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Roles of ERK and NF-κB in Interleukin-8 Expression in Response to Heat Shock Protein 22 in Vascular Smooth Muscle Cells

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Heat shock proteins (HSPs) serve as molecular chaperones and play a role in cell protection from damage in response to stress stimuli. The aim of this article is to investigate whether HSP22 affects IL-8 expression in vascular smooth muscle cells (VSMCs), and which cellular factors are involved in the HSP-mediated IL-8 induction in that cell type in terms of mitogen activated protein kinase (MAPK) and transcription element. Exposure of aortic smooth muscle cells (AoSMCs) to HSP22 not only enhanced IL-8 release but also induced IL-8 transcript via promoter activation. HSP22 activated ERK and p38 MAPK in AoSMCs. HSP22-induced IL-8 release was inhibited by U0126, but not by SB202190. A mutation in the IL-8 promoter region at the binding site of NF-κB, but not AP-1 or C/EBP, impaired promoter activation in response to HSP22. Delivery of IκB, but not dominant negative c-Jun, lowered HSP22-induced IL-8 release from AoSMCs. These results suggest that HSP22 induces IL-8 in VSMCs via ERK1/2, and that transcription factor NF-kB may be required for the HSP22-induced IL-8 up-regulation.

Key Words: Heat shock protein, Interleukin-8, Vascular smooth muscle cell

INTRODUCTION

Heat shock proteins (HSPs) are highly conserved molecules which participate in protein folding and assembly, and are crucial for correct transportation of proteins through the cell (Whitley et al. 1999). HSPs can be classified into several distinct families. Among them, HSP60 (HSP60, HSP65, and GROEL) and HSP70 (HSP72, HSP73, and GRP170) have been the subject of extensive studies with regard to immunogenetic and inflammatory properties (Pockley, 2003; Srivastava, 2002). The small HSPs (sHSPs), including αA crystallin, αB crystallin, HSP27, HSP22 (HSPB8), and HSP20, represent another class of HSP, ranging in size from 12 to 43 kDa (de Jong et al. 1993; MacRae, 2000). One of the pathological functions of HSPs is the association with inflammatory diseases and atherosclerosis (Pockley et al. 2003). In particular, HSP60 is expressed by the endothelial cells of stressed arteries and because of sequence homology between microbial and human HSP60, the immunity against microorganisms may be responsible for endothelial cell damage and early atherosclerosis (Perschinka et al. 2003; Wick et al. 2001). In contrast to HSP60 and HSP70, the roles of sHSP in inflammatory diseases have not yet been subjected to similar research.

Findings from clinical and animal studies indicate the involvement of IL-8 in the vascular disease. A recent clinical study has shown that IL-8 is the most powerful predictor of future cardiovascular events independent of other cytokines as well as high sensitive C reactive protein (Inoue et al. 2008). The knock-out mice of IL-8 homologue exhibit reduced levels of atherosclerotic lesions in animal experiments (Boisvert et al. 1998). This chemokine is believed to exacerbate atherosclerotic process by modifying cellular events during atherogenesis. IL-8 mediates proliferation of VSMCs (Yue et al. 1994), triggers monocyte arrest on early atherosclerotic endothelium (Huo et al. 2001), and recruits neutrophils to the site of tissue injury (Peveri et al. 1988; Schroder and Christophers, 1989). In addition, IL-8 can affect stability of atherosclerotic plaque by down-regulating tissue inhibitor of metalloproteinase-1 expression in cholesterol-laden macrophages (Moreau et al. 1999). Thus, understandings of IL-8 gene expression in vascular cells appear to be important in therapeutic strategies for vascular diseases.

VSMCs express very low level of IL-8 whose expression can be induced by pro-atherogenic factors. IL-1β augments transcription of the IL-8 gene (Ho et al. 2007), and IL-8 gene expression has been shown to be induced by tumor necrosis factor (Wang et al. 1991) and lysophosphatidylcholine (Aiyar et al. 2007), which is thought to play a major role in the proinflammatory effects of oxidized low density lipoprotein (oxLDL) (Steinberg et al. 1989). However, mechanisms behind these molecules to up-regulate IL-8 have not yet been described.

To explore the potential role of sHSP in vascular in-

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ABBREVIATIONS: AP-1, activator protein 1; AoSMC, aortic smooth muscle cell; HSP, heat shock protein; NF-κB, nuclear factor kappaB; VSMC, vascular smooth muscle cell; MAPK, mitogen activated protein kinase.

flammation, we investigated the ability of HSP22 to induce IL-8 using aortic smooth muscle cells and demonstrated that HSP22 up-regulated IL-8 via transcriptional activation. Furthermore, the involvement of mitogen activated protein kinase (MAPK) and transcription elements in HSP22-induced IL-8 up-regulation in VSMCs was investigated.

METHODS

Cell culture and reagents

Aortic smooth muscle cells (AoSMCs) of human origin, purchased from Cambrex (East Rutherford, NJ), were grown in Dulbecco's modified Eagle's medium-high glucose (DMEM) supplemented with 15% fetal bovine serum (FBS), 50 units/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2. The cells in between passages 6 and 9 were used. All cell culture media and supplements were purchased from Gibco-BRL (Grand Island, NY). A7r5 rat smooth muscle cell line (American Type Culture Collection, Manassas, VA) was cultured in the presence of 10% FBS. Recombinant human HSP22 was purchased from ProSpec-Tany Technogene Ltd (Rehovot, Israel). U0126 and SB202190 were purchased from Cell Signaling Technology (Danvers, MA). To exclude the contribution of contaminated LPS, HSP22 was treated with endotoxin removal resins (US Biological, Swampscott, MA). The reporter plasmid containing a part of human IL-8 promoter, ranging from nucleotide −416 to +44 upstream of the transcriptional start site, was kindly provided by Dr. Hsing-Jien Kung (University of California Davis Cancer Center, Sacramento, CA). Three additional constructs with mutations in the nuclear factor kappaB (NF-κB) [pNF-κB-mut], CCAAT/enhancer binding protein β (C/EBPβ) [pC/EBP β-mut], and activator protein (AP)-1 [pAP-1-mut] elements in the context of pIL8(-416)-Luc construct were created by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA). The sequences of the mutants were designed as previously (Wu et al. 2004).

Enzyme linked immunosorbent assay (ELISA) of IL-8

The cytokine content of culture supernatant was determined by using commercially available Biotrack ELISA kit according to the manufacturer's instructions (Amersham, Piscataway, NJ). Cells on 100 mm culture dishes were serum-starved for 6 h and exposed to HSP22 prior to isolation of culture medium. The isolated culture medium and IL-8 standards were added to a plate pre-coated with a monoclonal antibody against IL-8. After incubation for an hour, the plate was washed. Next, the plate was incubated with an enzyme-linked polyclonal antibody specific for IL-8. The substrate solution was added after several washes, and the color intensity was determined.

Reverse transcription (RT) - realtime polymerase chain reaction (PCR)

Total RNAs extracted from AoSMCs were reverse-transcribed for an hour at 42°C with 200 units of Moloney Murine Leukemia Virus reverse transcriptase, followed by quantitative PCR analysis. Real-time quantitative PCR was performed in triplicate in 384-well plates; each 20-μl reaction consisted of 10 μl of SYBR Green Master Mix, and 0.8 μl of 10 pM forward and reverse primers for IL-8 and GAPDH using Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermal cycling conditions comprised 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C for 30 sec, 60°C for 30 sec and a 72°C elongation period for 30 sec. Sequences of IL-8 primers were 5'-TCTGCAGCTCTGTGTGAAGG-3' (forward) and 5'- AATTTC TGTGTTGGCGCAGT-3' (reverse). Primers for GAPDH were 5'-ATGGGGAAG GTGAAGGTCG-3' (forward) and 5'-GGGGTCATTGATGGCAACAATA-3' (reverse). Each of the 384-well real-time PCR plate included serial dilutions (1, 1/2, 1/4, 1/8 and 1/16) of cDNA, which were used to generate relative standard curves for IL-8 and GAPDH. The fluorescence emission of each sample was collected by a charge-coupled device-camera and the quantitative data were analyzed using the Sequence Detection System software (SDS version 2.0, Applied Biosystems, Foster City, CA).

Transient transfection and luciferase assay

For reporter gene assays, A7r5 cells that had previously been transfected were used. A7r5 cells were seeded in 100-mm culture dishes 24 h before transfection. Cells were then transfected with 10 μg of IL-8 reporter plasmid and 3 μg of β-galactosidase plasmid using Lipofectamine (Invitrogen, Carlsbad, CA). Cells were then re-fed DMEM medium 6 h post-transfection and incubated overnight. Transfected cells were exposed to HSP22 for the indicated time periods. Luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI) with signal detection for 5 sec in a luminometer and normalized to β-galactosidase activity.

Construction of recombinant adenoviruses

Recombinant adenoviral DNAs were prepared via homologous recombination in E. coli, in accordance with AdEasyTM Adenoviral vector system (Stratagene, La Jolla, CA). The transactivation domain deletion mutant of c-Jun (TAM67) (Brown et al. 1994) and the hemagglutinin-tagged inhibitor of NF-κB (IκB) (32A/36A) (Brown et al. 1994) were cloned into the Shuttle vector and transfected with the pAdEasy-1 vector into E. coli BJ5183. Recombinant DNAs were then selected and transfected into 293 cells, and the replication-deficient recombinant adenoviruses were generated. Adenoviruses harboring genes of interest were isolated with a commercially available adenovirus purification kit (Cell Biolabs Inc., San Diego, CA) in accordance with the manufacturer's instructions. The purified viruses were aliquoted and maintained at −80°C. Viral titer was determined using QuickTiter™ Adenoviral Quantitation Kit (Cell Biolabs Inc., San Diego, CA).

Western blot analysis

Lysates of AoSMCs prepared in lysis buffer [50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% NP40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride] were resolved on SDS-PAGE gels, followed by transfer to nitrocellulose membranes. The membranes were blocked for 2 h at room temperature in Tris-buffered solution [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20] containing 5% non-fat dry milk, and then incubated for 2 h with appropriate pri-

mary antibodies. The membranes were washed and incubated for an hour with horseradish peroxidase-conjugated secondary antibodies diluted to 1 : 8,000. After three washes, the membranes were incubated with ECL reagents.

Statistical analysis

Statistical analyses were performed by ANOVA, and $p < 0.05$ was considered statistically significant.

RESULTS

The effects of HSP22 on IL-8 transcript and protein in human VSMCs

To investigate the effects of HSP22 on IL-8 gene expression in VSMCs, the level of IL-8 transcript was examined by non-quantitative and quantitative PCR after exposure of AoSMCs to HSP22. Fig. 1A shows that IL-8 transcripts were induced in the presence of HSP22. The induction was observed at 4 h post-treatment and increased up to 12 h after treatment. When the increase of IL-8 was analyzed by real-time PCR (Fig. 1B), IL-8 transcript was increased by approximately 10 fold in the presence of HSP22 in comparison with control exposed to identical concentration of bovine serum albumin (BSA). HSP90 was included as a positive control, since we observed that it increased IL-8 transcript in AoSMCs (data not shown). In the analysis, GAPDH mRNA was amplified as an internal control. We also examined by ELISA (Fig. 2A) whether VSMCs released IL-8 protein in response to the HSP, and found that the release of IL-8 was profoundly enhanced by HSP22 in comparison with control: The amount of IL-8 within the medium increased from 45 to 250 pg/ml in the presence of HSP22.

Reporter gene assays were carried out to investigate whether HSP22 increased IL-8 transcript via promoter activation. Thus, A7r5 cells were transfected with the IL-8 reporter plasmid described above, and then luciferase activity was assessed in the presence or absence of HSP22 (Fig. 2B). Compared with control exposed to BSA, luciferase activity which was driven by IL-8 gene promoter region was increased by 2.5-fold in the presence of HSP22, indicating that HSP22 promoted IL-8 transcriptional activity.

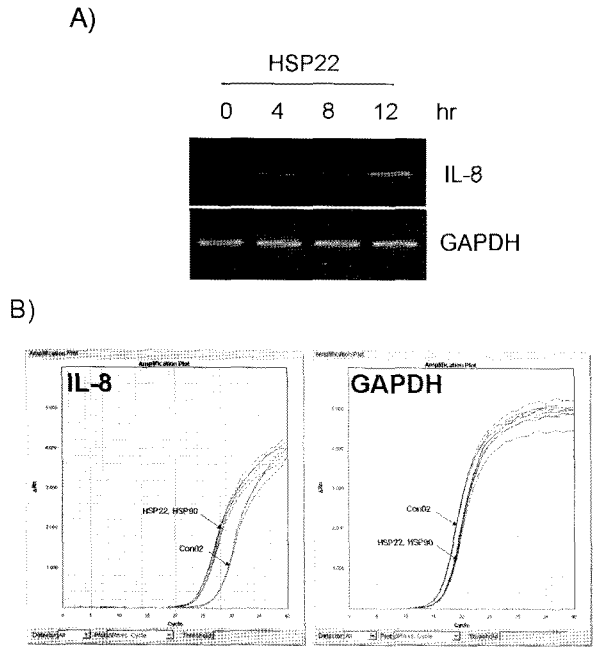

Fig. 1. Induction of IL-8 in VSMCs by HSP22. (A) AoSMCs were treated with 100 ng/ml HSP22 for indicated periods, and IL-8 transcript was amplified by RT-PCR. (B) IL-8 transcript was quantified by quantitative real-time PCR with BSA a control after AoSMCs were treated with 100 ng/ml of HSP22, HSP90 and BSA (con02) for 12 h.

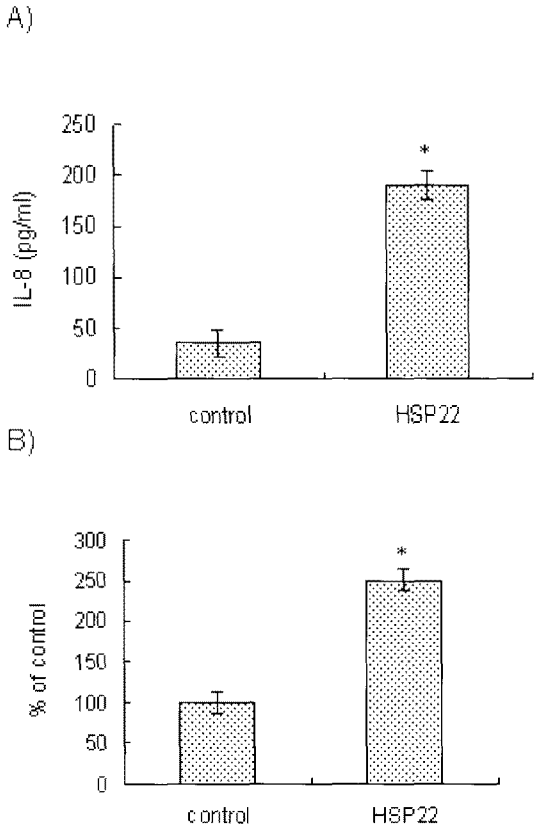

Fig. 2. Effects of HSP22 on IL-8 release and IL-8 promoter activity in VSMCs. (A) AoSMCs (1×10^6 cells/100-mm culture dish) cultured in growth media were incubated for 12 h in the presence of BSA (control) or HSP22 (100 ng/ml). The culture media were harvested. IL-8 secreted in the culture medium was measured by ELISA. Data are expressed as mean±SD (n=3 replicates/group). *$p < 0.01$ vs. control. (B) A7r5 cells were transfected with the wild-type pIL-8-Luc651 construct. The transfected cells were incubated in the presence of BSA (control) or HSP22 (100 ng/ml, for 8 h), and processed for luciferase and β-galactosidase assays. Luciferase activity ($\times10^6$) was normalized to β-galactosidase activity. Induction was calculated relative to the activity of control cells. Data are expressed as mean±SD (n=3 replicates/group). *$p < 0.01$ vs. control.

Involvement of ERK in IL-8 up-regulation by HSP22 in VSMCs

To investigate the effect of HSP22 on MAPK pathways, phosphorylated forms of extracellular signal-regulated kinase (ERK) and p38 MAPK were examined after exposure of VSMCs to HSP22 (Fig. 3A). Phosphorylated forms of ERK and p38 MAPK were elevated in response to HSP22: The phosphorylated ERK2 was evident at 10 min post treatment and was maintained up to 40 min post treatment. In case of p38 MAPK, the phosphorylated form was observed at 10 min post treatment. To understand the roles of the MAPKs in HSP22-induced IL-8 up-regulation, AoSMCs were pre-treated with U0126 (an ERK1/2 kinase inhibitor), or SB202190 (a p38 MAPK inhibitor) prior to stimulation with HSP22 (Fig. 3B) and IL-8 release was determined by ELISA. U0126 significantly inhibited HSP22-induced IL-8 release without affecting viability (data not shown). Pre-treatment of the cells with SB202190 slightly affected IL-8 release.

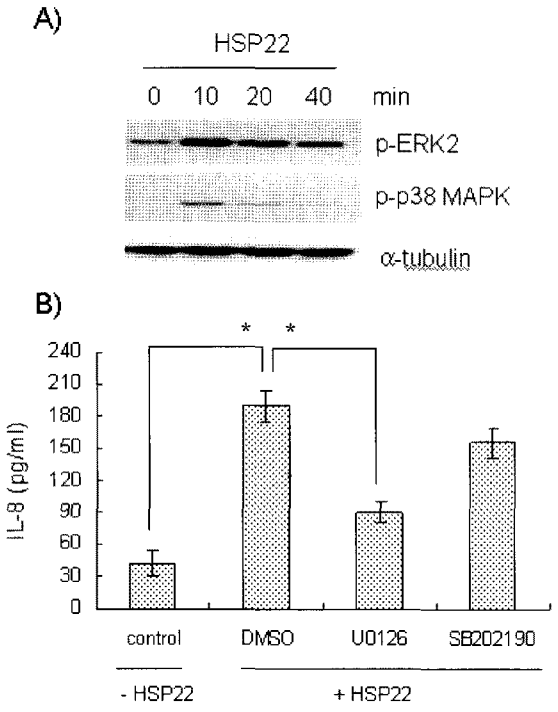

Fig. 3. Effects of MAPKs inhibitors on HSP22-induced IL-8 release. (A) Human aorta smooth muscle cells were exposed to HSP22 (100 ng/ml) for the indicated time periods, and cell lysates were then prepared. An equal amount of protein was subjected to Western blot analysis with antibodies for α-tubulin, phospho-ERK2 (p-ERK2), phospho-p38 MAPK (p-p38 MAPK) and α-tubulin. (B) AoSMCs were pre-treated with U0126 or SB202190 (10 μM each, for 2 h) and stimulated with (+) or without (−) HSP22 (100 ng/ml, for 12 h). Secreted IL-8 was measured by ELISA. Data are expressed as mean±SD (n=3 replicates/group). *$p<0.01$.

Role of NF-κB in HSP22-induced IL-8 promoter activation in VSMCs

To investigate the roles of transcription elements in HSP22-induced IL-8 up-regulation, IL-8 gene promoter activity was examined after transfection of A7r5 cells with an IL-8 reporter plasmid mutated at the NF-κB-, C/EBP-, or AP-1-binding site (Fig. 4A). HSP22 significantly increased lucifease activity, which is consistent with previous data. Mutation at the NF-κB- binding site of the promoter region significantly attenuated luciferase activity induced by HSP22, whereas mutation at the AP-1- or C/EBP-binding site showed slight effects on luciferase activity, indicating that the NF-κB-binding site is necessary for activation of IL-8 by HSP22 in VSMCs. To obtain further evidence for the role of NF-κB in HSP22-induced IL-8 up-regulation in VSMC, whether NF-κB was involved in IL-8 release was examined. Thus, the amount of IL-8 in response to HSP22

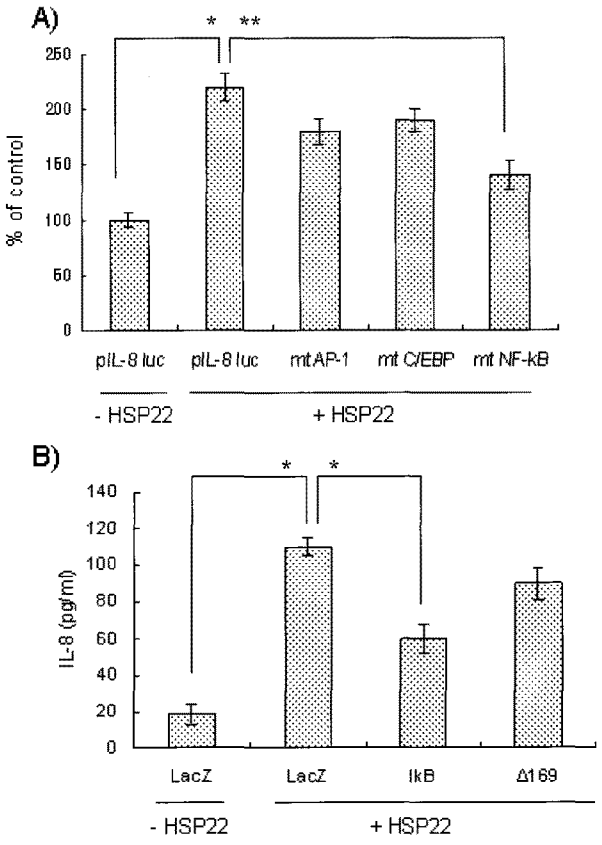

Fig. 4. Effects of transcriptional elements on HSP22-induced IL-8 up-regulation. (A) A7r5 cells were transfected with the wild-type pIL-8-Luc651 construct or the indicated mutant construct. The transfected cells were stimulated with (+) or without (−) HSP22 (100 ng/ml) for 8 h and processed for luciferase and β-galactosidase assays. Induction was calculated relative to the activity of cells incubated in the absence of HSP22. Data are expressed as mean±SD (n=3 replicates/group). *$p<0.01$, **$p<0.05$. (B) AoSMCs were infected with indicated recombinant adenoviruses (MOI: 100) and stimulated with (+) or without (−) HSP22 (100 ng/ml, for 12 h). The amount of secreted IL-8 was measured by ELISA. Data are expressed as mean±SD (n=3 replicates/group). *$p<0.01$.

was determined by ELISA after AoSMCs were infected with recombinant adenoviruses expressing IκB, TAM67 and β-galactosidase (Fig. 4B). In consistent with the previous finding, HSP22 elevated IL-8 release from VSMCs. The delivery of TAM67, which inhibits the activity of AP-1, slightly affected IL-8 release with no statistical significance. On the other hand, the HSP22-induced IL-8 release was significantly attenuated by delivery of IκB, an endogenous inhibitor of NF-κB.

DISCUSSION

Previous studies have demonstrated close association of IL-8 with atherosclerosis. Both transcript and protein of IL-8 are expressed in the normal and diseased arteries (Rus et al. 1996; Tedgui and Mallat, 2006). The pattern of IL-8 expression, however, is different between normal and diseased arteries. Fibrous plaques and intimal thickening show extended intracellular and extracellular deposits of IL-8, while only cellular deposits of IL-8 has been observed in the normal intima (Rus et al. 1996). The accumulation of IL-8 and MCP-1 at the luminal surface of the endothelium establishes an IL-8 and MCP-1 gradient across the endothelial monolayer, thereby enhancing the transendothelial migration of monocytes (Gerszten et al. 1999; Huo et al. 2001). Therefore, IL-8 participates in early events of atherogenesis by recruiting monocytes into vascular wall in conjunction with monocyte chemoattractant protein-1 (MCP-1) (Boisvert et al. 1998; Huo et al. 2001). These findings suggest that regulation of IL-8 is important in control of atherogenesis.

In the current study, we investigated the mechanisms of IL-8 induction in VSMCs. When VSMCs were exposed to HSP22, IL-8 transcript was increased and luciferase activity driven by the fragment of the human IL-8 gene promoter was enhanced, indicating that HSP22 transactivates IL-8 gene. HSP22 also enhanced the release of IL-8 protein from VSMCs. Taken together, these data indicate that HSP22 up-regulates IL-8 at transcriptional and translational levels. Because HSP22 activated ERK1/2 and p38 MAPK, the role of the kinases in HSP22-mediated IL-8 induction was investigated in conjunction with inhibition of ERK and p38 MAPK pathways with specific inhibitors. When the effects of the inhibitors on IL-8 release were investigated, the inhibitor of ERK pathway alone significantly inhibited IL-8 secretion. This finding indicates that ERK1/2 but not p38 MAPK plays a crucial role in HSP22-mediated IL-8 release from VSMCs. The IL-8 promoter region contains transcription element of NF-κB, C/EBP and AP-1 (Wu et al. 2004). Thus, we determined which transcription elements were responsible for promoter activation in response to HSP22 using IL-8 reporter plasmids mutated at the NF-κB-, C/EBP-, or AP-1-binding site. The mutation at NF-κB-binding site significantly attenuated promoter activity, whereas the mutation at AP-1- or C/EBP-binding site did not inhibit IL-8 promoter activity. Thus, the data indicate that HSP22 regulates IL-8 in mechanisms distinct from IL-1β which induces IL-8 gene transcription in human VSMCs via p38 MAPK and AP-1 (Jung et al. 2002). Next, whether the inhibition of transcription factor NF-κB played a role in IL-8 release was investigated. In contrast with dominant negative c-Jun, overexpression of IκB attenuated the IL-8 release. Taken together, these data indicate that NF-κB is necessary for HSP22-mediated IL-8 up-regulation in VSMCs.

It has been reported that HSP22 induces IL-8 via Toll-like receptors (TLRs), because some HSPs can act as endogenous ligands for the receptors (Akira and Takeda, 2004; Kawai and Akira, 2005). Among the subtypes of TLRs, expression of TLR-3, -4, and -6 has been found in human VSMCs (Yang et al. 2005; Yang et al. 2006), and TLR-4, which is the most abundant TLR in human VSMCs, transmits signals through MyD88-dependent and -independent pathways after recognition of lipopolysaccharide (LPS), an exogenous ligand (Akira and Takeda, 2004). In the MyD88-dependent pathway, which is also utilized by most TLRs, the signal from TLR is transduced via MyD88 and IL-1 receptor-associated kinase (IRAK), a serine/threonine kinase, and finally activates nuclear factor (NF)-κB (Akira and Takeda, 2004; Kawai and Akira, 2005). Moreover, the activation of TLR-4 enhances expression of cytokines, including IL-6 and MCP-1, in VSMCs (Yang et al. 2005). Further investigations are needed to elucidate association of TLR4 signaling with HSP22-mediated IL-8 induction.

In the present study, we showed that HSP22 up-regulates IL-8 in VSMCs via transcriptional activation, and that ERK1/2 and NF-κB play crucial role in the up-regulation. These findings suggest that HSPs which are produced during stress and cell damage contribute to elevation of IL-8 in VSMCs, thereby triggering inflammatory reaction in the vasculature.

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