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Interaction of SOCS3 with NonO attenuates IL-1β-dependent *MUC8* gene expression

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ABSTRACT

The intracellular negatively regulatory mechanism which affects IL-1β-induced *MUC8* gene expression remains unclear. We found that SOCS3 overexpression suppressed IL-1β-induced *MUC8* gene expression in NCI-H292 cells, whereas silencing of SOCS3 restored IL-1β-induced *MUC8* gene expression. Sequentially activated ERK1/2, RSK1, and CREB by IL-1β were not affected by SOCS3, indicating that SOCS3 has an independent mechanism of action. Using immunoprecipitaion and nano LC mass analysis, we found that SOCS3 bound NonO (non-POU-domain containing, octamer-binding domain protein) in the absence of IL-1β, whereas IL-1β treatment dissociated the direct binding of SOCS3 and NonO. A dominant-negative SOCS3 mutant (Y204F/Y221F) did not bind to NonO. Interestingly, SOCS3 overexpression dramatically suppressed *MUC8* gene expression in cells transfected with wild-type or siRNA of NonO. Moreover, silencing of SOCS3 dramatically increased NonO-mediated *MUC8* gene expression caused by IL-1β compared to NonO overexpression alone, suggesting that SOCS3 acts as a suppressor by regulating the action of NonO.

Understanding the mechanisms that lead to increased mucus hypersecretion in respiratory diseases is important for developing new therapeutic strategies. Even though the importance of MUC8 in airway mucosal inflammation has been emphasized in our previous studies [1–3], the regulation of *MUC8* gene expression is still unclear. Since *MUC8* mRNA levels were up-regulated in chronic sinusitis with polyps [4], expression may be related to mucus hypersecretion and/or hyperviscosity in airway mucosa. However, further molecular studies have been limited because only short partial sequences of the *MUC8* gene have been identified.

Suppressor of Cytokine Signaling (SOCS) proteins are members of negative feedback regulators of the Janus kinase (Jak)/signal transducer and activator of transcription (STAT), or receptor tyrosine kinase pathways [5,6]. To date, eight SOCS subtypes, cytokine-inducible SH2-containing protein (CIS), and SOCS1-7, have been identified [7] and they consist of a Src homology 2 (SH2) domain, SOCS box, and variable N-terminal region. The main physiological function of SOCS is the negative regulation of Jak/STAT-dependent IL-6 signaling [8,9]. IL-6-dependent Jak/STAT signaling, especially SOCS3, inhibits toll-like receptor (TLR) 3 signaling [10], IL-1 signaling [11,12], and interferon-γ signaling [13]. Thus, the concept of SOCS3 as a suppressor of IL-6-mediated Jak/STAT signaling should be added to its characterized roles as a modulator of biological functions [12].

The Non-POU-domain containing, octamer-binding domain protein (NonO) has been known as one of the coregulators of androgen receptors belonging to the nuclear receptor superfamily [14]. $p54^{nrb}$ (human) and NonO (mouse) are highly homologous to the C-terminus splicing factor, praline- and glutamine-rich (SFPQ, previously known as PSF). These proteins are members of the *Drosophila* behavior, human splicing (DBHS)-containing protein [15]. DBHS-containing proteins are involved in various nuclear events, such as DNA replication, transcription, and mRNA processing [16]. NonO protein forms a protein complex with various proteins to regulate gene expression in the nucleus [16].

In the present study, we examined an inhibitory effect of SOCS3 on IL-1β-induced *MUC8* gene expression and identified the SOCS3-binding partner for regulating mucus production. We show that SOCS3 negatively regulated IL-1β-induced *MUC8* gene expression. In addition, SOCS3 regulates the action of NonO to suppress *MUC8* transcriptional activation.

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Materials and methods

Materials. IL-1β and MG132 were purchased from R&D system (Minneapolis, MN) and Calbiochem (EMD Chemicals Inc.; Darmstadt, Germany), respectively. Phospho-specific antibodies were purchased from Cell Signaling (Beverly, MA), SOCS3 and NonO antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and Upstate (Lake Placid, NY), respectively. All siRNAs were synthesized by Bioneer (Daejeon, Korea) [GUUUACAAUCUGCCUCAAU(dTdT) for SOCS3, GUCCAACGAACUGCUGGAA(dTdT) for NonO, and CCUACGCCACCAAUUUCGU (dTdT) for negative control].

Nano LC. MS/MS analysis was performed on an agilent 1100 Series nano-LC and LTQ-mass spectrometer (Thermo Electron, San Jose, CA). The capillary column used for LC–MS/MS analysis (150 × 0.075 mm) was obtained from Proxeon (Odense M, Denmark) and slurry packed in house with 5 μm, 100 Å pore size Magic C18 stationary phase (Michrom Bioresources, Auburn, CA). The mobile phase A for the LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was set up to give a linear increase from 5% B to 35% B in 50 min and from 40% B to 60% B in 20 min and from 60% B to 80% B in 5 min. The flow rate was maintained at 300 nl/min after splitting. Mass spectra were acquired using data-dependent acquisition with full mass scan (400–1800 m/z) followed by MS/MS scans. Each MS/MS scan acquired was an average of one microscan on the LTQ. The temperature of the ion transfer tube was controlled at 200 °C and the spray was 1.5–2.0 kV. The normalized collision energy was set at 35% for MS/MS. Sequest software was used to identify the peptide sequence. For high confidence results, deltaCn $\geqslant$ 0.1 and Rsp $\leqslant$ 4 and Xcorr $\geqslant$ 1.5 with charge state 1+, Xcorr $\geqslant$ 2.0 with charge state 2+, and Xcorr $\geqslant$ 2.5 with charge state 3+, peptide probability > 0.1, were used as cutoff for protein identification. Peptides were allowed to be variably oxidized at methionine residues and to be variably carboxyamidomethylated and carboxymethylated at cystein.

Other methods. Other methods in this study have been described previously [1,17].

Statistical analysis. The data are presented as the means ± SD of at least three independent experiments. Where appropriate, statistical differences were assessed by Wilcoxon Mann–Whitney tests. A p value less than 0.05 was considered statistically significant.

Results

SOCS3 suppressed IL-1β-induced MUC8 gene expression

In our previous studies, IL-1β, a proinflammatory cytokine, could induce *MUC5AC* and *MUC8* gene expression in both normal human nasal epithelial (NHNE) cells and NCI-H292 cells [1,17]. Nonetheless, there is little published report regarding the negatively regulatory mechanism which affects IL-1β-induced *MUC5AC* and *MUC8* gene expression in the airway. Since many cytokine signaling pathways were inhibited by SOCS3 [18], we examined whether SOCS3 can negatively regulate IL-1β-induced *MUC5AC* and *MUC8* gene expression. First, to examine if IL-1β can alter *SOCS3* gene expression in NCI-H292 cells, PCR analysis was performed. Cells were treated with various doses of IL-1β for 24 h. IL-1β induced *MUC5AC* and *MUC8* gene expression in a dose-dependent manner, whereas *SOCS3* gene expression was dramatically suppressed in a dose-dependent manner (Fig. 1A). To determine the inhibitory effects of SOCS3 in IL-1β-mediated *MUC5AC* and *MUC8* gene expression, cells were transfected with wild-type SOCS3 and siRNA-SOCS3. Overexpressed SOCS3 dramatically suppressed IL-1β-induced *MUC5AC* and *MUC8* gene expression, whereas siRNA-SOCS3 much increased IL-1β-induced *MUC8* gene expression compared to IL-1β treatment alone (Fig. 1B). Interestingly, *MUC5AC* gene expression was not restored by siRNA-SOCS3.

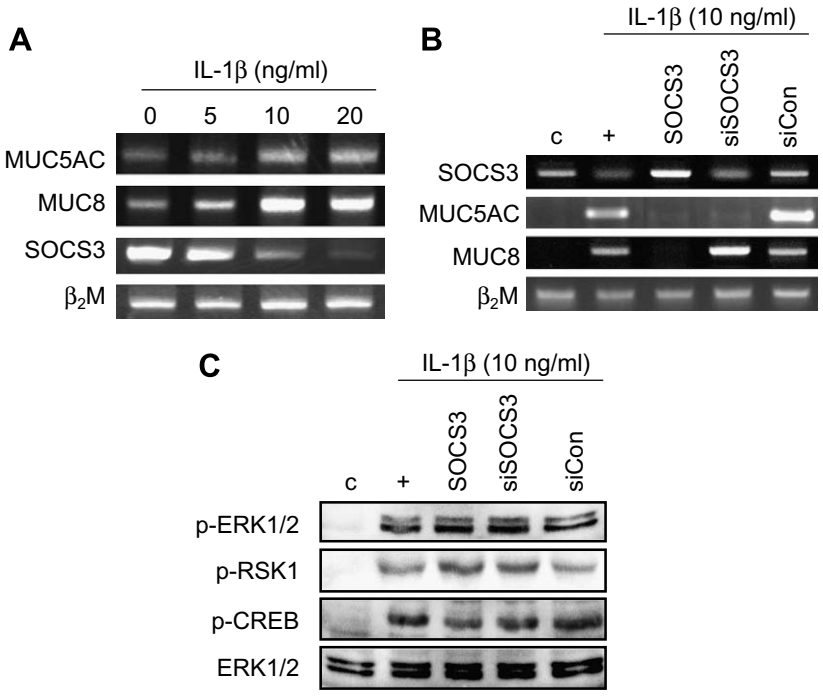

Fig. 1. SOCS3 overexpression suppressed IL-1β-induced *MUC8* gene expression in human airway epithelial cells. (A) NCI-H292 cells were treated for 24 h with IL-1β at the indicated concentrations. Cell lysates were harvested for RT-PCR. (B) Cells transfected with construct encoding wild-type SOCS3, siRNA-SOCS3, or siRNA-control were treated for 24 h with IL-1β. Cell lysates were harvested for PCR. The figures are representative of three independent experiments. (C) After cells were transfected with construct a wild-type SOCS3, siRNA-SOCS3, or siRNA-control, cells were treated with IL-1β for either 15 min (for ERK1/2 activation) or 30 min (for RSK1 and CREB activation; see ref. 1) prior to the collection of cell lysates. Total ERK1/2 expression was used as a loading control.

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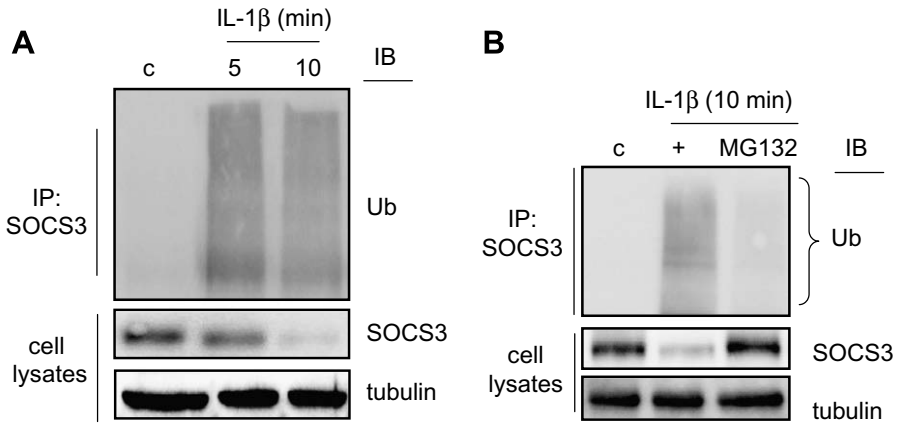

Fig. 2. IL-1β mediated the ubiquitination of SOCS3. (A) Confluent and quiescent cells were treated for the indicated times (min) with IL-1β. Total cell lysates were then immunoprecipitated with anti-SOCS3 antibody and blotted with phosphor-specific tyrosine antibody (upper panel). IP: immunoprecipitation; IB: immunoblotting. The cells lysates treated by IL-1β were harvest for Western blot analysis with SOCS3 and tubulin antibodies (lower panel). (B) Cells were treated with 1 μM of MG132 for 12 h in prior to treatment with IL-1β for 10 min. Cell lysates were harvested for immunoprecipitation assay (upper panel) and Western blot analysis (lower panel). Tubulin expression was used as a loading control. The figures are representative of three independent experiments.

These results suggest that IL-1β down-regulates *SOCS3* gene expression and SOCS3 can act as an intracellular suppressor for *MUC8* gene expression during airway mucosal inflammation, but not for *MUC5AC* gene expression. Accordingly, we selected the *MUC8* gene as our target gene to study the regulatory effects of SOCS3 in the present study. Furthermore, in our previous study [1], we reported that the signal pathway of ERK1/2, RSK1, and CREB was essential for IL-1β-induced *MUC8* gene expression. We wondered if SOCS3 could inhibit this pathway. Western blot analysis was performed with cells transfected with either wild-type SOCS3 or siRNA-SOCS3. For the activation of ERK1/2 MAPK, cells were treated with IL-1β for 15 min, whereas cells were treated for 30 min to activate RSK1 and CREB. As seen in Fig. 1C, SOCS3 did not inhibit the phosphorylation of ERK1/2, RSK1, and CREB, indicating that SOCS3 did not affect the pathway we reported previously and that SOCS3 has its own pathway to negatively regulate IL-1β-induced *MUC8* gene expression.

IL-1β induced the ubiquitination of SOCS3

SOCS3 is known as a suppressor of cytokine signaling and thereby plays a crucial role on cytokine-mediated biological phenomena [12]. Thus, we examined whether IL-1β can mediate the ubiquitination of SOCS3 to maintain IL-1β signaling. IL-1β significantly increased the ubiquitination of SOCS3 at a very early time point (5 min after IL-1β treatment) and IL-1β decreased SOCS3 gene expression (Fig. 2A). In addition, 26S proteosome inhibitor, MG132 [19], dramatically inhibited the ubiquitination of SOCS3, thus stabilizing the expression of SOCS3 (Fig. 2B). These results suggest that IL-1β ubiquinates SOCS3 to play its own potential roles.

SOCS3 bound to NonO to regulate IL-1-induced MUC8 gene expression

Since SOCS3 is a multi-functional protein, we thought it might form a protein complex to exert its potential roles. To identify SOCS3-binding partners in mammalian cells, we immunoprecipitated SOCS3 complex from IL-1β-treated cells. Coomassie Blue staining detected a unique protein band in the SOCS3-immune complex that was not present in IL-1β-treated immunoprecipitations (Fig. 3A). The SOCS3-specific band was analyzed by LC mass spectrometry and it was identified as NonO protein (Fig. 3B). To validate the association of endogenous SOCS3 with NonO, immunoprecipitation of the lysates showed that SOCS3 bound to NonO without IL-1β treatment, whereas this protein complex was dissociated by IL-1β treatment (Fig. 3C). To further characterize this protein complex, dominant-negative mutant SOCS3 Y204F/Y221F construct was employed. Tyr204 and Tyr221 of SOCS3 are located in the conserved SOCS box [20]. Association of SOCS3 and NonO was not detectable due to mutant SOCS3 (Fig. 3D). These results are noteworthy because there was no report on the interaction between SOCS3 and NonO in NCI-H292 cells. These results suggest that SOCS3 interacts selectively with NonO and SOCS3 box is essential for the interaction with NonO. Interestingly, this complex is disrupted by IL-1β signaling.

SOCS3 suppressed the transcriptional transactivation of NonO

Next, NonO has been known as a multiphosphorylated protein to exert a variety of nuclear processing [21]. Thus, we performed immunoprecipitation assay with anti-phospho-Tyr antibody. As seen in Fig. 4A, the phosphorylation of NonO by IL-1β was maximally activated at 60 min and then decreased at 120 min. In addition, we asked if SOCS3 may play a role in regulating the function of NonO. We used with wild-type and siRNA constructs of SOCS3 and NonO. Interestingly, overexpressed NonO drastically increased IL-1β-induced *MUC8* gene expression. Moreover, silencing of NonO had no affect *MUC8* gene expression, indicating that NonO has a transcriptional transactivation activity. In addition, cotransfection with both wild-type SOCS3 and NonO constructs suppressed IL-1β-induced *MUC8* gene expression, whereas cotransfection with siRNA-SOCS3 and wild-type NonO constructs increased it. In contrast, cotransfection with SOCS3 and either wild-type or siRNA-NonO constructs suppressed IL-1β-induced *MUC8* gene expression. However, cotransfection with both siRNA-SOCS3 and siRNA-NonO constructs did not affect IL-1β-induced *MUC8* gene expression (Fig. 4B). These findings suggest that SOCS3 leads to enhanced recruitment of NonO by SOCS box to suppress *MUC8* transcriptional activation.

Discussion

We reported previously that IL-1β and PGE_2 could induce *MUC8* gene expression in human airway epithelial cells [1,2]. Apart from these reports, there is little published data regarding the signal pathway for stimulant-induced *MUC8* gene expression. An important reason for this is that the promoter and cDNA sequence of the *MUC8* gene have not been fully characterized. Since the mech-

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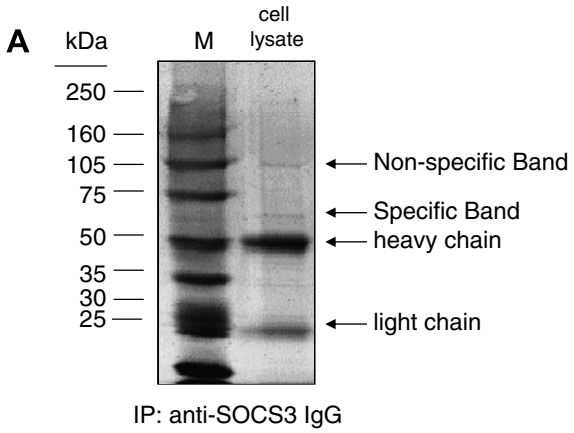

B

Band ID	Protein ID	Sequence of peptide	P value	Cross correlation value	DeltaCn	Molecular Weight (Dalton)	Accession number
NonO	Homo sapiens non-POU domain, Octamer-binding [synthetic construct]	KGIVEFSGKPAARK	3.48E-07	10.13	0.41	54344.4	30584547

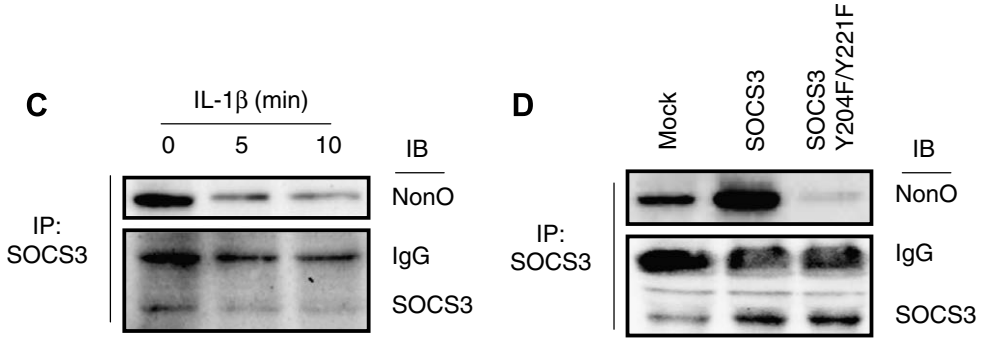

Fig. 3. Identification of NonO as a novel SOCS3-binding partner in NCI-H292 cells. (A) Confluent and quiescent cells were treated for the indicated times (min) with IL-1β. Total cell lysates were then immunoprecipitated with anti-SOCS3 antibody and were resolved by SDS–PAGE. The gel was stained with Coomassie-blue and nano LC analysis was performed. (B) The information of the identified specific band which is marked by the arrow on the right (Fig. 3A) was summarized. (C) Confluent and quiescent cells were treated for indicated times with IL-1β. Total cell lysates were immunoprecipitated with anti-SOCS3 antibody and blotted with anti-NonO antibody. The same membrane was stripped and reprobed with anti-SOCS3 antibody. IP: immunoprecipitation; IB: immunoblotting. (D) Cells were transfected with construct encoding either wild-type SOCS3 or dominant-negative SOCS3 mutant. This assay was performed in the absence of IL-1β treatment. Immunoprecipitation assay was performed with the same method (Fig. 3C). The figures are representative of three independent experiments.

anism of its negative regulation is also unknown, determining how *MUC8* gene expression is down-regulated by a suppressor to maintain homeostasis should provide additional insights into the phenomena of mucus overproduction during inflammation. Thus, we initially investigated SOCS3 as a candidate protein for down-regulating SOCS3 expression, because the main function of SOCS3 is the suppression of cytokine signaling. As Karlsen et al. reported, SOCS3 prevents IL-1β-mediated toxicity through the inhibition of iNOS- or the NF-κB-regulated proapoptotic pathway in pancreatic β cells [13,22]. Accordingly, we thought that SOCS3 may affect cytokine-induced *mucin* gene expression in human airway epithelial cells. To verify this, we investigated the inhibitory effect of SOCS3 on IL-1β-induced *MUC8* gene expression. IL-1β was shown to down-regulate *SOCS3* gene expression (Fig. 1). In contrast to our results, Yang et al. reported that SOCS3 expression was not altered by IL-1β in hepatocytes [12]. This discrepancy may be due to differences in the type of cell line studied. Thus, the question of why SOCS3 expression was decreased after treatment of IL-1β should then be addressed. It is possible that a regulatory protein activated by IL-1β accelerates SOCS3 degradation to increase *MUC8* gene expression during inflammation. We also showed that overexpressed SOCS3 has an inhibitory effect on *MUC8* gene expression, whereas silencing of SOCS3 expression impaired the inhibitory action of SOCS3 on *MUC8* gene expression (Fig. 1B), indicating that an important role of SOCS3 in the airway is the repression of IL-1β-induced MUC8 gene expression in airway epithelial cells. Our findings suggest that SOCS3 acts as an anti-inflammatory protein in human airway epithelial cells, leading us to examine the mechanism by which SOCS3 inhibited IL-1β-induced *MUC8* gene expression.

In mammalian cells, gene expression is regulated by several different processes at the transcriptional and post-transcriptional levels. Each processing is tightly controlled by a specific mechanism. Of these, ubiquitination plays critical roles for protein regulation. Recently, Ehlting et al. reported that cytokine TNF-α signaling mediated SOCS3 stabilization in RAW 294.7 cells [23]. However, in the present study, IL-1β signaling accelerated the ubiquitination of SOCS3 (Fig. 2A). This discrepancy is thought to be due to the difference in cell types studied. SOCS3 is relatively stable in 293 cells and mouse epithelial cell lines, whereas it is highly unstable in Ba/

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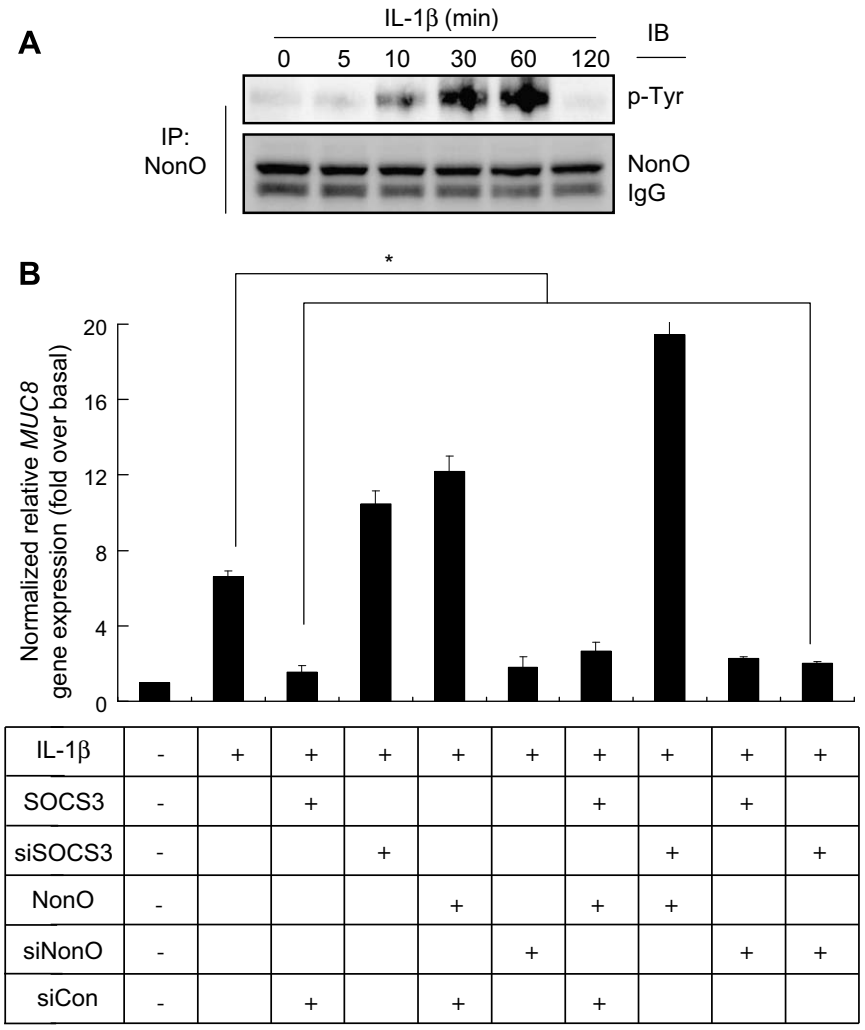

Fig. 4. Critical role of SOCS3 for the activating effect of NonO on IL-1β-induced *MUC8* gene expression in NCI-H292 cells. (A) Quiescent cells were treated for indicated times (min) with IL-1β (10 ng/ml). Total cell lysates were immunoprecipitated with NonO antibody and blotted with phosphor-specific tyrosine antibody. The same membrane was stripped and reprobed with NonO antibody. IP: immunoprecipitation; IB: immunoblotting. (B) Cells were transiently transfected with a wild-type or siRNA constructs of SOCS3 or NonO, and cells were then serum-starved and treated with 10 ng/ml of IL-1β for 24 h, after which cell lysates were harvested for real-time PCR. $^{*}p < 0.05$ compared to IL-1β alone. The figures are representative of three independent experiments.

F3, Raw, UT-7 and HepG2 cells [12,19]. It is noteworthy that IL-1β induces the degradation of SOCS3 at very early stage to eliminate the suppressor of IL-1β signaling and thereby exerts its own cytokine functions. Thus, studies on the ubiquitination of SOCS may be important for novel drug development strategies to treat cytokine-mediated respiratory diseases controlled by SOCS3.

Next, we tried to identify the SOCS3-binding partner to investigate how it regulates IL-1β-induced *MUC8* gene expression. Using immunoprecipitation and LC analysis, we identified a new SOCS3-binding partner, NonO, a known transcription factor [24]. This is reasonable because SOCS3 is localized to the nucleus [25]. However, there is no previous report regarding the interaction between SOCS3 and NonO in airway epithelial cells. According to our findings, IL-1β induced the disruption of SOCS3-NonO binding. In addition, a dominant-negative mutant (Y204F/Y221F) of SOCS box in SOCS3 did not bind to NonO (Fig. 3C and D). These results suggest that IL-1β induced dissociation of the interaction of SOCS3 with NonO and, consequently, NonO increased IL-1β-induced *MUC8* gene expression (Fig. 3). Interestingly, SOCS3 overexpression dramatically suppressed *MUC8* gene expression in cells transfected with wild-type or siRNA-NonO, whereas silencing of SOCS3 dramatically increased NonO-mediated *MUC8* gene expression caused by IL-1β compared to NonO overexpression alone. These results suggest that SOCS3 may be a key factor for negative regulation in the nucleus and may form a ternary complex to control *MUC8* gene expression in an inflammatory environment. Unfortunately, as mentioned before, full cDNA and promoter sequences of *MUC8* gene have not yet been unidentified. In addition, post-translational modification of NonO has not been fully defined [24]. These reasons make further studies of the mechanism by which *MUC8* gene expression is up/down-regulated by stimulants in the airway difficult to persue.

Taken together, these findings suggest that IL-1β induces SOCS3 degradation, resulting in increased *MUC8* gene expression. The ability of SOCS3 protein to interact with NonO is an important negative regulator of IL-1β-induced *MUC8* gene expression in the airway. This mechanistic study integrates the diverse signaling pathways involved in regulating *MUC8* gene expression.

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