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Calcium/Calmodulin-Dependent Protein Kinase (CaMK) IV Mediates Nucleocytoplasmic Shuttling and Release of HMGB1 during Lipopolysaccharide Stimulation of Macrophages

Xianghong Zhang,* David Wheeler,* Ying Tang,* Lanping Guo,* Richard A. Shapiro,* Thomas J. Ribar, † Anthony R. Means, † Timothy R. Billiar,* Derek C. Angus,* and Matthew R. Rosengart†‡

The chromatin-binding factor high-mobility group box 1 (HMGB1) functions as a proinflammatory cytokine and late mediator of mortality in murine endotoxemia. Although serine phosphorylation of HMGB1 is necessary for nucleocytoplasmic shuttling before its cellular release, the protein kinases involved have not been identified. To investigate if calcium/calmodulin-dependent protein kinase (CaMK) IV serine phosphorylates and mediates the release of HMGB1 from macrophages (Mφ) stimulated with LPS, RAW 264.7 cells or murine primary peritoneal Mφ/H9278 were incubated with either STO609 (a CaMKIV kinase inhibitor), KN93 (a CaMKIV inhibitor), or we utilized cells from which CaMKIV was depleted by RNA interference (RNAi) before stimulation with LPS. We also compared the LPS response of primary Mφ isolated from CaMKIV+/− and CaMKIV−/− mice. In both cell types LPS induced activation and nuclear translocation of CaMKIV, which preceded HMGB1 nucleocytoplasmic shuttling. However, Mφ treated with KN93, STO609, or CaMKIV RNAi before LPS showed reduced nucleocytoplasmic shuttling of HMGB1 and release of HMGB1 into the supernatant. Additionally, LPS induced serine phosphorylation of HMGB1, which correlated with an interaction between CaMKIV and HMGB1 and with CaMK IV phosphorylation of HMGB1 in vitro. In cells, both HMGB1 phosphorylation and interaction with CaMKIV were inhibited by STO609 or CaMKIV RNAi. Similarly, whereas CaMKIV+/+ Mφ showed serine phosphorylation of HMGB1 in response to LPS, this phosphorylation was attenuated in CaMKIV−/− Mφ. Collectively, our results demonstrate that CaMKIV promotes the nucleocytoplasmic shuttling of HMGB1 and suggest that the process may be mediated through CaMKIV-dependent serine phosphorylation of HMGB1. The Journal of Immunology, 2008, 181: 5015–5023.

High-mobility group box 1 (HMGB1) is initially identified as an architectural chromatin-binding factor that binds DNA and directs protein assembly on specific DNA targets. Recently it has been demonstrated to function as a proinflammatory cytokine and late mediator of mortality in murine endotoxemia and sepsis (1–3). HMGB1 appeared 8 h poststimulation and plateaued at 16–32 h, a time course very distinct from the acute rise and fall of early mediators of severe sepsis and septic shock (TNF-α, IL-1β) (3). Recombinant HMGB1 mimicked the lethality of high-dose LPS and induced the release of TNF-α by macrophages (Mφ) (3). Anti-HMGB1 Abs conferred a dose-dependent protection in animal models of endotoxia, even when the first dose of anti-HMGB1 Abs was delayed for at least 2 h (2, 3). Human studies have revealed that systemic HMGB1 levels are markedly elevated in patients that die of sepsis and during hemorrhagic shock (4, 5).

In the context of sepsis, current data indicate that HMGB1 is released by activated monocytes and Mφ via an active process that involves shuttling the protein from nucleus to cytoplasm (3, 6, 7). Acetylation of HMGB1 and of the histones to which it binds appears to be essential for its release (8). HMGB1 contains two nuclear localization signals (NLS) and two putative nuclear export signals, suggesting that HMGB1 shuttles between the nucleus and cytoplasm through a tightly controlled mechanism (6). Recently, serine phosphorylation has been demonstrated to be a requisite step for this process, although neither the kinase nor the mechanisms controlling nucleocytoplasmic shuttling have been identified (9). Considering the data suggesting that HMGB1 may serve as a target to reduce mortality from sepsis, identifying the mechanisms responsible for inducing and controlling its release is important.

We have recently demonstrated the integral role for members of the family of multifunctional calcium/calmodulin-dependent protein kinases (CaMKI, II, IV) in the release of HMGB1 by hepatocytes subjected to oxidant stress and hepatocellular injury in an in vivo model of hepatic ischemia/reperfusion, although we have yet to identify the specific members involved (10). The multifunctional CaMKs are serine/threonine kinases sensitive to changes in...
intracellular [Ca\(^{2+}\)] that coordinate a variety of cellular functions, including gene expression, cell cycle progression, apoptosis, differentiation, and ischemic tolerance (11, 12). Whereas isoforms of CaMKI and CaMKII are expressed in all mammalian cells, CaMKIV is present in only selective tissues, which include the bone marrow (13). CaMKIV is activated and translocates into the nucleus upon its phosphorylation by an upstream CaMKK in the cytoplasm (14, 15). The nuclear, autonomously active form of CaMKIV phosphorylates a number of proteins involved in the regulation of transcription (16). Additionally, it has recently been shown that CaMKIV would be an attractive candidate kinase to phosphorylate HMGB1 in macrophages and facilitate its translocation from nucleus to cytoplasm in response to LPS.

**Materials and Methods**

**Reagents**

*Escherichia coli* coli 0111:B4 LPS was obtained from Sigma-Aldrich. KN93, obtained from Calbiochem, was dissolved in sterile DMSO at a concentration of 10 mM. STO690 was obtained from Calbiochem. STO609 is selective for CaMKK: it has an in vitro IC\(_50\) of 0.13–0.38 \(\mu\)M for CaMKK and 32 \(\mu\)M for CaMKII with little or no inhibition of CaMKI, CaMKIV, protein kinase A, protein kinase C, ERK, or myosin light chain kinase (18). mAb against autonomously active, Thr\(^{196}\)-phosphorylated CaMKIV (anti-p-Thr\(^{196}\)-CaMKIV) was the generous gift of Dr. Naohito Nozaki (Kanagawa, Japan). Abs against total CaMKIV and HMGB1 were obtained from Abcam. Ab against phosphoserine was obtained from Promega. Ab against FLAG epitope was obtained from Sigma-Aldrich. DAPI was obtained from Molecular Probes.

**Cell isolation and treatment**

Murine monocyte/macrophage-like cells (RAW 264.7, American Type Culture Collection) were grown in DMEM (BioWhittaker) supplemented with 10% FCS (Sigma-Aldrich), 50 U/ml penicillin, and 50 \(\mu\)g/ml streptomycin (Cellgro/Mediatech). Primary murine peritoneal M\(_{φ}\) were isolated by lavaging the peritoneal cavity with five 3-ml aliquots of sterile PBS. After centrifugation at 300 \(×\) g for 10 min, the M\(_{φ}\) were resuspended in RPMI 1640 with 10% FCS, 50 \(\mu\)g/ml streptomycin. Selected cells were pretreated with varying concentrations of KN93 (5, 10, 20 \(\mu\)M) or STO609 (1, 2, 5, 10, 20 \(\mu\)M) for 1 h or subjected to RNA interference (RNAi) using nontarget or CaMKIV small interfering RNA (siRNA) (see below). Selected cells were then treated with increasing doses of LPS (1, 10, 100 ng/ml).

**Transfection of fluorescein-labeled cyclophilin, nontarget, and CaMKIV siRNA**

RAW 264.7 cells (2 \(×\) 10\(^5\)) or murine peritoneal M\(_{φ}\) (1 \(×\) 10\(^5\)) were plated in 0.5 ml of growth medium (without antibiotics) in each well of a 24-well
FIGURE 2. CaMK kinase cascade mediates LPS-induced HMGB1 release. A. RAW 264.7 cells were treated with LPS (100 ng/ml) for 16 h, at which time cellular supernatant was harvested, subjected to immuno blot analysis, and probed for HMGB1. Parallel populations were pretreated with STO609 (5 \( \mu \)M) for 1 h before LPS stimulation and subjected to similar immunoblot analysis. Representative blot of three individual experiments. B. RAW 264.7 cells were subjected to similar conditions as described above. Cell viability was evaluated by MTT assay and is shown as means \( \pm \) SEM fold change relative to control, untreated cell population (\( n = 4 \)). C. Murine peritoneal M\(_6\) were isolated and treated with LPS and STO609 as previously described. The supernatant was harvested, subjected to Western blot analysis, and probed for HMGB1. Representative blot of three individual experiments.

Plate, resulting in 30% or 80% confluence, respectively. Fluorescein-labeled cyclophilin control siRNA, nontarget siRNA, and CaMKIV siRNA obtained from Dharmacon were added to 30% or 80% confluent RAW 264.7 cells to a final concentration of 25 nM. We utilized the SMARTpool siRNA from Dharmacon that incorporates four separate siRNA sequences for CaMKIV: 5'-GAGAUCCUCUGGGCGAUUUU3'–5'-UCAAGGAAUAUGC-GAAACU3', 5'-GGUGCUACAUCCGUGUGUU3', and 5'-GG- GAUGAAGUGUCUUAAAU3'. In a separate tube, 3 \( \mu \)l HiPerFect was diluted in 50 \( \mu \)l serum-free DMEM and incubated at room temperature for 5 min. These two solutions were combined, and the final transfection mixture was incubated for 20 min at room temperature. This transfection mixture was applied to each well and incubated for 6 h, after which it was replaced by 500 \( \mu \)l of cell medium and incubated for 72 h. Transfection efficiency was determined at 24 h by fluorescence microscopy. For each experiment at least three microscopic visual fields (\( \times 200 \) magnification) were counted to facilitate calculation of the ratio of fluorescent cyclophilin-expressing cells to nonfluorescent cells. Inhibition of each targeted protein was determined by immunoblot or RT-PCR. All experiments and cell number determinations were performed in triplicate.

Plasmid construction and transfection

Plasmids encoding a constitutively active CaMKIV (CaMKIV-dCT) or a kinase-inactive CaMKIV-dCTK75E mutant were the generous gifts of Dr. Douglas Black (19). CaMKIV-dCT contains a C-terminally truncated version of the human CaMKIV-encoding gene, truncated to Leu\(^{317}\), and an N-terminal FLAG epitope (19, 20). CaMKIV-dCTK75E was constructed by changing Lys\(^{75}\) to glutamate in CaMKIV-dCT, which negatively affects ATP binding at the catalytic site (19, 20). For transient transfection, RAW 264.7 cells were seeded in a 24-well plate at 3 x 10\(^5\) cells/well. After 2 h of adhesion, M6 were transfected with 1 \( \mu \)g of plasmid CaMKIV-dCT or CaMKIV-dCTK75E using the Lipofectamine 2000 reagent according to the instructions specified by the manufacturer (Invitrogen). Following transfection, cells were handled as detailed in the figure legends.

Cellular protein extraction

Total cellular protein was extracted at 4°C in 500 \( \mu \)l of lysis buffer (20 mM Tris, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 \( \mu \)M sodium orthovanadate, 100 \( \mu \)M DTT, 200 \( \mu \)M PMSF, 10 \( \mu \)g/ml leupeptin, 0.15 U/ml aprotinin, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 2.5 \( \mu \)g/ml peptatin A, 1 mM benzamidine, and 40 mM \( \alpha \)-glycerolphosphate). Protein concentration was determined using a bicinchoninic acid protein assay (Pierce).

Nuclear and cytoplasmic protein isolation

Cells were harvested and washed with PBS, followed by centrifugation at 300 \( \times \) g for 10 min. The cell pellet was lysed with NE-PER nuclear and cytoplasmic extraction reagent according to the instructions specified by the manufacturer (Thermo Scientific).

Immunoprecipitation

Equal amounts of cellular protein obtained from each experimental condition were used for immunoprecipitation. Five microliters of Ab was added to 500

FIGURE 3. CaMKIV mediates LPS-induced HMGB1 release. A. RAW 264.7 cells were transfected with fluorescent cyclophilin B siRNA for 24 h and imaged by confocal microscopy. Transfection efficiency was determined by determining the ratio of fluorescent cells to total number of cells. B. RAW 264.7 cells were transfected with either nontarget (NT) or CaMKIV siRNA for 72 h, at which time total cell lysate was harvested, subjected to immuno blot analysis, and probed for CaMKIV. Representative blots of three individual experiments. C. RAW 264.7 cells were transfected with either NT or CaMKIV siRNA for 72 h and then stimulated with LPS (100 ng/ml) for 16 h. The supernatant was harvested, subjected to immunoblot analysis, and probed for HMGB1. Representative blots of three individual experiments. D. RAW 264.7 cells were subjected to similar conditions as described above. Cell viability was evaluated by MTT assay and is shown as means \( \pm \) SEM fold change relative to control, untreated NT cell population (\( n = 4 \)).
μg of isolated cellular protein within lysis buffer and incubated at 4°C overnight on a rotator. Fifty microliters of 50% slurry of prewashed protein G-agarose beads (Abcam) were then added to each sample, followed by incubation for an additional 2 h at 4°C. The samples were spun briefly in a microcentrifuge at 14,000 rpm and washed four times in lysis buffer. Samples were then resuspended in 30 μl of lysis buffer for future analysis.

FIGURE 4. Spatial and temporal relationship of LPS-induced CaMKIV Thr\(^{196}\) phosphorylation and HMGB1 nucleocytoplasmic shuttling. A, RAW 264.7 cells were stimulated with LPS (100 ng/ml) for 15 min and 1, 2, and 8 h, at which time they were fixed, permeabilized, stained for HMGB1 (green) or p-Thr\(^{196}\)-CaMKIV (red), and imaged by confocal microscopy at ×630. Green, HMGB1; red, p-Thr\(^{196}\)-CaMKIV; blue, DAPI. Representative image of three individual experiments. B, RAW 264.7 cells were subjected to similar conditions as described above, stained for total CaMKIV, and imaged by confocal microscopy at ×630. Red, CaMKIV; blue, DAPI. Representative image of three individual experiments.

FIGURE 5. CaMKIV mediates HMGB1 nucleocytoplasmic shuttling. A, RAW 264.7 cells underwent RNAi using nontarget (NT) siRNA or CaMKIV siRNA. Cells were stimulated with LPS (100 ng/ml) for 8 h and then fixed, permeabilized, stained for HMGB1 (green), and imaged by confocal microscopy at ×630. Green, HMGB1; blue, DAPI. Representative image of three individual experiments. B, RAW 264.7 cells were subjected to STO609 (5 μM) or KN93 (10 μM) or underwent NT or CaMKIV RNAi before stimulation with LPS (100 ng/ml) as detailed above. Nuclear and cytoplasmic protein were isolated, subjected to immunoblot analysis, and probed for HMGB1. Representative blots of three separate experiments.
Immunoblots

Total cellular protein was electrophoresed in a 10% SDS-PAGE gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked for 1 h at room temperature with 5% milk and was then incubated with primary Ab for 12 h at 4°C. Blots were then incubated in a HRP-conjugated secondary Ab against the primary Ab at room temperature for 1 h. The blot was developed using the SuperSignal chemiluminescent substrate (Pierce) and exposed on KAR-5 film (Eastman Kodak). In addition to utilizing the primary anti-threonine phosphorylated (Thr196) CaMKIV Ab, the active threonine phosphorylated form of CaMKIV was determined using CaMK-immunoprecipitated protein. Densitometry was performed by the National Institutes of Health (Bethesda, MD) image program to quantify OD.

Immunocytochemistry and confocal microscopy

Cells were cultured on glass coverslips until 80% confluent. The coverslips were washed in PBS and fixed for 20 min in 4% paraformaldehyde in PBS at 4°C. Cells were permeabilized with 5% BSA, 0.1% Triton X-100 for 1 h at 4°C and then stained with the appropriate Ab (1 μg/ml) overnight at 4°C. Cells were further treated with anti-rabbit IgG conjugated with FITC (1/1000), anti-mouse IgG conjugated with tetramethylrhodamine isothiocyanate (1/1000), and DAPI (5 mg/ml) in 5% nonfat milk for an additional 2 h at room temperature. The cells were then washed four times with PBS, mounted with Gel/Mount (Biomeda) and examined by confocal microscopy.

In vitro kinase assay

Recombinant HMGB1 (1 μg) was incubated in the presence or absence of activated p-Thr196-CaMKIV (25 ng) for 10 min at 30°C with the following additions: 10 mM MgCl₂, 0.2 mM ATP, 1 mM CaCl₂, and 1 μM CaM. Reactions were terminated by boiling in SDS-2-ME dissociation solution, subjected to 10% SDS-PAGE, and probed with anti-phosphoserine, anti-CaMKIV, or anti-HMGB1 Ab.

Cell viability and morphologic features

Representative cell populations from each condition were examined under light microscopy. Cell viability was also confirmed by MTT assay. Cells were incubated in 96-well plates (Costar). After a 24-h incubation in 100 μl RPMI 1640 medium containing the stimulus, 50 μl of a 5 mg/ml MTT (Sigma-Aldrich) solution in PBS was added, and cells were incubated at 37°C for 2 h. The cells were then lysed by addition of 100 μl per well extraction buffer (20% (w/v) SDS, 50% (v/v) N,N-dimethyl formamide, 2% (v/v) acetic acid (pH 4.7)). After overnight incubation with extraction buffer, the OD at 562 nm was measured.

Statistic analysis

Values are expressed as means ± SEM. Groups are compared by Mann-Whitney. A p value of <0.05 was considered statistically significant.
Results

**LPS activates CaMKIV**

The phosphorylation of CaMKIV on Thr\(^{196}\) generates an autonomously active p-CaMKIV (11). We first established the time course for CaMKIV activation in RAW 264.7 cells following LPS stimulation. LPS activated CaMKIV, with Thr196 phosphorylation occurring at 15 min and returning toward baseline by 60 min after stimulation (Fig. 1A). Upstream inhibition of CaMKIV kinase with STO609 prevented CaMKIV phosphorylation (Fig. 1A). We did observe two bands in our assessment of CaMKIV phosphorylation, and two CaMKIV isoforms have been described (21). This time course of CaMKIV phosphorylation was consistent across all independent experiments, yielding a mean CaMKIV activation time course that peaked 15–30 min after LPS (Fig. 1B). We repeated these experiments with primary peritoneal M\(\phi\)/H9278. LPS induced Thr196 phosphorylation of CaMKIV within 15–30 min (Fig. 1C). However, even at 60 min after LPS exposure, CaMK IV remained phosphorylated (Fig. 1C). Again, CaMKIV activation was dependent upon CaMKIV kinase, as inhibition with STO609 markedly reduced the LPS-induced increase in p-Thr\(^{196}\)-CaMKIV (data not shown).

**The CaM kinase cascade mediates LPS-induced HMGB1 release**

Consistent with previous studies, we found that LPS induced the release of HMGB1 by RAW 264.7 cells, with detectable concentrations of supernatant HMGB1 occurring as early as 8 h (data not shown) and peaking at 16 h after LPS stimulation as shown (Fig. 2A, lanes 1 and 2). Incubation with STO609 markedly reduced supernatant HMGB1 concentration (Fig. 2A, lane 3). As shown in Fig. 2B, the HMGB1 release was not due to differential cell death since similar cell viability was observed for treated cell populations.

We confirmed that the CaM kinase cascade also mediated LPS-induced HMGB1 release in primary peritoneal M\(\phi\). Similar to RAW 264.7 cells, LPS induced HMGB1 release by peritoneal M\(\phi\) that was inhibited by upstream CaM kinase inhibition with STO609 (Fig. 2C).

**CaMKIV mediates LPS-induced HMGB1 release**

The previous observations suggested that the CaM kinase cascade is integral to LPS-induced M\(\phi\) HMGB1 release. We utilized RNAi to study the individual role of downstream CaMKIV in these events. As determined by fluorescent microscopy, cell transfection with fluorescent cyclophilin B siRNA was efficacious (Fig. 3A). As determined by SDS-PAGE, CaMKIV RNAi markedly reduced CaMKIV expression (Fig. 3B). Control, nontarget RNAi-treated cells released HMGB1 in response to LPS similar to untreated RAW 264.7 cells and peritoneal M\(\phi\) (Fig. 3C, lanes 1 and 2). By contrast, CaMK IV RNAi almost completely inhibited LPS-induced HMGB1 release (Fig. 3C, lanes 3 and 4). The effects of CaMKIV RNAi were not related to cell death, as the MTT assay demonstrated similar viability for all cells regardless of how they were treated (Fig. 3D).

**CaMKIV mediates nuclear export of HMGB1 after LPS stimulation**

HMGB1 release is an active process by which HMGB1 is shuttled from nucleus to cytoplasm and then out of the cell. Because most
of the identified functions of CaMKIV occur within the nucleus, we hypothesized that CaMKIV plays an integral role in the translocation of HMGB1 from nucleus to cytoplasm. We initially explored the spatial and temporal relationships of CaMKIV phosphorylation and HMGB1 release. As shown in Fig. 4A, HMGB1 (green) is predominantly intranuclear under basal, unstimulated conditions (Fig. 4A, top row). Within 120 min of LPS stimulation, HMGB1 is observed within the cytoplasm and continues to increase within the cytoplasm over the ensuing 8 h (Fig. 4A, top row). Unstimulated RAW 264.7 cells demonstrate minimal CaMKIV Thr196 phosphorylation (red), but within 15 min of LPS stimulation, phosphorylation increases and continues to increase with time, peaking at 120 min after LPS stimulation (Fig. 4A, middle row). Although some active CaMKIV is apparent within the cytoplasm, most of this p-Thr196-CaMKIV seems to be concentrated within the nucleus. This localization appears to result from translocation of active CaMKIV to the nucleus as demonstrated by the increased fluorescence of both p-Thr196-CaMKIV (Fig. 4A, middle row) and total CaMKIV (Fig. 4B, top row) within the nucleus after LPS stimulation. Inhibition of CaMKIV kinase with STO609 inhibits the Thr196 phosphorylation of CaMKIV and the translocation of HMGB1 to the cytoplasm (Fig. 4A, last column).

To confirm that this translocation process was CaMKIV dependent, we conducted similar experiments using cells treated with CaMKIV RNAi. RAW 264.7 cells transfected with control, non-target siRNA demonstrated nucleocytoplasmic shuttling of HMGB1 (green) at 8 h after LPS stimulation (Fig. 5A, left). On the other hand, CaMKIV RNAi inhibited LPS-induced HMGB1 translocation with strong nuclear and minimal cytoplasmic staining of HMGB1 at 8 h after LPS stimulation (Fig. 5A, right). This inhibition was similar to that induced by pretreatment with the CaMKIV kinase inhibitor STO609 (Fig. 4A, last column).

We confirmed these immunofluorescent microscopic observations by analyzing the HMGB1 concentration of nuclear and cytoplasmic protein isolated from Mφ subjected to similar conditions as detailed above. As shown in Fig. 5B, LPS induced the translocation of HMGB1 from the nucleus to the cytoplasm. By comparison to LPS-stimulated Mφ, there was a significant reduction in HMGB1 translocation to the cytoplasm in response to either KN93 (which inhibits CaMKIV) or STO609 (which inhibits CaMKIV kinase) (Fig. 5B). Similarly, by comparison to control, nontarget RNAi, CaMKIV RNAi reduced the LPS-induced nucleocytoplasmic shuttling of HMGB1 (Fig. 5B). These results confirm our idea that a CaMKK/CaMKIV cascade regulates LPS-induced nuclear-to-cytoplasmic translocation of HMGB1.

**CaMKIV directly serine phosphorylates HMGB1 after LPS stimulation**

Our results demonstrate that CaMKIV mediates LPS-induced HMGB1 release, in part, through processes guiding the nuclear exportation of HMGB1. Recently, HMGB1 translocation to the cytoplasm has been demonstrated to require serine phosphorylation within its NLSs (9). We first confirmed that LPS induced serine phosphorylation of HMGB1. As shown in Fig. 6A, stimulation of RAW 264.7 cells with LPS results in serine phosphorylation of HMGB1 within 2 h. Additionally, this phosphorylation event may require CaMKIV, as preincubation of primary peritoneal Mφ with STO609 inhibited LPS-induced serine phosphorylation of HMGB1 (Fig. 6B). Similarly, CaMKIV RNAi treatment of Mφ also reduced LPS-induced HMGB1 serine phosphorylation (Fig. 6B), and the mean degree of inhibition determined by densitometry is illustrated in Fig. 6C. Finally, we compared the degree of serine phosphorylated HMGB1 in peritoneal Mφ isolated from either CaMKIV+/− or CaMKIV−/− mice. As shown in Fig. 6D, control CaMKIV+/− Mφ show serine phosphorylation of HMGB1 after LPS stimulation, whereas the extent of this induced HMGB1 phosphorylation is reduced in CaMKIV−/− Mφ.

Our immunocytochemistry data suggest colocalization of CaMKIV and HMGB1 after LPS stimulation, and thus we investigated whether CaMKIV interacts with HMGB1. As shown in Fig. 7A, LPS stimulation markedly increased the degree to which HMGB1 coimmunoprecipitated with CaMKIV. One hour after stimulation with LPS, CaMKIV and HMGB1 coimmunoprecipitation was markedly increased and peaked by 2 h. Reciprocal coimmunoprecipitation experiments yielded similar results (data not shown). These LPS-stimulated interactions likely require the phosphorylation and activation of CaMKIV, as preincubation of cells with STO609 before addition of LPS inhibited the coimmunoprecipitation of HMGB1 with CaMKIV (Fig. 7A).

To determine whether CaMKIV could directly serine phosphorylate HMGB1 we conducted in vitro kinase assays. Active p-Thr196-CaMKIV was unable to phosphorylate HMGB1 in the absence of Mg2+/ATP (Fig. 7B, lane 1). Similarly, in the absence of p-Thr196-CaMKIV, there was minimal serine phosphorylation of HMGB1 (Fig. 7B, lane 2). However, activated p-Thr196-CaMKIV, in combination with Mg2+/ATP, was able to serine phosphorylate HMGB1 (Fig. 7B, lane 4).
Active CaMKIV is sufficient for HMGB1 release

To assess whether active CaMKIV is sufficient for HMGB1 release, we transfected RAW 264.7 cells with plasmids expressing either a truncated, constitutively active form of CaMKIV (CaMKIV-dCT) or a similarly truncated kinase-inactive mutant of CaMKIV (CaMKIV-dCTK75E). Both plasmids were successfully transfected and expressed, although expression of the active CaMKIV-dCT was greater than that of CaMKIV-dCTK75E (Fig. 8A), confirming previously published observations that substitutions that disrupt kinase activity, including K75E, result in less protein expression in mammalian cells compared with wild-type CaMKIV-dCT (Fig. 8A) (20). As shown in Fig. 8B, expression of the active CaMKIV-dCT induced the secretion of HMGB1 from the cells, whereas CaMKIV-dCTK75E failed to do so (Fig. 8B). This 6-fold induction of HMGB1 release in response to CaMKIV-dCT was highly reproducible in three independent experiments (Fig. 8C).

Discussion

During sepsis, perturbations in cellular calcium homeostasis are postulated to mediate the aberrant inflammation underlying organ dysfunction and death (22, 23). Recently, HMGB1, an architectural chromatin-binding factor that bends DNA and directs protein assembly on specific DNA targets, has been demonstrated to function as a late mediator of mortality in murine endotoxia and sepsis (1–3). Monocytes and Mφ have been demonstrated to be a primary source of HMGB1, and evidence is accumulating that production of this inflammatory mediator is Ca2+/CaM dependent. Herein, we characterize that LPS-induced HMGB1 release is mediated by a Ca2+/CaM-dependent signaling cascade involving a CaMKIV kinase and CaMKIV. Our data suggest that this series of reactions may terminate in the nucleus where CaMKIV phosphorylates HMGB1, an event that is required to facilitate the translocation of HMGB1 from the nucleus to the cytoplasm (24).

Substantial work has been conducted to elucidate the mechanisms by which HMGB1 is released, in part, because interventions that inhibit systemic HMGB1 concentrations reduce mortality in murine models of sepsis (2). Current data support an active process initiated by HMGB1 histone disengagement, HMGB1 hyperacetylation, and shuttling of the protein from the nucleus to the cytoplasm (6). Recently, serine phosphorylation of HMGB1 has been demonstrated to be essential for this translocation event, although the kinase responsible had not been identified (9). We hypothesized that CaMKIV mediates this process, in part, because this enzyme has been shown to function downstream of TLR4 in the pathway by which LPS induces the Ca2+-dependent survival of dendritic cells (17). Additionally, prior studies demonstrated that activation of CaMKIV by Ca2+/CaM and threonine phosphorylation (Thr196 in mice, Thr200 in humans) by an upstream CaMK kinase generates increased and autonomous CaMKIV activity that enables the kinase to translocate to the nucleus where it modulates gene expression (11, 25, 26).

Indeed, our data demonstrate that LPS induces this Thr196-phosphorylated and autonomous species of CaMKIV in Mφ, which then enters the nucleus where it interacts with and phosphorylates HMGB1. The evidence to support this contention is as follows: 1) CaMKIV translocation to the nucleus preceded HMGB1 translocation to the cytoplasm; 2) CaMKIV RNAi reduced cytoplasmic HMGB1 concentration by retaining HMGB1 in the nucleus; 3) CaMKIV coimmunoprecipitated with HMGB1 in response to LPS; and 4) activated CaMKIV directly phosphorylated HMGB1 in vitro kinase assays. The modest degree of HMGB1 phosphorylation observed in our in vitro kinase assay suggests that, perhaps, additional protein components may be necessary to assemble a signaling complex that facilitates CaMKIV phosphorylation of HMGB1. The minimum consensus CaMKIV substrate recognition sequence Hyd-X-R-X-(S/T) provides only a rough template for CaMKIV-mediated phosphorylation. In HMGB1 only Ser209 and Ser46 in NLS1 meet the hydrophilic requirement at the -5 position amino acid, and Ser209 carries a lysine substitution at -3, which is also present in the well-characterized substrate oncprotein 18 (27).

Interestingly, HMGB1 has two NLS sequences, and current evidence suggests that phosphorylation of both sequences is important for the cytoplasmic localization of HMGB1 (9). Our data support a role for CaMKIV in the mechanism that controls the nuclear export of HMGB1 (9). Regarding this mechanism, Bianchi et al. contend that the strength of HMGB1-histone binding is inversely related to the histone acetylation status, and that increased histone acetylation may enable HMGB1-histone disengagement and subsequent cellular release of HMGB1 (6, 8). The CaMKs have been demonstrated to mediate the nuclear export of various class II histone deacetylates HDAC4 and HDAC5, a process that could favor histone acetylation (28–31). Additionally, HMGB1 requires direct hyperacetylation before export from the nucleus (8). Further investigation of how CaMKIV activation and histone and HMGB1 acetylation are related will be an important aspect of future studies on HMGB1 nuclear export.

Although LPS stimulation of TLR4 can clearly activate CaMKIV, as shown here and in a prior study, the signaling events that occur between receptor ligation and CaMKIV activation have yet to be determined (17). Prior studies have established the dependency of monocye/Mφ function on Ca2+/CaM activation, and although it remains unclear precisely how the Ca2+ transients are generated, the multifunctional CaMKs respond to relatively small changes in intracellular [Ca2+] (32). Regarding CaMKIV activation and generation of autonomous activity, published data have established a requirement for binding of the Ca2+/CaM complex to both CaMKK and CaMKIV (11, 12, 25). We note the presence of nuclear and cytoplasmic activated CaMKIV in resting cells and an increasing concentration of activated total CaMKIV within the nucleus after LPS stimulation. These observations support a LPS-mediated increase in the cytoplasmic Ca2+ signal that is sufficient to activate CaMKIV and induce its nuclear translocation. Alternatively, it is possible that CaMK may be activated by reactive oxygen species in the absence of a Ca2+ rise. For example, Howe et al. suggested that IxB phosphorylation in lymphocytes in response to hydrogen peroxide is mediated by Ca2+-independent activation of CaMKs (33, 34). Hence, additional studies are necessary to determine the spatiotemporal patterns of Ca2+ signaling and CaMKIV activation following LPS stimulation as well as the TLR4 dependence of such events.

In summary, CaMKIV kinase/CaMKIV mediates Mφ LPS-induced HMGB1 production by translocating to the nucleus after activation and phosphorylating HMGB1 in a manner that enables nuclear export of HMGB1 that must occur in order for the protein to be secreted from the cell. The combined observations of investigations conducted by our laboratories and those of others are beginning to highlight the integral role of the CaMK transduction cascade in Mφ function and cytokine production and inflammation. Future studies will elucidate the sites on both proteins that participate in the CaMKIV-HMGB1 interaction and serine phosphorylation of HMGB1. Interestingly, precedent exists for CaMKIV forming a stable complex with PP2A that is competitive with Ca2+/CaM and CaMKKβ, one of the two CaMKs that has been shown to form stable complexes with CaMKIV and PP2A or...
AMPK (15, 35). In the latter but not the former case, the interaction requires the “RP” domain of CaMKKβ (35). Perhaps, HMGB1 will also utilize a unique sequence of CaMKKβ to form a complex. At any rate, these combined observations suggest that modulation of CaMKK and/or CaMKIV activity may be of potential utility in diseases characterized by heightened and aberrant inflammation.

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