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Effect of 3-mercaptopyruvate Sulfurtransferase Deficiency on the Development of Multiorgan Failure, Inflammation, and Wound Healing in Mice Subjected to Burn Injury

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The gaseous transmitter hydrogen sulfide (H_2S) has been implicated in various forms of critical illness. Here, we have compared the outcome of scald burn injury in wild-type mice and in mice deficient in 3-mercaptopyruvate sulfurtransferase (3-MST), a mammalian H_2S-generating enzyme. Outcome variables included indices of organ injury, clinical chemistry parameters, and plasma levels of inflammatory mediators. Plasma levels of H_2S significantly increased in response to burn in wild-type mice, but remained unchanged in 3-MST$^{-/-}$ mice. The capacity of tissue homogenates to produce H_2S from 3-mercaptopyruvate was unaffected by burn injury. In 3-MST$^{-/-}$ mice, compared to wild-type controls, there was a significant enhancement in the accumulation of polymorphonuclear cells (as assessed by the quantification of myeloperoxidase) in the liver (but not heart, lung, or skin) at 7 days postburn. Oxidative tissue damage (as assessed by malon dialdehyde content) was comparable between wild-type and 3-MST-deficient mice in all tissues studied. 3-MST$^{-/-}$ and wild-type mice exhibited comparable burn-induced elevations in circulating plasma levels of hepatic injury; however, 3-MST$^{-/-}$ mice exhibited a higher degree of renal injury (as reflected by elevated blood urea nitrogen levels) at 7 days postburn. Inflammatory mediators (eg, TNF-α, IL-1β, IL-2, IL-6, IL-10, and IL-12) increased in burn injury, but without significant differences between the 3-MST$^{-/-}$ and wild-type groups. The healing of the burn wound was also unaffected by 3-MST deficiency. In conclusion, the absence of the H_2S-producing enzyme 3-MST slightly exacerbates the development of multiorgan dysfunction but does not affect inflammatory mediator production or wound healing in a murine model of burn injury.

Hydrogen sulfide (H_2S) is an endogenous gasotransmitter, which regulates multiple physiological and pathophysiological functions in the vascular, immune, and nervous system.[1–6] H_2S biogenesis in mammals is catalyzed by three enzymes: cystathionine-gamma-lyase (CSE), cystathionine-beta-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST).[1–6]

The regulatory roles of H_2S are emerging in various forms of critical illness[7–22] including burn injury.[23–36] In some forms of critical illness (eg, endotoxic shock, septic shock, as well as in burn injury), increased circulating H_2S levels have been reported, and in many—but not all—published studies, pharmacological inhibition of H_2S production, or genetic deficiency of H_2S-producing enzymes exerted beneficial effects. In other forms of critical illness (eg, ischemia-reperfusion, or hemorrhagic shock), H_2S degradation appears to be increased, resulting in local or systemic H_2S deficiency; accordingly, in these conditions H_2S donors (ie, pharmacological supplementation of H_2S) was found to be therapeutically advantageous. However—and in line with the characteristic bell-shaped concentration-response of H_2S, where low vs high concentrations of this mediator can exert opposing biological effects in modulating cell injury and inflammatory mediator production—in several preclinical models of critical illness, including murine burn models—both H_2S donors and H_2S synthesis inhibitors have been shown to exert beneficial effects.[23–26]

It was recently demonstrated that deficiency in CSE exerts beneficial effects in a mouse model of burn injury.[36] However, CSE is only one of the enzymes that contributes to the biosynthesis of H_2S; two other enzymes, CBS and 3-MST also play important roles. So far, the pathophysiological role of 3-MST has been relatively less studied, in part because, until recently, specific experimental tools (eg, mice deficient in 3-MST or pharmacological inhibitors of 3-MST) have not been available.[1] The goal of the current study, therefore, was to elucidate the potential role of 3-MST on the development of multiorgan injury and inflammatory responses in a mouse model of burn injury.

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METHODS

Materials

Unless indicated otherwise, all other all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals and Experimental Design

Male wild-type and 3-MST$^{-/-}$ mice[37] (a kind gift of Dr. Noriyuki Nagahara, Isotope Research Center, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113–8602, Japan) (10–12 weeks of age) were housed at 24 to 26°C on a 12:12 light:dark cycle and subjected to burn injury as described.[38,39] Briefly, mice were anesthetized by isoflurane (3–5% inhalation). After an intraperitoneal (i.p.) injection of buprenorphine (0.1 mg/kg), ~40% of the dorsum was shaved and approximately 1 ml Ringer's lactate solution was injected under the skin along the spinal column. Subsequently, the dorsa were subjected to ~95°C water for 10 seconds. This produces a full thickness scald wound (~30% of the total BSA). The animals received 2 ml Ringer's lactate for resuscitation. In order to minimize animal suffering, pain, or distress, animals were scored twice daily and evaluated based on an IACUC-approved Rodent Intervention Score Sheet. Buprenorphine was administered when needed indicated to reduce pain and distress.

N = 30 wild-type and N = 30 3-MST$^{-/-}$ animals were used in the current study. Groups of animals (n = 10 wild-type and n = 10 3-MST$^{-/-}$) were sacrificed at 24 hours, 7 days, or 21 days under anesthesia and blood, heart, lung, liver, and kidney tissues and skin tissue around the burn were harvested for analysis. Additional 10 wild-type animals were used as wild-type sham and additional 10 3-MST$^{-/-}$ animals were used as 3-MST$^{-/-}$ sham animals (all killed at 24 hours).

The current investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Eight Edition, 2011) and was performed in accordance with the IACUC, University of Texas Medical Branch, Galveston, TX, USA.

Measurement of Plasma H_2S Levels

Plasma H_2S levels at 24 hours postburn were measured using the fluorescent dye 7-azido-4-methylcoumarin (AzMC) as described.[29] Plasma H_2S concentrations were calculated against a Na_2S standards reacted with AzMC and were expressed as µM.

Measurement of Tissue H_2S Production

H_2S production in tissue homogenates was measured using AzMC as described.[33] Briefly, tissues were homogenized and AzMc (10 µM), and either the CBS substrates L-cysteine and homocysteine (each at 2.5 mM final concentration) or the 3-MST substrate 3-mercaptopyruvate (2 mM final concentration) was added to the homogenate and H_2S fluorescence was measured at 3 hours. The L-cysteine/homocysteine-induced H_2S production was used to estimate CBS activity and the 3-mercaptopyruvate-induced H_2S production was used to estimate 3-MST activity. H_2S production was calculated against Na_2S standards reacted with AzMC and expressed as femtomoles of H_2S produced/mg total tissue protein/min.

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity, an indicator of tissue polymorphonuclear leukocyte accumulation, was measured in heart, lung, liver, and kidney homogenates using a commercially available myeloperoxidase fluorometric detection kit (Enzo Life Sciences, , Farmingdale, NY) as described.[39]

Malondialdehyde Assay

Tissue malondialdehyde (MDA) levels, an index of cellular injury/oxidative stress, were measured in heart, lung, liver, and kidney homogenates using a fluorimetric MDA-specific lipid peroxidation assay kit (Enzo Life Sciences) as described.[39]

Measurement of Biochemical Parameters of Organ Dysfunction

Blood samples were collected via cardiac puncture and were analyzed by using a Vetscan analyzer as described[39] for various biochemical parameters within 1 hour of collection.

Quantification of Plasma Cytokine Levels

Blood from all groups were collected into heparinized blood collection tubes and processed within 30 minutes of collection. Blood was centrifuged at 4°C for 10 minutes at 1000g. Plasma collected and stored at –80°C until analysis, using the Luminex system and Invitrogen's Mouse Cytokine Magnetic 10-plex Panel kit, as described.[39] Simultaneous quantification of the following analytes was conducted: tumor-necrosis factor α (TNF-α), interleukin (IL)-1β , IL-2, IL-4, IL-6, IL-10, IL-12 p70, interferon γ-induced protein 10 kDa (IP 10), chemokine (C-X-C motif) ligand 1/keratinocyte chemoattractant (KC), monocyte chemoattractant protein 1 (MCP-1), regulated on activation, normal T cell expressed and secreted/chemokine (C-C motif) ligand 5 (RANTES), vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF).

Quantification of Wound Size

Wound areas at 24 hours after burn (to obtain a baseline value) and at 21 days were quantified as previously described,[39] using a transparent sheet and the NIS Elements Imaging Software (Nikon).

Statistical Analysis

The study was conducted in such a way that the person taking the measurements was blinded to the identity of the treatment groups. Numerical values in the text and figures are expressed as mean ± SEM for n observations. Student's t-test, one-way and two-way analysis of variance with Tukey's post hoc test were used to detect difference between groups using the Graph Pad software. $P < .05$ was considered statistically significant.

RESULTS

H_2S Production and Activity of H_2S-producing Enzymes

Burn injury in wild-type mice resulted in an increase in circulating H_2S levels, as assessed by the fluorescent dye AzMC. However, no increase in circulating H_2S was seen in the 3-MST$^{-/-}$ mice (Figure 1), suggesting that 3-MST contributes to the burn-induced elevation in circulating H_2S levels.

Next, the H_2S-producing capacity of liver and kidney homogenates was measured. H_2S production in response to cysteine/homocysteine was considered as an estimator of CBS activity; H_2S production in response to 3-mercaptopyruvate was used as an estimator of 3-MST activity (Figure 2). In the liver,

maximal H_2S production in response cysteine/homocysteine (ie, CBS-dependent H_2S production) was higher than maximal H_2S production in response to 3-mercaptopyruvate (ie, 3-MST-dependent H_2S production), while in the kidney, 3-MST activity was approximately twice as high as CBS activity (Figure 2). In 3-MST$^{-/-}$ liver homogenates, as expected, 3-mercaptopyruvate-induced H_2S production was completely absent. However, surprisingly, in 3-MST$^{-/-}$ tissues, the cysteine/homocysteine-dependent H_2S production was lower than the corresponding levels in wild-type tissues, perhaps indicating an activating interaction between the 3-MST and the CBS system in these tissues.

Burn failed to affect either 3-mercaptopyruvate-catalyzed H_2S production in liver and kidney homogenates; however, cysteine+homocysteine-dependent H_2S production appeared to increase over baseline at 7 days postburn (Figure 2).

Effect of CSE Deficiency on Multiorgan Injury and Systemic Inflammatory Response in Burns

Burn induced marked increases in lung, liver, heart, and kidney MPO and MDA levels, indicating, respectively, the infiltration of these tissues with polymorphonuclear cells, and increased oxidative burden of the tissues (Figures 3 and 4). The patterns and magnitudes of these burn-induced increases were, generally, comparable in wild-type mice and 3-MST$^{-/-}$ mice, with the exception of the liver, where MPO levels 7 days postburn were higher in the 3-MST$^{-/-}$ mice than in the wild-type control mice (Figure 3), possibly indicating a slight protective role of endogenously produced, 3-MST-derived H_2S.

Burn injury also induced marked changes in the clinical chemistry parameters and organ injury markers of the animals. There was an increase in the plasma levels of the hepatic/bone injury marker alkaline phosphatase, the hepatic injury marker alanine aminotransferase, the pancreatic injury marker amylase, and the renal dysfunction markers creatinine and blood urea nitrogen (Figures 5 and 6). Many of these parameters peaked at 24 hours, but alkaline phosphatase levels remained increased by 21 days postburn. Plasma blood urea nitrogen levels tended to be higher in 3-MST$^{-/-}$ mice even under baseline conditions, and they became significantly more elevated in 3-MST$^{-/-}$ mice than in wild-type mice 7 days postburn, perhaps indicative of a slight protective role of endogenous, 3-MST-derived H_2S against renal dysfunction (Figure 5).

Consistent with the systemic inflammatory response syndrome associated with burns, burn markedly increased the plasma levels of multiple chemokines, cytokines, and colony-stimulating and angiogenic hormones; however, plasma levels of these mediators were unaffected by the absence of 3-MST (Figures 7 and 8).

There were no differences in plasma troponin-I levels between wild-type and 3-MST-deficient mice postburn, nor were any statistically significant differences in the rate of wound healing between the wild-type and the 3-MST$^{-/-}$ animals (Figure 9).

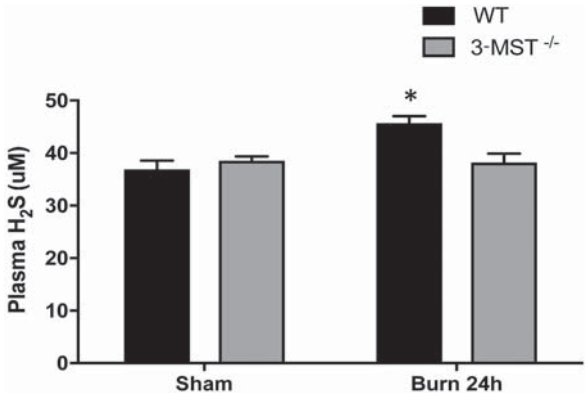

Figure 1. Effect of burn on plasma H_2S levels in wild-type and 3-MST$^{-/-}$ mice. Data show that H_2S plasma levels in sham mice and in wild-type (WT) and 3-MST$^{-/-}$ mice subjected to 24 hours of burn. H_2S plasma levels increased in wild-type animals after burn; however, in 3-MST$^{-/-}$ mice, sham and postburn plasma H_2S levels are same. Data are shown as mean ± SEM of 10 animals for each group; $*P < .05$ shows significant increase in H_2S levels in response to burn, compared to the sham group.

DISCUSSION

It been previously proposed that the role of H_2S in burn may be time-dependent, with pro-inflammatory actions dominating in the early stages of the disease, and anti-inflammatory and wound-healing-modulating effects dominating in later stages.[30] However, the relative role of the various H_2S-producing enzymes in burns remains incompletely understood. Based on the beneficial effect of the CBS/CSE inhibitor aminooxyacetic acid,[25] it was previously hypothesized that CBS-derived H_2S plays a pathophysiological, deleterious role in burns. Moreover, based on the protection seen in CSE-deficient mice,[36] it was previously concluded that CSE-derived H_2S also plays a pathogenetic role in this model. Other studies have measured H_2S-producing enzyme expression levels and circulating H_2S concentrations, and demonstrated that in rodent models of burn, CSE mRNA expression is upregulated[26] and both in rodent models of burn as well as in patients with burn, circulating H_2S levels are increased.[26,29] Taken together, these experiments indicate that CSE- and CBS-derived H_2S both play pathogenetic (deleterious) roles in burns, by promoting inflammatory mediator production and exacerbating end-organ injury. However, the role of 3-MST in the pathogenesis of burn injury has not yet been elucidated, most likely because, until recently, neither mice deficient in 3-MST, nor sufficiently potent or selective pharmacological inhibitors of 3-MST were available.

The measurement of circulating H_2S levels in the current study confirms and extends earlier studies[25,29,36] showing that burn induces an increase in circulating H_2S levels and suggests that 3-MST is involved in this increase, because burn failed to increase circulating H_2S levels in 3-MST$^{-/-}$ mice (Figure 1). The tissue source of the increased 3-MST-derived H_2S production was not identified in the current study, as the liver and kidney tissues studied by us did not exhibit any increases in the activity of 3-MST, at least at the maximal (saturating) substrate (3-mercaptopyruvate) concentrations employed in this assay (Figure 2). These results do not, however, exclude the possibility that 3-MST (and/or other H_2S-synthesizing enzymes) become upregulated in response to burn in other cells or tissues that were not examined by us.

One interesting observation is that in tissues from 3-MST$^{-/-}$ mice, the L-cysteine/homocysteine-induced H_2S production (which estimates CBS activity) was lower than the

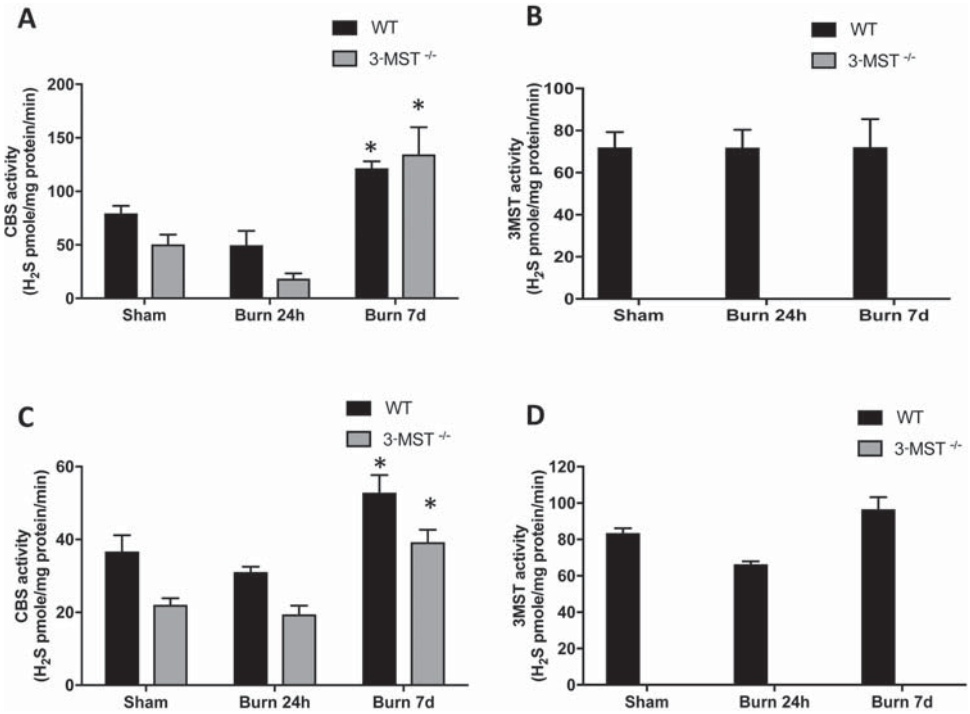

Figure 2. Effect of burn on the enzymatic activity of cystathionine-beta-synthase (CBS) and 3-MST in wild-type and 3-MST$^{-/-}$ mice. Data show H_2S production by liver homogenates in response to cysteine/homocysteine (an index of CBS activity) and in response to 3-mercatopyruvate (an index of 3-MST activity) in wild-type (WT) sham mice, in wild-type mice subjected to 24 hours and 7 days of burn, in 3-MST$^{-/-}$ sham mice and in 3-MST$^{-/-}$ mice subjected to 24 hours and 7 days of burn. Burn significantly increased CBS activity in liver (A) and kidney (C) samples from wild-type and 3-MST$^{-/-}$ mice at 7 days. 3-MST activities were comparable in liver (B) and kidney (D) samples from wild-type mice subjected to burn. Data are shown as mean ± SEM of 10 animals for each group; *$P < .05$ shows significant increase in CBS activity in response to burn, compared to the sham group.

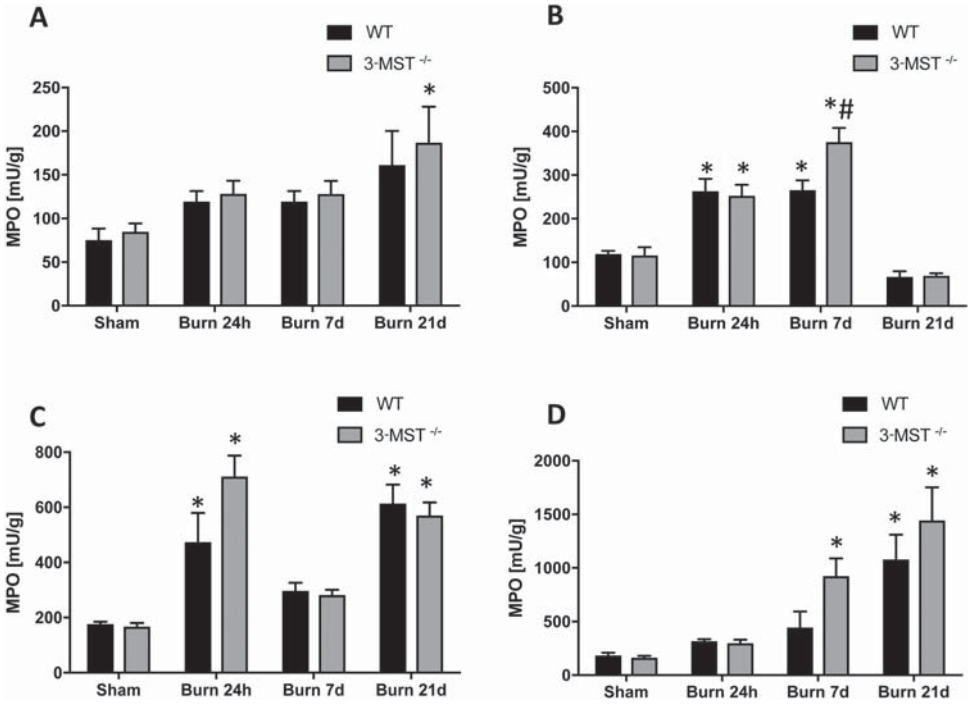

Figure 3. Burn injury increases in lung, liver, skin, and kidney myeloperoxidase (MPO) levels. Lung (A), liver (B), skin (C), and kidney (D) MPO levels (expressed as milliunits [mU]/g tissue) are shown in wild-type (WT) mice and in 3-MST$^{-/-}$ mice subjected to sham injury or to burn injury (24 hours, 7 days, and 21 days). Burn significantly increased MPO levels at 24 hours, 7 days, and 21 days ($P < .05$) compared to the values measured in sham animals. MPO levels in 3-MST$^{-/-}$ burn mice were significantly increased as compared to wild-type burn mice in liver homogenates at 7 days. Data are shown as mean ± SEM of 10 animals for each group; *$P < .05$ shows significant increase in MPO in response to burn mice, compared to the sham group; #$P < .05$ shows significant increases in 3-MST$^{-/-}$ burn mice compared to wild-type burn mice.

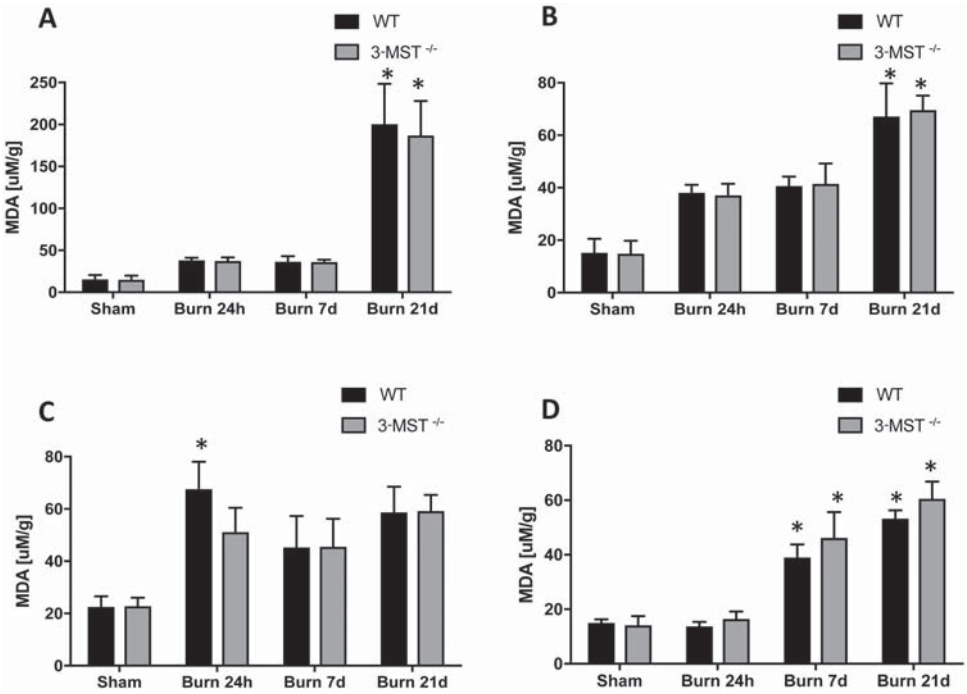

Figure 4. Burn injury increases in lung, liver, skin, and kidney malondialdehyde (MDA) levels. Lung (A), liver (B), skin (C), and kidney (D) MDA levels are shown in wild-type (WT) mice and in 3-MST$^{-/-}$ mice subjected to sham injury or to burn injury (24 hours, 7 days, and 21 days). Burn significantly increased MDA levels at 24 hours, 7 days, and 21 days ($P < .05$) compared to the value measured in sham animals. Data are shown as mean ± SEM of 10 animals for each group; *$P < .05$ shows significant increase in MDA in response to burn, compared to the sham group.

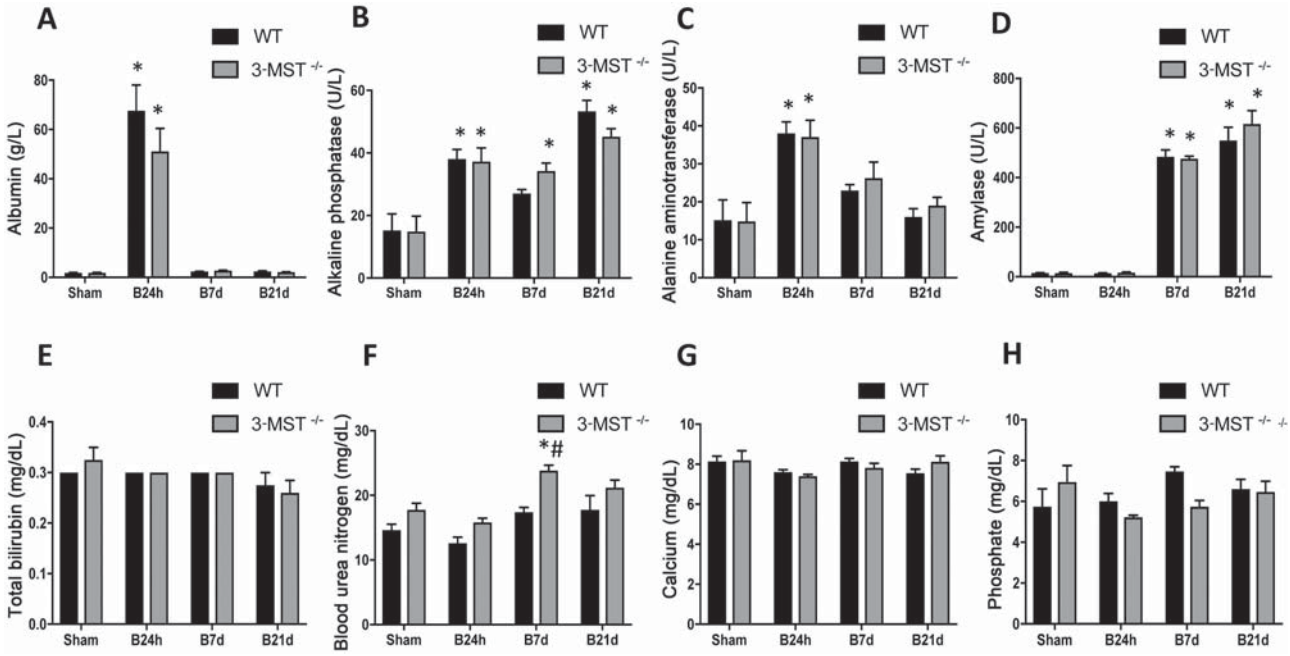

Figure 5. Burn injury increases selected parameters of organ injury. Various physiological and organ injury marker levels (albumin (A), alkaline phosphatase (B), alanine aminotransferase (C), amylase (D), total bilirubin (E), plasma blood urea nitrogen (F), plasma calcium (G) and plasma phosphate (H)) measured by Vetscan analysis, are shown in wild-type (WT) mice and in 3-MST$^{-/-}$ mice subjected to sham injury or to burn injury (24 hours, 7 days, and 21 days). Burn significantly increased the indicated parameter at 24 hours, 7 days, and 21 days ($P < .05$) compared to the value measured in sham animals. The blood urea nitrogen level in 3-MST$^{-/-}$ burn mice was significantly higher than the corresponding values in wild-type burn mice at 7 days. Data are shown as mean ± SEM of 10 animals; *$P < .05$ shows significant changes in response to burn, compared to the sham group; #$P < .05$ shows a significantly higher increase in 3-MST$^{-/-}$ burn mice compared to wild-type burn mice.

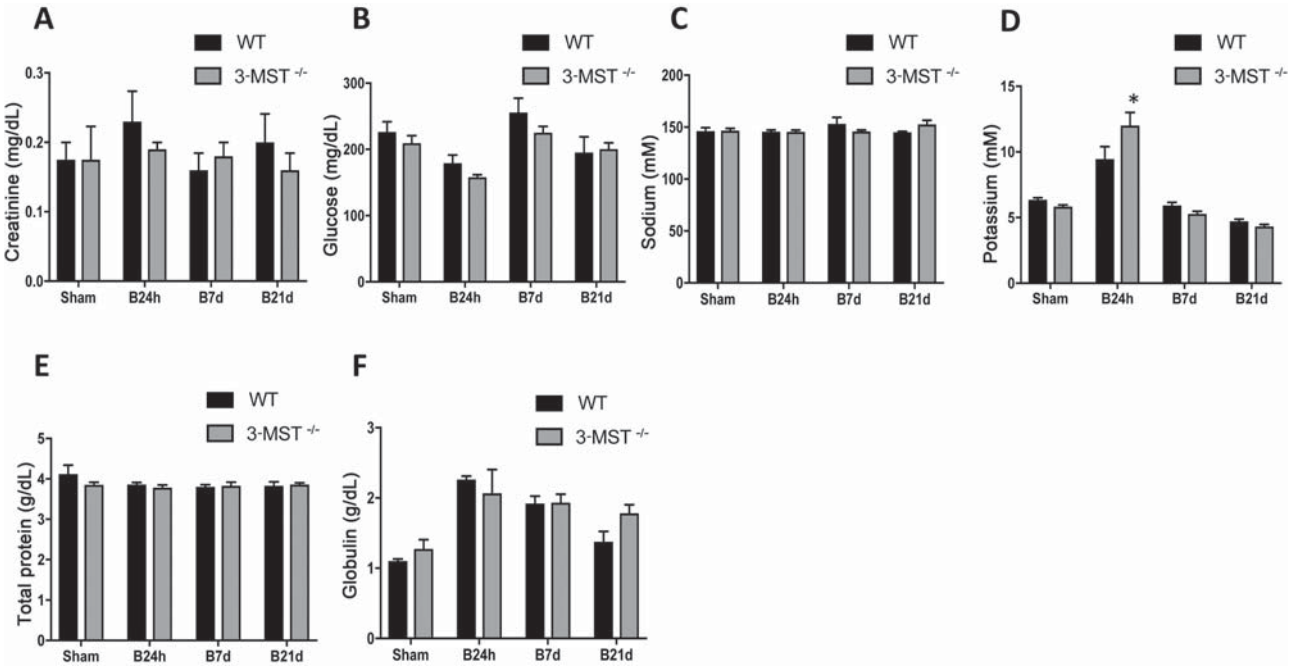

Figure 6. Burn injury increases in selected parameters of organ injury. Various physiological and organ injury marker levels (plasma creatinine (A), plasma glucose (B), plasma sodium (C), plasma potassium (D), plasma total protein (E) and plasma globulin (F)) measured by Vetscan analysis, are shown in wild-type (WT) mice and in 3-MST$^{-/-}$ mice subjected to sham injury or to burn injury (24 hours, 7 days, and 21 days). Burn significantly increased the indicated parameter at 24 hours, 7 days, and 21 days ($P < .05$) in wild-type and 3-MST$^{-/-}$ mice compared to the value measured in sham animals. Data are shown as mean ± SEM of 10 animals; *$P < .05$ shows significant changes in response to burn, compared to the sham group.

corresponding H_2S production in wild-type tissues. This is an unexpected finding, because the two enzymes have previously not been linked functionally. Although the substrate of 3-MST (3-mercaptopyruvate) is produced from cysteine, and cysteine is also a substrate of CBS, this commonality will also not explain the reduced tissue CBS activity in the 3-MST$^{-/-}$ mice. The mechanism and the potential functional importance of these changes remain to be further explored.

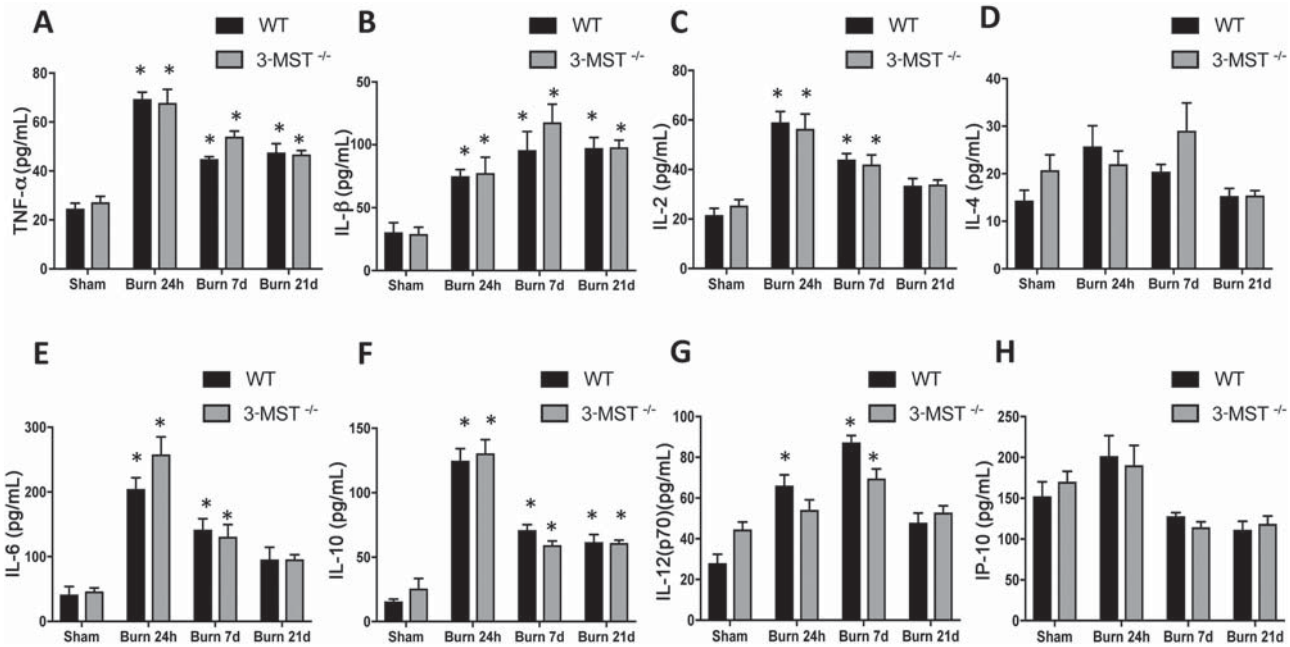

Figure 7. Burn injury increases cytokine levels in plasma samples. Plasma cytokine levels, measured by Luminex analysis, are shown in wild-type (WT) mice and in 3-MST$^{-/-}$ mice subjected to sham injury or to burn injury (24 hours, 7 days, and 21 days). Burn markedly increased plasma TNF-α (A), IL-β (B), IL-2 (C), IL-4 (D), IL-6 (E), IL-10 (F), IL-12(p70) (G), and IP-10 (H) levels in wild-type and 3-MST$^{-/-}$ mice at 24 hours, 7 days, and 21 days. Data are shown as mean ± SEM of 10 animals; *$P < .05$ shows significant changes in response to burn, compared to the sham group.

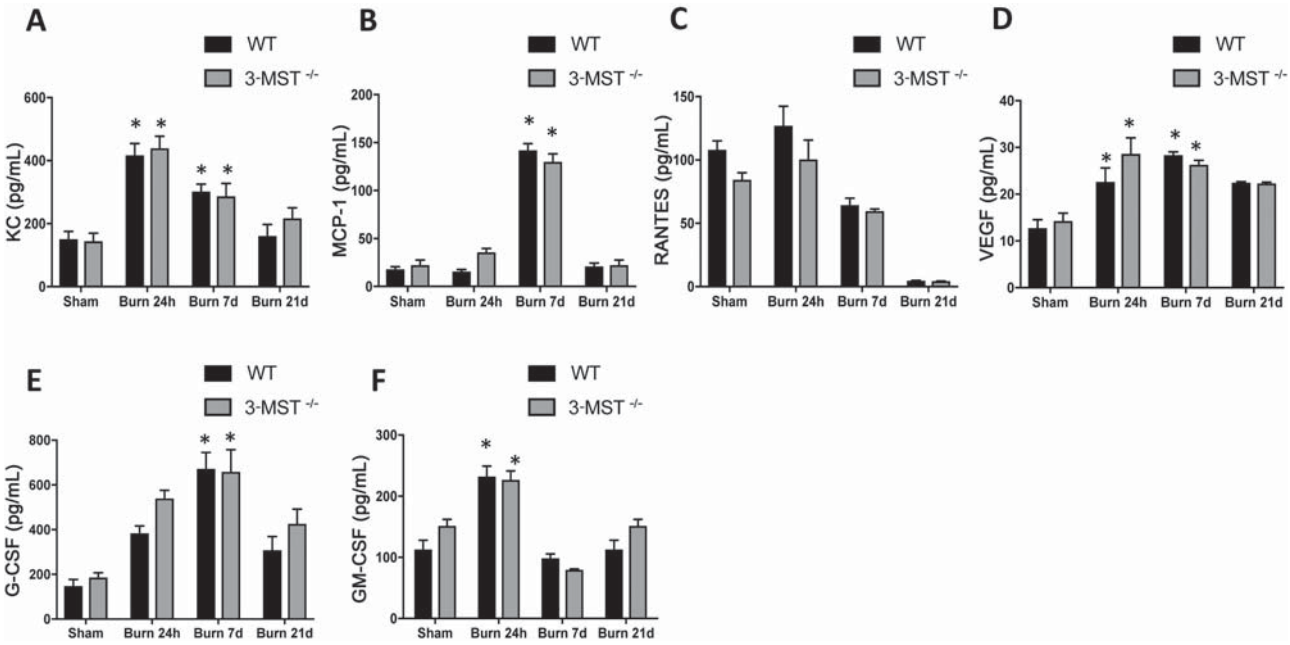

Figure 8. Burn injury increases cytokine levels in plasma samples. Plasma cytokine levels, measured by Luminex analysis, are shown in wild-type (WT) mice and in 3-MST$^{-/-}$ mice subjected to sham injury or to burn injury (24 hours, 7 days, and 21 days). Burn markedly increased plasma KC (A), MCP-1 (B), RANTES (C), VEGF (D), G-CSF (E), and GM-CSF (F) levels in wild-type and 3-MST$^{-/-}$ mice at 24 hours, 7 days, and 21 days. Data are shown as mean ± SEM of 10 animals; *$P < .05$ shows significant changes in response to burn, compared to the sham group.

Based on prior studies focusing on CBS and CSE, our working hypothesis was that 3-MST deficiency will also exert protective effects in burns. However, unexpectedly, 3-MST deficiency did not provide any protective effects in burn in various tissues, nor did it affect circulating inflammatory mediator production. On a few selected parameters, at a few time points, we have even observed an exacerbation of the burn-induced pathophysiological changes in the 3-MST$^{-/-}$ mice, although we must keep in mind that these differences are relatively minor, and, overall, probably the correct conclusion should be that wild-type and 3-MST-deficient mice behave, in almost all respects, in the same manner when subjected to burn injury.

It should be noted that a recent study compared wild-type and 3-MST-deficient mice on hemodynamic or metabolic parameters and mortality in an experimental model of traumatic-hemorrhagic shock and observed no differences.[40] Taken together, the available evidence so far suggests that 3-MST plays no major role in the pathogenesis of critical illness.

What, then, are the differences between 3-MST and CBS and CSE in terms of H_2S production and related pathways that may explain these differences? First of all, 3-MST—in contrast to CBS and CSE—has a significant mitochondrial localization (although it is also present in the cytosol).[41] Thus, the intracellular localization of the H_2S it produces may be different, and therefore, the cellular pathways/targets affected by H_2S may also be different. Second, 3-MST—in contrast to CBS and CSE—produces a significant amount of polysulfides (rather than, or in addition to "free" H_2S).[41–43] Polysulfides

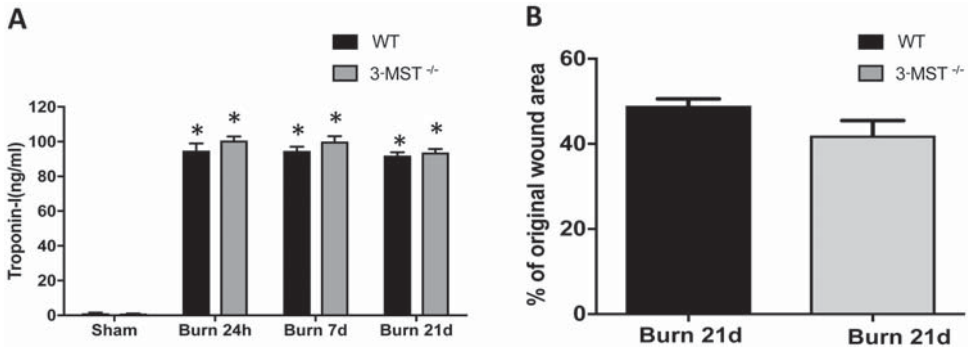

Figure 9. Effect of burn injury on plasma troponin levels and burn wound area at Day 21. (A) Plasma troponin levels are shown in wild-type (WT) mice and in 3-MST$^{-/-}$ mice subjected to sham injury or to burn injury (24 hours, 7 days and 21 days). Burn markedly increased plasma troponin levels at 24 hours, 7 days, and 21 days; there was no statistically significant difference between the values measured in the wild-type and the 3-MST$^{-/-}$ burn group. *$P < .05$ shows significant increases in response to burn, compared to the sham group. (B) % wound area on Day 21 is shown in the wild-type and 3-MST$^{-/-}$ burn group. Wound healing was comparable in the two groups of mice. Data are shown as mean ± SEM of 10 animals.

have their own, very distinct regulatory roles in biological systems, which are fairly different from the roles of H_2S, due to the different array of chemical reactions polysulfides can catalyze (most importantly, a post-translational modification of proteins via S-sulfhydration).[44] As far as the net output of H_2S from 3-MST vs CBS and CSE, no marked differences were noted (at least in the highly artificial conditions of tissue homogenates incubated with saturating concentrations of the respective substrates utilized in the current model). 3-MST is broadly expressed in all cells and tissues[40,45] and therefore the lack of effect on MDA or MPO levels in the various organs studied here cannot be due to the absence of 3-MST in those organs. It is also not the case that 3-MST, in general, is not biologically relevant: multiple studies, in multiple models, have already implicated functional roles of this enzyme in various biological systems ranging from neuronal roles to vascular and metabolic roles.[41–53]

Taken together, the data presented in the current paper do not find a major role of 3-MST in burn injury and support the concept that different H_2S-producing enzymes (CBS, CSE, 3-MST) can play substantially different roles in the pathogenesis of burn injury and other forms of critical illness.

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