $p < 0.05$ burn SB vs. burn vehicle (ANOVA).

Figure 3.

In vitro measurement of peak sarcomere shortening values (A, D), contraction velocity (B, E) and relaxation velocity (C, F) in normal cardiomyocytes. Shown in the left panel are CMs exposed to homogenates from normal skin or burn skin treated with activated p38 MAPK-inhibitor (SB) or vehicle (applied locally at the time 0, 8, and 16 hours after burn or sham injury). Shown in the right panel are CMs exposed to serum isolated from normal or burn injured rats treated topically with vehicle or SB at time 0, 8, and 16 hours after burn or sham injury. The serum was collected 24 hours after burn or sham injury. Cardiomyocytes were harvested from healthy rats and incubated with 200 μ L of skin homogenate or 100 μ L serum for 18 hours. Using IonWizard[®] Software and the IonOptix[®] Acquisition System, 10–15

sarcomeres were included in a rectangle-shaped region of interest (ROI) and contractions were recorded for 75 s, averaged and analyzed. The total number of analyzed cells per group and time point was n=30. *p<0.05 burn SB vs. burn vehicle (ANOVA).

A. Western blots of cardiomyocyte homogenates

B. Densities of Western blots expressed as ratio to GAPDH

Figure 4.

Expression of base p38 MAPK, phosphorylated p38 MAPK and phosphorylated MAPKAPK protein expression in cardiomyocytes using Western blotting. Ventricular cardiomyocytes were harvested from sham or burn injury rats 24 hours after injury. Burn injury animals were treated with vehicle or topical SB at time 0, 8, and 16 hours after burn injury. Results for cardiomyocytes from sham rats are representative for all time points. Representative gel patterns from Western blots are shown in the lower part of Figure 4B. Results were quantified using digital pixel density and image analysis software, normalized to housekeeping GAPDH and expressed as ratios of protein/GAPDH pixel density.