Original Contribution

Functional dissection of Nrf2-dependent phase II genes in vascular inflammation and endotoxic injury using Keap1 siRNA

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ABSTRACT

Keap1 is a cytoplasmic repressor of the transcription factor Nrf2, and its degradation induces Nrf2 activation, leading to upregulation of antioxidant phase II genes. We investigated the roles of phase II genes in vascular inflammation and septic injury using Keap1 siRNA and elucidated its underlying mechanism. Selective knockdown of Keap1 with siRNA promoted Nrf2-dependent expression of phase II genes in endothelial cells, such as heme oxygenase-1 (HO-1), glutamate-cysteine ligase (GCL), and peroxiredoxin-1 (Prx1), resulting in the elevation of cellular glutathione levels and suppression of tumor necrosis factor (TNF-α)-induced intracellular H2O2 accumulation. Keap1 knockdown inhibited TNF-α-induced expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by suppressing NF-κB activation via inhibition of its upstream modulators, Akt, NIK, and IKK, resulting in the elevation of monocyte adhesion to endothelial cells. Importantly, these events were reversed by HO-1 and GCL inhibitors and Prx1-specific siRNA. Keap1 knockdown also inhibited endotoxin-induced expression of inducible nitric oxide synthase (iNOS) and TNF-α by upregulating HO-1, GCL, and Prx1 expression in macrophages. Moreover, in vivo Keap1 knockdown increased the expression of phase II genes and suppressed the expression of ICAM-1, VCAM-1, iNOS, and TNF-α in an endotoxemic mouse model, resulting in significant protection against liver and lung injuries and lethality. Our results indicate that Keap1 knockdown prevents NF-κB-mediated vascular inflammation and endotoxic shock by suppressing NF-κB-mediated inflammatory gene expression via upregulation of Nrf2-mediated antioxidant genes. Thus, siRNA targeting Keap1 may provide a new therapeutic approach for inflammation-associated vascular diseases and sepsis.

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Introduction

Inflammation is closely associated with the pathogenesis of many human diseases, including atherosclerosis, cardiovascular diseases, and sepsis. Endothelial cells are important participants in vascular inflammation [1]. Inflammatory stimuli, including tumor necrosis factor-α (TNF-α), increase the expression of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on the surface of endothelial cells, which bind circulating immune cells resulting in leukocyte recruitment and infiltration, cytokine production, and induction of tissue inflammation [2–4]. The expression of adhesion molecules and other inflammation-associated genes, such as TNF-α and inducible nitric oxide synthase
(iNOS), is tightly regulated by the activation of nuclear factor-kB (NF-kB). Therefore, selective inhibition of NF-kB activation/activity prevents hyperinflammation and is used as a therapeutic treatment for various human inflammatory diseases [5,6].

Keap1 (Kelch-like ECH-associated protein 1) is a cytoplasmic repressor protein essential for complex formation with the transcription factor Nrf2, leading to inhibition of its nuclear translocation [7]. Keap1 contains redox-sensitive cysteine residues at positions 273 and 288 and is associated with ubiquitin E3 ligase (Cul3), which is responsible for ubiquitination of Nrf2. Under normal conditions, Keap1 promotes Nrf2 degradation via the Cul3-dependent ubiquitin–proteasome pathway [7]. However, upon exposure to oxidative or electrophilic stress, the reactive cysteine residues of Keap1 become modified, leading to a decline in E3 ligase activity, stabilization of Nrf2, and its subsequent translocation into nucleus. This translocation leads to heterodimer formation with small MAF proteins that bind to the antioxidant/electrophile response element (ARE/EpRE) sequence in the promoters of phase II genes, which stimulates their expression.

Among the phase II enzymes, heme oxygenase-1 (HO-1), glutamate–cysteine ligase catalytic subunit (GCLC), glutamate–cysteine ligase modifier subunit (GCLM), peroxiredoxin-1 (Prx1), NAD(P)H:quinone oxidoreductase 1 (NQO1), and glutathione S-transferase Mu 1 (GSTM1) play important roles in maintaining NAD(P)H:quinone oxidoreductase 1 (NQO1), and glutathione S-transferase Mu 1 (GSTM1) play important roles in maintaining redox homeostasis and regulating inflammatory response [8]. Indeed, some phase II gene products, including HO-1, GCL, and Prx1, have been demonstrated to suppress intracellular accumulation of reactive oxygen species (ROS), which play an important role in the redox-sensitive NF-kB activation pathway [9–12]. Thus, these enzymes/proteins are critically involved in the regulation of NF-kB-dependent inflammatory gene expression. These observations indicate that phase II enzymes contribute to the regulation of NF-kB-mediated inflammatory responses. Many cytoprotective and anti-inflammatory compounds have been shown to suppress cytotoxic gene expression mostly by upregulation of HO-1 expression; however, the protective role of additional Nrf2-dependent genes has not been extensively studied. We hypothesize that Keap1 downregulation promotes Nrf2-induced gene expression and inhibits vascular inflammation and leukocyte infiltration into inflamed sites by suppressing the expression of inflammatory cytokine and adhesion molecule expression, resulting in the prevention of human inflammatory diseases.

In this study, we investigated the effects of short interfering RNA (siRNA)-mediated Keap1 knockdown on inflammation and cytoprotection in both cell culture and an animal model to elucidate its underlying mechanism in these processes. Our results showed that Keap1 knockdown inhibited NF-kB activation and inflammatory gene expression in macrophages and endothelial cells by increasing Nrf2-dependent expression of ARE/EpRE-regulatory phase II genes, particularly HO-1, GCL, and Prx1, resulting in protection of mice from LPS-induced organ injury and lethality.

Materials and methods

Chemicals and reagents

Medium 199 (M199), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). RPMI 1640, Dulbecco’s modified Eagle medium (DMEM), and TRizol reagent kit were purchased from Invitrogen (Grand Island, NY, USA). Recombinant human TNF-α and ELISA kit for TNF-α were obtained from R&D Systems (Minneapolis, MN, USA). Basic fibroblast growth factor was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Luciferase assay system, poly(dI–dC), and NF-κB-specific oligonucleotide were purchased from Promega (Madison, WI, USA). Antibodies for iNOS, p-IκKα/β, HO-1, and PARP were purchased from BD Transduction Laboratories (San Diego, CA, USA), Cell Signaling Technology (Beverly, MA, USA), Stressgen (Madison, WI, USA), and Calbiochem (San Diego, CA, USA), respectively. Other antibodies used were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2’, 7’-Dichlorofluorescin diacetate (DCFH2–DA) was purchased from Molecular Probes (Eugene, OR, USA). The siRNAs for Keap1, Prx1, and GSTM1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) isolated from human umbilical cord veins were cultured as previously described [13] and used for experiments in passages 3 to 6. The immortalized murine macrophage RAW264.7 cells were grown in DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 mM Hapes at 37 °C in humidified 5% CO2/95% air. U937 cells were grown in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a CO2 incubator. HUVECs and RAW264.7 cells were transfected respectively with 100 nM human and mouse siRNA for Keap1, Prx1, or GSTM1 using a microinjection (Digital Bio Technology, Suwon, Korea). Cells were pretreated with 20 µM enzyme inhibitors (tin-protoporphyrin IX (SnPP), buthionine sulfoximine (BSO), and dicumarol) for 1 h before stimulation with TNF-α (10 ng/ml) or LPS (1 µg/ml).

Measurement of NO, TNF-α, ROS, and GSH

The levels of nitrite and total nitrite plus nitrate (NO3−), stable oxidized products of NO, were measured in culture media and sera using Griess reagents and a reductase-based colorimetric assay kit (Alexis, San Diego, CA, USA), respectively. The serum level of TNF-α was determined in culture media and sera using an ELISA kit (R&D Systems). Intracellular ROS accumulation was determined using the H2O2–sensitive fluorescent dye DCFH2–DA [14]. HUVECs transfected with Keap1 siRNA for 24 h were pretreated with 20 µM SnPP, BSO, or dicumarol for 1 h, followed by stimulation with 10 ng/ml TNF-α for 1 h. Cells were further incubated with DCFH2–DA (10 µM) for 30 min. Cells were washed twice with phosphate-buffered saline (PBS), and intracellular levels of ROS were analyzed by confocal microscopy. After HUVECs were treated with TNF-α for 1 h, levels of GSH and total GSH (GSH+GSSG) were determined in cell lysates using a glutathione detection kit from Abcam (Cambridge, MA, USA).

Western blot analysis

Whole-cell lysates as well as cytosolic and nuclear fractions were prepared as previously described [14]. Liver and lung tissues were homogenized in ice-cold protein-extraction buffer containing 100 mM Hapes (pH 7.9), 10% glycerol, 5% Triton X-100, 250 mM NaF, 5 mM Na2VO4, and a protease inhibitor cocktail (10 µg aprotinin, 10 µg leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 15,000g at 4 °C for 10 min, and supernatants were collected for detecting specific proteins. Samples (50 µg protein) were electrophoretically separated on SDS–PAGE and transferred onto nitrocellulose membranes. Western blot was performed as previously described [14].
Reverse transcription–polymerase chain reaction (RT-PCR)

Isolated RNA and complementary DNA were prepared as described previously [14]. PCR was performed in 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 μM each target primer for human and mouse (Table 1). Amplification conditions were as follows: denaturation at 94°C for 5 min for the first cycle and for 45 s starting from the second cycle; annealing of human HO-1 at 60°C for 30 s; annealing of human Keap1 and GAPDH and mouse HO-1, Keap1, and actin at 56°C for 30 s; annealing of NQO1, GCLC, GCLM, GSTM1, GSTA4, ICAM-1, and VCAM-1 at 55°C for 30 s; annealing of mouse iNOS at 65°C for 1 min; and annealing of mouse TNF-α at 51°C for 45 s; and extension at 72°C for 45 s for 30 cycles. Final extension was performed at 72°C for 10 min, and the PCR products were determined by agarose gel electrophoresis.

**Analyses of Nrf2 and NF-κB activation pathways**

Activation pathways of the transcription factors Nrf2 and NF-κB were analyzed according to our previous report [14]. Electrophoretic gel mobility-shift assay (EMSA) was performed for the measurement of binding activities of Nrf2 and NF-κB to 32P labeled consensus oligonucleotides in the nuclear extracts from HUVECs, which were cotransfected with 100 nM Keap1 siRNA for 24 h or further treated with 10 ng/ml TNF-α in the presence of 20 μM enzyme inhibitors. Specific binding of transcription factors was analyzed in the reaction mixtures, which were preincubated with an excess (×100) of cold probe or antibody for each transcription factor at room temperature for 30 min. Luciferase reporter gene assay was measured in lysates from cells cotransfected with 1 μg/ml pGL3-ARE/EpRE-Luc (or pGL3-HO-1-Luc) and 100 nM Keap1 siRNA for 36 h, as well as in lysates from cells treated with TNF-α for 12 h in the presence or absence of 20 μM enzyme inhibitors after cotransfection with 1 μg/ml pGL3-NF-κB-Luc and 100 nM Keap1 siRNA for 24 h. Nuclear translocation of Nrf2 and NF-κB was examined using an immunocytochemical method. Briefly, after transfection with Keap1 siRNA for 24 h, cells were pretreated with 20 μM enzyme inhibitors, followed by simulation with TNF-α for 1 h. Cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% saponin. Cells were incubated with rabbit polyclonal antibodies (1:100) for Nrf2 and NF-κB for 2 h at 4°C and then incubated with anti-rabbit IgG–TRITC (1:200; Sigma). For nuclear staining, cells were further incubated for 30 min with DAPI (1 μg/ml; Sigma). After mounting, nuclear translocation of transcription factors was observed by confocal microscopy.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was performed as previously described [14]. To analyze the binding activity of Nrf2 to the HO-1 promoter, HUVECs were transfected with Keap1 siRNA for 24 h. For assaying binding activity of NF-κB to promoters of ICAM-1 and VCAM-1, HUVECs were transfected with Keap1 siRNA for 24 h and pretreated with 20 μM enzyme inhibitors for 1 h, followed by stimulation with TNF-α for 1 h. Both cell lysates were prepared for ChIP assay and immunoprecipitated with an antibody against Nrf2 or NF-κB p65. Immunoprecipitated DNA samples were purified by phenol extraction. Targeted promoter sequences of HO-1, ICAM-1, and VCAM-1 were identified on a 2% agarose gel. The primer sequences were as follows: forward 5'-AGAAATGCGCTGCTAAGCT-3' and reverse 5'-GGGATTAAACCCTGGAGCAGC-3' for ICAM-1; forward 5'-GCCAACAGAGAGAGGAG-3' and reverse 5'-CCACCAATTCCACGGGAG-3' for VCAM-1; and forward 5'-GGGATCAAGGTGAGAGGACAG-3' and reverse 5'-TTTTCCTGCTGAGTACCCAGG-3' for HO-1.

**HO activity assay**

HO activity in endothelial cells was measured by bilirubin generation [15]. Cells were transfected with Keap1 siRNA using a microporator and recovered in fresh medium for 24 h, followed by further incubation with 20 μM SnPP. The cells were then washed with PBS and harvested. Cell pellets were suspended in MgCl2 (2 mM)–phosphate (100 mM) buffer (pH 7.4), lysed by three cycles of freezing and thawing, and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was added to a reaction mixture containing NADPH (0.8 mM), mouse liver cytosol (2 mg) as a source of biliverdin reductase, the substrate hemin (10 μM), glucose 6-phosphate (2 mM), and glucose-6-phosphate dehydrogenase (0.2 units) in a final volume of 400 μl. The reaction was performed in the dark for 1 h at 37°C and the bilirubin formed was extracted with chloroform (400 μl) and calculated by the difference in absorbance between 464 and 530 nm using the

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extinction coefficient of 40 mM\(^{-1}\) cm\(^{-1}\) for bilirubin. HO activity was expressed as pmol of bilirubin formed/mg of protein/h.

**Monocyte adhesion assay**

HUVECs transfected with or without Keap1 siRNA were plated on 0.2% gelatin-coated 24-well plates at a density of 1 \times 10^5 cells/well. After 36 h, cells were treated with TNF-\(\alpha\) for 8 h after treatment with chemical inhibitors for 1 h. Cells were washed three times with PBS, replenished with M199 containing 5% FBS, and incubated with human U937 cells (5 \times 10^5 cells/well) for 30 min. After three washes with PBS, the attached cells were fixed, stained with Diff-Quick solution (Baxter Healthcare, IL, USA), and then counted in five randomly selected microscopic fields for each well [16].

**Flow cytometric analysis**

HUVECs transfected with or without Keap1 siRNA for 24 h were pretreated with 20 \mu M enzyme inhibitors for 1 h, followed by stimulation with TNF-\(\alpha\) for 12 h. After being washed twice with PBS, HUVECs were detached gently by treating with PBS containing 10 mM EDTA. After wash with PBS, the cells were blocked with 3% bovine serum albumin for 1 h and then washed two times with PBS. Subsequently, the cells were incubated with rabbit polyclonal antibody (1:50) for ICAM-1 or VCAM-1 for 1 h at 4 \(^\circ\)C. The cells were washed and incubated with anti-rabbit IgG–TRITC (1:100) for 30 min in ice-cold PBS containing 2% bovine serum albumin, fixed with 2% paraformaldehyde, and analyzed by flow cytometry in a fluorescence-activated cell sorter.

**Animal treatment**

Male BALB/c mice (6 to 8 weeks of age) were obtained from Orient (Sungnam, Korea) and maintained at the specific-pathogen-free housing facility. All procedures performed on these animals were in accordance with the guidelines of the University Animal Care and Use Committee. For lethality assay, mice were injected i.p. with LPS (35 mg/kg) at 24 h after intravenous injection with a mixture (400 \mu l) of Keap1 siRNA (0.5 mg/kg) and in vivo-jetPEI reagent (Polyplus-Transfection, NY, USA). Lethality was monitored every 6 h. In addition, whole blood and organs were obtained from mice treated with LPS for 12 h by cardiac puncture and surgical operation. The organs and serum prepared from whole blood by centrifugation at 3000 \(g\) at 4 \(^\circ\)C were quickly frozen and kept at -70 \(^\circ\)C until use.

**Histological examination**

Mouse liver and lung tissues were fixed in 10% formaldehyde and embedded in paraffin. Five-micrometer-thick tissue sections were placed on polylysine-coated slides and stained with hematoxylin and eosin. The slides were examined under an optical microscope.

**Statistical analysis**

The data are presented as means ± standard deviation (SD) of at least three separate experiments. Comparisons between two groups were analyzed using the Student \(t\)-test, and significance was established at \(P < 0.05\).

**Fig. 1.** Transfection of HUVECs with Keap1 siRNA increases ARE/EpRE promoter activity by stabilizing Nrf2 and its nuclear translocation. HUVECs were transfected with Keap1 siRNA using a microporator and allowed to recover in fresh medium for 30 h. (A) Whole-cell lysates and (B) cytosolic and nuclear fractions were electrophoretically separated on SDS–PAGE. Target proteins were determined by Western blot analysis. (C) Transfected cells recovered in fresh medium for 24 h; nuclear translocation of Nrf2 was determined by immunohistochemistry using an antibody for Nrf2 and DAPI. (D) Nuclear extracts were analyzed for Nrf2–DNA binding activity by EMSA in the presence or absence of cold probe. (E) Soluble chromatin from cells was immunoprecipitated with anti-Nrf2 antibody. The region of the ARE/EpRE sequence of the human HO-1 promoter was amplified by PCR. (F) Luciferase gene reporter activity was analyzed in lysates from cells cotransfected with 100 nM Keap1 siRNA and 1 \mu g/ml pARE/EpRE-Luc for 48 h. The data shown are the means ± SD (n = 3). **\(P < 0.01\) versus control.
Results

Specific knockdown of Keap1 increases Nrf2 activation

We first conducted experiments to determine the effects of the Keap1 siRNA on Nrf2 stabilization and its translocation into the nucleus, where it activates the transcriptional expression of phase II enzymes. Transfection of HUVECs with Keap1 siRNA inhibited Keap1 protein expression and elevated the level of Nrf2 protein in a dose-dependent manner (Fig. 1A). Western blot and immunohistochemical analyses revealed that Keap1 knockdown decreased the cytosolic level of Nrf2 compared with control, resulting in an increase in its nuclear level (Fig. 1B and C). EMSA revealed a significant increase in DNA-binding activity of Nrf2 in nuclear extracts from HUVECs transfected with Keap1 siRNA, and this binding specificity was confirmed by competition assay using an excessive amount of cold probe (Fig. 1D). Moreover, ChIP assay also showed an increase in functional binding of Nrf2 to the HO-1 promoter in Keap1 siRNA-transfected HUVECs (Fig. 1E). We next examined the effect of Keap1 knockdown on the transcriptional activity of Nrf2 using an ARE/EpRE-luciferase reporter gene. Transfection with Keap1 siRNA resulted in a significant increase in the transcriptional activity of the ARE/EpRE promoter in a dose-dependent manner compared with control cells (Fig. 1F). These data suggest that Keap1 knockdown increases the expression of phase II genes by promoting Nrf2 activation and its translocation into the nucleus.

Keap1 knockdown increases the expression of ARE/EpRE-regulatory phase II genes in HUVECs

We determined the effect of Keap1 knockdown on the mRNA levels of Nrf2-dependent phase II genes, such as NQO1, GCLC, GCLM, GSTM1, GSTA4, and HO-1. Keap1 knockdown increased the mRNA levels of these specific genes (Fig. 2A) and also upregulated the protein level of HO-1, a typical target of phase II genes (Fig. 2B). Furthermore, cells transfected with Keap1 siRNA showed a significant increase in HO-1 promoter and enzyme activities, and its catalytic activity was inhibited by treatment with the HO-1 inhibitor SnPP (Fig. 2C and D). Because Keap1 knockdown increased the transcriptional expression of GCL, a rate-limiting enzyme in GSH synthesis, we examined intracellular GSH levels in HUVECs transfected with Keap1 siRNA, by using a GSH assay kit. Keap1 knockdown increased intracellular levels of total GSH (GSH + GSSG) and reduced GSH, and these effects were reversed by the GCL inhibitor BSO (Fig. 2E and F). These results indicate that Keap1 knockdown induces antioxidant gene expression.

Keap1 knockdown suppresses TNF-α-induced adhesion molecule expression

We next examined whether Keap1 knockdown regulates the expression of vascular adhesion molecules, such as ICAM-1 and VCAM-1, which play an important role in vascular inflammatory responses as well as the pathogenesis of a variety of inflammatory diseases [3]. Keap1 knockdown suppressed TNF-α-mediated increases in the total protein and mRNA levels of ICAM-1 and VCAM-1 in HUVECs, and these suppressive effects were reduced by treatment with HO-1 inhibitor (SnPP) and GCL inhibitor (BSO), but not with NQO1 inhibitor (dicumarol) (Fig. 3A). We did not find any cytotoxic effects of these inhibitors under our experimental conditions (data not shown). We further examined the effect of Keap1 siRNA on the cell surface levels of these adhesion molecules in TNF-α-stimulated HUVECs using flow cytometric analysis. Keap1 knockdown suppressed TNF-α-induced increases in cell surface levels of ICAM-1 and VCAM-1 in HUVECs, and these increases were reduced by treatment with SnPP and BSO, but not with dicumarol (Fig. 3B–D). It is well known that the adhesion of leukocytes from circulating blood to vascular endothelial cells is the earliest and most essential process in vascular inflammatory responses as well as in the initiation of vascular diseases [3]. As such, we examined whether Keap1 knockdown would regulate...
leukocyte adhesion to TNF-α-stimulated HUVECs. Keap1 knockdown effectively suppressed TNF-α-stimulated increase in the adhesion of monocytes to HUVECs, and this suppressive effect was significantly reversed by treatment with SnPP and BSO, but not with dicumarol (Fig. 3E and F). Taken together these results indicate that Keap1 knockdown may suppress vascular inflammatory responses and potentially vascular disorders.

Keap1 knockdown suppresses TNF-α-induced NF-κB activation

The nuclear transcription factor NF-κB is critically involved in the expression of the inflammation-associated genes, including ICAM-1 and VCAM-1 [17]. We investigated the effects of Keap1 siRNA on the transcriptional activity of both gene promoter constructs containing the proximal NF-κB-binding sites located at −200 bp (ICAM-1) and at 65 and 75 bp (VCAM-1) upstream of their transcription start sites [18]. Stimulation of HUVECs with TNF-α resulted in significant increases in both promoter activities, which were suppressed by Keap1 knockdown (Fig. 4A). We next performed a ChIP assay to determine the involvement of NF-κB in the activation of both promoters. Binding of NF-κB to both promoters was significantly increased in TNF-α-stimulated cells compared with control, and these interactions were inhibited by Keap1 knockdown (Fig. 4B). Moreover, these suppressive effects were significantly reversed by treatment with SnPP and BSO, but not with dicumarol (Fig. 4A and B). Similar inhibitory effects of SnPP or BSO on NF-κB reporter activity were also observed in TNF-α-stimulated HUVECs after transfection with Keap1 siRNA (Fig. 4C). Furthermore, Keap1 knockdown inhibited nuclear translocation of the NF-κB subunit p65 in TNF-α-stimulated HUVECs, as confirmed by Western blotting and confocal microscopy (Fig. 4D and E). EMSA further demonstrated that Keap1 knockdown suppressed NF-κB–DNA binding activity in the nuclear extract from HUVECs treated with TNF-α. The specific interaction between DNA and NF-κB was demonstrated by a competition assay with cold probe and a supershift experiment using a NF-κB p65 antibody (Fig. 4F). These inhibitory effects of Keap1 siRNA on TNF-α-induced NF-κB activation were significantly reversed by treatment with SnPP and BSO, but not with dicumarol (Fig. 4D–F). These results suggest that Keap1 knockdown inhibits the expression of ICAM-1 and VCAM-1 by suppressing nuclear translocation and activation of NF-κB via the induction of HO-1 and GCL expression.

Keap1 knockdown inhibits TNF-α-induced NF-κB signaling pathway

The IkBα degradation is required for NF-κB nuclear migration [19]. Western blot analysis revealed that Keap1 knockdown suppressed TNF-α-induced IkBα phosphorylation and degradation, and these effects were reversed by treatment with SnPP and BSO, but not with dicumarol (Fig. 5A). Because phosphorylation of IkBα is largely dependent on IKK activity, which is regulated by the upstream signal mediators NIK and Akt [20–22], we examined the effect of Keap1 siRNA on phosphorylation-dependent activation of NIK, Akt, and IKK. Keap1 knockdown effectively inhibited TNF-α-induced increases in the phosphorylation of NIK, Akt, and IKK (Fig. 5A). Keap1 knockdown was also shown to decrease the phosphorylation level of NF-κB p65, as shown by Western blotting (Fig. 5B).

Fig. 3. Keap1 knockdown suppresses TNF-α-induced adhesion molecule expression. HUVECs were transfected with 100 nM Keap1 siRNA and allowed to recover in fresh medium for 30 h. (A) Transfected cells were treated with TNF-α (10 ng/ml) for 12 h after pretreatment with 20 μM SnPP, 20 μM BSO, and 20 μM dicumarol (DC) for 1 h. The levels of ICAM-1 and VCAM-1 protein and mRNA were determined by Western blotting and RT-PCR. Under the same experimental conditions, the cell surface levels of (B and C) ICAM-1 and (D) VCAM-1 were determined by flow cytometric analysis. Data shown are the relative mean channel fluorescence (MCF) ± SD (n = 3). *P < 0.05 and **P < 0.01. (E and F) Transfected cells were incubated with TNF-α for 12 h in the presence or absence of SnPP, BSO, and DC. Cells were incubated with U937 cells for 30 min, and the levels of monocyte adhesion were determined after staining with Diff-Quick solution. (a) Control, (b) TNF-α, (c) siRNA+TNF-α, (d) siRNA+TNF-α+SnPP, (e) siRNA+TNF-α+BSO, (f) siRNA+TNF-α+DC. Data shown in (F) are the means ± SD (n = 3). **P < 0.01.
not with dicumarol (Fig. 5B). ROS are important second messengers for the regulation of NF-κB activation, by activating its upstream signal modulators, including Akt, NIK, and IKK [14,20,22]. Consequently, we sought to determine the effects of Keap1 knockdown on the TNF-α-induced ROS production. Keap1 knockdown effectively inhibited TNF-α-induced intracellular ROS accumulation, and this inhibitory effect was reversed by treatment with SnPP and BSO, but not with dicumarol (Fig. 5C and D). Moreover, the suppressive effect of Keap1 knockdown on TNF-α-induced NF-κB reporter activity was reduced by the addition of the heme degradation product CORM-2 and the antioxidant N-acetylcysteine (Fig. 5E). These results indicate that Keap1 knockdown blocked the TNF-α-induced NF-κB pathway in endothelial cells through inhibition of intracellular ROS accumulation by inducing HO-1 and GCL expression.

Transfection with siRNA for Prx1, but not GSTM1, attenuates Keap1 knockdown-mediated suppression of adhesion molecule expression

Because Keap1 knockdown elevated the expression of Prx1 and GSTM1 (Fig. 2), we further examined the roles of these enzymes and proteins on TNF-α-induced adhesion molecule expression in HUVECs transfected with Keap1 siRNA. Keap1 knockdown resulted in increases in Prx1 and GSTM1 expression, which were suppressed by transfection with siRNAs for Prx1 and GSTM1 (Fig. 6A). Specific knockdown of Prx1, but not GSTM1, reduced the suppressive effect of Keap1 knockdown on TNF-α-mediated increases in ICAM-1 and VCAM-1 expression, monocyte adhesion, and intracellular ROS accumulation (Fig. 6B–D). Moreover, Prx1 knockdown attenuated Keap1 siRNA-mediated nuclear translocation of NF-κB and NF-κB-Luc reporter activity in HUVECs (Fig. 6E and F). These results suggest that induction of Prx1, but not GSTM1, by Keap1 knockdown also plays an important role in the regulation of NF-κB activation and inflammatory gene expression.

Keap1 knockdown inhibits LPS-induced inflammatory response in macrophages

Because NF-κB is also a key transcription factor for eliciting immune responses to LPS [23], we next investigated whether Keap1 knockdown regulates the production of cytotoxic mediators, such as NO and TNF-α, which are responsible for septic shock. Transfection of RAW264.7 cells with Keap1-specific siRNA inhibited the levels of Keap1 mRNA and protein, leading to the induction of HO-1, GCL, and Prx1 (Fig. 7A). Furthermore, transfection with Keap1 siRNA suppressed the mRNA and protein levels of iNOS and TNF-α in LPS-stimulated RAW264.7 cells (Fig. 7B). Under the same experimental conditions, Keap1 knockdown inhibited LPS-induced secretion of NO and TNF-α into the culture medium of LPS-stimulated RAW264.7 cells (Fig. 7C and D). These data indicate that Keap1 knockdown suppressed LPS-induced NO and TNF-α production in macrophages via the induction of HO-1, GCL, and Prx1.

Keap1 knockdown inhibits LPS-induced in vivo inflammation, tissue damage, and lethality

To further investigate a role for Keap1 knockdown in in vivo inflammatory processes, we determined the expression levels of inflammation-associated genes in liver and lung tissues from mice, which were treated ip with LPS after intravenous injection with mixture of in vivo-jetPEI reagent and Keap1 siRNA. Administration
of Keap1 siRNA resulted in the reduction of Keap1 expression; elevation of HO-1, GCL, and Prx1 expression; and suppression of ICAM-1, VCAM-1, TNF-α, and iNOS expression in the liver and lung of LPS-treated mice (Fig. 8A). In addition, in vivo Keap1 knockdown significantly suppressed LPS-mediated increases in serum levels of NO2 and TNF-α (Fig. 8B and C). We next examined the functional role of Keap1 in LPS-mediated tissue damage in a mouse model. Administration with Keap1 siRNA prominently inhibited LPS-induced tissue injuries of liver and lung, showing distinct injury and immune cell infiltration (Fig. 8D). Furthermore, administration of LPS (35 mg/kg) resulted in a 24-h mortality rate of 100% in mice; however, preinjection with Keap1 siRNA significantly reduced LPS-induced mice lethality (Fig. 8E). These results provide evidence for the protective effects of Keap1 knockdown on endotoxic tissue injury and lethality by inhibiting inflammatory and cytotoxic gene expression.

Discussion

This study was undertaken to elucidate the functional role of Nrf2-dependent phase II gene products in NF-κB activation and inflammatory responses in vitro and in vivo using Keap1-specific siRNA and phase II enzyme inhibitors. We found that specific knockdown of Keap1 effectively induced Nrf2 activation and ARE/EpRE-dependent expression of phase II genes (HO-1, GCL, and Prx1), which were highly correlated with suppression of TNF-α-induced ROS accumulation and NF-κB-mediated adhesion molecule expression in HUVECs. Moreover, Keap1 knockdown suppressed LPS-mediated iNOS and TNF-α expression in cultured macrophages and in a mouse model, as well as protecting mice from LPS-induced lethality. These results indicate that Nrf2 activation prevents NF-κB-mediated vascular inflammation and endotoxic shock by suppressing NF-κB-mediated inflammatory gene expression via upregulation of Nrf2-mediated antioxidant genes.

Keap1 contains a number of redox-sensitive cysteine residues [24]. Of them, three residues, C151, C273, and C288, are critical to the function of Keap1, which is a substrate adaptor for the Keap1–Cul3–E3 ubiquitin ligase complex. Under normal conditions, this complex induces the ubiquitination of Nrf2 and its subsequent degradation. However, modification of these cysteine residues by oxidants, heavy metals, and alkylating agents is considered to impair the structural integrity of the Keap1–Cul3–E3 ligase complex, resulting in a decline in ubiquitination activity, thereby facilitating accumulation of Nrf2 in the cytosol. The liberated and stabilized Nrf2 translocates into the nucleus and activates transcription of its downstream target genes by binding to ARE/EpRE present in the promoters of antioxidant and cytoprotective phase II detoxifying genes.

Nrf2 is known to induce phase II genes, such as HO-1, Prx1, GCL, GST, and NQO1. HO-1 breaks heme down to carbon monoxide (CO), ferrous iron, and biliverdin/bilirubin. These by-products function as antioxidants under physiological and pathological conditions [25–27]. Thus, induction of HO-1 is directly associated with
cytoprotection and suppression of NF-κB-mediated inflammatory gene expression [28–30]. Prx1 is a thiol-containing small redox protein involved in the suppression of intracellular ROS accumulation [31]. GCL is a rate-limiting enzyme in cellular GSH synthesis, leading to an increase in cellular redox potential [32]. Although NQO1 does not possess direct antioxidant activity, its major function is to detoxify quinones and their derivatives, thus protecting cells from oxidative stress by removing compounds capable of generating ROS [33]. GST family enzymes catalyze the conjugation of GSH to toxic compounds, resulting in more water-soluble and less biologically active products that may easily be excreted [34]; however, GSTM1, an isoform of the GST family, has been shown to inhibit intracellular accumulation of ROS [35]. Thus, phase II gene products regulate cellular redox potential by blocking intracellular ROS accumulation through two distinct mechanisms, suppression of ROS generation and elevation of ROS removal activity. We here found that Keap1 knockdown effectively induces Nrf2-dependent phase II gene expression, subsequently resulting in inhibition of TNF-α-induced intracellular accumulation of ROS, which is responsible for NF-κB activation (Figs. 2, 4, 5, and 6). Importantly, this

Fig. 6. Keap1 knockdown induces Prx1, which is responsible for regulation of NF-κB-dependent adhesion molecule expression. (A) The mRNA and protein levels of Keap1, Prx1, and GSTM1 were determined in HUVECs transfected with 100 nM Keap1 siRNA for 30 h by Western blotting and RT-PCR. (B) Cells cotransfected with 100 nM siRNAs for Keap1, Prx1, and GSTM1 were treated with TNF-α for 12 h. The protein levels of ICAM-1 and VCAM-1 were determined by Western blotting. (C) Monocyte adhesion, (D) intracellular levels of ROS, (E) nuclear translocation of NF-κB, and (F) NF-κB promoter activity were determined in HUVECs transfected with siRNA against Keap1, Prx1, and GSTM1 as described for Figs. 3E, 5C, 4E, and, and 5E, respectively. Data shown in (C, D, and F) are the means ± SD (n = 3). **P < 0.01.

Fig. 7. Keap1 knockdown inhibits inflammatory gene expression in LPS-activated RAW264.7 cells. (A) RAW264.7 cells were transfected with 50 and 100 nM Keap1 siRNA for 30 h. The expression levels of Keap1 and some phase II genes were determined by Western blotting and RT-PCR. (B–D) Cells were transfected with 100 nM Keap1 siRNA for 30 h and treated with LPS (1 μg/ml) for 12 h. (B) The expression levels of iNOS and TNF-α were determined by Western blotting and RT-PCR. The levels of (C) nitrite and (D) TNF-α were determined by Griess reaction and ELISA, respectively. **P < 0.01.
suppressive effect was reduced by treatment with HO-1 and GCL inhibitors and Prx1 siRNA, but not with NQO1 inhibitor and GSTM1 siRNA. These effects are likely to be associated with production of CO, biliverdin/bilirubin, and iron from heme by HO-1 [25–27]; de novo synthesis of GSH by GCL [32]; and expression of thiol-containing Prx1 [31]. These biological molecules are involved in the inhibition of NAD(P)H oxidase activity [36,37] and promotion of ROS-scavenging activity [38]. However, NQO1 and GST are not implicated in the suppression of intracellular ROS accumulation in TNF-α-stimulated HUVECs. These findings suggest that induction of these phase II genes by Keap1 knockdown, with the exception of NQO1 and GST, is responsible for regulating cellular redox potential in TNF-α-activated endothelial cells.

Although high levels of ROS are toxic to cells, physiological levels of ROS serve a signaling function to regulate various biological processes, such as cell proliferation and migration and gene expression [39]. ROS produced by immune stimulants, including TNF-α and LPS, play a critical role in inflammatory gene expression via activation of the NF-κB signaling pathway. Our previous study demonstrated that the antioxidant lutein inhibits NF-κB activation and inflammatory gene expression in LPS-stimulated macrophages by suppressing redox-sensitive activation of the PTEN/Akt and NIK-dependent pathways through removal of intracellular ROS [14]. Both NIK and Akt are involved in NF-κB activation in TNF-α- and LPS-stimulated cells through the phosphorylation of Iκκβ. This promotes Iκκβ phosphorylation in a reciprocal activation mode, demonstrating an involvement of Akt and NIK in the canonical NF-κB activation pathway [22,40,41]. These observations indicate that ROS are involved in the activation of the canonical pathway via a reciprocal cross talk between Iκκks and Iκκβ by activating Akt and NIK. We here found that Keap1 knockdown promoted Nrf2-dependent antioxidant gene expression and removal of intracellular ROS, which is responsible for NF-κB activation and inflammatory gene expression (Figs. 2 and 5). These findings indicate that Nrf2 activation exerts anti-inflammatory effects through inhibition of NF-κB activation by inducing phase II antioxidant gene expression.

Endothelial cells play an important role in host immune responses for vascular homeostasis during inflammation and infection. Endothelial cells activated by immune stimulants upregulate the adhesion molecules ICAM-1 and VCAM-1, which are involved in the migration of peripheral blood leukocytes to the sites of inflammation or infection [42]. Although leukocyte recruitment is important for eliminating invading pathogens, excessive leukocyte accumulation in the site of inflammation can lead to tissue damage [42]. The expression of ICAM-1 and VCAM-1 in endothelial cells can be tightly regulated by NF-κB activation. Antioxidants have been shown to inhibit NF-κB activation and block leukocyte infiltration into sites of inflammation by suppressing the expression of adhesion molecules, implicating endothelial cells as a target for regulating inflammatory processes [43]. We here found that Keap1 knockdown attenuated monocyte adhesion to TNF-α-stimulated HUVECs by inhibiting NF-κB-dependent expression of ICAM-1 and VCAM-1 (Fig. 3), which participate in the pathogenesis of sepsis and atherosclerosis. These inhibitory effects were highly correlated with the expression levels of HO-1, GCL, and Prx1 and were suppressed by selective inhibition of these enzymes/proteins (Figs. 3 and 6).
These results suggest that phase II gene products are involved in inhibiting TNF-α-mediated expression of adhesion molecules in HUVECs via suppression of ROS-mediated NF-κB activation. Moreover, systemic delivery of Keap1 siRNA decreased the expression of adhesion molecules and infiltration of leukocytes into liver and lung, which are major organs of septic injury, in an endotoxemic mouse model (Fig. 8). These findings indicate that Keap1 siRNA can prevent endotoxic shock by suppressing NF-κB-dependent adhesion molecule expression.

LPS, a prime initiator of septic shock, activates macrophages and endothelial cells and upregulates a variety of NF-κB-dependent inflammatory genes, leading to production of the cytotoxic mediators TNF-α, interleukin-1β, and NO. These mediators are known to increase tissue damage and lethality [14,44]. Thus, NF-κB inhibitors have clinical potential for controlling various inflammation-associated human diseases [56]. We found that Keap1 knockdown suppressed the expression of NF-κB-dependent inflammatory genes, TNF-α and iNOS, in LPS-stimulated RAW264.7 cells (Fig. 7). On the other hand, delivery of Keap1 siRNA significantly induced Nrf2-dependent expression of phase II genes and suppressed the expression of TNF-α, iNOS, and adhesion molecules in LPS-administered mice (Fig. 8). Furthermore, in vivo Keap1 knockdown reduced organ injuries and lethality in a mouse model of endotoxic shock. These findings suggest that siRNA targeting Keap1 can be used as a therapeutic agent for treating various inflammatory diseases, including septic or endotoxic shock.

Recent genome-wide analysis demonstrated that Nrf2 regulates hundreds of genes that are involved in the cytoprotective response against oxidative stress [45]. Although we did not determine the expression levels of all these genes in this study, it is feasible that Keap1 knockdown could induce the expression of many other antioxidant-related genes. Although not shown, Keap1 knockdown did not significantly increase other antioxidant enzymes, such as superoxide dismutase-1, catalase, and peroxidase-1, as determined by RT-PCR. Therefore, we excluded the notion that these enzymes could not contribute to the regulation of NF-κB-mediated inflammatory gene expression under our experimental conditions. Anti-inflammatory effects induced by Keap1 knockdown or Nrf2-activating compounds may be elicited by cross talk of multiple pathways or synergistic interaction among Nrf2-targeting gene pathways. Therefore, the beneficial effect of Keap1 knockdown cannot be fully explained by upregulation of three antioxidant gene products, HO-1, GCL, and Prx1; however, these enzymes' proteins may play an important role in the immune-suppressive effect of siRNA-based Keap1 knockdown.

Taken together, our data show that siRNA-mediated knockdown of Keap1 increased Nrf2-dependent expression of the phase II genes HO-1, GCL, and Prx1, which are implicated in the suppression of intracellular ROS accumulation in immune-activated cells. This suppressive effect is directly associated with inhibition of the signal mediators Akt, NIK, and IKK, which are upstream of NF-κB activation, resulting in the suppression of inflammation-associated gene expression in TNF-α-stimulated endothelial cells and LPS-treated macrophages. Moreover, systemic delivery of Keap1 siRNA protected mice from LPS-induced organ injury and lethality. These findings provide a rationale for novel therapeutic approaches utilizing Keap1-specific siRNA for human inflammatory diseases, such as sepsis and atherosclerosis.

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