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Original Article

Inhibition of ERK1/2 by silymarin in mouse mesangial cells

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ABSTRACT The present study aimed to show that pro-inflammatory cytokines [tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β] synergistically induce the production of nitric oxide (NO) production in mouse mesangial cells, which play an important role in inflammatory glomerular injury. We also found that co-treatment with cytokines at low doses (TNF-α; 5 ng/ml, IFN-γ; 5 ng/ml, and IL-1β; 1.25 U/ml) synergistically induced NO production, whereas treatment with each cytokine alone did not increase NO production at doses up to 100 ng/ml or 50 U/ml. Silymarin, a polyphenolic flavonoid isolated from milk thistle (*Silybum marianum*), attenuates cytokine mixture (TNF-α, IFN-γ, and IL-1β)-induced NO production. Western blot and RT-PCR analyses showed that silymarin inhibits inducible nitric oxide synthase (iNOS) expression in a dose-dependent manner. Silymarin also inhibited extracellular signal-regulated protein kinase-1 and -2 (ERK1/2) phosphorylation. Collectively, we have demonstrated that silymarin inhibits NO production in mouse mesangial cells, and may act as a useful anti-inflammatory agent.

INTRODUCTION

Chronic kidney disease is characterized by immunological and non-immunological injury of the glomerulus [1,2]. Previous studies have demonstrated an essential role of mesangial cells, specialized cells around blood vessels in the kidneys, in the immunological injury of the glomerulus [3]. Glomerular mesangial cells are involved in inflammation through their response to inflammatory factors, resulting in secretion of pro-inflammatory molecules, such as numerous low-molecular-weight substances (O_2^-, H_2O_2, NO, platelet-activating factor, and eicosanoids) [4]; proteins (proteinases, matrix components, interleukins (IL)1 and 6, colony-stimulating factors, and growth factors); and cell-surface molecules [5]. Mesangial cells also contribute to chronic inflammatory injury and autoimmune reactions by a direct interaction with immune cells such as monocytes or lymphocytes [3].

Glomerulonephritis, one of the most important causes of renal failure, is accompanied by the production of nitric oxide (NO), which is synthesized by inducible nitric oxide synthase (iNOS) [6,7]. NO has also been proposed as a mediator of acute renal dysfunction in sepsis [7]. Transgenic mice overexpressing GTP cyclohydrolase-1, the rate-limiting enzyme for the production of a cofactor of NOS (tetrahydrobiopterin), demonstrated a significant increase in serum NO and IL6 levels when treated with LPS than the wild-type mice [7]. Transgenic mice showed a significantly decreased renal function parameters including glomerular filtration rate and renal blood flow [7]. Because non-selective NOS inhibition leads to further deterioration of renal function [8], the use of specific iNOS inhibitors may be an alternative to avoid endothelial dysfunction [8,9]. Accumulated experimental evidence suggest that pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-1β, increase NO production and glomerulus destruction by stimulating the

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production of oxygen radicals [4]. Further, IFN-γ, TNF-α, and IL-1β synergistically increase iNOS expression and NO generation [10].

Silymarin, a standardized extract isolated from the fruit and seeds of milk thistle, *Silybum marianum*, is known to protect against hepatotoxicity caused by a variety of agents [11-14]. Protective effects of silymarin against nephrotoxic drugs including chloroform and ferric nitrilotriacetate have been reported [15]. Silymarin also prevented ischemia/reperfusion-induced renal injury and morphology changes in Sprague-Dawley rats, and has exhibited anticancer activities against renal cell carcinoma [16-18]. Possible mechanisms for the anticancer effects of silymarin include inhibition of cell proliferation, enhancement of apoptosis, decrease of angiogenesis, and blockage of cell cycle regulators. Silymarin attenuated diabetic nephropathy in streptozotocin-induced diabetic rats [19] and led to recovery of the endocrine function of damaged pancreatic tissue in alloxan-induced diabetic rats [20]. Silymarin treatment increased catalase and glutathione peroxidase activities and reduced lipid peroxidation in the renal tissue [19] and increased the expression of both Pdx-1 and insulin genes, while increasing β-cell proliferation, in the pancreatic tissue [21]. Although its mechanisms of action are largely unknown, silymarin does exert a direct antioxidant activity by scavenging free radicals and modulating antioxidant and inflammatory enzymes [22,23]. In the present study, we investigated the synergistic effects of cytokines on NO production and the effects of silymarin on the regulation of iNOS and p44/42 in pro-inflammatory cytokine-stimulated mesangial cells.

METHODS

Materials

Mesangial cells purchased from ATCC (Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. For each experiment, cells (5×10^5 cells/ml) were plated in 100-mm dishes. Silymarin was purchased from Sigma (St. Louis, MO) and CalBiochem (San Diego, CA). The anti-iNOS antibody and antibodies against phospho-p44/42, p44/42, phospho-p38, and p38 were purchased from Upstate Biotechnology (Lake Placid, NY) and Cell Signaling Technology, Inc. (Beverly, MA), respectively.

Nitrite determination

Mesangial cells were treated with the indicated concentrations of silymarin in the presence of cytokine mixture (CM 1×: TNF-α, 20 ng/ml; IFN-γ, 20 ng/ml; IL-1β, 5 U/ml) for 24 h. Culture supernatants were collected, and the accumulation of nitrate in culture supernatants was measured as an indicator of NO production in the medium as previously described [24,25].

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). Forward and reverse primer sequences were as follows: iNOS: 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3', 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3', respectively; and β-actin: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3', respectively. Equal amounts of RNA were reverse-transcribed into cDNA with oligo(dT)15 primers. PCR was performed using cDNA and each of the primers. PCR reaction conditions were as follows: 94°C for 5 min, 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and 94°C for 1 min, followed by an additional extension step at 72°C for 5 min. PCR products were separated using 8% SDS-polyacrylamide gels, followed by staining with ethidium bromide. The iNOS and β-actin primers produced amplified products of 311 and 349 bp, respectively.

Western immunoblot analysis

Cell lysates were separated by 10% SDS-polyacrylamide gels and then electro-transferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were incubated for 1 h at room temperature in Tris-buffered saline (TBS) pH 7.6, containing 0.05% Tween-20 and 3% bovine serum albumin, followed by incubation with iNOS, phosphorylated p44/42, and phospho-p38antibodies. Immunoreactive bands were detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagent (Amersham).

Statistical analysis

Data were expressed as the mean±SD for each treatment group in a given experiment. When significant differences were noted, treatment groups were compared to the respective vehicle controls using a Student's two-tailed *t*-test.

RESULTS

Synergistic induction of nitrite production by cytokines in mesangial cells

Cytokines, including IL-1β, IFN-γ, and TNF-α, are known to induce or potentiate iNOS expression and NO production [26,27]. To investigate the effects of cytokines on NO production in mouse mesangial cells, we prepared a cytokine mixture (CM 1×: TNF-α,

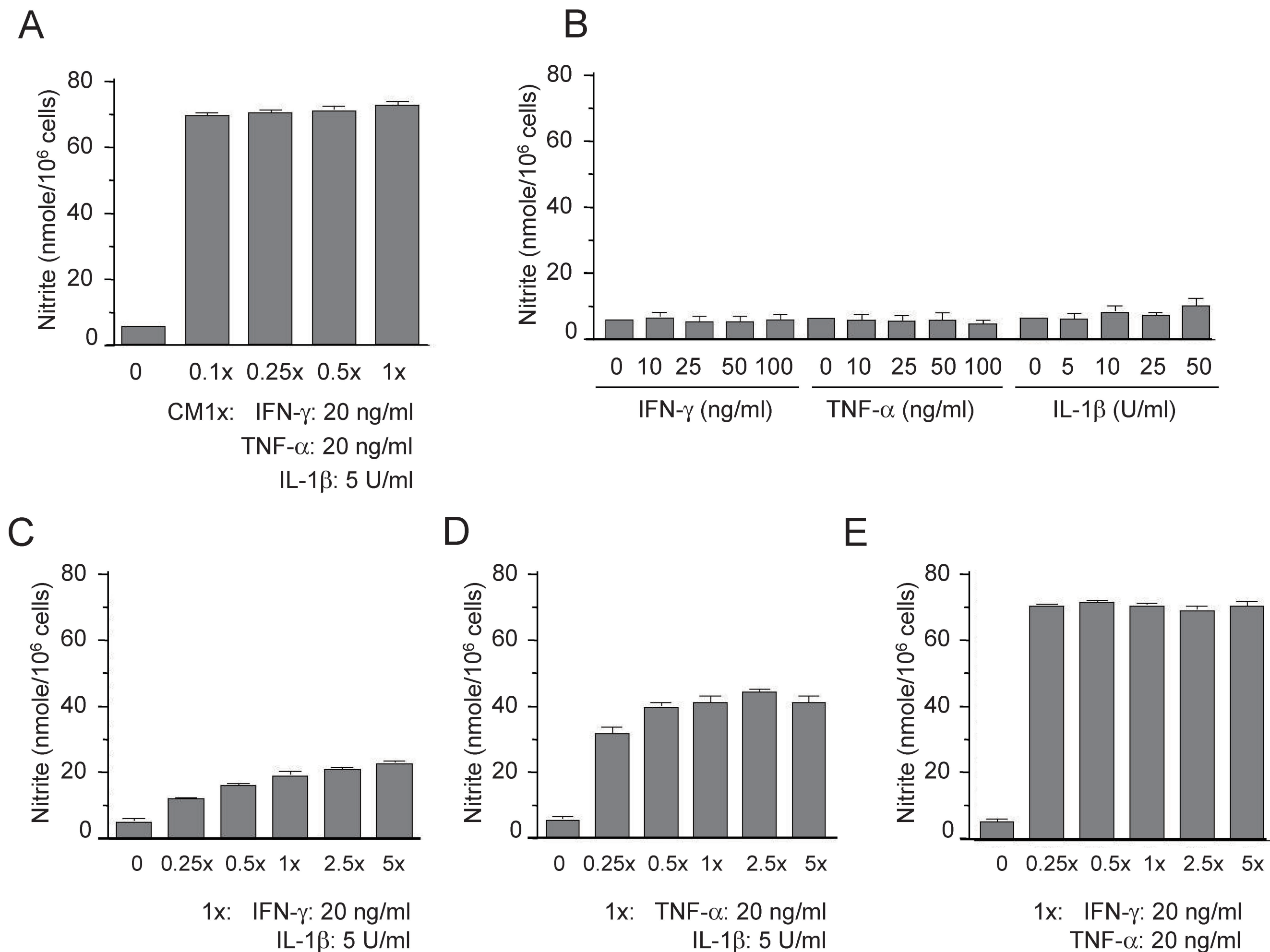

Fig. 1. Synergistic induction of nitrite production by cytokines in mesangial cells. (A) Mesangial cells were treated with the indicated concentrations of cytokine mixture (CM: TNF-α, IFN-γ, and IL-1β) for 24 h. (B) Mesangial cells were treated with each cytokine for 24 h. (C) Cells were co-treated with IFN-γ and IL-1β for 24 h. (D) Cells were co-treated with TNF-α and IL-1β for 24 h. (E) Cells were co-treated with TNF-α and IFN-γ for 24 h. Supernatants were subsequently isolated and analyzed for nitrite. Each column shows the mean±SD of triplicate measurements. *p<0.05 compared with the control group, as determined by Student's two-tailed *t*-test.

20 ng/ml; IFN-γ, 20 ng/ml; IL-1β, 5 U/ml), and treated mesangial cells with various concentrations of CM (0.1×, 0.25×, 0.5×, and 1×) for 24 h. Supernatants were then prepared and analyzed for NO production by measuring nitrite, a stable end-product of NO. Treatment with CM increased the production of nitrite ≥ 10-fold over basal levels in mesangial cells (Fig. 1A). However, treatment with each cytokine alone could not increase NO production, even when applied at a dose 5–10 fold higher than that present in 1× CM (Fig. 1B). When we co-treated mesangial cells with two cytokines together, we noted a synergistic induction of NO production (Fig. 1C, 1D, and 1E). Co-treatment with TNF-α and IFN-γ showed a strong synergistic effect on the induction of NO production (Fig. 1E), and the level of NO induced was similar to that induced after three-cytokine co-treatment. These results demonstrate that TNF-α, IFN-γ, and IL-1β synergistically interact to stimulate NO production in mouse mesangial cells, and that the NO induction stimulated by TNF-α and IFN-γ co-treatment is as strong as the induction by treatment with CM containing the three cytokines.

Inhibition of iNOS expression by silymarin in cytokine-stimulated mesangial cells

We investigated the effects of silymarin on NO production and iNOS expression in cytokine-stimulated mouse mesangial cells. Mesangial cells were treated with silymarin in the presence of CM for 24 h, and nitrite generation was analyzed. CM-induced nitrite generation was inhibited by silymarin in a dose-dependent manner (Fig. 2A). To test the cytotoxic effect of silymarin, we performed an MTT assay for evaluating tetrazolium dye reduction activity by mitochondrial succinate dehydrogenase. The viability of all the silymarin-treated cells exceeded 90%, except for those treated with the highest dose of 100 μg/ml, which showed slight cytotoxicity (Fig. 2B).

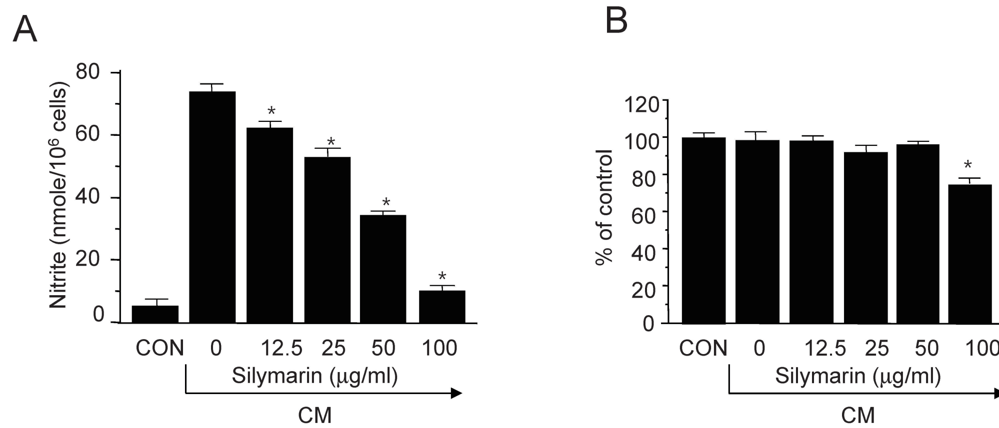

Fig. 2. Inhibition of nitrite production by silymarin in CM-stimulated mesangial cells. Mesangial cells were treated with the indicated concentrations of silymarin in the presence of cytokine-mixture (CM) for 24 h. (A) Supernatants were subsequently isolated and analyzed for nitrite. (B) Cells were analyzed for viability by MTT assay. Each column shows the mean±SD of triplicate measurements. *p<0.05 compared with the control group, as determined by Student's two-tailed *t*-test.

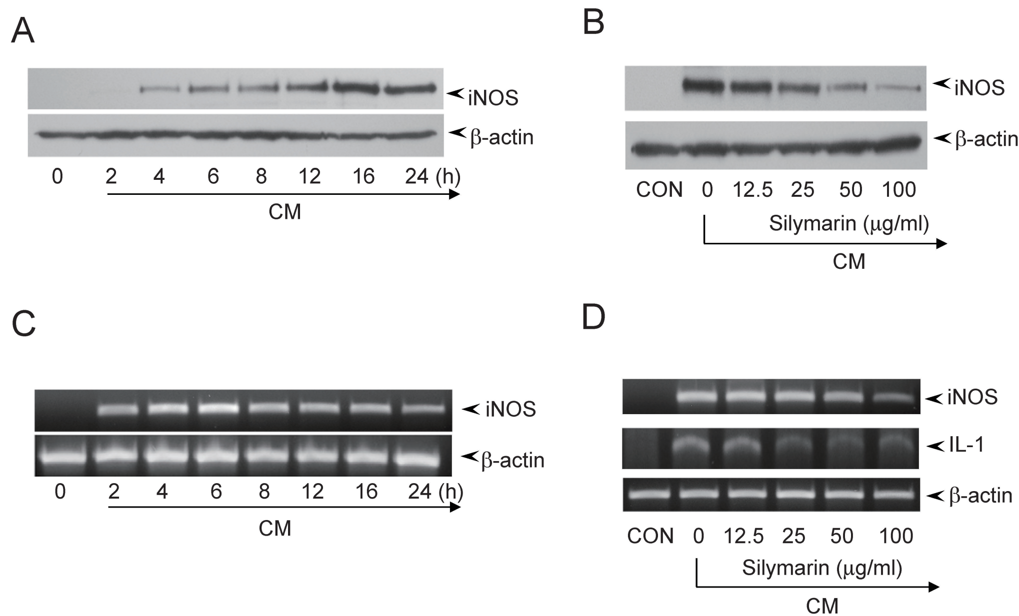

Fig. 3. Inhibition of iNOS gene expression by silymarin in CM-stimulated mesangial cells. (A and B) Mesangial cells were treated with CM for the indicated duration. (A) Cell lysates were prepared and the expression of iNOS were analyzed by Western blot using an antibody specific for murine iNOS. (B) Total RNA was isolated and mRNA expression levels of iNOS and β-actin were analyzed by RT-PCR. (C and D) Mesangial cells were treated with the indicated concentrations of silymarin in the presence of CM for 16 h (C) or 6 h (D). Cell lysates (C) or total RNA (D) were prepared and analyzed by Western blot or RT-PCR, respectively.

We further analyzed the effects of silymarin on iNOS expression by Western blot and RT-PCR analyses. Western blot analysis showed that the expression of iNOS protein was detected 4 h after CM treatment, peaked at 16 h, and was maintained until 24 h (Fig. 3A). RT-PCR analysis showed that iNOS mRNA expression was detected 2 h after CM treatment, peaked at 6 h, and was maintained at a slightly lower level until 24 h (Fig. 3B). To analyze the effect of silymarin on iNOS expression, we treated mouse mesangial cells with silymarin and CM for 16 h and 6 h for Western blot and RT-PCR analyses, respectively. Silymarin inhibited the CM-induced iNOS protein expression in a dose-dependent manner (Fig. 3C). The β-actin loading control was constitutively expressed and was not affected by silymarin treatment. Silymarin inhibited iNOS mRNA expression, although the sensitivity of iNOS mRNA to inhibition was relatively lower than that of iNOS protein (Fig. 3D). These results showed that silymarin decreased the expression of iNOS, which is involved in chronic kidney disease.

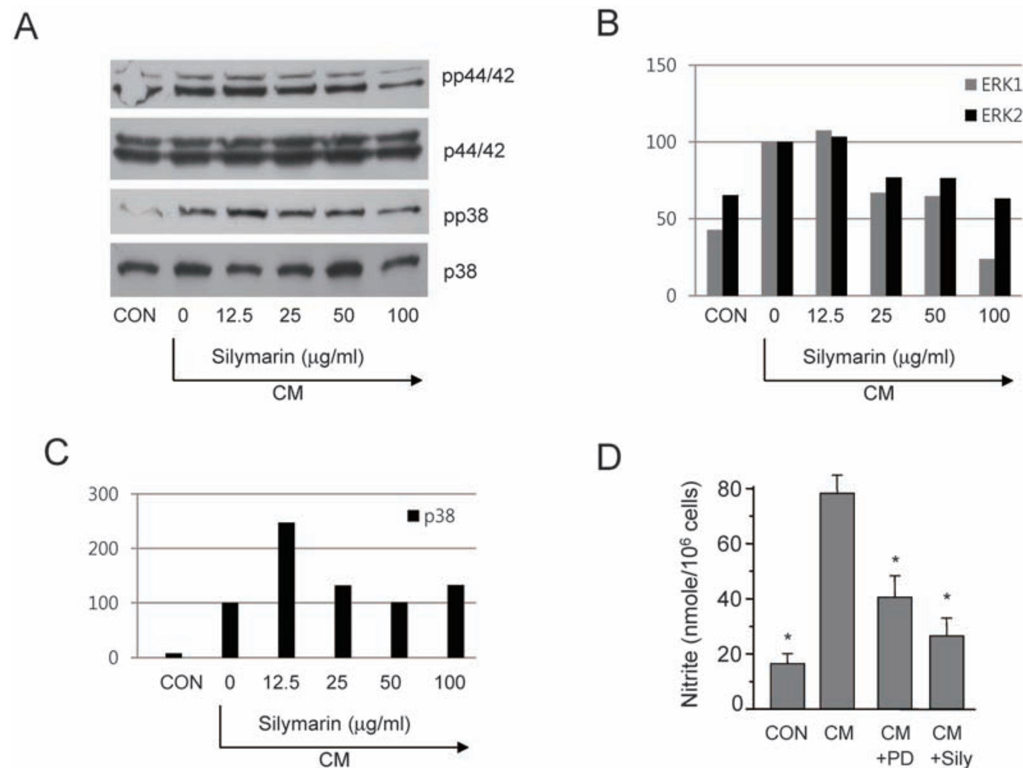

Fig. 4. Inhibition of p44/42 phosphorylation by silymarin in CM-stimulated mesangial cells. (A) Mesangial cells were pretreated with the indicated concentrations of silymarin for 1 h and then incubated in the presence of CM for 20 min. The phosphorylation of p44/42 and p38 was analyzed by Western blot. The relative band densities of phosphorylated p44/42 (B) and p38 (C) were analyzed using Image J software. (D) Cells were treated with PD98059 (50 μM) or silymarin (50 μg/ml) for 48 h in the presence of CM. The supernatants were subsequently isolated and analyzed for nitrite. Each column shows the mean±SD of triplicate measurements. *p<0.05 compared with the control group, as determined by Student's two-tailed *t*-test.

Inhibition of p44/42 (ERK1/2) phosphorylation by silymarin in CM-stimulated mesangial cells

Because p44/42 kinase is important for NO generation in CM-stimulated mesangial cells and is a possible target of silymarin, we further determined the role of p44/42 in NO inhibition by silymarin. Mesangial cells were pretreated with silymarin for 1 h and then incubated for 20 min in the presence of CM. MAPK phosphorylation was analyzed by Western blot. The phosphorylation of p44/42 was strongly increased by CM treatment, while silymarin pretreatment decreased the phosphorylation of p44/42 in a dose-dependent manner (Fig. 4A). We also analyzed the effect of silymarin on the phosphorylation of p38, another important MAPK in the production of NO. CM significantly induced the phosphorylation of p38, and silymarin inhibited this phosphorylation (Fig. 4A). We analyzed relative band densities of phosphorylated p44/42 (Fig. 4B) and p38 (Fig. 4C) using Image J software. These results demonstrate that silymarin inhibits p44/42 and p38, which are important factors in signal transduction pathways that regulates iNOS expression in CM-stimulated mouse mesangial cells. We tested the role of ERK1/2 in iNOS expression using PD98059, a specific inhibitor of mitogen activated protein kinase/extracellular signal-regulated kinase 1 (MEK-1), which is responsible for ERK1/2 activation. PD98059 inhibited CM-induced production of nitrite (Fig. 4D).

DISCUSSION

In the present study, we reported that silymarin, isolated from milk thistle (*Silybum marianum*), is a potent anti-inflammatory agent in mouse mesangial cells. Silymarin inhibited cytokine-induced production of NO, an important mediator of inflammatory responses. The inhibition of NO generation is related to the attenuation of iNOS gene expression. The protective role of silymarin against cytokines, including TNF-α and IL-1β, in human mesangial cells is demonstrated [28]. Silymarin inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and monocyte chemoattractant protein-1 (MCP-1) expression through the inhibition of increase in intracellular calcium levels [28]. Inhibition of iNOS gene expression by silymarin has been reported in various cells including macrophages, pancreatic beta cells, fibroblasts, and mesenchymal stem cells. Because NO production by mesangial cells, macrophages, and pancreatic beta cells plays critical roles in the pathogenesis of diabetes and diabetic nephropathy, the

protective role of silymarin against these conditions is likely due, at least in part, to an anti-inflammatory effect. In addition, silymarin protects pancreatic beta cells from pro-inflammatory-cytokine-induced NO production and cell death [29,30]. Pro-inflammatory cytokines are known to induce the expression of iNOS mRNA and production of NO, resulting in death of beta cells [26,27].

A relationship between diabetic nephropathy and NO production has been suggested to exist [31]. During the development of diabetic nephritis, immune cells, including macrophages, invade and initiate local inflammatory responses. Cytokines produced by infiltrated cells induce the expression of iNOS gene and the production of NO [32,33], which then enhances glomerular mesangial cell relaxation and hyperfiltration [34]. Hyperfiltration exacerbates glomerular damage and causes fibrotic changes that lead to renal dysfunction.

In the present study, we showed that IFN-γ and TNF-α synergistically induce mouse mesangial cells to express iNOS. Co-treatment with low doses of IFN-γ (5 ng/ml) and TNF-α (5 ng/ml) synergistically induced NO production, whereas treatment with each cytokine alone did not increase production of NO at doses up to 100 ng/ml (Fig. 1B). Because similar induction of NO production was observed following co-treatment of mesangial cells with IFN-γ and TNF-α as with the three-cytokine mixture (IFN-γ, TNF-α, and IL-1β), the contribution of IL-1β towards NO induction was concluded to be minimal. However, co-treatment with IL-1β together with either IFN-γ or TNF-α clearly showed the synergistic induction of NO (Fig. 1C, 1D). Synergistic induction of NO by IFN-γ and TNF-α is further supported by the results of a previous study [10]. Although the mechanism for the synergistic induction of NO by cytokines is not known, signal transduction pathways involving their membrane receptors might play a role in regulating NO production. Cytokine binding to their receptors initiate signal transduction events leading to the activation of transcription factors such as NF-κB, signal transducers and activators of transcription family of transcription factors (STAT)1, and interferon regulatory factor (IRF)-1. Activated transcription factors cooperate to produce the maximal induction of iNOS gene expression (Fig. 5).

TNF-α binds to TNF receptors (TNFR1 and TNFR2) that are preferentially expressed in the glomeruli in the normal kidney [35]. The role of TNF-α-TNFR1 signal transduction was shown using TNFR-deficient mice [36]. Chemokine secretion was absent and glomerular leukocyte infiltration was abrogated in TNF-stimulated TNFR1-deficient glomeruli. Signal transduction through TNFR1 activates NF-κB, an important transcription factor of inflammation-regulating genes [37]. Following its activation, NF-κB binds to the promoters of many inflammatory genes, including iNOS [38]. The involvement of IFN-γ in glomerulonephritis was shown by animal studies using IFN-γ-receptor-deficient mice and soluble IFN-γ receptor treatment [39,40]. Signal transduction through the IFN-γ receptor induces

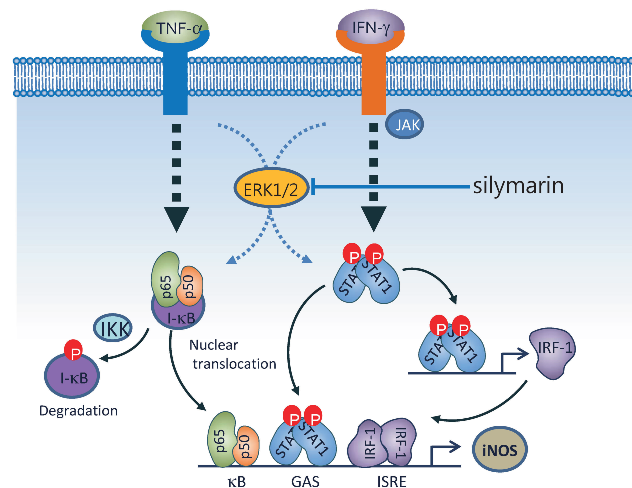

Fig. 5. Diagram showing cytokine-induced signal transduction pathways and possible targets of silymarin. TNF-α and IFN-γ binding to their receptors activate signal transduction pathways, including MAPKs, STAT1, NF-κB, and IRF-1. The iNOS gene promoter contains binding sites for NF-κB, STAT1, and IRF-1. Some possible targets of silymarin including ERK1/2 are shown.

STAT1 and IRF-1, which then bind to the gamma-interferon activated site (GAS) motif and IFN-stimulated response elements (ISRE) of the iNOS promoter, respectively [10]. Use of a reporter gene assay demonstrated that the binding sites of IRF-1, STAT1, and NF-κB were essential for the synergistic response. Ectopic expression experiments further showed that these transcription factors synergistically induce NO accumulation [10].

We demonstrated that silymarin inhibits the p44/42 pathway in cytokine-stimulated mesangial cells. Silibinin, a major component of silymarin, has been shown to inhibit TPA- or TNF-α-induced MMP-9 expression through inhibition of the MAPK pathway [41-43]. ERK activity is required for iNOS gene expression in insulin-producing INS-1E cells and beta cells [31,44].

In summary, these experiments demonstrated that silymarin inhibits CM-induced iNOS gene expression in mouse mesangial cells. Based on our findings, the most likely mechanism that can account for this biological effect involves inhibition of the ERK1/2 kinase pathway. Owing to the critical roles that NO and ERK1/2 play in mediating inflammatory responses in glomerular mesangial cells, inhibition of these activities by silymarin is potentially a useful strategy for protecting against chronic kidney disease.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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