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Acquisition of Anergy to Proinflammatory Cytokines in Nonimmune Cells through Endoplasmic Reticulum Stress Response: A Mechanism for Subsidence of Inflammation

Kunihiro Hayakawa,* Nobuhiko Hiramatsu,* Maro Okamura,* Hiroaki Yamazaki,* Shotaro Nakajima,* Jian Yao,* Adrienne W. Paton,† James C. Paton,† and Masanori Kitamura**

Acute endoplasmic reticulum (ER) stress causes induction of inflammatory molecules via activation of NF-κB. However, we found that, under ER stress conditions, renal mesangial cells acquire anergy to proinflammatory stimuli. Priming of the cells with ER stress inducers (tunicamycin, thapsigargin, A23187, and AB5 subtilase cytotoxin) caused blunted induction of MCP-1 in response to TNF-α, IL-1β, macrophage-derived factors, or bystander macrophages. The magnitude of suppression was closely correlated with the level of GRP78, an endogenous indicator of ER stress. The suppression of MCP-1 under ER stress conditions was reversible and observed in general regardless of cell types or triggers of ER stress. The decrease in the level of MCP-1 mRNA was ascribed to transcriptional suppression via unexpected inhibition of NF-κB, but not to accelerated mRNA degradation. Subsequent experiments revealed that TNF receptor-associated factor 2, an essential component for TNF-α signaling, was down-regulated by ER stress. We also found that, under ER stress conditions, expression of NF-κB suppressor A20 was induced. Overexpression of A20 resulted in suppression of cytokine-triggered NF-κB activation and knockdown of A20 by RNA interference significantly attenuated induction of anergy by ER stress. In contrast, other ER stress-inducible/-related molecules that may suppress NF-κB (e.g., GRP78, NO, reactive oxygen species, and IκB) were not involved in the inhibitory effects of ER stress. These results elucidated ER stress-dependent mechanisms by which nonimmune cells acquire anergy to inflammatory stimuli under pathological situations. This self-defense machinery may play a role in halting progression of acute inflammation and in its spontaneous subsidence. The Journal of Immunology, 2009, 182: 1182–1191.

Macrophage-glomerular cell interaction plays a crucial role in the pathogenesis of glomerulonephritis (1, 2). Under pathological conditions, activated macrophages secrete a variety of inflammatory mediators and stimulate resident cells toward activation (3). Once activated, resident glomerular cells express chemokines and accelerate accumulation of macrophages, leading to progression of glomerular injury (4). Depletion of monocytes/macrophages or inhibition of macrophage infiltration attenuates glomerular injury, suggesting the crucial role of macrophages in the pathogenesis of glomerulonephritis (5–7).

During glomerulonephritis, monocytes/macrophages are attracted to glomeruli via chemokines produced by resident cells. In the expression of chemokines in glomerular cells, NF-κB plays a pivotal role (8–10). For example, exposure of glomerular mesangial cells to inflammatory cytokines induces rapid activation of NF-κB and consequent induction of MCP-1 (8). However, we recently reported that, once exposed to macrophage-derived cytokines, mesangial cells become insensitive to subsequent activation triggered by macrophages and cytokines, leading to attenuated expression of MCP-1 (11). This phenomenon may be involved in the spontaneous subsidence of acute glomerular inflammation. However, molecular mechanisms involved in this finding have not been elucidated.

Mammalian stress responses are evolutionarily conserved mechanisms to protect cells against adverse environmental conditions via induction of specific sets of stress proteins. For example, thermal stress induces expression of the heat shock protein (HSP) family of molecules that attenuate cellular damage. The “heat shock response” is induced not only by thermal stress but also by a variety of other triggers including oxygen radical species (ROS), transition heavy metals, inhibitors of energy metabolism, and growth factors (12). It is also caused during development, inflammation, ischemia, infection, mechanistic tissue injury, and aging (12). Under inflammatory situations, up-regulation of HSPs by inflammatory stimuli may lead to attenuation of inflammatory responses. Indeed, several previous studies suggested this possibility. For example, Cahill et al. (13) reported that heat shock factor...
1 was a transcriptional repressor that inhibits expression of proIL-1β. Other investigators also reported that overexpression of HSPs, especially HSP70, attenuated production and/or secretion of cytokines, chemokines, or adhesion receptors via, at least in part, suppression of NF-κB (14–16).

The HSP family consists of the HSP150, HSP110, HSP90, HSP70, HSP60, HSP40, and small HSP subfamilies (12). HSPs have critical roles in the attenuation of cellular stress and in the recovery from injury. In addition to their “emergency” roles, constitutively expressed HSPs also function as “housekeeping” chaperones and contribute to quality control of cellular proteins. That is, these molecules facilitate folding of newly synthesized proteins in the endoplasmic reticulum (ER) and assist translocation of proteins across intracellular membranes (17). In particular, 78-kDa glucose-regulated protein (GRP78) and other glucose-regulated proteins (GRPs) are important resident chaperones located in the ER, and these GRPs belong to the HSP family. GRPs, especially GRP78, are markedly induced under a particular stress condition, so-called ER stress, which is defined as accumulation of unfolded/misfolded proteins in the ER (18).

ER stress is implicated in a wide range of pathologies including viral infection, ischemic injury, neurodegenerative disorders, and metabolic diseases (19). Once the ER is functionally perturbed under pathological situations, unfolded proteins accumulate in the ER, leading to a specialized stress response, namely, the unfolded protein response (UPR) (20). Three major transmembrane transducers for sensing ER stress are identified in the ER; i.e., RNA-dependent protein kinase-like ER kinase, activating transcription factor 6, and IRE1, resulting in activation of their downstream signaling cascades for UPR (21).

As described, heat shock responses have the potential to inhibit induction of proinflammatory molecules by cytokines. ER stress responses could also confer cellular insensitivity to inflammatory stimuli. Several previous reports suggested that ER stress per se activates NF-κB (22–24). However, there are few reports that investigated whether and how ER stress responses affect activation of NF-κB and NF-κB-regulated gene expression by inflammatory stimuli. The present study was initiated to examine this issue, especially focusing on the effects of ER stress on macrophage-triggered activation of NF-κB and expression of NF-κB-dependent chemokines in glomerular cells.

Materials and Methods

Cells and stable transfectants

The rat mesangial cell line SM43 and stable transfectants (SM/Neo, SM/NFκB-SEAP, SM/GRP78) were established as described previously (25–27). SM/NFκB-SEAP cells produce secreted alkaline phosphatase (SEAP) under the control of the NF-κB enhancer elements (26). Conventionally immortalized murine podocytes were provided by Dr. K. Endlich (University of Heidelberg, Heidelberg, Germany) and cultured as described before (28). The normal rat alveolar macrophage cell line NR8383 was a gift from Dr. S. Hirano (National Institute for Environmental Studies, Tsukuba, Japan). The murine hematoma cell line Hpa-1c1c7 and the rat renal tubal epithelial cell line NRK-52E were purchased from American Type Culture Collection. Hpa-1c1c7 cells were maintained in o-MEM (B-27). Other cells were cultured in DMEM/F-12 (Life Technologies). Medium containing 1% FBS was generally used for studies.

Reagents and pharmacological manipulation

Confluent cells were pretreated with tunicamycin (0.1 ng/ml to 10 μg/ml; Sigma-Aldrich), thapsigargin (0.8–100 nM; Sigma-Aldrich), A23187 (1 μM; Sigma-Aldrich), or AB, subtilase cytotoxin (SubAB; 1 μg/ml) (29) for 6 h and exposed to TNF-α (human recombinant, 10 ng/ml; Genzyme) or IL-1β (human recombinant, 1 ng/ml; Genzyme) for 18 h. To examine degradation of mRNA, cells were pretreated with or without thapsigargin (100 nM) for 1 h and exposed to actinomycin D (5 μg/ml; Sigma-Aldrich) for 0–6 h. To investigate involvement of ROS and NO, cells were pretreated with N-acetylcysteine (0–5 mM; Sigma-Aldrich) or 5′-nitro-1-arginine methyl ester (1 mM; Sigma-Aldrich) for 0.5 h, treated with tunicamycin (10 μg/ml) or thapsigargin (10–100 nM) for 6 h and exposed to IL-1β or TNF-α for 18 h. Cells were also treated with 5′-nitroso-N-acetylpenicillamine (100 μM; Sigma-Aldrich) for 6 h to prevent IL-1β or TNF-α for 18 h.

Preparation of conditioned medium

Macrophage-conditioned medium (MoCM) was prepared as described before (11). In brief, NR8383 macrophages (5 × 10^6 cells) in medium containing 10% FBS were stimulated with LPS (1 μg/ml; Escherichia coli 0111; B4; Sigma-Aldrich) for 6 h. After washing twice, the macrophages were incubated in 10 ml of medium containing 1% FBS for 24 h. The conditioned medium were filtered through 0.45-μm filters to remove macrophages completely and stored at −20°C until use. Cross-feeding studies using MoCM were performed as follows. Confluent SM/NFκB-SEAP cells were pretreated with tunicamycin (10 μg/ml) or thapsigargin (100 nM) for 6 h, exposed to MoCM in the presence or absence of tunicamycin or thapsigargin for an additional 18 h, and subjected to Northern blot analysis, as described later.

Conditioned medium from ER stress-primed cells was prepared as follows. Confluent SM43 cells (3 × 10^6) in medium containing 1% FBS were treated with tunicamycin or thapsigargin for 6 h. After washing three times, the mesangial the cells were incubated in 10 ml of medium containing 1% FBS for 24 h. The conditioned medium was filtered and stored at −20°C until use. In some experiments, conditioned medium was heated at 80°C for 30 min and its effects were retested. Recombinant GRP78 (Stressgen) was also used for experiments at 100 nM.

Coculture

NR8383 macrophages were activated with LPS (1 μg/ml) for 6 h. After washing twice, activated macrophages (5 × 10^6 cells/well) were seeded onto confluent cultures of SM/NFκB-SEAP cells (5 × 10^5 cells/well) pretreated with thapsigargin (100 nM). After 18 h, cells were subjected to Northern blot analysis.

Construction of micro-RNA (miRNA) expression plasmids

To create pcDNA6.2-GW/EmGFp-miR-A20(750) and pcDNA6.2-GW/EmGFp-miR-A20(1060) encoding rat A20 miRNAs, single-strand oligonucleotides (5′-tgctgtTGTTACTGACTAAGTACCCATCGCCGTTTtgcacgtctgacggcagctgtggtctgacgcacacaccGCGGAGTGTCCTACTGACACAC3′ for miR-A20(750); 5′-tggtaACAGCTCTGAATTTCCAGGGCGCTTTtgcacgtctgacggcagctgtggtctgacgcacacaccGCGGAGTGTCCTACTGACACAC3′ for miR-A20(1060)) were designed using RNA Designer (Invitrogen), annealed, and cloned into pcDNA6.2-GW/EmGFp-miR vector (Invitrogen) using the BLOCK-IT Pol II miR RNA Expression Kit (Invitrogen) according to the manufacturer’s instruction.

Transient transfection

Using GeneJuice (Novagen), SM43 mesangial cells were transiently co-transfected with pNFκB-Luc (Panomics) along with pcDNA3.1 (Invitrogen), pcDNA3.1-GRP78 (provided by Dr. R. C. Austin, Henderson Research Center, Ontario, Canada) (30), pCAGGS-FLAGmA20 (BCCM/LMBP) (31), pcDNA6.2-GW/EmGFp-miR-A20(750), or pcDNA6.2-GW/EmGFp-miR-A20(1060) at a 1:2–4 ratio. pcDNA6.2-GW/EmGFp-miR-A20(1060) was used for 8–24 h, and subjected to luciferase assay, as described later. Assays were performed in quadruplicate.

Northern blot analysis

Total RNA was extracted by a single-step method and Northern blot analysis was performed as described before (11). CDNAs for SEAP (BD Biosciences), MCP-1 (32), GRP78 (provided by Dr. K. Imaizumi, University of Miyazaki, Miyazaki, Japan) (33), CCAAT/enhancer-binding protein homologous protein (CHOP) (provided by Dr. D. Ron, New York University School of Medicine, NY) (34), and A20 (BCCM/LMBP) (31) were used to
prepare radiolabeled probes. Expression of GAPDH was used as a loading control. Densitometric analysis was performed using Scion Image.

Western blot analysis

Western blot analysis was performed by the ECL system (Amersham Biosciences) as described before (11, 35). Primary Abs used were anti-IκBα Ab (1/200 dilution; Santa Cruz Biotechnology), anti-IκBβ Ab (1/200 dilution; Santa Cruz Biotechnology), anti-KDEL Ab (1/1000 dilution; Stressgen), anti-phospho-specific IκB kinase (IKK) α/β Ab (1/1000 dilution; Cell Signaling), and anti-TNFR-associated factor (TRAF) 2 Ab (1/100 dilution; Santa Cruz Biotechnology). As a loading control, identical filters were reprobed for -actin using anti-actin Ab (1/30,000 dilution; Sigma-Aldrich). For Western blot analysis of GRP78, culture medium from tunicamycin- or thapsigargin-treated cells was concentrated using molecular cutoff membranes (Microcon Centrifuge Filter YM-30; Nihon Millipore).

Evaluation of DNA-binding activity of NF-κB

The DNA-binding activity of NF-κB p65 subunit was evaluated using the ELISA-based TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif) as described previously (36). Assays were performed in triplicate.

Luciferase assay

Activity of luciferase was evaluated by luciferase assay system (Promega) according to the manufacturer’s protocol (37). Assays were performed in quadruplicate.

Statistical analysis

Data are expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. A value of p < 0.05 was considered to be a statistically significant difference.

Results

Blunted expression of MCP-1 in response to proinflammatory cytokines in ER stress-primed mesangial cells

To examine whether ER stress-primed cells become either sensitive or insensitive to subsequent exposure to inflammatory stimuli, mesangial cells were pretreated with several ER stress inducers including tunicamycin, thapsigargin, or A23187 for 6 h and exposed to IL-1β (1 ng/ml) or TNF-α (10 ng/ml) for 18 h. Expression of MCP-1 and GRP78 was examined by Northern blot analysis. Expression of GAPDH is shown at the bottom as a loading control. B–E, Cells were treated with tunicamycin (0.1–100 ng/ml; B and D) or thapsigargin (0.8–100 nM; C and E), exposed to IL-1β or TNF-α (B and C), and subjected to Northern blot analysis. F, Mesangial cells were pretreated with thapsigargin for 1 h, exposed to actinomycin D (Act D; 5 μg/ml) for 0–6 h, and subjected to Northern blot analysis (left panel). The level of MCP-1 was normalized by the level of GAPDH, and relative reduction of MCP-1 mRNA is shown in the right graph. Closed circle and solid line, in the absence of actinomycin D; closed triangle and dotted line, in the presence of actinomycin D.
was observed by tunicamycin only at concentrations ≥10 ng/ml, and it was inversely correlated with dose-dependent suppression of MCP-1 by 10–100 ng/ml tunicamycin. Similarly, dose-dependent induction of GRP78 and dose-dependent suppression of MCP-1 were observed in parallel by the treatment with thapsigargin at concentrations ≥20 nM (Fig. 1C). Furthermore, basal expression of MCP-1 was also attenuated by the treatment with the ER stress inducers (Fig. 1, D and E).

The down-regulation of the steady-state level of MCP-1 is caused by transcriptional suppression or enhanced degradation of mRNA. To examine the latter possibility, mesangial cells were pretreated with or without thapsigargin for 0.5 h and incubated further in the presence of an inhibitor of RNA synthesis, actinomycin D, for an additional 0.5–6 h. Northern blot analysis showed that the level of MCP-1 mRNA was reduced by actinomycin D in a time-dependent manner (Fig. 1F, left panel). Densitometric analysis revealed that the degradation of MCP-1 mRNA was not enhanced under the ER stress condition (Fig. 1F, right graph), suggesting that the down-regulation of MCP-1 mRNA by ER stress was caused by transcriptional suppression.

### Blunted activation of NF-κB in response to proinflammatory cytokines in ER stress-primed mesangial cells

We previously reported that, in rat mesangial cells, cytokine-inducible MCP-1 expression was dependent on NF-κB and independent of AP-1, whereas constitutive expression of MCP-1 was dependent on both NF-κB and AP-1 (38). The suppression of MCP-1 expression by ER stress may be caused via inhibition of NF-κB, although several previous reports suggested that ER stress per se activated NF-κB (22–24). To examine our hypothesis, SM/NFκB-SEAP reporter mesangial cells that express SEAP under the control of the κB enhancer elements (26) were used. The reporter cells were pretreated with tunicamycin, thapsigargin, or A23187 for 6 h, exposed to IL-1β or TNF-α, and subjected to Northern blot analysis for 18 h, and subjected to analysis of SEAP mRNA to evaluate NF-κB activity. Northern blot analysis revealed that expression of SEAP was induced in unprimed cells following the exposure to IL-1β or TNF-α. This induction was attenuated in ER stress-primed cells markedly in TNF-α-treated cells (Fig. 2A). Of note, after the treatment with tunicamycin, thapsigargin, or A23187 for 24 h, ER stress (indicated by GRP78) was induced, whereas activation of NF-κB (indicated by SEAP) was not observed in cytokine-unprimed cells (Fig. 2A, lanes 1–4). Basal activity of NF-κB was rather suppressed by ER stress, which was consistent with our result showing that basal expression of MCP-1 was also attenuated by the treatment with the ER stress inducers (Fig. 1, D and E).

Using serial concentrations of tunicamycin and thapsigargin, correlation between induction of ER stress and suppression of NF-κB was confirmed. As shown in Fig. 2, B and C, substantial suppression of NF-κB was observed by 10–100 ng/ml tunicamycin and 20–100 nM thapsigargin, the doses of which suppressed expression of MCP-1 (Fig. 1, B and C). Of note, TNF-α-triggered NF-κB activation was more susceptible to ER stress than IL-1β-triggered NF-κB activation (Fig. 2, A–C). It was consistent with the higher susceptibility of TNF-α-triggered MCP-1 expression to the ER stress inducers (Fig. 1, A–C).

Activated forms of NF-κB are heterodimers, which typically consist of p65 and p50 subunits. The blunted activation of NF-κB by ER stress was further confirmed by evaluation of DNA-binding activity of the p65 subunit. In unprimed cells, the DNA-binding
activity of p65 was significantly increased by the treatment with TNF-α or IL-1β. Consistent with the results shown in Fig. 2, A–C, however, this increase was attenuated by ER stress significantly in TNF-α-treated cells and only modestly (not statistically significant) in IL-1β-treated cells (Fig. 2D). This result provided additional evidence for blunted activation of NF-κB in ER stress-primed cells.

IκBα and IκBβ proteins are endogenous inhibitors of NF-κB and are located downstream of IKK in the NF-κB signaling pathway. Under unstimulated conditions, NF-κB locates in the cytoplasm as complexes with IκBs. When cells are stimulated, IKKs phosphorylate IκBα and cause rapid degradation of IκBα by the proteasome pathway. It allows for translocation of NF-κB into the nucleus and its binding to the κB sites. We examined whether ER stress-initiated anergy to cytokines is associated with blockade of IKK-mediated IκBα degradation. Mesangial cells were pretreated with tunicamycin or thapsigargin for 6 h and exposed to IL-1β or TNF-α for 0.5 h. Western blot analysis revealed that treatment of unprimed cells with IL-1β or TNF-α rapidly reduced the levels of IκBα and IκBβ. However, in ER stress-primed cells, degradation of IκBα either by IL-1β or TNF-α was abrogated (Fig. 2E). Of note, treatment with tunicamycin or thapsigargin alone for 6.5 h did not cause degradation of IκBα (Fig. 2E, lanes 1–3).

Degradation of IκBα is regulated by phosphorylation of IKKα/β in the presence of IKKγ (39). We examined whether ER stress-initiated anergy to cytokines is associated with altered phosphorylation and/or stability of IKKs. Mesangial cells were pretreated with or without thapsigargin for 6 h and exposed to TNF-α for 0–10 min. Treatment of the cells with the cytokine rapidly caused phosphorylation of IKKα/β. However, under the ER stress condition, phosphorylation of IKKα/β was attenuated (Fig. 2F). Western blot analysis showed that the protein level of IKKαγ was not affected by the treatment with thapsigargin (Fig. 2G). These results suggested the potential of ER stress for suppression of the NF-κB pathway, possibly targeting upstream of IKK.

Reversibility and generality of anergy to cytokines in ER stress-primed cells

To examine whether the anergy in ER stress-primed cells is reversible, SM/NFxβB-SEAP reporter cells were pretreated by thapsigargin for 6 h, incubated further without thapsigargin for 3 days, and exposed to IL-1β or TNF-α for 0.5 h. Northern blot analysis revealed that treatment of unprimed cells with IL-1β or TNF-α rapidly reduced the levels of GRP78. Mesangial cells were pretreated with or without thapsigargin for 6 h and exposed to TNF-α for 0–10 min. Treatment of the cells with the cytokine rapidly caused phosphorylation of IKKα/β. However, under the ER stress condition, phosphorylation of IKKα/β was attenuated (Fig. 2F). Western blot analysis showed that the protein level of IKKαγ was not affected by the treatment with thapsigargin (Fig. 2G). These results suggested the potential of ER stress for suppression of the NF-κB pathway, possibly targeting upstream of IKK.

The cells were then exposed to IL-1β or TNF-α for 18 h. Northern blot analysis revealed that, like mesangial cells, expression of GRP78 was rapidly reduced the levels of GRP78. Mesangial cells were pretreated with or without thapsigargin for 6 h and exposed to TNF-α or IL-1β for 6 h. Northern blot analysis showed that SubAB markedly induced expression of GRP78 and CHOP mRNAs, confirming induction of ER stress (Fig. 4A, second and third rows). Under this experimental condition, induction of MCP-1 by TNF-α was abrogated and the effect by IL-1β was partially suppressed (Fig. 4A, top row). It was consistent with the results using tunicamycin, thapsigargin, and A23187 (Fig. 1A).

To examine whether or not the acquisition of anergy by ER stress is specific to mesangial cells, we tested other cell types including a murine hepatoma cell line Hepa-1c1c7 and a rat renal tubular cell line NRK-52E. Hepa-1c1c7 cells and NRK-52E cells were pretreated with thapsigargin for 6 h and exposed to IL-1β or TNF-α for 18 h. Northern blot analysis revealed that, like mesangial cells, expression of MCP-1 was induced by cytokines in unprimed Hepa-1c1c7 cells and NRK-52E cells and the induction was abolished in thapsigargin-pretreated cells, except for IL-1-stimulated NRK-52E cells (Fig. 4B). Similar results were also obtained in SubAB-pretreated NRK-52E cells and murine glomerular podocytes; i.e., priming of these cells by SubAB caused expression of GRP78 and CHOP and blunted induction of MCP-1 by IL-1β and TNF-α (Fig. 4C). These results suggested that acquisition of anergy to cytokines via ER stress is a general phenomenon, but some uncommon exception may exist under particular situations.

Activation of glomerular cells by macrophages plays a crucial role in the pathogenesis of glomerulonephritis (1, 2). Under pathological conditions, activated macrophages secrete a variety of inflammatory mediators and stimulate resident cells toward activation (3). IL-1β and TNF-α are pivotal cytokines produced by macrophages, but other inflammatory mediators from macrophages may also be important for activation of resident cells.
Therefore examined whether ER stress–primed mesangial cells become insensitive to activated macrophages. First, SM/NfXb-SEAP reporter cells were pretreated with thapsigargin for 6 h. After washing out thapsigargin, the cells were cocultured with activated rat macrophages for 6 h and cotransfected with activated Nrf3383 macromolecules (M3M) for 18 h. Expression of SEAP and GRP78 was examined by Northern blot analysis. E, Reporter mesangial cells were pretreated with tunicamycin or thapsigargin, treated with culture medium conditioned by activated M3M (M3MCM) for 18 h and subjected to Northern blot analysis.

### Molecular mechanisms involved in the blunted responses to cytokines in ER stress-primed cells

As described, ER stress suppressed activation of NF-κB in response to inflammatory stimuli. A previous report showed that in thapsigargin- or tunicamycin-treated tumor cells, the level of TRAF2 protein, the essential component for the TNF-α signaling (41), was selectively down-regulated (42). The blunted response of NF-κB to TNF-α under ER stress may be ascribed to down-regulation of TRAF2. To examine this possibility, mesangial cells were treated with tunicamycin or thapsigargin for 8–12 h and subjected to Western blot analysis of TRAF2. B–D, Mesangial cells (B and C) and NRK-52E cells (D) were treated with thapsigargin (B), tunicamycin (C), or SubAB (C and D) for the indicated time periods, and expression of A20 as well as GRP78 was examined by Northern blot analysis. E, Mesangial cells were transiently cotransfected with pNFκB-Luc along with control vector or pCAGGS-mA20 encoding wild-type A20. After 16 h, the cells were treated with or without TNF-α for 8 h and subjected to a luciferase assay. F, Mesangial cells were transiently cotransfected with pNFκB-Luc along with pcDNA6.2-GW/EmGFP-miR-A20(750), pcDNA6.2-GW/EmGFP-miR-A20(1060), or pcDNA6.2-GW/EmGFP-miR-A20(1060) that introduces a scrambled miRNA, A20(750) miRNA, or A20(1060) miRNA, respectively. After 16 h, the cells were pretreated with or without thapsigargin (50 nM) for 6 h, exposed to IL-1β or TNF-α for 24 h without thapsigargin, and subjected to luciferase assay. In E and F, assays were performed in quadruplicate and data are expressed as means ± SE. Asterisks indicate statistically significant differences (p < 0.05).

### FIGURE 4. Generality of anergy to cytokines in ER stress-primed cells.

A, Mesangial cells were pretreated with or without SubAB (1 μg/ml) for 3 h and exposed to IL-1β or TNF-α for 6 h. Expression of MCP-1, GRP78, and CHOP was examined by Northern blot analysis. B, Hepa-1c1c7 cells and NRK-52E cells were pretreated with 50–100 nM thapsigargin for 6 h, exposed to IL-1β or TNF-α for 18 h, and subjected to Northern blot analysis. C, NRK-52E cells and podocytes were pretreated with SubAB (100 ng/ml) for 6 h, exposed to IL-1β or TNF-α for 6 h, and subjected to Northern blot analysis. D, SM/NFκB-SEAP reporter cells were pretreated with thapsigargin for 6 h and cotransfected with activated Nrf3383 macromolecules (M3M) for 18 h. Expression of SEAP and GRP78 was examined by Northern blot analysis. E, Reporter mesangial cells were pretreated with tunicamycin or thapsigargin, treated with culture medium conditioned by activated M3M (M3MCM) for 18 h and subjected to Northern blot analysis.
stress caused by tunicamycin and thapsigargin. This result suggested that TRAF2 is a target of ER stress for blockade of the cytokine-induced NF-κB activation.

ER stress response may induce some endogenous inhibitors of NF-κB. A20 is a major molecule involved in the negative feedback loop of NF-κB activation (43). Previous reports showed that overexpression of A20 inhibited NF-κB activation and that, in A20-deficient cells, prolonged activation of NF-κB was observed in response to TNF-α (44, 45). A20-deficient mice develop severe multiorgan inflammation and are susceptible to sublethal doses of TNF-α (45). The inhibitory effect of A20 on NF-κB may be observed not only in TNF-α-stimulated cells but also in IL-1β-exposed cells (46). Although there are no reports that indicate a relationship between ER stress and A20, we examined involvement of A20 in ER stress-mediated blunting of NF-κB activation. First, mesangial cells were treated with thapsigargin for 1–9 h and expression of A20 was evaluated by Northern blot analysis. As shown in Fig. 5B, expression of A20 was induced by ER stress within 3 h and sustained for at least 9 h. It was in parallel with induction of GRP78. The induction of A20 via ER stress was observed not only by thapsigargin but also by other ER stress inducers, including tunicamycin and SubAB (Fig. 5C). The similar effect was also observed in NRK-52E cells treated with SubAB (Fig. 5D), suggesting that the induction of A20 by ER stress is a general phenomenon. Reporter assays showed that transfection with A20 markedly suppressed activation of NF-κB in TNF-α-stimulated mesangial cells (Fig. 5E). To confirm involvement of A20, we constructed two types of expression plasmids encoding rat A20 miRNAs. Overexpression of either miR-A20(750) or miR-A20(1060) efficiently down-regulated the level of endogenous A20 mRNA in NRK-52E cells (data not shown). Mesangial cells were transiently cotransfected with pNFκB-Luc along with miR-Neg (scrambled miRNA), miR-A20(750), or miR-A20(1060). After the transfection, the cells were pretreated with or without thapsigargin for 6 h, exposed to IL-1β or TNF-α for 24 h, and subjected to luciferase assay. As shown in Fig. 5F, knockdown of A20 by these miRNAs modestly but significantly attenuated the induction of energy by thapsigargin in IL-1β- or TNF-α-stimulated cells. These results indicated involvement of A20 in the blunted activation of NF-κB under ER stress conditions.

To further identify other mechanisms involved in the blunted responses to cytokines in ER stress-primed cells, we focused on GRP78 that is abundantly induced by ER stress. In general, GRP78 is considered as a major chaperone located in the ER and contributes to folding of newly synthesized proteins. However, in some situations, GRP78 may be transported to the cytoplasm (47), expressed on the cell surface (48), or secreted into the extracellular space (49). The ectopic GRP78 may have distinct biological function. Because GRP78 in the cytoplasm can interact with IKK (47), it could affect NF-κB signaling. We first tested whether overexpression of GRP78 can confer anergy to inflammatory cytokines. Mesangial cells were transiently transfected with GRP78 along with pNFκB-Luc and stimulated by TNF-α. Although overexpression of GRP78 conferred significant resistance to ER stress-induced apoptosis in mesangial cells (27), transfection with GRP78 did not attenuate TNF-α-induced NF-κB activation (Fig. 6A). Previous reports showed that GRP78 was present in synovial fluid and that extracellular GRP78 caused expression and secretion of anti-inflammatory molecules in monocytes (50, 51). Secretion of GRP78 could be caused by ER stress and contribute to acquisition of anergy to inflammatory stimuli. Indeed, Western blot analysis revealed that tunicamycin or thapsigargin triggered secretion of GRP78 in mesangial cells (Fig. 6B). However, the conditioned medium from ER stress-primed cells failed to attenuate expression of MCP-1 and activation of NF-κB in IL-1β- or TNF-α-stimulated reporter cells (Fig. 6C). Furthermore, consistent with a previous report showing that addition of recombinant GRP78 induced production of IL-6 and TNF-α in microglia (52), our results also showed that externally added GRP78 rather enhanced TNF-α-triggered MCP-1 expression and NF-κB activation (Fig. 6D), excluding the above-mentioned possibility.

NO and ROS have the potential to modulate activity of NF-κB positively or negatively (53). These molecules may be generated under ER stress conditions (54). We examined involvement of these substances in the ER stress-induced anergy to cytokines. The results showed that treatment of mesangial cells with NO donor S-nitroso-N-acetylpenicillamine did not induce ER stress and did not inhibit induction of MCP-1 and activation of NF-κB by IL-1β or TNF-α (data not shown). Consistently, pretreatment with an inhibitor of NO synthase, Nω-nitro-L-arginine methyl ester, did not affect tunicamycin- or thapsigargin-induced anergy to cytokines (data not shown). Similarly, responsiveness of thapsigargin-primed cells to TNF-α was not recovered by the treatment with antioxidant N-acetylcysteine (data not shown). These results suggested lack of involvement of NO and ROS in ER stress-induced acquisition of anergy to inflammatory stimuli.
Discussion
Perturbation of ER function, so-called ER stress, causes activation of inflammation-related transcription factors including NF-κB (55). In response to ER stress, IRE1α binds to the IKK complex and activates NF-κB by promoting degradation of IκB. IRE1α also binds to TRAF2 and activates apoptosis signal-regulating kinase 1, leading to activation of IKK (56). Other investigators also suggested that, under ER stress, TNFR1 is accumulated in the ER and forms a complex with TRAF2 and TNFR-interacting protein (RIP). This molecular event may lead to induction of the TNF signaling, especially activation of IKK, even without TNF-α (41, 57). In contrast to this current concept, in the present investigation, we demonstrated that preceding ER stress causes blunted activation of NF-κB and attenuates expression of NF-κB-regulated MCP-1 in response to bystander macrophages, macrophage-de facto factors, and inflammatory cytokines including IL-1β and TNF-α.

In this report, we showed for the first time that A20, one of the major negative regulators for NF-κB, was induced by ER stress, suggesting a possible involvement of this molecule in the blunted responses to inflammatory stimuli under ER stress conditions. Indeed, knockdown of A20 by RNA interference partially but significantly reversed the blunted response of NF-κB (Fig. 5F). A20 is known to act as a deubiquitinating enzyme, which removes Lys63-linked ubiquitin chains from RIP, the crucial component for the TNF-α signaling. A20 also functions as an ubiquitin ligase by polyubiquitinating RIP with Lys48-linked ubiquitin chains and thereby targets RIP to proteosomal degradation (58). The degradation of RIP consequently interferes with the signaling triggered by TNF-α. Interestingly, however, the inhibitory effect of A20 on NF-κB was observed not only in TNF-α-stimulated cells but also in IL-1β-exposed cells. The latter effect may be caused by interfering with TRAF6, the crucial component for the IL-1 signaling, through removal of its ubiquitin moieties (46, 59).

The fact that A20 interferes with the cytokine signaling at the levels of RIP/TRA2 and TRAF6 is consistent with our current finding that the target(s) of ER stress in the NF-κB pathway should be located upstream of IKK (Fig. 2F). Currently, it is unclear how A20 is induced by ER stress, but previous reports showed that expression of A20 is up-regulated by NF-κB (60). Because ER stress causes activation of NF-κB in the early phase (22–24, 55), the induction of A20 by ER stress could be caused by the early, transient activation of NF-κB.

In addition to A20, some other molecules can also be involved in the negative feedback loop of NF-κB activation. Active export of nuclear NF-κB by IκBα may be another major mechanism for the suppression of NF-κB (61). After NF-κB activation, IκBα is transcribed in a NF-κB-dependent manner. The synthesized IκBα protein enters the nucleus and shuttles NF-κB back to the cytoplasm, leading to repression of NF-κB activation (62). To examine a possibility that ER stress increases IκBα protein and thereby inhibits cytokine responses, protein levels of IκBα were evaluated after the exposure to ER stress inducers. However, we could not detect any alterations in the level of IκBα following the treatment with either tunicamycin or thapsigargin (Fig. 2E, lanes 1–3), excluding this possibility. The lack of involvement of IκBα is consistent with our current finding that ER stress interfered with the NF-κB pathway upstream of IKK.

In this report, we found that activation of NF-κB by TNF-α was more susceptible to ER stress than that by IL-1β. It indicates that some mechanism that preferentially targets the TNF signaling may be involved in the suppressive effect of ER stress. In the TNF-α signaling, TNFR1, TNFR1-associated death domain, RIP, and TRAF2 are essential for NF-κB activation. In contrast, these molecules are not required for the IL-1 signaling. Recently, Hu et al. (42) reported that, in thapsigargin- or tunicamycin-treated MCF-7 cells and L929 cells, TNFR1, TNFR1-associated death domain, and RIP proteins were maintained at the same levels as those in untreated controls, whereas the level of TRAF2 protein was selectively decreased. The decrease in TRAF2 protein was not due to transcriptional suppression or increased turnover of mRNA but due to enhanced protein degradation (42). Consistent with this report, we found in the present study that TRAF2 protein was downregulated by ER stress triggered by tunicamycin or thapsigargin in mesangial cells (Fig. 5A). The preferential, blunted response of NF-κB to TNF-α may be, in part, caused by down-regulation of TRAF2.

GRP78 is the most famous, multifunctional molecule induced by ER stress. A previous report indicated that GRP78 may affect the NF-κB signaling via binding to IKK (47). We tested whether GRP78 can confer insensitivity to inflammatory cytokines, but overexpression with GRP78 did not attenuate TNF-α-induced NF-κB activation in mesangial cells. Previous reports showed that GRP78 was present in synovial fluid and the extracellular GRP78 caused expression and secretion of anti-inflammatory molecules including IL-10, soluble TNFR2 (p75-TNFR) and IL-1R antagonist in human monocytes (50, 51). We found that ER stress increased the level of extracellular GRP78 in mesangial cells. However, using conditioned medium from ER stress-primed cells and recombinant GRP78, we could not obtain any supportive evidence for suppressive effects of extracellular GRP78 on TNF-α-triggered MCP-1 expression and NF-κB activation.

A number of previous reports demonstrated the potential of NO for negative regulation of NF-κB, as reviewed by Janssen-Heininger et al. (53). It is believed that NO is involved in a negative feedback loop to block prolonged activation of NF-κB. The inhibitory effect of NO on NF-κB may occur through nitrosylation at cysteine 62 of p50 NF-κB subunit, leading to prevention of NF-κB binding to the κB site (63, 64). IKK may also be a potential target of NO, because multiple cysteines are present in the kinase domain of IKK (65). However, our results showed that NO donor did not inhibit induction of MCP-1 and activation of NF-κB by cytokines and 2) inhibition of NO synthesis did not affect tunicamycin- or thapsigargin-induced anergy to cytokines.

Recent reports showed that ER stress is induced under inflammatory situations. For example, we recently reported that systemic inflammation caused by LPS in mice resulted in ER stress in various organs including lung, liver, kidney, and spleen (37). Shkoda et al. (47) also reported that expression of GRP78 is increased in intestinal epithelial cells of mice under chronic inflammation. They also showed that in intestinal epithelial cells from patients with inflammatory bowel diseases, including ulcerative colitis and Crohn’s disease, the level of GRP78 increased. Epithelial cells from patients with sigmoid diverticulitis also exhibited increased GRP78 protein, suggesting that ER stress responses may be a general part of intestinal inflammation (47). Based on these findings, our current results suggest that, although ER stress may induce proinflammatory molecules via transient activation of NF-κB in the early phase, consequent ER stress responses may suppress NF-κB in the later phase. This mechanism may play a role in halting progression of acute inflammation and in its spontaneous subsidence.

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References


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