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Tanshinone IIA sodium sulfonate facilitates endocytic HMGB1 uptake

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Abstract

Our seminal discovery of high mobility group box 1 (HMGB1) as a late mediator of lethal systemic inflammation has prompted a new field of investigation for the development of experimental therapeutics. We previously reported that a major Danshen ingredient, tanshinone IIA sodium sulfonate (TSN-SS), selectively inhibited endotoxin-induced HMGB1 release and conferred protection against lethal endotoxemia and sepsis. To investigate the underlying mechanisms by which TSN-SS effectively inhibits HMGB1 release, we examined whether TSN-SS stimulates HMGB1 uptake by macrophages and whether genetic depletion of HMGB1 receptors [e.g., toll-like receptors (TLR)2, TLR4, or the receptor for advanced glycation end product (RAGE)] or pharmacological inhibition of endocytosis impairs TSN-SS-facilitated HMGB1 cellular uptake. TSN-SS stimulated internalization of exogenous HMGB1 protein into macrophage cytoplasmic vesicles that subsequently co-localized with microtubule-associated protein light chain 3 (LC3)-positive punctate structures (likely amphisomes). Meanwhile, it time-dependently elevated cellular levels of internalized HMGB1, leading to elevated LC3-II production and aggregation. Although genetic depletion of TLR2, TLR4, and/or RAGE did not impair TSN-SS-mediated HMGB1 uptake, specific inhibitors of the clathrin- and caveolin-

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Competing financial interests

A.E.S. and H.W. are co-inventors of a patent application entitled "Inhibition of inflammatory cytokine production with tanshinones". However, the intellectual property disclosed above did not directly or significantly affect the design, conduct or reporting of relevant mechanistic findings in this manuscript.

Author contributions

Y.Z. and W.L. performed most experiments; S.Z., A.J. performed some experiment J.L. prepared recombinant HMGB1 protein; H.Y. and K.J.T. contributed HMGB1 receptor knock-out mice; S.F., P.W., and A.E.S. provided scientific discussions; H.W. and Y.Z. designed the study, analyzed the data and wrote the manuscript.

dependent endocytosis significantly impaired TSN-SS-mediated HMGB1 uptake. Co-treatment with a lysosomal inhibitor, bafilomycin A1, led to enhanced accumulation of endogenous LC3-II and internalized exogenous HMGB1 in TSN-SS/rHMGB1-treated macrophages. Taken together, these findings suggest that TSN-SS may facilitate HMGB1 endocytic uptake, and subsequently delivered it to LC3-positive vacuoles (possibly amphisomes) for degradation via a lysosome-dependent pathway.

1. Introduction

Innate immune cells (such as macrophages, monocytes, and neutrophils) constitute the front line of defense against microbial infections by engulfing and killing invading pathogens. In addition, these phagocytes are equipped with receptors [such as the Toll-like receptors (TLRs) TLR2, TLR3, TLR4, and TLR9] [1–5] for various pathogen-associated molecular patterns (PAMPs, such as bacterial peptidoglycan, double-stranded RNA, endotoxin, and CpG-DNA) [6;7], and can sequentially release early (e.g., TNF, IL-1, IFN- γ) and late (e.g., HMGB1) proinflammatory mediators [8]. If the invading pathogens can be efficiently eliminated, the infection-elicited inflammatory response resolves to restore immunologic homeostasis. Otherwise, exogenous pathogens and/or endogenous pro-inflammatory mediators can leak into the blood stream, triggering widespread systemic inflammatory responses such as sepsis [9].

Previously, we discovered that HMGB1 was secreted from macrophages / monocytes in response to various PAMPs (e.g., ds-RNA, CpG-DNA, and endotoxin) or cytokines [e.g., interferon (IFN)- γ] [8;10–12]. Upon binding to the receptor for advanced glycation end products (RAGE), TLR2 or TLR4 [13–16], HMGB1 induces various cytokines, chemokines, and adhesion molecules [13;17–19], thereby sustaining rigorous and potentially injurious inflammatory responses. In animal models of endotoxemia or sepsis, circulating HMGB1 increases to plateau levels between 24–36 h [8;20], distinguishing itself from tumor necrosis factor (TNF) and other early cytokines [21]. Furthermore, HMGB1-neutralizing antibodies confer protection against lethal endotoxemia [8] and sepsis [20;22] even when given 24 h after the onset of sepsis, suggesting HMGB1 as a critically important late mediator of lethal systemic inflammation [9;23]. Notably, HMGB1 can also be passively leaked by necrotic cells [24;25], thereby functioning as an early mediator of ischemia-induced myocardial or cerebral ischemic injury [26–28]. Thus, therapeutic agents capable of inhibiting HMGB1 release may hold potential for the treatment of infection- or injury-elicited inflammatory responses.

Recently, we have discovered a number of herbal components [e.g., nicotine, epigallocatechin-3-gallate (EGCG), and tanshinones] [29–32] that effectively inhibited endotoxin-induced HMGB1 release, and conferred protection against lethal endotoxemia and sepsis. For instance, a major Green tea component, EGCG, dose-dependently suppressed endotoxin-induced HMGB1 release by stimulating its aggregation and autophagic degradation [30;31]. Similarly, a popular Chinese cardiovascular medicine [33], tanshinone IIA sodium sulfonate (TSN-SS), effectively inhibited endotoxin-induced HMGB1 release, and rescued mice from lethal sepsis [32]. Furthermore, it conferred protection against sepsis-induced cardiovascular dysfunction [32] or cerebral ischemic injury [34] partly through inhibiting HMGB1 release or expression. Notably, TSN-SS effectively attenuated HMGB1 release even when given 2–6 h post endotoxin stimulation [32], long after the initiation of nuclear-cytoplasmic HMGB1 translocation. It was previously unknown, however, whether TSN-SS inhibits HMGB1 release through additional mechanisms other than blocking cytoplasmic HMGB1 translocation [32].

In addition to the capacity to engulf microbes (e.g., phagocytosis), macrophages can also uptake extracellular macromolecules via endocytosis by internalizing cytoplasmic membrane vesicles with the aid of coat proteins (e.g., clathrin and caveolin) [35]. These clathrin- and caveolin-dependent endocytic pathways can be distinguished by using specific pharmacological inhibitors (such as chlorpromazine, nystatin and indomethacin) [36]. When endocytosis and autophagy occur simultaneously, these two cellular processes can converge on a common lysosome-dependent degradation pathway. Specifically, endosomes can fuse with autophagosomes to form prelysosomal vesicles termed amphisomes [37;38], which can merge with lysosomes and mature into autolysosomes, where the amphisome contents are digested by lysosomal enzymes [39]. In the present study, we provided the evidence to support a novel mechanism by which a popular Chinese cardiovascular drug, TSN-SS, inhibits HMGB1 release. It effectively facilitates HMGB1 uptake via a clathrin and caveolin-dependent endocytosis, and delivers internalized HMGB1 to LC3-positive cytoplasmic vesicles (likely amphisomes) for possible degradation via a lysosome-dependent mechanism.

2. Materials and methods

2.1. Materials

Tanshinone IIA sodium sulfonate (TSN-SS) was obtained from the Shanghai No. 1 Biochemical & Pharmaceutical LTD (Shanghai, P. R. China), and its purity was determined by HPLC as previously described [32]. Chlorpromazine (Cat# C8138), Indomethacin (Cat# I7378), Nystatin (Cat# N4014), and Bafilomycin A1 (Cat# B1793) were purchased from Sigma-Aldrich (St. Louis, MO). Chlorpromazine was dissolved in water to make a 40 mM stock solution; whereas indomethacin, nystatin and bafilomycin A1 were dissolved in DMSO to prepare 100 mM stock solutions. HMGB1-specific polyclonal antibodies were generated in rabbits as previously described [8]. LC-3 mouse monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti- β -actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of recombinant HMGB1

The cDNA encoding for rat HMGB1 was cloned onto a pCAL-n vector, and the recombinant CBP-HMGB1 (rHMGB1) was expressed in *E. coli* BL21 (DE3) pLysS cells as previously described [8]. Contaminating endotoxin was removed from the HMGB1 preparation by Triton X-114 extraction as previously described [40]. Highly purified HMGB1 was biotinylated using a Pierce EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Cat. # 21430, Thermo Scientific, Waltham, MA, USA), and the biotinylated protein was purified by gel filtration chromatography using Sephadex G-25 column as previously described [30].

2.3. Cell culture

Murine macrophage-like RAW 264.7 cells and human HepG2 hepatoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). GFP-LC3-transfected RAW 264.7 cells were established as previously described [31], and maintained in RPMI 1640/10%FBS/2 mM glutamine supplemented with puromycin (2 μ g/ml, Sigma, P9620, St. Louis, MO) to retain clonal homogeneity. Primary peritoneal macrophages were isolated from wild-type C57BL/6 (male, 7–8 weeks, 20–25 grams) or HMGB1 receptor knock-out mice at 3 days after intraperitoneal injection of 2 ml thioglycollate broth (4%) as previously described [10;41]. TLR2, TLR4, RAGE knock-out (KO), TLR2/RAGE or TLR4/RAGE-double KO C57BL/6 mice were obtained from Helena Erlandsson-Harris (Department of Medicine, Karolinska Institute, Stockholm, Sweden) and the genotypes were confirmed as previously described [42]. Age- and sex-matched wild-type C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All animal procedures were approved

by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research.

2.4. Fluorescence Immunostaining

To examine HMGB1 uptake, RAW 264.7 cells were incubated with biotinylated rHMGB1 in the absence or presence of TSN-SS (100 μ M) for 6 h. Subsequently, cells were fixed with 2% formalin for 10 min, and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS, 1 min, room temperature). After extensive washing with PBS, cells were incubated with streptavidin-conjugated Alexa fluor 594 (Invitrogen, Carlsbad, CA, USA) to visualize exogenous HMGB1 as previously described [30]. Images were captured using a fluorescence microscope (Carl Zeiss Microimaging).

2.5. Visualization of LC3-containing cytoplasmic vesicles

To visualize LC3-containing cytoplasmic vesicles, Green Fluorescent Protein (GFP)-LC3-transfected RAW 264.7 cells were treated with biotinylated rHMGB1 in the absence or presence of TSN-SS (100 μ M) for various time periods. Subsequently, cells were stained respectively with streptavidin-conjugated Alexa fluor 594 to visualize exogenous HMGB1, and with 4',6-diamidino-2-phenylindole (DAPI, H-1200, Vector Lab, Burlingame, CA) to visualize cell nuclei. The formation of GFP-LC3 punctate structures were observed under a fluorescence microscope as previously described [31].

2.6. Western blotting analysis

The levels of internalized rHMGB1 in whole-cell lysate were determined by Western blotting analysis with reference to β -actin as previously described [8;10;41]. Briefly, equal amounts of cellular proteins were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk, the membrane was incubated with respective antibodies (anti-HMGB1, 1:2000; anti-LC3, 1:500; anti- β -actin, 1:2000) overnight. Subsequently, the membrane was incubated with the appropriate secondary antibody, and the immunoreactive bands were visualized by chemiluminescence technique.

The ratio between the 18-kD cytosolic LC3-I and 16-kD lipidated autophagosome-bound LC3-II was determined by Western blotting analysis as previously described [31]. The autophagic flux was measured by evaluating the effects of TSN-SS/rHMGB1 on LC3-II turnover in the absence or presence of an autophagy inhibitor, bafilomycin A1 at saturate concentrations. Specifically, macrophage cultures were stimulated with TSN-SS/rHMGB1 for 12 h, and bafilomycin A1 was added at a saturate concentration (100 nM) [31]. At 4 h post bafilomycin A1 addition, cells were harvested and assayed for LC3 concentrations by Western blotting analysis.

2.7. Statistical analysis

Data are expressed as mean $\pm$ SD of 2–3 independent experiments ($n = 2-3$). One-way ANOVA was used for comparison among all different groups. When the ANOVA was significant, post-hoc testing of differences between groups was performed using Tukey's test. A P value <0.05 was considered significant.

3. Results

3.1. TSN-SS facilitated internalization of exogenous HMGB1 to macrophage cytoplasmic vesicles

We recently demonstrated that TSN-SS effectively inhibited LPS-induced HMGB1 release even when given 2–6 h post endotoxin stimulation, a time frame that nuclear-cytoplasmic HMGB1 translocation had already initiated (data not shown). Moreover, TSN-SS (100 μ M) was also capable of selectively inhibiting HMGB1-induced release of nitric oxide (by >90%) in murine macrophage cultures [32]. To gain insight into the novel mechanisms by which TSN-SS effectively inhibits HMGB1 release or action, we first determined whether TSN-SS interferes with HMGB1 cell surface binding. We used biotin-labeled recombinant calmodulin-binding peptide (CBP)-HMGB1 fusion protein (rHMGB1) to distinguish between the exogenous rHMGB1 (33 kDa) from the endogenous HMGB1 protein (30 kDa). In the absence of exogenous rHMGB1, staining with streptavidin-conjugated Alexa594 revealed rather weak and diffuse background fluorescence (Figure 1, **left panels**). Consistent with our earlier report [30], prolonged treatment with rHMGB1 (for 6 h) led to punctuate rHMGB1 accumulation predominantly on cell surfaces (Figure 1, **middle panels**). In a sharp contrast to EGCG, which prevented macrophage cell surface HMGB1 accumulation [30], TSN-SS treatment led to a dramatic internalization of exogenous rHMGB1 into cytoplasmic vesicles (Figure 1, **right panels**), suggesting that TSN-SS enhances HMGB1 endocytic uptake by macrophages.

3.2. TSN-SS enhanced HMGB1 uptake in a time- and dose-dependent fashion

To test the above possibility, murine macrophage-like RAW 264.7 cells were incubated with unlabelled biotin-free rHMGB1 in the absence or presence of TSN-SS at indicated doses (10, 50, and 100 μ M) for various time periods, and cellular levels of exogenous rHMGB1 were determined by Western blot analysis. In the absence of TSN-SS, exogenous rHMGB1 accumulated on macrophage cell surfaces, and cellular rHMGB1 levels increased slightly in a time-dependent fashion (Figure 2A). However, the co-addition of TSN-SS led to a time- (Figure 2A, **left panels**) and dose-dependent (Figure 2A, **right Panel**) enhancement of cellular rHMGB1 levels, peaking around 4–6 hours in the presence of TSN-SS at 50 μ M (data not shown) or 100 μ M (Figure 2A, **left panels**). Interestingly, even in non-immune cells (such as human HepG2 hepatocytes), TSN-SS also time- (Figure 1B, **left panel**) and dose-dependently (Figure 2B, **right panel**) increased cellular levels of exogenous rHMGB1. Taken together, these findings suggest that TSN-SS can facilitate uptake of extracellular HMGB1 possibly via endocytosis.

3.3. TLR4 and RAGE were not involved in TSN-SS-mediated HMGB1 cellular uptake

HMGB1 can bind to multiple cell surface receptors including RAGE [43], TLR2, TLR4 [13;15;16], TLR9 [11;43], cluster of differentiation 24 (CD24)/Siglec-10 [44], Mac-1 [45], thrombomodulin [46], as well as single transmembrane domain proteins (e.g., syndecans) [47]. To evaluate the involvement of TLR2, TLR4 or RAGE in TSN-SS-mediated HMGB1 uptake, we compared the levels of TSN-SS-mediated HMGB1 uptake between wild-type and receptor-deficient peritoneal macrophages. The depletion of TLR2 or TLR4 did not impair TSN-SS-mediated elevation of rHMGB1 uptake in peritoneal macrophages (data not shown). Moreover, the depletion of both TLR4 and RAGE did not affect TSN-SS-mediated HMGB1 uptake either (Figure 3), suggesting that TLR2, TLR4, or RAGE are not involved in TSN-SS-mediated HMGB1 internalization. It is presently unknown whether other HMGB1-binding cell surface proteins (such as Mac-1, thrombomodulin or syndecan) are required for TSN-SS-mediated HMGB1 uptake.

3.4. Clathrin or caveolin inhibitors partly impaired TSN-SS-mediated HMGB1 uptake

To gain insights into the mechanisms of endocytosis, we determined the effects of specific inhibitors for clathrin- and caveolin-dependent endocytosis on TSN-SS-mediated HMGB1 uptake. Chlorpromazine, an inhibitor of clathrin-dependent endocytosis, did not affect the basal level of HMGB1 cell surface binding or uptake in macrophage cultures (Figure 4A). However, it persistently impaired TSN-SS-mediated HMGB1 uptake over a wide time frame (Figure 4A). Similarly, two specific inhibitors of the caveolin-dependent endocytosis, nystatin and indomethacin, also significantly attenuated TSN-SS-mediated HMGB1 uptake (Figure 4B). To appreciate the significance of this finding, we determined the effects of these inhibitors on TSN-SS-mediated HMGB1 uptake in other non-immune cells. In human HepG2 hepatocytes, both groups of inhibitors similarly diminished TSN-SS-mediated HMGB1 uptake (Figure 4C), supporting the possibility that TSN-SS enhances extracellular HMGB1 uptake through clathrin- and caveolin-dependent endocytosis pathways.

3.5. TSN-SS enhanced HMGB1-induced LC3-II production and aggregation

It was recently suggested that cytoplasmic HMGB1 can interact with beclin-1, thereby functioning as an important regulator of autophagy [48;49]. To evaluate the impact of HMGB1 cytoplasmic internalization on autophagy, we determined whether TSN-SS enhanced HMGB1-induced LC3-II production in macrophage cultures. By itself, TSN-SS (up to 100 μ M) did not increase cellular LC3-II levels (data not shown); whereas exogenous HMGB1 (2.0 μ g/ml) only slightly elevated LC3-II levels at 8 h post treatment (Figure 5A). However, TSN-SS not only elevated cellular HMGB1 uptake (Figure 2A), but also significantly enhanced HMGB1-induced increase in LC3-II levels in macrophage cultures (Figure 5A).

Following endocytosis, internalized HMGB1 was destined to endosomes, which then fuse with other cytoplasmic vesicles such as lysosomes or autophagosomes (to form amphisomes). To determine the fate of internalized HMGB1, we examined whether large HMGB1-containing cytoplasmic vesicles co-localize with LC3-positive vacuoles in GFP-LC3-transfected RAW 264.7 cells. In the absence of TSN-SS, exogenous HMGB1 was barely internalized into macrophage cytoplasmic vesicles (Figure 5B, **top panels**), and it induced the formation of a limited number of small LC3-positive vesicles (likely autophagosomes, Figure 5B, **top panels**). In the presence of TSN-SS, however, exogenous HMGB1 was detected in dramatically higher numbers of larger cytoplasmic vesicles (Figure 5B, **bottom panels**). Interestingly, these large HMGB1-containing cytoplasmic vesicles co-localized with LC3-positive punctate structures (Figure 5B, **bottom panels**), suggesting that HMGB1-containing endosomes may have fused with other cytoplasmic vesicles (such as autophagosomes to form amphisomes).

To distinguish between the possibilities whether TSN-SS/HMGB1 elevated LC3-II production or merely decreased LC3-II degradation (i.e., autophagic flux), we determined the effect of bafilomycin A1, an inhibitor of lysosome fusion, on TSN-SS/HMGB1-induced elevation of LC3-II levels. Even in the presence of bafilomycin A1 at saturating concentrations for LC3-II accumulation (100 nM), TSN-SS/HMGB1 treatment still triggered a significant increase of LC3-II levels (Figure 6, **top panel**), suggesting that TSN-SS/HMGB1 increased autophagosome synthesis, rather than merely inhibiting LC3-II degradation. To test the possibility that internalized HMGB1 is possibly degraded via the lysosome-dependent pathway, we determined the effects of a lysosomal inhibitor, bafilomycin A1, on cellular accumulation of exogenous HMGB1. At the concentration that effectively elevated cellular LC3-II levels (by inhibiting LC3-II degradation), bafilomycin A1 also significantly elevated exogenous rHMGB1 levels in TSN-SS/rHMGB1-treated cells possibly by blocking HMGB1 degradation (Figure 6, **bottom panel**). Taken together, these

data support the possibility that TSN-SS facilitated endocytic HMGB1 uptake, and delivered it to cytoplasmic vesicles for possible degradation via a lysosome-dependent mechanism.

4. Discussion

In the present study, we provided the evidence for a novel mechanism by which a popular Chinese cardiovascular medicine, TSN-SS, effectively inhibits HMGB1 release. TSN-SS facilitated internalization of exogenous HMGB1 possibly via clathrin- and caveolin-dependent endocytosis into cytoplasmic vesicles that eventually mature into endosomes (Figure 7). Subsequently, these HMGB1-containing endosomes could be fused with other cytoplasmic vesicles (such as autophagosomes) to form amphisomes, where the internalized HMGB1 was likely degraded via a lysosome-dependent mechanism (Figure 7).

HMGB1 contains a continuous stretch of negatively charged (aspartic and glutamic acids) residues in the C-terminus, and two internal repeats of positively charged domains (the HMG boxes) in the N-terminus. As a highly charged di-polar molecule, HMGB1 is not likely to be internalized into macrophage vesicles via passive diffusion. Instead, it may be engulfed via active endocytosis through clathrin- and caveolin-dependent mechanisms. This possibility was supported by the observations that specific endocytosis inhibitors significantly impaired TSN-SS-mediated HMGB1 uptake. For instance, chlorpromazine, a specific inhibitor of the clathrin-coated pit formation [36], partly impaired TSN-SS-mediated enhancement of HMGB1 uptake in macrophages (by 50–90%) and hepatocytes (by 40%) cultures. Similarly, specific inhibitors of the caveolin-dependent endocytosis (e.g., nystatin and indomethacin) also significantly inhibited TSN-SS-mediated increase of HMGB1 uptake. Taken together, these findings suggested a possibility that TSN-SS facilitated HMGB1 endocytic uptake through clathrin- and caveolin-dependent pathways.

The mechanism by which TSN-SS effectively facilitates HMGB1 endocytosis remains a subject of future investigation. Although TLR2, TLR4, and RAGE may be involved in HMGB1-mediated immune cell recruitment and activation [40;45;50;51], these receptors were not critical in TSN-SS-mediated HMGB1 cellular uptake. Notably, HMGB1 can bind to many other cell surface proteins including thrombomodulin [46], CD24/Siglec-10 [44], Mac-1 [45], as well as single transmembrane domain proteins (e.g., syndecans) [47]. For instance, syndecans can interact with various cationic molecules (via electrostatic interactions with heparin sulfate), thereby facilitating internalization of the resulting ligand-syndecan complex via clathrin- or caveolin-mediated endocytosis [52]. It will thus be interesting to determine whether syndecans or other cell surface proteins are involved in TSN-SS-mediated HMGB1 endocytosis. Similarly, it will also be important to identify the cell-surface receptors or proteins (e.g., enzymes or transcription factors) targeted by TSN-SS in future studies.

Given the capacity of cytoplasmic HMGB1 in triggering autophagy [48;49], we propose that the internalized HMGB1 may contribute to the elevation of LC3-II production and aggregation, two specific markers of autophagy [53]. Indeed, co-treatment of macrophage cultures with TSN-SS and rHMGB1 led to a time-dependent elevation of LC3-II production and aggregation (Figure 5A). Bafilomycin A1 is a specific inhibitor of vacuolar type H⁺-ATPase, and can inhibit autophagosome trafficking to, and fusion with, lysosomes. In the presence of bafilomycin A1 at saturate concentrations, TSN-SS/rHMGB1 treatment still significantly increased cellular LC3-II levels, suggesting that TSN-SS/HMGB1 stimulated LC3-II production, rather than merely inhibiting its degradation. Taken together, these results suggested that TSN-SS facilitated endocytosis of exogenous HMGB1, leading to subsequent degradation via a lysosome-dependent pathway.

When endocytosis and autophagy occur simultaneously, the endocytosed molecules (e.g., HMGB1) and autophagically sequestered proteins (LC3-II) could be delivered to the same prelysosomal vacuole called amphisomes [37;38], where both exogenous and endogenous proteins are degraded [39] (Figure 7). Indeed, in the presence of TSN-SS, exogenous HMGB1 was dramatically internalized into cytoplasmic vesicles (possibly endosomes) within 4–6 h (Figure 1). Subsequently, these HMGB1-containing “puncta” intercepted with LC3-containing vesicles in TSN-SS/rHMGB1-treated macrophages (Figure 5B), supporting a possibility that some HMGB1-containing endosomes may have fused with other cytoplasmic vesicles (e.g., autophagosomes) to form amphisomes (Figure 7). These amphisomes may subsequently merge with lysosomes and mature into autolysosomes, where the amphisome contents are digested by lysosomal enzymes [39]. Consistently, cellular levels of internalized rHMGB1 started to decrease at a late stage, which paralleled with a decrease in the number of rHMGB1-positive vesicles in TSN-SS/rHMGB1-treated macrophages (data not shown). Furthermore, co-treatment with a lysosomal inhibitor, bafilomycin A1, led to elevated accumulation of exogenous rHMGB1 in TSN-SS/HMGB1-treated macrophages. Thus, TSN-SS is capable of stimulating HMGB1 endocytic uptake, and delivers the internalized HMGB1 into cytoplasmic vesicles (likely amphisomes) for possible degradation via a lysosome-dependent pathway (Figure 7).

In addition, we found that TSN-SS elevated endocytic HMGB1 uptake in non-immune cells (such as hepatocytes) via a similar clathrin- and caveolin-dependent mechanism. Carrying receptors for various endogenous proteins such as the low-density lipoprotein [LDL] [54], epidermal growth factor [55], asialoglycoproteins [56], coagulation proteases (e.g., factor VIIa) [57], or exogenous substances (e.g., LPS) [58], hepatocytes can internalize and destroy various endogenous and exogenous substances, thereby fulfilling the liver’s physiological function to endocytose and metabolize a variety of ligands. The significance of TSN-SS-mediated endocytosis of proinflammatory mediators (e.g., HMGB1) by hepatocytes remains an interesting subject of on-going investigation. Notably, TSN-SS (100 μ M) failed to enhance cellular HMGB1 uptake in other type of cells (e.g., human alveolar epithelial A549 cells, data not shown). It will thus be important to investigate whether TSN-SS, a Chinese cardiovascular medicine, facilitates endocytic HMGB1 uptake in cardiomyocytes or vascular endothelial cells in future studies.

Because HMGB1 is not only secreted by activated innate immune cells, but also leaked by necrotic cells, therapeutic agents capable of facilitating endocytic HMGB1 uptake back to professional phagocytes or hepatocytes may hold potential for the treatment of infection- and injury-elicited inflammatory diseases. The discovery of a novel mechanism underlying TSN-SS-mediated HMGB1 inhibition may pave the road for developing novel endocytosis-modulating therapeutic strategies. It is now possible to search for other pharmacological agents that can “recycle” injurious proinflammatory mediators (such as HMGB1) for the clinical management of infection- or injury-elicited inflammatory responses.

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Abbreviations used in this paper

HMGB1	high mobility group box 1
LC3	microtubule-associated protein1 light chain 3
TSN-SS	tanshinone IIA sodium sulfonate.

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Figure 1. TSN-SS facilitated HMGB1 internalization into macrophage cytoplasmic vesicles
 Murine macrophage-like RAW 264.7 cells were incubated with biotin-labeled recombinant HMGB1 (“+ rHMGB1”, 2 μ g/ml) alone or in the presence of TSN-SS (100 μ M) for 6 h, and exogenous rHMGB1 was visualized by staining with streptavidin-conjugated Alexa 594. Bar, 10 μ m.

Figure 2. TSN-SS enhanced HMGB1 uptake in a time- and dose-dependent fashion

Murine RAW 264.7 macrophages (**Panel A**) or human HepG2 hepatocytes (**Panel B**) were incubated with rHMGB1 (2 μ g/ml) in the absence or presence of TSN-SS at indicated doses (10, 50 and 100 μ M) for indicated time periods. The relative cellular levels of exogenous rHMGB1 were determined by Western blotting analysis with reference to a house-keeping gene product, β -actin. *, $P < 0.05$ versus control (“+rHMGB1 alone”).

Figure 3. Role of TLR4 and RAGE in TSN-SS-mediated HMGB1 cellular uptake

Primary peritoneal macrophages were isolated from wild-type or TLR4/RAGE-deficient mice, and treated with rHMGB1 (2 μg/ml) in the absence or presence of TSN-SS (50 μM) for 4h. Cellular rHMGB1 levels were determined by Western blotting analysis with reference to β-actin.

A. Murine Macrophages

B. Human Hepatocytes

Figure 4. Clathrin or caveolin inhibitors partly impaired TSN-SS-mediated HMGB1 uptake Murine RAW 264.7 macrophages (**Panel A, B**) or human HepG2 hepatocytes (**Panel C**) were pre-treated with various clathrin (chlorpromazine, 40 µM or caveolin inhibitors (indomethacin, 300 µM nystatin, 50 µM) for 30 min, and subsequently incubated with rHMG1 (2 µg/ml) in the absence or presence of TSN-SS (100 µM) for indicated time periods. Cellular levels of exogenous HMGB1 were determined by Western blotting analysis with reference to β-actin. *, $P < 0.05$ versus positive control (“+rHMG1 + TSN-SS”).

Figure 5. TSN-SS enhanced HMGB1-induced LC3-II production and aggregation

A). TSN-SS enhanced HMGB1-induced LC3-II production. Murine RAW 264.7 cells were treated with rHMGB1 (2 µg/ml) in the absence or presence of TSN-SS (100 µM) for various time periods, and cellular LC3-II levels were determined by Western blotting analysis with reference to β -actin as a loading control. *, $P < 0.05$ versus negative control (“-rHMGB1 - TSN-SS”); #, $P < 0.05$ versus positive control (“+rHMGB1” alone). B) TSN-SS enhanced HMGB1-induced LC3-II aggregation. GFP-LC3-transfected RAW 264.7 cells were treated with biotin-labeled rHMGB1 (2 µg/ml) in the absence or presence of TSN-SS (100 µM) for 8 h, and were then examined for exogenous rHMGB1 or LC3-II aggregation under a

fluorescence microscope. Note that exogenous rHMGB1 co-localized with LC3-containing cytoplasmic vesicles in rHMGB1/TSN-SS-treated cells. Bar, 10 μ m

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Figure 6. A lysosomal inhibitor, Bafilomycin A1, led to elevated accumulation of endogenous LC3-II and exogenous HMGB1

Macrophage cultures were pre-treated with rHMGB1 (2 $\mu\text{g/ml}$) in the presence or absence of TSN-SS (100 μM) for 6–8 h, and bafilomycin A1 was added at a saturating concentration (100 nM). After incubation for an additional 4 h, cells were harvested and cellular levels of endogenous LC3-II and exogenous rHMGB1 were determined by Western blotting analysis with reference to β -actin. *, $P < 0.05$ versus negative control (“- rHMGB1 – TSN-SS”). #, $P < 0.05$ versus positive controls (“+ Baf” alone or “+rHMGB1 + TSN-SS”).

Via clathrin- or caveolin-dependent endocytosis, TSN-SS facilitates cellular uptake of exogenous HMGB1, which triggers a cellular degradation process, autophagy. Subsequently, endosomes may merge with autophagosomes to form amphisomes, which then fuse with lysosomes to degrade the amphisome contents.

Figure 7. Proposed model for TSN-SS-mediated HMGB1 cellular uptake

TSN-SS facilitates cellular HMGB1 uptake via clathrin- and/or caveolin-dependent endocytosis, and delivers HMGB1 into cytoplasmic vesicles (such as endosomes). Consequently, it likely triggers another cellular degradation process, autophagy, during which cytoplasmic macromolecules are engulfed by double-membraned cytoplasmic vesicles termed autophagosomes. Subsequently, autophagosomes can fuse with other cytoplasmic vesicles such as lysosomes (to form autophagolysosomes) and endosomes (to form amphisomes). Similarly, amphisomes may fuse with lysosomes to form degradative autolysosomes, where amphisome contents are digested by lysosomal enzymes.