Identification of Candidate Genes Downstream of TLR4 Signaling after Ozone Exposure in Mice: A Role for Heat-Shock Protein 70

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BACKGROUND: Toll-like receptor 4 (TLR4) is involved in ozone (O3)-induced pulmonary hyperpermeability and inflammation, although the downstream signaling events are unknown.

OBJECTIVES: The aims of our study were to determine the mechanism through which TLR4 modulates O3-induced pulmonary responses and to use transcriptomics to determine potential TLR4 effector molecules.

METHODS: C3H/HeJ (HeJ; Tlr4 mutant) and C3H/HeOuJ (OuJ; Tlr4 normal) mice were exposed continuously to 0.3 ppm O3 or filtered air for 6, 24, 48, or 72 hr. We assessed inflammation using bronchoalveolar lavage and molecular analysis by mRNA microarray, quantitative RT-PCR (real-time polymerase chain reaction), immunoblots, immunostaining, and ELISAs (enzyme-linked immunosorbent assays). B6-Hspa1a/Hspa1b1m1Dis/NIEHS (Hsp70−/−) and C57BL/6 (B6; Hsp70+/+ wild-type control) mice were used for candidate gene validation studies.

RESULTS: O3-induced TLR4 signaling occurred through myeloid differentiation protein 88 (MyD88)-dependent and -independent pathways in OuJ mice and involved multiple downstream pathways. Genomewide transcript analyses of lungs from air- and O3-exposed HeJ and OuJ mice identified a cluster of genes that were significantly up-regulated in O3-exposed OuJ mice compared with O3-exposed HeJ mice or air-exposed controls of both strains; this cluster included genes for heat-shock proteins (e.g., Hspa1a, Hsp70). Moreover, O3-induced inflammation, MyD88 up-regulation, extracellular-signal–related kinase-1/2 (ERK1/2) and activator protein-1 (AP-1) activation, and keratinocyte-derived chemokine (KC) protein content were significantly reduced in Hspa1a/Hspa1b1m1Dis (Hsp70−/−) compared with Hsp70+/+ mice (p < 0.05).

CONCLUSIONS: These studies establish that Hsp70 is an effector molecule downstream of TLR4 and is involved in the regulation of O3-induced lung inflammation by triggering similar pathways to TLR4. These novel findings may have therapeutic and preventive implications for inflammatory diseases resulting from environmental exposures.


Ozone (O3) is a principal oxidant of pollution and is generated when nitrous oxides and volatile organic compounds react with sunlight and molecular oxygen in the presence of ultraviolet light (Mudway and Kelly 2000). In the United States, an estimated one-half of individuals exposed to O3 are at risk of developing pulmonary disease (American Lung Association 2011), and approximately 118 million U.S. residents live in cities out of attainment (i.e., in which the O3 levels are higher than federal regulations) or in regions that approach or exceed the National Ambient Air Quality Standard (NAAQS) set for O3 [U.S. Environmental Protection Agency (EPA) 2011]. O3 exposure may lead to premature death (Bell et al. 2005; Jerrett et al. 2009), dyspnea (Horstman et al. 1990), wheezing and coughing (Triche et al. 2006), increased susceptibility to lung infection (Hollingsworth et al. 2007), increased risk of asthma attacks (Burnett et al. 1997; Desqueyroux et al. 2002; Gent et al. 2003), reduced birth weight (Salam et al. 2005), and new-onset asthma in children living in regions with high concentrations of O3 (McConnell et al. 2002). In 2008, the NAAQS was reduced from 0.080 ppm to 0.075 ppm because of increased concern about human health risks, and further reduction of O3 levels in the U.S. is currently under review (U.S. EPA 2010).

In mice, O3 causes pulmonary inflammation [indicated by polymorphonuclear leukocyte (PMN) infiltration] and hyperpermeability [indicated by increased proteins in bronchoalveolar lavage fluid (BALF)] (Kleeberger et al. 1993a, 1993b, 1997). A genomewide linkage analysis found a susceptibility quantitative trait locus for O3-induced hyperpermeability (Kleeberger et al. 2000), and toll-like receptor 4 (Tlr4) was identified as a candidate gene. Tlr4 has been implicated in innate immunity and endotoxin (specifically lipopolysaccharide [LPS]) susceptibility (Kopp and Medzhitov 1999; Poltorak et al. 1998; Qureshi et al. 1999). Significantly higher BALF protein concentrations and lung tissue Tlr4 mRNA were found in C3H/HeOuJ (OuJ; Tlr4 normal) mice after exposure to O3, compared with C3H/HeJ (HeJ; Tlr4 dominant negative mutant) mice (Kleeberger et al. 2000). In addition, Tlr4 deficiency protected against O3-induced airway hyperresponsiveness (Hollingsworth et al. 2004). Together, these results indicate that the chromosome 4 quantitative trait locus explains a substantial portion of the genetic variance in O3-induced hyperpermeability and support Tlr4 as a susceptibility gene.

TLR4 protein is activated by the binding of ligand(s), which leads to the recruitment of adaptor molecules, including myeloid differentiation protein 88 (MyD88) (Akira and Takeda 2004). For example, LPS in a complex with LPS-binding protein, CD14, and myeloid differentiation protein-2 binds to TLR4 and is therefore an exogenous ligand (Poltorak et al. 1998). The MyD88-dependent pathway then signals through mitogen-activated protein kinase (MAPK), nuclear factor κB (NFκB), and/or activator protein-1 (AP-1) pathways to induce downstream genes such as TNFα (tumor necrosis factor α), ILβ (interleukin-1β), and KC (keratinocyte-derived chemokine) in response to ligands such as LPS (Akira and Takeda 2004). A MyD88-independent pathway signals through TRAM (Toll-interleukin-1 receptor domain containing adaptor protein–inducing interferon-β (TRIF)-related adaptor molecule) and TRIF binding, which mediates the activation of interferon regulatory factor 3, leading to the induction of interferon-α/β–inducible genes, such as Ifn10 (IFNγ-inducible 10 kDa protein) (Akira and Takeda 2004).

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The objective of this study was to identify the pathways through which TLR4 mediates O\textsubscript{3}-induced lung inflammation and injury and to validate the functional role of downstream effectors. We used a transcriptomics approach to address the hypothesis that TLR4-specific changes in gene expression associate with differential susceptibility to O\textsubscript{3}-induced pulmonary responses in OuJ and HeJ mice. We then tested one of these pathways [heat-shock protein 70 (HSP70)] using a knockout mouse model to confirm the functional importance of HSP70 to O\textsubscript{3} responsiveness.

**Materials and Methods**

*Animals and\ O\textsubscript{3} exposures*. C3H/HeJ (HeJ) and C3H/HeOuJ (OuJ) mice (males 6 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME). B6;129S7-Hspa1a\textsuperscript{-/-}\textsuperscript{tm1Dix}Mmcdd mice were crossed onto the B6 background (Jackson Laboratories) until the seventh generation (N6) [called B6-Hspa1a\textsuperscript{-/-}\textsuperscript{tm1Dix}Mmcdd (NIEHS (Hsp70+/+))], when they were intercrossed with siblings. These mice are thus 96.9% B6 (Silver 1995). Maleagematched (6–8 weeks of age) wild-type controls (C57BL/6J; Hsp70+/+) (Jackson Laboratories) were also used for these studies. Mice were fed laboratory chow (NIH-07; Zeigler Brothers, Gardeners, PA) and given water *ad libitum* before and during exposures. OuJ and HeJ mice were exposed to O\textsubscript{3} as described previously (Yoon et al. 2007), and all other exposure studies were done at the Michigan State University Containment Facility as described previously (Wagner et al. 2003). Mice were exposed continuously in whole-body chambers to filtered air or 0.3 ppm O\textsubscript{3} for 6, 24, 48, or 72 hr (23.5 hr/day). Immediately after exposure, mice were euthanized by sodium pentobarbital (104 mg/kg). Time points used for each phenotype were based on kinetics identified in previous studies [chemokines (Yoon et al. 2007) and transcription factors, gene expression, and MAPK (Cho et al. 2007)]. All mice were treated humanely and with regard for alleviation of suffering. All animal use and procedures were approved by the institutional animal care and use committees of the National Institute of Environmental Health Sciences and Michigan State University.

**BALF analysis.** For some OuJ and HeJ mice and all Hsp70+/+ and Hsp70−/− mice, the left lobe of the lung was clamped and the right lung lobes were lavaged based on body weight (17.5 mL/kg). We performed lavage analysis to determine cell differentials and total protein (indicator of lung hyperpermeability) as described elsewhere (Bauer et al. 2010; Kleeberger et al. 1997, 2000). The left lobes (all strains) were either snap frozen in liquid nitrogen or inflation fixed in 10% neutral buffered formalin and processed for histology.

**RNA extraction and Affymetrix GeneChip array processing.** Total RNA was extracted from left lobes of lung from OuJ and HeJ mice exposed to air or 6, 24, or 48 hr O\textsubscript{3} (n = 3/treatment group) and homogenized in TRIZOL reagent (Invitrogen, Gaithersburg, MD [for details, see Supplemental Material, p. 3 (doi:10.1289/ehp.1003326)]). Right lung lobes from the same animals were used for quantitative real-time polymerase chain reaction (qRTPCR) confirmation. Processing of templates for GeneChip Analysis followed methods described in the GeneChip Expression Analysis Technical Manual, Revision Three (Affymetrix Inc. 2005–2009).

**Transcriptomic analysis.** CEL format files were imported into GeneSpring (version 7.0; Silicon Genetics, Redwood City, CA) for statistical analyses and characterization of data [for details, see Supplemental Material pp. 3–5 (doi:10.1289/ehp.1003326)]. All samples were normalized in GeneSpring to OuJ (wild-type) air controls (Cho et al. 2005), and k-means cluster analyses were performed.

**qRTPCR confirmation of array data and TLR4 downstream adaptors.** qRTPCR was performed using either the Taqman assay or the Sybr green assay (both from Applied Biosystems, Foster City, CA) following the manufacturer’s instructions [for details of qRTPCR, see Supplemental Material, p. 5; for primers, see Supplemental Material, Table 2 (doi:10.1289/ehp.1003326)].

**Immunohistochemical detection of HSP70.** HSP70 was detected in lung sections from O\textsubscript{3}-exposed OuJ and HeJ mice using a specific HSP70 antibody (EMD Chemicals, Gibbstown, NJ) and a labeled streptavidin-biotin (LSAB) secondary antibody (DAKO, Carpinteria, CA). Immunodetection of HSP70 was evaluated as previously described (Cho and Kleeberger 2007; Cho et al. 2005).

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**Figure 1.** Differential response (mean ± SE) to air or 0.3 ppm O\textsubscript{3} in lungs of OuJ and HeJ mice. Total protein concentration (a marker of lung permeability; A) and number of PMNs recovered (B) from the BALF (n = 3–7 mice/group, repeated once). Expression of MydbB (C) and Trif mRNA (D) (normalized RNA; n = 3–5 mice/group, repeated once). Differential NF-kB (E) and AP-1 (F) DNA binding activity (measured at a wavelength of 450 nm) (n = 3–4 mice/group, repeated once).

*p < 0.05 compared with air-exposed controls. **p < 0.05 compared with O\textsubscript{3}-exposed HeJ mice.
NFκB and phosphorylated c-Jun (p-c-Jun) nuclear binding activity. We used 8 μg nuclear protein prepared from left lung lobes of mice 6 and 24 hr after O₃ exposure (Active Motif, Carlsbad, CA) to determine specific binding of p-c-Jun and NFκB p65 proteins using transcription factor ELISA (enzyme-linked immunosorbent assay; TransAM kit, Active Motif), similar to that described for previous studies (Rondini et al. 2010).

Immunoblot detection of MAPK and HSP70. For immunoblot detection of MAPK and HSP70, we used primary antibodies specific for MAPK (Cell Signaling, Danvers, MA), β-actin (Sigma, St. Louis, MO), and HSP70 (EMD Chemicals), followed by horseradish peroxidase secondary antibodies ( Pierce: Thermo Fisher, Rockford, IL). Following protein extraction and separation, we used 75–100 μg protein from control and O₃ exposed lungs for immunoblotting according to previously published methods (Bauer et al. 2005; Cho et al. 2005; Rondini et al. 2010). Immunoblotting was then performed using the BioRad ChemiDoc illumination system with Quality One software (Bio-Rad, Carlsbad, CA).

ELISA for keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2). We analyzed KC (CXCL1) and MIP-2 in BALF using ELISA kits from R&D Systems (Minneapolis, MN) according to manufacturer’s instructions.

Statistics. Data are expressed as mean ± SE. We used two-way analysis of variance to evaluate the effects of exposure and strain on BALF phenotypes, qRT-PCR, ELISAs, transcription factors, and immunoblotting studies. Student Newman-Keuls test was used for a posteriori comparisons of means; statistical significance was defined as p < 0.05. All analyses were performed using a commercial statistical analysis package (SigmaStat, version 3.5; Jandel Scientific Software, San Rafael, CA).

Results

TLR4 signaling in response to O₃. We found significantly greater mean total protein (at 24, 48, and 72 hr) and numbers of BALF PMNs (at 48 hr) in O₃-exposed OuJ mice compared with O₃-exposed HeJ mice (Figure 1A,B), as described previously (Kleeberger et al. 2000). Epithelial cell numbers in BALF were also significantly different (24 and 72 hr) between OuJ and HeJ strains (see Supplemental Material, Table 3 [doi:10.1289/ehp.1003326]). After 24 hr O₃ exposure, transcript levels of the adaptor molecules Myd88 and Traf were upregulated in OuJ mice compared with the three other treatment groups (OuJ controls and both air- and O₃-exposed HeJ mice; Figure 1C,D), similar to changes found previously for Tlr4 mRNA expression (Kleeberger et al. 2000).

Transcription factors NFκB (p65 subunits) and AP-1 (p-c-Jun) were likewise significantly higher after 24 hr O₃ exposure in OuJ mice compared with O₃-exposed HeJ mice or air-exposed controls of both strains (Figure 1E,F; p < 0.05).

Three primary MAPKs—ERK1/2 (extracellular-signal–related kinase-1/2), JNK (c-Jun N-terminal kinase), and p38—are involved in response to O₃ (Cho et al. 2007) and LPS-mediated TLR4 signaling in the lung (Chanteux et al. 2007; Fang et al. 2007). After 24 hr O₃ exposure, ERK1/2 and p38 proteins were significantly elevated in OuJ mice compared with O₃-exposed and air-exposed HeJ mice, but after 48 hr of exposure ERK1/2 and p38 were significantly increased in O₃-exposed HeJ mice compared with O₃-exposed OuJ mice or controls of both strains (Figure 2A,B). JNK activity was unchanged in both strains after O₃ exposure (data not shown). Furthermore, neutrophil chemoattractant KC (CXCL1) protein levels were significantly elevated in OuJ compared with HeJ mice after 24 and 48 hr O₃ exposure (Figure 2C); KC transcript levels were also elevated after 24 hr O₃ (data not shown). We found no effects of O₃ on protein levels of MIP-2, another neutrophil chemoattractant, in either strain (data not shown).

Transcriptomic analysis to identify TLR4 effector genes. k-Means clustering identified five clusters after the initial filtering, which determined 200 transcripts with significant interactions for strain and time with 2-fold changes in gene expression (p < 0.05; data not shown). We focused subsequent analyses on 39 genes that were distributed in three distinct cluster patterns [clusters 2, 4, and 5; see Supplemental Material, Excel Table 1A–C (doi:10.1289/ehp.1003326)]. In cluster 2 (24 genes; see Supplemental Material, Table 4A and Supplemental Material, Figure 1A), expression of transcripts was significantly greater in OuJ mice after 24 and 48 hr O₃ exposure compared with air-exposed controls of both strains and O₃-exposed HeJ mice. Analysis using the Database for Annotation, Visualization, and Integrated Discovery [DAVID (Huang et al. 2009a, 2009b); see Supplemental Material, Excel Table 1C] identified protein folding, response to heat and stress, response to temperature stimulus, chaperone, and response to protein stimulus (p-values ranged from 4.35 × 10⁻⁹ to 4.0 × 10⁻²) as major functional categories. Five heat-shock proteins in the antigen processing and presentation KEGG pathway [Hsp10, Hsp90aa1, Hsp90ab1, Hsp5, Hsp70; (Kyoto Encyclopedia of Genes and Genomes; Kanehisa Laboratories 2010)] were particularly notable (see Supplemental Material, Figure 1B).

In HeJ mice, cluster 4 transcripts [10 genes; see Supplemental Material, Table 4B (doi:10.1289/ehp.1003326)] were expressed at significantly higher levels in mice exposed to O₃ for 24 hr compared with air-exposed controls, and most genes were decreased after 48 hr. In contrast, we found minimal changes in OuJ mice (data not shown). DAVID analysis categorized 6 of the 10 genes in this cluster...
Candidate gene validation. To confirm some of the genes identified using expression profiling, we performed qRTPCR on genes from all three focus clusters in the same samples used for the microarray analysis [Supplemental Material, Figure 2 (doi:10.1289/ehp.1003326)]. Hspa1b mRNA expression did not significantly change after 48 or 72 hr O₃ exposure in HeJ mice, in contrast to the array results (see Supplemental Material, Figure 2B). Because a significant number of genes were identified in the heat-shock protein functional category, we focused on HSP70 (the protein encoded by Hspa1b) for further validation. In HeJ mice, HSP70 protein expression was not changed after O₃ exposure. However, HSP70 protein expression was significantly elevated in O₃-exposed OuJ mice compared to OuJ controls and both air- and O₃-exposed HeJ mice (Figure 3A). HSP70 immunostaining confirmed up-regulation and localization in alveolar macrophages and epithelial cells in OuJ mice (Figure 3B).

HSP70 involvement in O₃-induced responses. To further investigate the role of HSP70 in this model, we exposed Hspa1b−/− and Hspa1b+/+ mice to air and 0.3 ppm O₃. Relative to Hspa1b+/+ mice, BALF total protein (at 24 and 48 hr), PMNs (at 24 and 48 hr), and macrophages (at 48 and 72 hr) were significantly reduced in Hspa1b−/− mice after O₃ exposure (Figure 4). Epithelial cell numbers were not significantly different between strains [see Supplemental Material, Table 3 (doi:10.1289/ehp.1003326)]. Histopathology also demonstrated increased cellularity and thickening of the airways in Hspa1b−/− mice (data not shown). We found significantly increased Hspa1b gene expression in Hspa1b−/− mice after 48 hr of O₃ compared with Hspa1b+/+ controls (data not shown).

After 24 hr exposure, transcript levels of Myd88 were significantly increased in O₃-exposed Hspa1b−/− mice compared with O₃-exposed Hspa1b+/+ mice and controls of both strains (Figure 5B). Trif mRNA expression was significantly higher in Hspa1b−/− mice than in Hspa1b+/+ mice (24 hr O₃ exposure), but it was not significantly different from controls of either strain (Figure 5D).

Figure 3. Immunodetection of 70 HSP70 protein in lungs of OuJ and HeJ mice in response to 48 hr exposure to air or 0.3 ppm O₃. (A) HSP70 expression detected by immunoblotting; HSP70 protein was normalized to β-actin and is expressed as fold change relative to the OuJ air controls (mean ± SE; n = 3 mice/group; repeated once). (B) Photomicrographs showing immunohistochemical staining of HSP70. Bar = 20 µM.

Figure 4. Inflammatory parameters measured in BALF from Hspa1b−/− and Hspa1b+/+ mice after exposure to air or 0.3 ppm O₃. (A) Total protein concentration. (B) Total number of PMNs. (C) Total number of macrophages in BAL from Hspa1b−/− and Hspa1b+/+ mice. Values shown are mean ± SE; n = 3–6 mice/group, repeated four times.

*p < 0.05, compared with air-exposed controls. **p < 0.05, compared with O₃-exposed HeJ mice.

*p < 0.05, compared with air-exposed controls. **p < 0.05, compared with 72 hr O₃ exposure. ***p < 0.05, compared with 24 hr O₃ exposure.
TLR4 was also significantly increased in the Hsp70+/+ but not Hsp70−/− mice after 6 and 24 hr O3 exposure (Figure 5A). NFkB p65 binding activity was significantly increased in both genotypes after 24 hr exposure to O3 compared with controls, but we found no significant differences between genotypes (Figure 5D). Binding activity of p-c-Jun was significantly increased in O3-exposed animals of both genotypes compared with controls, but was significantly higher in Hsp70+/+ mice than in Hsp70−/− mice (Figure 5E). ERK1/2 protein was also significantly elevated in O3-exposed Hsp70+/+ mice compared with Hsp70−/− controls and both air- and O3-exposed Hsp70−/− mice (Figure 5F), whereas p38 was elevated in both strains after 24 hr exposure to O3 compared with controls (Figure 5G). JNK was unchanged in both strains (data not shown), similar to OuJ and HeJ mice. In the 24-hr exposure group, KC protein levels were significantly elevated in O3-exposed Hsp70+/+ mice compared with O3-exposed Hsp70−/− mice and air controls of both genotypes (Figure 5H; p < 0.05).

In the 48-hr exposure group, KC levels in O3-exposed mice of both genotypes were significantly greater than those in respective air controls. We found a significant decrease in MIP-2 protein levels in O3-exposed Hsp70−/− mice compared with the corresponding controls in the 24-hr group, but MIP-2 levels were significantly increased in both genotypes after 48 hr of O3 exposure compared with air controls of both genotypes (Figure 5I).

**Discussion**

The objective of the present study was to determine the mechanisms through which TLR4 modulates O3-induced inflammation and injury. TLR4 signaling in response to O3 involved significant elevations in MyD88-dependent and -independent (TRIF) pathways in Tlr4 (OuJ) but not Tlr4 mutant (HeJ) mice, suggesting that both effector pathways are involved in mediating O3-induced inflammation and hyperpermeability. Additionally, we observed significant strain differences in NFkB and AP-1 transcription factors, MAPKs, and chemokines, in control and O3-exposed Hsp70+/+ and Hsp70−/− mice. Expression of Tlr4 (A), Myd88 (B), and Trif (C) transcripts (n = 3–5 mice per treatment group; repeated twice). Nuclear binding activity of p65 NFkB (D) and p-c-Jun (E) (n = 3–4 mice per treatment group; repeated once). Quantitation of phosphorylated MAPK activity [pERK1/2 (F) and pp38 (G)]. Proteins were normalized to the unphosphorylated form of the same protein (total MAPK), n = 3 mice/group, repeated three times (duplicate samples per study were run on the same gel followed by normalization to total MAPK). Protein levels of KC (H) and MIP-2 (I) in BALF (n = 3–4 mice/group; repeated once). Values shown are mean ± SE. *p < 0.05, compared with air-exposed controls. vs O2. **p < 0.05, compared with O3-exposed Hsp70−/− mice.

This is the first study to suggest a panel of HSFs (Dnaja1, Dnaja4, Hsp90aa1, Hsp90ab1, and Hspa1b) are involved in mediating O3-induced pulmonary injury and inflammation. However, it is important to note that although there are many similarities in O3 sensitivity between the B6 (Hspa1b wild-type) and OuJ (Tlr4 wild-type) mice, genes other than Tlr4 likely contribute to the enhanced O3 sensitivity in these two strains relative to Hspa1b-deficient and Tlr4 mutant mice.

![Figure 5](image-url)
Hspa1b, Hspa5, and Hspa8) as effector genes in response to O3-induced TLR4 signaling. The HSP70 family of proteins is highly conserved evolutionarily and includes multiple genes (e.g., Hspa9, Hspa11, Hspa4a, Hspa1b, Hspa8) (Daugaard et al. 2007; Huang et al. 2001). Hspa1a and Hspa1b are located on mouse chromosome 17 and human chromosome 6, respectively (Daugaard et al. 2007; Huang et al. 2001) and encode nearly identical proteins (Hsp70.1 and Hsp70.3, 99% homologous). Hspa1b was also recently identified as a candidate susceptibility gene in the O3 susceptibility quantitative trait locus Linf2 on chromosome 17 (Bauer et al. 2010). Hsp70.1 and Hsp70.3 (HSP70) have been implicated in stress regulation, including heat shock, oxidative stress, and other environmental stresses, such as ultraviolet light exposure (Daugaard et al. 2007; Su and Gordon 1997; Zhou et al. 1998).

HSP70 is ubiquitously expressed in mouse and human lung (Sartori and Scherrer 2003), and is localized intra- and extracellularly, although the mechanism involved in HSP70 secretion is unclear (Mambula et al. 2007; Wheeler and Wong 2007). Augmented HSP70 expression has been reported in the lungs of individuals with asthma, acute lung injury, respiratory syncytial virus infection, and cancer (Sartori and Scherrer 2003). HSP70 protein expression was also elevated in isolated lavage cells (primarily macrophages) and whole-lung guinea pig and rat homogenates (Su and Gordon 1997) after acute O3 exposure (Servais et al. 2005; Su and Gordon 1997). Interestingly, acute O3 exposure did not trigger the induction of the Hsp70-1 promoter in a transgenic mouse model (Wirth et al. 2002); however, higher O3 levels and different mouse strains were used than in the studies noted above. Rats exposed chronically to O3 also had increased HSP70 (Wong et al. 1996); however, primates exposed chronically to O3 had repressed HSP70, among other HSPs (Wu et al. 1999). The discrepancy between acute and chronic O3 exposure models may reflect differences in species, strain, and/or exposure protocol.

O3-induced oxidative stress likely results from cyclical and self-generating reactions forming highly unstable radical and nonradical reactive oxygen species, such as aldehydes, hydroxyl radical, and hydrogen peroxide (Pryor et al. 1995). However, the role of HSP70 in O3-induced oxidative stress responses is unclear. Pulmonary HSP70 is induced by cadmium in mice (Wirth et al. 2002), sodium arsenite in guinea pigs (Su and Gordon 1997), and hyperthermia in rats (Wheeler and Wong 2007), among other oxidative stress-inducers, and is hypothesized to elicit a protective or adaptive response in the respiratory epithelium after initial exposure (Wheeler and Wong 2007). The present study has implicated HSP70 as important to the progression of O3-induced lung injury and underscores the complex roles of HSPs in mediating oxidant-induced lung injury.

We also found that, in mice with impaired TLR4 signaling (H6J), the genes involved in mediating the downstream effects in response to O3 were primarily in the metal-binding functional category (C4H1N, M11, M21, M12 and M2- deficient mice are more susceptible to O3-induced responses; thus, metallo-oxin 1 and 2 appear to be protective against the effects of O3 (Inoue et al. 2008). Another gene that we previously identified as differentially expressed between Ouj and H6J mice (Marco) is also protective against O3-induced lung injury (Dahl et al. 2007).

In vivo administration of HSP70 induced the TLR4 pathway (Chase et al. 2007); when applied to human bone-marrow–derived PMNs in vitro, HSP70 induced the production of KC in a TLR4-dependent manner (Wheeler et al. 2009). In our model, we hypothesize that HSP70 is in part responsible for triggering the initial increase in BALF inflammatory phenotypes observed in the Ouj and Hsp70−/− mice. We provide evidence that after the initial activation, HSP70 signals through MyD88 only; thus, another pathway is likely involved in TLR4 signaling involving the MyD88-independent (TRIF) pathway. We observed a delayed ERK1/2 activation response in both models (Tlr4 and Tlr4−/−), another indication that the two models are linked temporally. p38 was differentially expressed between Ouj and H6J mice but not between Hsp70−/− and Hsp70−/− mice, suggesting that p38 is induced through the MyD88-independent pathway. However, unlike the involvement of JNK downstream of TLR4 inactivation in the OuJ and SR-AI/II. J Clin Invest 117(3):757–764.

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