Functional link between TNF biosynthesis and CaM-dependent activation of inducible nitric oxide synthase in RAW 264.7 macrophages


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Submitted 20 October 2005; accepted in final form 11 January 2006

Weber, Thomas J., Heather S. Smallwood, Loel E. Kathmann, Lye Meng Markillie, Thomas C. Squier, and Brian D. Thrall. Functional link between TNF biosynthesis and CaM-dependent activation of inducible nitric oxide synthase in RAW 264.7 macrophages. Am J Physiol Cell Physiol 290: C1512–C1520, 2006. First published January 18, 2006; doi:10.1152/ajpcell.00527.2005. Inflammatory responses stimulated by bacterial endotoxin LPS involve Ca\(^{2+}\)-mediated signaling, yet the cellular sensors that determine cell fate in response to LPS remain poorly understood. We report that exposure of RAW 264.7 macrophage-like cells to LPS induces a rapid increase in CaM abundance, which is associated with the modulation of the inflammatory response. Increases in CaM abundance precede nuclear localization of key transcription factors (i.e., NF-κB p65 subunit, phospho-c-Jun, Sp1) and subsequent increases in the proinflammatory cytokine TNF-α and inducible nitric oxide synthase (iNOS). Cellular apoptosis after LPS challenge is blocked upon inhibition of iNOS activity using the pharmacological inhibitor 1400W. LPS-mediated iNOS expression and apoptosis also were inhibited by siRNA-mediated silencing of TNF induction, indicating TNF induction both precedes and is necessary for subsequent regulation of iNOS expression. Increasing the level of cellular CaM by stable transfection results in reductions in LPS-induced expression of TNF and iNOS, along with reduced activation of their transcriptional regulators and concomitant protection against apoptosis. Thus the level of CaM available for Ca\(^{2+}\)-dependent signaling regulation plays a key role in determining the expression of the proinflammatory and proapoptotic cascade during cellular activation by LPS. These results indicate a previously unrecognized central role for CaM in maintaining cellular homeostasis in response to LPS such that, under resting conditions, cellular concentrations of CaM are sufficient to inhibit the biosynthesis of proinflammatory mediators associated with macrophage activation. Although CaM and iNOS protein levels are coordinately increased as part of the oxidative burst, limiting cellular concentrations of CaM due to association with iNOS (and other high-affinity binders) commit the cell to an unchecked inflammatory cascade leading to apoptosis.

inflammation; apoptosis; macrophage activation

A CENTRAL ROLE for CaM in mediating macrophage activation and bacterial killing has been apparent for some time, consistent with the known role of CaM-dependent inducible nitric oxide (NO) synthase (iNOS) in conjunction with the multicomponent NADPH oxidase system in the generation of NO and O\(_2^•\) (14, 25). However, CaM is able to interact with and regulate a multitude of different binding partners; thus how CaM functions to regulate macrophage activation and bacterial killing remains poorly understood. For example, defective activation of CaM-dependent pathways contributes to the ability of Mycobacterium tuberculosis to parasitize macrophages (36), suggesting a key role for CaM in modulating macrophage activation. In contrast, the downregulation of CaM function through the addition of inhibitors and binding peptides can activate macrophages and block bacterial infections (53a). Thus, whereas prior measurements implicated a key role for CaM in mediating macrophage activation, the roles of specific signaling pathways have not been identified, owing to the involvement of >50 CaM-binding proteins, including both CaM-dependent kinases, phosphatases, and critical proteins associated with bacterial killing, such as iNOS (7, 20, 49, 52, 63). Furthermore, CaM-dependent signaling pathways function both to modulate target protein function directly and to induce changes in gene expression on longer time scales, emphasizing that time-dependent changes in CaM signaling pathways are critical in defining cell function.

Macrophage activation upon exposure to bacterial endotoxin involves coordinated increases in intracellular Ca\(^{2+}\) levels and the release of TNF and other primary response cytokines that function to initiate an inflammatory response (31, 32, 46–48, 50, 62). The cellular responses initiated by TNF overlap with many LPS-mediated responses, consistent with their activation of common transcriptional regulators, including members of the NF-κB and activator protein (AP)-1 families (1, 5, 27, 33). For instance, NF-κB-dependent expression of iNOS is associated with both TNF and LPS signaling (27), and association of iNOS with CaM contributes to the oxidative burst associated with bacterial killing (27, 49). However, unchecked activation of these pathways leads to both necrosis and apoptosis through proteolytic activation of caspase cascades and loss of cellular Ca\(^{2+}\) balance (40, 61). In contrast, the proapoptotic effects of LPS and TNF are often counterbalanced by their cyt protective functions, which have been associated with gene expression regulated by NF-κB and other signaling mediators (35, 40). In support of this concept, the inhibition of gene expression by cotreatment with inhibitors of transcription or protein translation can unmask the apoptotic effects of TNF (35). In addition to NF-κB, complementary pathways involving, for example, signal transducer and activator of transcription 3 (STAT3) have an important role in the protection of inflammation-induced damage (21). Likewise, the CaM-dependent expression of inhibitor of apoptosis-2 (IAP2) has been shown to prevent cellular apoptosis after monocyte activation (38), suggesting that coordinated signaling mechanisms control the time course of macrophage activation (30). Such observations suggest that

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cell fate in response to these agents depends on whether homeostatic mechanisms involving Ca\textsuperscript{2+}-initiated signaling events and gene regulation remain in balance.

In the present study, we have examined how the abundance of CaM in response to bacterial endotoxin LPS affects the inflammatory response and cellular apoptosis in an effort to understand how Ca\textsuperscript{2+}-dependent signaling and cytokine release coordinate macrophage activation. These measurements were obtained using RAW 264.7 cells, a well-established model system in systems biology approaches to the study of macrophage signaling (see the Alliance for Cellular Signaling’s Signaling Gateway web site at http://www.signalingsignaling-gateway.org/). In response to LPS, a rapid increase in CaM abundance precedes nuclear localization of key transcription factors (TFs) (i.e., NF-κB p65 subunit, active c-Jun, and (Sp1), as well as subsequent increases in protein levels of TNF and iNOS. Cellular apoptosis is blocked after inhibition of iNOS activity, regardless of whether block is accomplished using the pharmacological inhibitor 1400W, through silencing of TNF induction, or by increasing the amount of available CaM through gene transfection. Furthermore, changes in CaM abundance result in dramatic reductions in the amounts of key inflammatory mediators (i.e., TNF, iNOS) and the nuclear localization of TFs that regulate the expression of these inflammatory mediators, indicating a previously unrecognized central role for CaM in modulating both macrophage activation and cell fate in response to endotoxin challenge.

**MATERIALS AND METHODS**

**Materials.** Bacterial LPS from *Escherichia coli* strain O127:B8 was purchased from Sigma Chemical (St. Louis, MO). FuGene 6 transfection reagent was obtained from Roche (Indianapolis, IN). DMEM, sodium pyruvate, FBS, l-glutamine, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). All other chemicals were of cell culture grade or higher. Antibodies used included PAb against CaM, Sp1, xeroderma pigmentosum group A (XPA), phospho-c-Jun, iNOS, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); TNF (Biosource International, Camarillo, CA); NF-κB subunit p65 (RelA; Rockland Immunochemicals, Gilbertsville, PA); and active caspase-3 (Promega, Madison, WI). Zenon antibody labeling kits were purchased from Molecular Probes (Eugene, OR).

**Cell culture.** The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM (no. 11960-044; Gibco-BRL) supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO\textsubscript{2}-95% air at 37°C. Treatment with LPS occurred in DMEM with glucose levels comparable to those that occur under normal physiological conditions (no. 11885-084; Gibco-BRL), supplemented as indicated above.

**Western blot analysis.** Total cellular extracts were obtained for Western blot analysis by lysing cells in ice-cold buffer containing 1% Nonidet P-40, 20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM Na\textsubscript{2}VO\textsubscript{4}, 1 mM EGTA, and anti-protease cocktail (Roche Molecular Biochemicals, Indianapolis, IN), followed by centrifugation in a microfuge (10,000 rpm) for 1 min to pellet cellular debris. Nuclear protein lysate samples were prepared using procedures described previously (60). Protein concentrations were determined using a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). Aliquots of equal amounts of protein (30 μg) were diluted in loading buffer [0.125 mM Tris·HCl (pH 7.4), 4% SDS, and 20% glycerol], and the samples were denatured by boiling them for 5 min and resolved by performing 12% SDS-PAGE. After being electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) and blocked with 5% Carnation nonfat milk (Nestlē, Wilkes-Barre, PA) in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20), the membranes were incubated with the indicated primary antibodies overnight at 4°C, followed by incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. The proteins were subsequently detected using LumiGLO (Cell Signaling Technology, Beverly, MA) and a chemiluminescence imager (LumiImager, Roche Molecular Biochemicals). In experiments with nuclear extracts, membranes were probed multiple times to determine target nuclear protein expression levels. Expression level measurement was accomplished by incubating membranes in 1.0 mM sodium azide solution in TBST for 2 h at room temperature to inhibit bound HRP activity, followed by reprobing of the membrane with the HRP-labeled primary antibody of interest as described above. Primary antibodies were labeled with HRP using the appropriate Zenon labeling kit. All assays were repeated three times, and representative blots are shown in the figures.

**Cell transfection and siRNA.** RAW 264.7 cells in which CaM levels were increased (RAWCs) were constructed by stably transfecting RAW 264.7 cells using a pcDNA3.1 vector (Invitrogen) encoding CaM. The CaM gene was placed under the control of the human cytomegalovirus immediate-early promoter for expression with a neomycin resistance gene for the selection of stable cell lines. The ligated vector-containing insert was transformed into *E. coli*. Transformants were selected on Luria-Bertani plates containing 100 μg/ml ampicillin, followed by DNA purification with Qiagen minikits (Qiagen, Valencia, CA) and restriction enzyme digestion to verify the correct insertion of CaM into the vector. The transformant with CaM was selected and sequenced to verify intact CaM within the pcDNA3.1 vector. FuGene transfection reagent (Roche, Indianapolis, IN) was used to transfect the CaM-pcDNA3.1 vector into RAW 264.7 cells. The control plasmid, pcDNA3.1-CAT (chloramphenicol acetyl transferase), was treated as indicated above for use as a positive control for transfection and expression. Stable transfectants were maintained in medium supplemented with geneticin (800 μg/ml) as a selection agent (GIBCO-BRL, Carlsbad, CA).

A stable small interfering RNA (siRNA) expression vector used to silence TNF mRNA induction was constructed with the pSuppressor-NEO vector (Ingenex). Briefly, the vector was modified by adding linker regions containing SalI, KmiI, and XbaI restriction enzyme sites, and a synthetic duplex oligonucleotide (sense, 5′-TGAGAACCACTACGTTGCGAGTACTGGCCACACATGTGTTGTCCCACTGCTGCCAGATTCTGACACCTAGGTGGTGTCTTCTT-3′; antisense, 5′-AAAAGACACAACTAGTGTTGCGAGTACTGGCCACACATGTGTTGTCCCACTGCTGCCAGATTCTGACACCTAGGTGGTGTCTTCTT-3′) was ligated into the SalI and XbaI restriction enzyme sites. The sequences for the oligonucleotides were derived from the mouse TNF sequences and included a spacer (underlined sequences above) to permit hairpin formation. Correct orientation of the final vector was verified by sequence analysis. Transfections were performed using FuGene transfection reagent, and stable transfectants were isolated on the basis of resistance to geneticin (800 μg/ml). For TNF siRNA, a direct correlation between the level of TNF silencing and inhibition of iNOS induction in response to LPS treatment was observed with multiple transfected clones (data not shown). *Clone 8c-8* was used in the current study.

**Apopotic index and lysosomal integrity.** Initially, control or LPS (10–1,000 ng/ml)-treated cells (24 h) were processed as follows. Cells were 1) harvested using a rubber blade scraper and pooled with the medium fraction to include floating cells in the measurement; 2) centrifuged at 1,800 g for 5 min and medium was aspirated; 3) resuspended in ice-cold methanol for 2 min, followed by centrifuga-
tion at 1,800 g for 5 min and aspiration of methanol; 4) resuspended in 100 μl of PBS, spread onto a microscope slide, and air dried; 5) cross linked onto the slide by incubation in formaldehyde for 10 min, followed by three washes in PBS (10 mM sodium phosphate, pH 7.4, and 150 mM NaCl); and 6) stained with 50 μg/ml 4,6-diamidino-2-phenylindole in PBS for 5 min at room temperature, followed by three washes in PBS. Individual nuclei (total and apoptotic) were counted using epifluorescence microscopy to derive the apoptotic index (%apoptotic cells) from a minimum of 100 cells/sample. In subsequent experiments, we observed that comparable trends in the apoptotic index could be obtained using a simplified protocol whereby cells were maintained in Nunc chambers, fixed in ice-cold methanol for 2 min, and stained as described above to count total and apoptotic nuclei. Comparable measurements were made using a neutral red assay to measure lysosome integrity as a measure of cell viability as described previously (58, 59).

**NO measurement.** The activity of iNOS was determined by measuring the release of the stable nitrite end product using Griess reagent (Sigma Chemical) and monitoring absorbance at 540 nm using a microtiter plate reader at a 24-h time point. Nitrite concentrations in conditioned media were determined on the basis of standard curves calibrated using sodium nitrite as a standard.

**ELISA analysis.** For analysis of soluble (shed) TNF levels, conditioned media supernatants were collected after 4 h of LPS treatment, centrifuged at 10,000 rpm for 1 min to remove residual debris, and frozen promptly at −80°C until analysis. Shed TNF was quantitated by performing ELISA (R&D Systems, Minneapolis, MN) according to the supplier's instructions, and data were normalized to the number of viable cells.

**Statistics.** Individual comparisons were conducted using Student's t-test or ANOVA, together with a post hoc Student-Newman-Keuls test as appropriate. P < 0.05 was accepted as statistically significant.

**RESULTS**

**LPS-dependent increase in CaM abundance.** After exposure to LPS, RAW 264.7 macrophages were lysed and membrane and cytosolic fractions were isolated and probed using PAb against CaM to assess changes in CaM abundance. In all cases, >80% of the total CaM was present in the cytosolic fraction, with no evidence of LPS-induced cellular redistribution between soluble and insoluble fractions. A time-dependent increase in cellular CaM levels occurred in stimulated cells relative to unstimulated cells, reaching 2.5-fold elevation within 45 min after exposure to LPS (10 ng/ml) (Fig. 1). LPS-induced increases in CaM protein levels were inhibited by actinomycin D, indicating that the LPS-dependent induction of cellular CaM levels involves transcription. This rapid increase in CaM abundance in response to LPS exposure is consistent with the known central role of Ca2+ signaling pathways in mediating the inflammatory response, which results in a rapid increase in cytosolic Ca2+ levels on the time scale of ~10 min (12, 18, 34, 39). Furthermore, CaM functions as an activator of iNOS and is therefore required for the generation of reactive nitrogen species necessary for bacterial killing. Because total CaM abundance increases during macrophage activation in conjunction with CaM-dependent enzymes, which typically exceed the amount of available CaM (9, 44, 55), these results indicate a need to compare how the kinetics of increases in the abundance of key proteins involved in the inflammatory response correlate to changing CaM levels and whether this correlation modulates cell fate in response to LPS challenge.

![Fig. 1. LPS-dependent increase in CaM abundance. A and B: Western blot analysis showing rapid upregulation of CaM protein levels in response to LPS (10 ng/ml). Relative CaM abundance changes after LPS treatment for 45 min were quantified using Western blot analysis with a chemiluminescence imager, and data were normalized to GAPDH levels (see B). C: mean effects of LPS in presence or absence of cotreatment with transcriptional inhibitor actinomycin D (Act D; 10 μg/ml) on CaM levels from 6 independent measurements. *P < 0.05 vs. control (vehicle alone). [CaM]t = level of CaM in the treated sample; [CaM]c = level of CaM in control.](image)

**Elevated CaM expression inhibits apoptosis.** Although inhibitors of CaM-dependent protein interactions are known to affect inflammatory responses, the effect of directly altering the amount of cellular CaM available for such interactions has not been evaluated. Direct manipulation of cellular CaM concentrations was achieved through stable transfection approaches, because cellular CaM concentrations are regulated by three distinct genes, making siRNA silencing approaches impractical. Stably transduced RAW 264.7 cells (i.e., RAWCs) were selected, which resulted in a twofold increase in CaM abundance (Fig. 2A) and approximated the increase in CaM abundance after LPS exposure (see above). No significant cell proliferation rate changes were observed in unstimulated RAWCs relative to wild-type RAW 264.7 cells, indicating that the moderate elevation in basal CaM expression does not cause significant perturbations in cell cycle control (data not shown). Vector controls represent cells transfected with the empty vector to account for selective pressures related to transfection and antibiotic selection. Among the vectors we examined in the present study, no differences were observed between vector control cells and wild-type RAW 264.7 cells, indicating that cells transfected with empty vector are appropriate controls for these experiments. As expected, the amount of expressed CaM in RAWCs was largely unchanged upon treatment with LPS (data not shown), permitting a determination of how changes in the abundance of CaM affect cellular responses to LPS. Compared with wild-type cells (or empty vector controls) in which ~30% of the cell population displayed apoptotic nuclei 24 h after exposure to LPS (10 ng/ml), a substantial reduction in cellular apoptosis was observed in RAWCs in which CaM levels were increased, such that only ~12% of the cellular population contained apoptotic nuclei (Fig. 2B). The increase in apoptotic nuclei observed in LPS-stimulated cells was par-
alleled by an increase in active caspase-3 relative to untreated controls as determined using Western blot analysis and immunocytochemistry (Fig. 2C). Immunocytochemical analysis demonstrated that the appearance of active caspase-3 was restricted to cells exhibiting apoptotic nuclear morphology (Fig. 2C), consistent with classic apoptotic cell death under these conditions. In RAWCs, a substantial decrease in apoptosis across a broad range of LPS concentrations was observed on the basis of both the apoptotic index assessment of changes in nuclear morphology and complementary measurements of cell viability using a neutral red assay to evaluate lysosomal integrity (Fig. 2D). These observed large reductions in the extent of LPS-mediated apoptosis suggest an important role for CaM in mediating cell survival.

Increasing cellular CaM levels diminish inflammatory response. Macrophage activation and the associated inflammatory response result in the upregulation of iNOS and TNF expression as well as biosynthesis, which mediate the oxidative burst associated with bacterial killing and the recruitment of other immune cells (31, 61). iNOS stabilization and activation are dependent on their association with CaM- and LPS-dependent increases in cytosolic Ca^2+ levels (17), suggesting that LPS-dependent changes in CaM levels contribute to the modulation of iNOS function. To further identify possible links between Ca^2+ signaling pathways and the stabilization of iNOS with autocrine responses mediated by TNF, we have investigated how increasing cellular CaM levels affects LPS-dependent increases in TNF and iNOS. In the absence of LPS stimulation, TNF and iNOS protein levels were not detectable on the basis of Western blot analysis, regardless of the basal levels of cellular CaM. In cells treated with LPS (10 ng/ml), we observed a time-dependent increase in the protein abundance of membrane and soluble (i.e., released) TNF that preceded the appearance of iNOS (Fig. 3). The latter results are consistent with prior measurements demonstrating that LPS induces the transcriptional activation of these proteins through the activation of NF-κB and the requirement that membrane-bound TNF be processed further proteolytically by TNF-α-converting enzyme to release the soluble (i.e., active) species associated with autocrine and paracrine signaling (10, 27). In cells in which CaM levels are constitutively elevated, the kinetics associated with LPS-dependent increases in iNOS and TNF protein abundance after LPS treatment were similar to those observed in control cells, but the magnitude of the increases was dramatically reduced. The latter results indicate that increases in the protein abundance of CaM (Fig. 1) function to downregulate the levels of expressed iNOS and TNF and correlate with the observed decreases in cellular apoptosis (Fig. 2), suggesting that CaM may function in the coordinated regulation of TNF release and iNOS activation.

Enhanced cellular viability upon inhibition of iNOS activity. The functional linkage between iNOS expression and apoptosis was further assessed through the use of 1400W, which is a selective inhibitor of iNOS activity (22). iNOS activity was measured using the Griess reagent, which monitors total NO release as a stable end product that couples with nitrite (45). A linear decrease in iNOS activity occurred upon titration with 1400W, and we observed complete inhibition when the concentration of 100 μM 1400W was reached (Fig. 4A). The selective inhibition of iNOS activity by 1400W permits assessment of the possible involvement of iNOS in modulating cell,
viability. LPS-dependent decreases in cellular viability are largely blocked after inhibition of iNOS with 100 μM 1400W (Fig. 4B), suggesting that iNOS is a significant contributor to the apoptotic response to LPS in RAW 264.7 cells under these conditions. Interestingly, low concentrations of LPS appear to stimulate cell survival in an iNOS-independent manner, consistent with the notions that priming concentrations of LPS are cytoprotective and that apoptosis predominates only when endogenous cytoprotective mechanisms are overcome (35).

Direct linkage between release of TNF, iNOS activation, and apoptosis. To identify possible links between Ca2+ -activated CaM signaling pathways associated with the activation of iNOS and LPS-dependent increases in TNF, TNF-specific siRNA was used to suppress TNF induction selectively in response to LPS. Using either immunoblot analysis or TNF-specific ELISA measurement techniques, we found that TNF induction by LPS was markedly reduced in TNF siRNA cells relative to vector and wild-type controls across a wide range of LPS concentrations (Fig. 5A). We quantified the induction of iNOS using Western blot analysis with an authentic iNOS standard in response to LPS exposure for wild-type and TNF siRNA cells across a range of LPS concentrations. Suppression of TNF induction by siRNA resulted in significant inhibition of iNOS induction after LPS treatment relative to that observed in either wild-type or vector control cells (Fig. 5B). Thus TNF expression is required for the induction of iNOS, in agreement with prior reports for other systems (3, 24, 26, 53). Furthermore, LPS-induced apoptosis was significantly reduced in TNF siRNA cells relative to vector controls (Fig. 5C). The latter results are consistent with the inhibition of apoptosis observed in association with cotreatment with 1400W. Furthermore, these results indicate that the reduced apoptotic response after LPS exposure associated with elevated CaM concentrations in RAWCs is the result of a decrease in TNF-dependent iNOS induction by LPS. Collectively, these results suggest that iNOS activation and the associated generation of reactive oxygen species play a central role in mediating apoptosis in response to LPS exposure.

Increasing cellular CaM levels inhibits TF nuclear localization. Linkages between the transcriptional regulation of TNF and iNOS and CaM expression were explored further to gain an understanding of whether classic transcriptional regulators of these inflammatory mediators are influenced by CaM levels. TNF and iNOS expression are known to be regulated at the transcriptional level through nuclear localization of the TF NF-κB p65 subunit (RelA) and after phosphorylation of c-Jun to initiate oligomerization and transcriptional activation of the AP-1 complex (2, 15, 41, 64). In addition to mediating CaM gene expression, Sp1 has been reported to potentiate c-Jun-mediated gene regulation and play an important role in NF-κB-dependent transcriptional regulation in some cell types (2,

Fig. 4. Critical involvement of iNOS in apoptosis. A: LPS (10 ng/ml)-induced iNOS activity as measured by nitrite accumulation was specifically inhibited by cotreatment with 1400W. B: RAW 264.7 cells were treated with either LPS alone at concentrations indicated (•) or with LPS combined with 100 μM 1400W to inhibit iNOS activity (○). Viability was measured 48 h after exposure. Values are normalized as percentages of respective controls (without LPS treatment). Results are means ± SD of triplicate measurements. Similar results were obtained in 3 different experiments.

Fig. 5. Requirement of TNF induction (TNFI) for iNOS activation and apoptotic response. Western blot analysis of shed TNF (A) and total cellular iNOS (B) for wild-type (WT) or vector control RAW 264.7 cells and RAW 264.7 cells stably transfected with TNF small interfering RNA (siRNA). Quantitation of TNF shedding (ELISA) and iNOS expression as a function of LPS concentration are shown at bottom for wild-type cells (●) and TNF siRNA cells (○). C: apoptotic index (24 h) as determined using DAPI nuclear staining and microscopy in untreated cells (open bars) or after LPS exposure (10 ng/ml; filled bars) for vector control RAW 264.7 cells and RAW 264.7 cells stably transfected with TNF siRNA vector. *P < 0.05 vs. untreated control. † P < 0.05 vs. LPS-treated vector controls.
Nuclear extracts were prepared from cells treated with LPS (10 ng/ml; 0.25–3 h) and subjected to Western blot analysis of the p65 subunit (RelA) of NF-κB, phospho-c-Jun, Sp1, or XPA. XPA protein was used as a control because nuclear levels of this protein are expressed constitutively and are not known to be highly inducible. Consistent with the expected roles of these TFs in modulating TNF and iNOS gene expression, we observed a time-dependent increase in the nuclear concentrations of p65 (RelA), phospho-c-Jun, and Sp1 in response to LPS exposure; significant increases in the nuclear concentration of these TFs were apparent within the first 30 min after LPS exposure in control cells (Fig. 6). Levels of XPA protein were unchanged by any treatment.

To determine whether increased cellular CaM levels modulate transcription activation through these TFs to control apoptosis, we measured the relative nuclear concentrations of p65, phospho-c-Jun, and Sp1 in response to LPS exposure in RAWCs, in which CaM levels are elevated. In the case of p65 (RelA), the kinetics of the cellular response to LPS were similar to those observed in vector control cells, albeit with a markedly reduced amplitude. In contrast, levels of phospho-c-Jun were suppressed after CaM overexpression, whereas Sp1 nuclear concentrations progressively diminished after LPS stimulation in RAWCs. Nevertheless, in all cases, marked decreases were observed in the nuclear translocation of these activated TFs, indicating a diminished inflammatory response to LPS exposure relative to vector control counterparts. These observations suggest that elevation of cellular CaM levels reduces LPS toxicity, which is mediated at least in part via effects on transcriptional complexes that regulate TNF and iNOS gene expression and corresponding decreases in the levels of expressed proteins.

DISCUSSION

Activation of RAW 264.7 cells by LPS was associated with distinct kinetic processes involving the rapid upregulation of CaM protein levels and the activation of key TFs associated with the control of TNF and iNOS expression, which occurred within the first hour after LPS activation (Figs. 1 and 6). Subsequent expression of membrane-associated and shed TNF were apparent at 2 and 3 h after LPS exposure (Fig. 3). On the basis of Western blot analysis and siRNA experiments, TNF both preceded and was necessary for the expression of iNOS, which became apparent at ~4 h after LPS activation (Figs. 3 and 5). There was a direct correlation between the activity or abundance of iNOS and the extent of cellular apoptosis observed for longer intervals. Specifically, apoptosis was blocked by the selective inhibition of iNOS by 1400W (Fig. 4B). Likewise, the amount of cellular apoptosis was significantly reduced after the downregulation of iNOS abundance resulting from the use of siRNA to downregulate TNF autocrine signaling or after the overexpression of CaM (Figs. 2, 3, and 5). These results indicate an important role for iNOS and the associated generation of reactive oxygen species in mediating apoptosis, which may occur through the oxidative modification of redox-sensitive proteins (6).

The coordinated regulation of CaM protein levels involving the expression of three separate gene products remains poorly understood. Although the use of an inducible expression vector to manipulate cellular CaM levels is feasible, the rapid upregulation of CaM after LPS exposure (Fig. 1) relative to TNF and iNOS induction would be difficult to discern because of the complexity of competing kinetic processes associated with experimental manipulation vs. LPS-stimulated responses.

Fig. 6. Downregulation of transcriptional components of inflammatory response after CaM overexpression. Western blot analysis (top) and chemiluminescent quantitation (bottom) associated with nuclear concentrations of transcription factors (TFs) NF-κB p65 subunit, phospho-c-Jun (P-c-Jun), and Sp1 for vector control (○) and RAWCs expressing elevated CaM levels (●) after LPS exposure (10 ng/ml) for the times indicated. RCU, relative chemiluminescent units. Values are normalized to nuclear xeroderma pigmentosum group A (XPA; used as loading control). Similar results were observed in 3 separate experiments.
Thus, by mimicking the moderate elevation of CaM levels observed during LPS stimulation through stable transfection, the relative order of signaling events initiated by LPS could be determined in addition to demonstrating that tight regulation of cellular CaM levels has important consequences for cell survival.

The ability of constitutive CaM expression to block apoptosis through the suppression of transcriptional activation of key proteins associated with the inflammatory response is consistent with earlier measurements demonstrating a role for CaM and CaMKII in mediating the expression of both the adhesion protein CD44 and IAP2 to modulate the inflammatory response in monocytes (38). Furthermore, CaM was previously shown to modulate NF-κB activation in other cell types (4, 19, 23). In the case of NF-κB family members p65 (RelA) and c-Rel, a direct association with CaM was demonstrated after the release of these TFs from the endogenous inhibitor IκB. In the case of c-Rel, nuclear localization after cellular activation has been shown to be inhibited by CaM (4). Our results demonstrating that increasing cellular CaM levels prevents LPS-dependent nuclear accumulation of p65 (RelA) and other TFs (i.e., phospho-c-Jun, Sp1) associated with the inflammatory response suggest that CaM plays an important role in mediating the coordinated regulation of multiple promoters and their transcriptional products (Fig. 6). Indeed, this regulation is consistent with earlier, well-studied, CaM-dependent regulatory mechanisms in which levels of CaM activation selectively modulated the relative activities of the CaM-dependent phosphatase calcineurin and CaM-dependent kinases to modulate the TF cAMP response element-binding protein, a critical component of the LPS-stimulated TNF enhancer complex (8, 13, 56). Likewise, Ca$^{2+}$ activation modulates the nuclear localization of the TF NF-E2-related factor 2 through the modulation of CaM binding to a cytosolic protein complex that regulates the redox-dependent modulation of key antioxidant enzymes (60). Because CaM levels are normally substantially lower than the levels of CaM protein binding partners (9, 43, 44, 54), these measurements suggest that during the initial phases of LPS stimulation, increases in CaM abundance promote the activation of low-affinity CaM binding proteins (e.g., CaMKII, CaMKIV) that are necessary to modulate the induction of downstream inflammatory genes.

Prior results demonstrated that under normal conditions, calcineurin activity is required to induce NF-κB activation associated with iNOS expression in RAW 264.7 cells (27). After iNOS expression, CaM binding is also necessary for the stabilization and activation of the enzyme; in the absence of sufficient CaM, newly synthesized iNOS is rapidly degraded through the calpain and proteasome systems (16, 29, 57). Upon CaM association, the calpain cleavage site on iNOS is sterically blocked to stabilize the iNOS-CaM complex, whose activation is further modulated by high-affinity Ca$^{2+}$ binding (17, 52). Thus transcriptional regulation and the associated inflammatory response depend on the stoichiometries and relative kinetics of CaM expression compared with those associated with iNOS and other CaM-binding proteins.

The repression of transcriptional regulation of inflammatory mediators by CaM has practical implications, including potential anti-inflammatory therapeutic applications through selective inhibition of CaM binding partners (53a). In addition, this CaM-dependent mechanism may be manifested physiologically differently at early and late times after bacterial endotoxin challenge. Our results indicate that under resting conditions or in association with low levels of antigen (i.e., LPS), CaM levels normally function to prevent the premature or prolonged activation of macrophages through the inhibition of the nuclear localization of TFs (Fig. 7). Under these conditions, there is a low level of TNF and iNOS synthesis and iNOS expression is minimized without sufficient TNF induction (Fig. 5). TNF is both controlled by NF-κB activation and is also a strong activator of NF-κB, the latter regulation is important in its ability to potentiate iNOS expression. However, iNOS binds CaM with high affinity ($K_d < 0.1$ nM), effectively competing with lower-affinity binding partners. Thus, at critical levels of iNOS accumulation, the amount of available CaM needed to suppress TF activation in response to LPS may be sequestered. The release of CaM-mediated NF-κB suppression, for example, is expected to further promote TNF autocrine signaling and iNOS expression, committing the cell to an apoptotic response. The tight regulation associated with CaM and apoptosis may provide a mechanism for minimizing the nonspecific collateral damage to surrounding tissues after pathogen clearance. Thus key to understanding the regulation of macrophage activation is an appreciation of the coordinated regulation of Ca$^{2+}$-dependent signaling through changes in the levels of CaM activity and associated changes in TNF release and the associated upregulation of iNOS activity. We suggest that this cross talk between Ca$^{2+}$ and cytokine-dependent signaling pathways provides the necessary stability to ensure the ability to initiate macrophage activation rapidly and correctly in response to pathogen exposure.

**GRANTS**

This research was conducted under the Laboratory-Directed Research and Development Program at the Pacific Northwest National Laboratory and was supported by National Institute on Aging Grant AG-12993. Pacific Northwest National Laboratory is a multiprogram national laboratory operated by Battelle for the Department of Energy under Contract DE-AC05-76RL01830.
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