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Blunted activation of NF-κB and NF-κB-dependent gene expression by geranylgeranylacetone: Involvement of unfolded protein response

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Abstract

Geranylgeranylacetone (GGA), an anti-ulcer agent, has anti-inflammatory potential against experimental colitis and ischemia-induced renal inflammation. However, molecular mechanisms involved in its anti-inflammatory effects are largely unknown. We found that, in glomerular mesangial cells, GGA blocked activation of nuclear factor-κB and consequent induction of monocyte chemoattractant protein 1 (*MCP-1*) by inflammatory cytokines. It was inversely correlated with induction of unfolded protein response (UPR) evidenced by expression of 78 kDa glucose-regulated protein (*GRP78*) and suppression of endoplasmic reticulum stress-responsive alkaline phosphatase. Various inducers of UPR including tunicamycin, thapsigargin, A23187, 2-deoxyglucose, dithiothreitol, and AB_5 subtilase cytotoxin reproduced the suppressive effects of GGA. Furthermore, attenuation of UPR by stable transfection with *GRP78* diminished the anti-inflammatory effects of GGA. These results disclosed a novel, UPR-dependent mechanism underlying the anti-inflammatory potential of GGA.

Keywords: Geranylgeranylacetone; Mesangial cell; Tumor necrosis factor-α; Unfolded protein response; Endoplasmic reticulum stress; Monocyte chemoattractant protein 1; Nuclear factor-κB

Geranylgeranylacetone (GGA), also known as teprenone, has been used in clinics for the treatment of gastric ulcer and gastritis. However, recent investigation revealed the potential of this compound as a general cytoprotective agent. It is currently believed that GGA induces 70 kDa heat shock protein (HSP70) and thereby protects various cells from apoptosis [1,2]. The therapeutic utility of GGA for non-gastric diseases has been documented by several investigators. For example, GGA attenuated ischemic brain injury and endotoxin shock [3,4]. Other reports also showed anti-inflammatory potential of GGA; e.g., GGA attenuated experimental colitis and ischemia-triggered renal inflammation [5,6]. However, it is currently unclear whether these therapeutic effects are ascribed only to up-regulation of HSP70. GGA might exert the beneficial effects through other mechanisms.

Recently, we reported novel potential of GGA to induce unfolded protein response (UPR) [7]. We found that GGA caused selective expression of 78 kDa glucose-regulated protein (*GRP78*), a HSP70 family member induced by endoplasmic reticulum (ER) stress, as well as CCAAT/enhancer-binding protein-homologous protein (*CHOP*), another marker of ER stress, in several cell types. In mesangial cells, GGA triggered selective branches of UPR including the activating transcription factor 6 (ATF6) pathway and the inositol-requiring ER-to-nucleus signal kinase 1 (IRE1)—X-box binding protein 1 (XBP1) pathway [7]. Although several previous reports suggested that ER stress and consequent UPR may trigger cellular activa-

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tion [8], information is limited regarding how UPR modulates cellular responses to inflammatory stimuli.

In the process of glomerulonephritis, infiltration of leukocytes, especially monocytes/macrophages, plays a decisive role [9]. Activated macrophages secrete inflammatory mediators including tumor necrosis factor-α (TNF-α) and IL-1β and stimulate resident cells toward activation [10]. Once activated, resident cells express chemokines and accelerate recruitment of monocytes/macrophages, leading to progression of glomerular inflammation [11]. In the expression of chemokines, nuclear factor-κB (NF-κB) plays a crucial role. For example, exposure of mesangial cells to cytokines induces rapid activation NF-κB and consequent induction of monocyte chemoattractant protein 1 (*MCP-1*) [12].

In the present investigation, we examine how GGA-initiated UPR modulates responses of glomerular cells to inflammatory stimuli. Using cultured mesangial cells, we aim at elucidating whether GGA attenuates activation of NF-κB and consequent expression of *MCP-1* in response to cytokines, and if so, how individual UPRs are involved in the suppressive effects.

Materials and methods

Reagents. GGA was provided by Eisai Co. Ltd. (Tokyo, Japan). Human recombinant IL-1β and human recombinant TNF-α were purchased from Genzyme (Cambridge, MA). Other reagents were obtained from Sigma–Aldrich Japan (Tokyo, Japan). AB_5 subtilase cytotoxin (SubAB) that specifically degrades GRP78 was prepared as described previously [13].

Cells and stable transfectants. The rat mesangial cell clone SM43 was established as described before [14]. Stably transfected mesangial cells SM/SV-SEAP, SM/Neo, SM/GRP78, and SM/NFκB-SEAP were established as described previously [7,15,16]. SM/SV-SEAP cells and SM/NFκB-SEAP cells express secreted alkaline phosphatase (SEAP) under the control of the simian virus 40 early promoter or the NF-κB binding site, respectively. All assays were performed in the presence of 1% fetal bovine serum (FBS).

ER stress-responsive alkaline phosphatase (ES-TRAP) assay. Induction of ER stress was evaluated by ES-TRAP assay [15] using SM/SV-SEAP.

Transient transfection. Using GeneJuice (Novagen, Madison, WI), SM43 cells were transiently co-transfected with pNFκB-Luc (Panomics, Fremont, CA) together with pcDNA3.1, pcDNA3.1-dnXBP1 encoding a dominant-negative mutant of XBP1 (XBP1-DN) [17], pcDNA-ATF6(373)ΔAD encoding a dominant-negative mutant of ATF6 (ATF6-DN) [18] or pCAG-hIRE1α-K599A encoding a dominant-negative mutant of IRE1α (IRE1α-DN) [19] at 1:3 ratio. After incubation for 24 h, the cells were seeded in 96-well plates in the presence of 1% FBS. After incubation overnight, the cells were pretreated with GGA, exposed to TNF-α and subjected to luciferase assay. The luciferase assay was performed using Luciferase Assay System (Promega, Madison, WI).

Northern blot analysis. Northern blot analysis was performed as described before [7]. cDNAs for MCP-1, GRP78 [20], CHOP [21], and SEAP (BD Biosciences) were used for preparation of radio-labeled probes. Expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control.

Formazan assay. The number of viable cells was assessed by a formazan assay using Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan).

Statistical analysis. Except for Northern blot analysis, assays were performed in quadruplicate. Data are expressed as means ± SE. Statistical analysis was performed using the non-parametric Mann–Whitney *U* test to compare data in different groups. *P* value <0.05 was considered to indicate a statistically significant difference.

Results

Induction of UPR and suppression of cytokine-induced MCP-1 expression by GGA

MCP-1 is an inflammatory mediator triggered by macrophage-derived cytokines including IL-1β and TNF-α. In glomerular cells, expression of this molecule is induced *in vivo* under inflammatory situations and contribute to progression of glomerular injury [22]. Using SM43 mesangial cells, we first examined effects of GGA on the induction of *MCP-1* by IL-1β and TNF-α. Northern blot analysis revealed that IL-1β and TNF-α substantially induced expression of *MCP-1*. Treatment of the cells with GGA diminished the induction of this gene. The suppressive effect of GGA was dose-dependent and more pronounced in the cells stimulated by TNF-α (Fig. 1A).

Recently, we reported novel potential of GGA to induce UPR in mesangial cells [7]. Indeed, as shown in Fig. 1B, GGA up-regulated the levels of *GRP78* and *CHOP*, the endogenous markers for UPR. Induction of ER stress by GGA was further confirmed using an exogenous indicator for ER stress, ES-TRAP [15]. The ES-TRAP assay is based on the fact that activity of SEAP constitutively produced by transfected cells is rapidly and selectively down-regulated by ER stress. SM/SV-SEAP cells constitutively expressing SEAP were treated with serial concentrations of GGA for 4 h, and culture media were subjected to chemiluminescent assay. To exclude influence of altered cell viability, the activity of SEAP was normalized by the number of viable cells evaluated by formazan assay. Consistent with the result in Fig. 1B, GGA reduced activity of SEAP dose-dependently (Fig. 1C).

Induction of UPR may alter activity of NF-κB and mitogen-activated protein kinases [8], both of which contribute to basal and/or inducible expression of *MCP-1* in mesangial cells [23]. Although, in general, UPR *per se* may activate NF-κB [8], it is unknown how preceding UPR modulates activation of NF-κB triggered by inflammatory cytokines. To examine a possibility that UPR is involved in the suppression of *MCP-1* by GGA, we investigated the relationship between UPR and cytokine responses in GGA-pretreated, cytokine-stimulated cells. As shown in Fig. 1D, close, inverse correlation was observed between the level of *MCP-1* and the level of *GRP78*.

Involvement of UPR in the suppression of cytokine-induced MCP-1 expression by GGA

To further investigate the relationship between induction of UPR and suppression of cytokine response by GGA, we examined whether other known inducers of

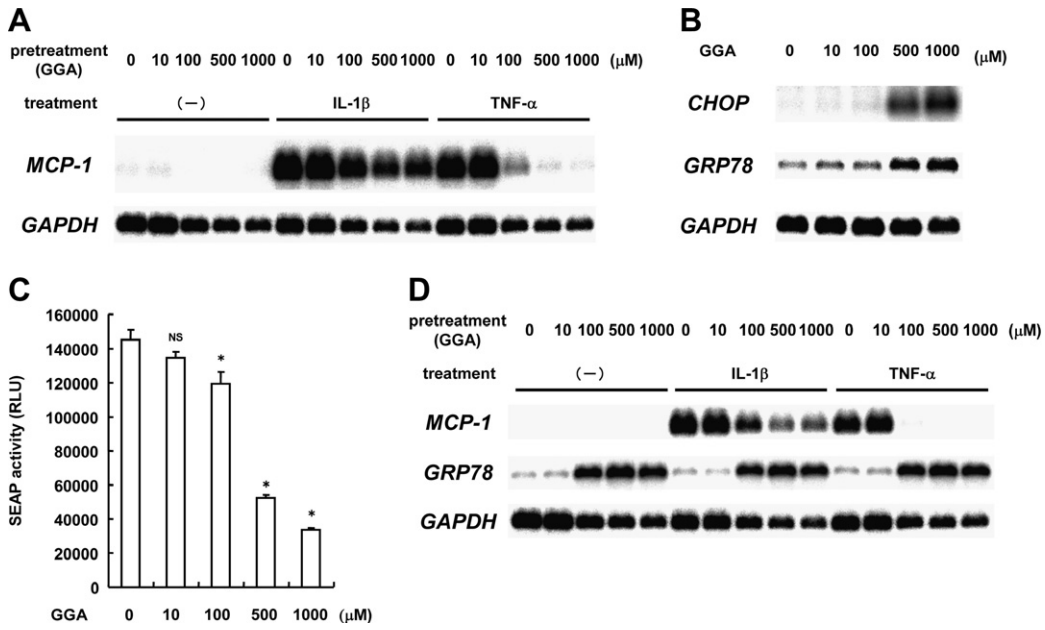

Fig. 1. Induction of unfolded protein response (UPR) and suppression of cytokine-induced monocyte chemoattractant protein 1 (*MCP-1*) expression by geranylgeranylacetone (GGA). (A,D) Mesangial cells were pretreated with indicated concentrations of GGA for 4 h and exposed to IL-1β (1 ng/ml) or tumor necrosis factor-α (TNF-α) (10 ng/ml) for 6 h. Expression of *MCP-1* and 78 kDa glucose-regulated protein (*GRP78*) was examined by Northern blot analysis. Expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was shown at the bottom as a loading control. (B) Cells were treated with serial concentrations of GGA for 4 h, and expression of CCAAT/enhancer-binding protein-homologous protein (*CHOP*) and *GRP78* was examined. (C) Mesangial cells constitutively expressing secreted alkaline phosphatase (*SEAP*) were treated with GGA for 4 h, and culture media and cells were subjected to chemiluminescent assay and formazan assay, respectively. The activity of SEAP was normalized by the number of viable cells evaluated by formazan assay, and the resultant values were shown. Assays were performed in quadruplicate. Data are expressed as means ± SE, and asterisks indicate statistically significant differences ($P < 0.05$). NS, not statistically significant. RLU, relative light unit.

UPR reproduce the effect of GGA. Mesangial cells were pretreated with several inducers of UPR including tunicamycin, thapsigargin, A23187, 2-deoxyglucose, and dithiothreitol (DTT) and stimulated by TNF-α. All five inducers of UPR completely mimicked the suppressive effect of GGA; i.e., these agents caused induction of *GRP78* and abrogated expression *MCP-1* in response to TNF-α (Fig. 2A).

GRP78 is one of the most important ER chaperons [24], and its knockdown leads to selective induction of ER stress and UPR. SubAB is a subtilase cytotoxin that has been identified as a specific inhibitor of GRP78 by rapid cleavage of this protein [13]. To confirm that UPR is causative of the blunted response to TNF-α, mesangial cells were pretreated with SubAB to induce UPR selectively and exposed to TNF-α. Northern blot analysis revealed that SubAB markedly induced expression of *GRP78* mRNA, confirming induction of UPR. Under this experimental setting, induction of *MCP-1* by TNF-α was abolished, as shown in Fig. 2B.

Up-regulated GRP78 promotes protein folding in the ER and attenuates ER stress and consequent UPR [24]. To further confirm that UPR is indeed responsible for the suppression of *MCP-1* by GGA, mesangial cells were stably transfected with a gene encoding GRP78, and SM/GRP78 cells were established. The established cells expressed GRP78 mRNA at a higher level (S-Fig. 1) and exhibited resistance to ER stress-induced apoptosis when compared with mock-transfected SM/Neo cells (S-Fig. 2). SM/Neo and SM/GRP78 cells were pretreated with GGA and exposed to IL-1β or TNF-α. Northern blot analysis showed that, in SM/Neo cells, GGA caused blunted expression of *MCP-1* in response to IL-1β or TNF-α. However, in SM/GRP78 cells, suppression of *MCP-1* by GGA was not observed in IL-1β-stimulated cells and attenuated in TNF-α-treated cells (Fig. 2C). These results provided evidence that UPR was responsible for suppression of *MCP-1* expression by GGA.

Involvement of UPR in the suppression of cytokine-induced NF-κB activation by GGA

Induction of *MCP-1* by proinflammatory cytokines is regulated by NF-κB [23,25]. We examined whether or not the blunted induction of *MCP-1* by GGA is caused by inhibition of NF-κB. For this purpose, SM/NFκB-SEAP reporter cells that express *SEAP* under the control of the κB enhancer elements were used [16]. Northern blot analysis revealed that activation of NF-κB evidenced by induction of *SEAP* mRNA was observed in the cells after the exposure to TNF-α. The activation of NF-κB was attenuated by the treatment with GGA in a dose-dependent manner (Fig. 3A). The suppression was closely correlated with the down-regulation of *MCP-1*. We also examined the effect of GGA on the activation of NF-κB using another reporter system. SM43 cells were tran-

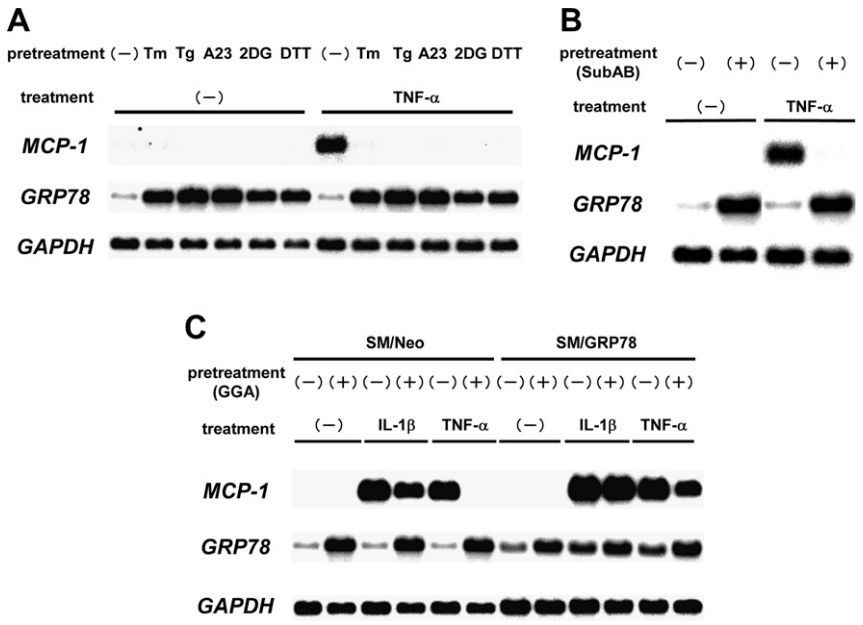

Fig. 2. Involvement of UPR in the suppression of cytokine-induced *MCP-1* expression by GGA. (A) Mesangial cells were pretreated with 10 μg/ml tunicamycin (Tm), 100 nM thapsigargin (Tg), 1 μM A23187 (A23), 6 mg/ml 2-deoxyglucose (2DG) or 1 mM dithiothreitol (DTT) for 4 h, stimulated by TNF-α for 6 h and subjected to Northern blot analysis of *MCP-1* and *GRP78*. (B) Cells were pretreated with (+) or without (−) 0.5 ng/ml AB_5 subtilase cytotoxin (SubAB) for 6 h, stimulated by TNF-α for 6 h and subjected to Northern blot analysis. (C) SM/GRP78 cells overexpressing *GRP78* and mock-transfected SM/Neo cells were pretreated with or without 100 μM GGA, exposed to IL-1β or TNF-α and subjected to Northern blot analysis.

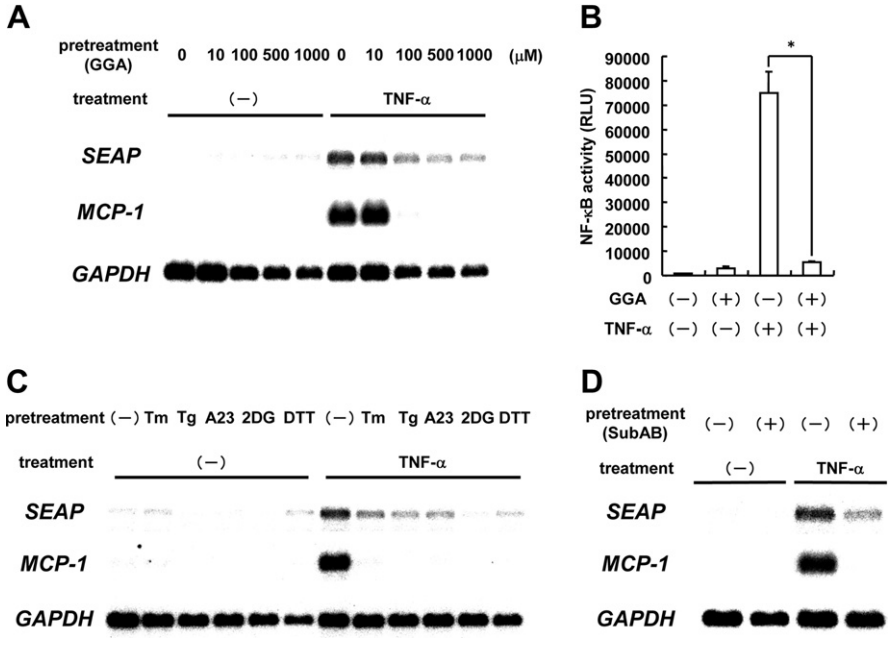

Fig. 3. Involvement of UPR in the suppression of cytokine-induced nuclear factor-κB (NF-κB) activation by GGA. (A,C, and D) SM/NFκB-SEAP cells were pretreated with GGA (A), tunicamycin (Tm), thapsigargin (Tg), A23187 (A23), 2-deoxyglucose (2DG) or dithiothreitol (DTT) (C) or SubAB (D) for 4–6 h, stimulated by TNF-α and subjected to Northern blot analysis. (B) SM43 cells were transiently transfected with pNFκB-Luc, pretreated with 100 μM GGA for 4 h and exposed to TNF-α for 6 h. Cells were then subjected to luciferase assay to evaluate activity of NF-κB.

siently transfected with pNFκB-Luc, pretreated with or without GGA and stimulated by TNF-α. Consistent with the result shown in Fig. 3A, pretreatment with GGA dramatically suppressed activation of NF-κB by TNF-α (Fig. 3B).

To confirm that UPR is causative of the blunted response of NF-κB to TNF-α, we examined whether other known inducers of UPR can reproduce the effect of GGA. SM/NFκB-SEAP cells were pretreated with tunicamycin, thapsigargin, A23187, 2-deoxyglucose or DTT and stimu-

lated by TNF-α. All inducers of UPR reproduced the suppressive effect of GGA; i.e., these agents attenuated activation of NF-κB (expression of *SEAP*), which was correlated with blunted induction of *MCP-1* in response to TNF-α (Fig. 3C). To further confirm our conclusion, the reporter cells were pretreated with the more specific inducer of UPR, SubAB, and exposed to TNF-α. Consistent with the results shown in Fig. 3C, SubAB reduced activation of NF-κB, and it was correlated with blunted induction of *MCP-1* in response to TNF-α (Fig. 3D).

Lack of involvement of the IRE1 and ATF6 pathways in the suppressive effect of GGA

In general, ER stress triggers UPR initiated by IRE1, ATF6 and PERK. We previously reported that, among the three major branches of UPR, the IRE1 pathway and the ATF6 pathway, but not the PERK pathway, were rapidly activated by GGA [7]. To investigate involvement of particular UPR in the anti-inflammatory effects of GGA, reporter assays were performed. Mesangial cells were transiently co-transfected with pNFκB-Luc together with a plasmid encoding IRE1α-DN, XBP1-DN or ATF6-DN. The cells were then treated with GGA, exposed to TNF-α and subjected to luciferase assay. As shown in Fig. 4A, treatment with TNF-α markedly induced activation of NF-κB, and pretreatment with GGA abrogated this effect in mock-transfected cells. Dominant-negative inhibition of either IRE1 or downstream XBP1 did not affect the suppressive effect of GGA (Fig. 4B and C). Similarly, dominant-negative inhibition of ATF6 did not attenuate the effect of GGA (Fig. 4D). These results indicated that GGA suppressed cytokine-induced activation of NF-κB through induction of UPR, whereas neither IRE1 nor ATF6 may be involved in this suppressive effect.

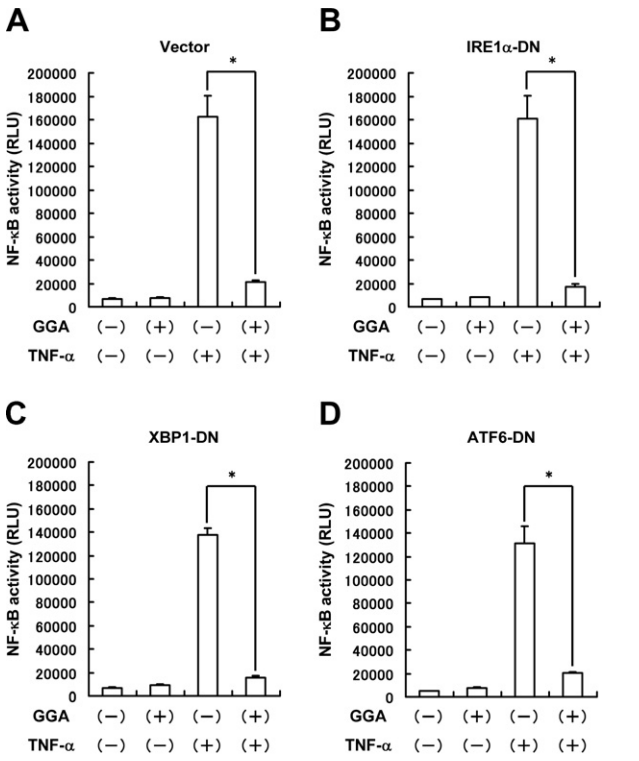

Fig. 4. Lack of involvement of the inositol-requiring ER-to-nucleus signal kinase 1 (IRE1)—X-box binding protein 1 (XBP1) pathway and the activating transcription factor 6 (ATF6) pathway in the suppressive effect of GGA. Mesangial cells were transiently co-transfected with pNFκB-Luc together with empty vector (Vector) (A), pCAG-hIRE1α-K599A (IRE1α-DN) (B), pcDNA3.1-dnXBP1 (XBP1-DN) (C), or pcDNA-ATF6(373)-ΔAD (ATF6-DN) (D) and treated with 100 μM GGA for 4 h. The cells were then exposed to TNF-α for 6 h and subjected to luciferase assay to evaluate activity of NF-κB.

Discussion

GGA has been reported to possess anti-inflammatory potential against experimental colitis and ischemia-induced renal inflammation [5,6]. However, the molecular mechanisms involved have not been fully elucidated. In the present report, we describe a novel, UPR-dependent mechanism underlying the anti-inflammatory effects of GGA. We found GGA blocked activation of NF-κB and consequent induction of *MCP-1* by inflammatory cytokines. It was inversely correlated with the induction of UPR, and various agents that trigger UPR reproduced the suppressive effects of GGA. Furthermore, attenuation of UPR by overexpression of the ER chaperone GRP78 diminished the suppressive effects of GGA.

Several previous reports showed that ER stress caused activation of inflammation-related transcription factors including NF-κB [8]. The activation of NF-κB by ER stress may be caused via IRE1 [26] or eukaryotic translation initiation factor 2α [27]. In contrast to this current concept, we could not detect activation of NF-κB and NF-κB-dependent gene expression by any inducers of UPR in mesangial cells (Figs. 2 and 3). Furthermore, preceding UPR rather caused blunted activation of NF-κB and NF-κB-dependent gene expression in response to cytokines. The reason for the discrepancy between our current results and previous reports is unclear. However, in the majority of previous investigation, effects of the early phase of UPR on the basal activity of NF-κB have been studied. In contrast, our studies examined effects of the late phase of UPR on the activation of NF-κB triggered by other stimuli. Of note, the suppressive effect of UPR on NF-κB was not a phenomenon specific to mesangial cells and was also observed in other cell types including murine podocytes and rat tubular epithelial cells (our unpublished data). UPR might have bidirectional effects on NF-κB depending on distinct cellular contexts.

Currently, molecular mechanisms involved in the suppression of NF-κB by UPR are unknown. As shown in this report, the IRE1 pathway and the ATF6 pathway activated by GGA may not be involved in its suppressive effect. The fact that UPR suppressed activation of NF-κB triggered by TNF-α, rather than by IL-1β, indicated a possibility that UPR interfered mainly with the TNF-α signaling upstream of the common pathway to NF-κB activation by TNF-α and IL-1β, i.e., upstream of IκB kinase (IKK) activation.

Indeed, our preliminary data showed that activation of IKK by TNF-α was significantly attenuated by UPR (our unpublished data).

In the TNF-α signaling, TNF receptor-associated factor 2 (TRAF2) is essential for NF-κB activation. TRAF2 recruits the IKK complex into the TNF receptor 1 signaling complex, leading to activation of IKK [28]. Recently, Hu et al. reported that, in thapsigargin- or tunicamycin-treated MCF-7 cells and L929 cells, the level of TRAF2 protein was significantly decreased. The decrease in TRAF2 protein was not due to transcriptional suppression or increased turnover of mRNA but due to enhanced protein degradation [29]. The suppression of NF-κB activation by GGA, observed in the present report, could be ascribed to depletion of TRAF2 protein.

In the present investigation, we showed that UPR triggered by GGA suppressed cytokine signaling. This result indicated a possibility that, although ER stress could activate NF-κB in the early phase, UPR may suppress cellular responses to subsequent inflammatory stimuli in the later phase. This novel, anti-inflammatory aspect of UPR may contribute to resolution of inflammation by GGA.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.10.115.

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