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Thiocyanate-Dependent Induction of Endothelial Cell Adhesion Molecule Expression by Phagocyte Peroxidases: A Novel HOSCN-Specific Oxidant Mechanism to Amplify Inflammation

Jian-Guo Wang, Shawn A. Mahmud, Julia Nguyen, and Arne Slungaard

Both eosinophil peroxidase (EPO) and neutrophil myeloperoxidase (MPO) preferentially oxidize SCN\(^-\) to generate HOSCN, a weak, sulfhydril-reactive oxidant, as a major physiologic product. We here show that HOSCN is a uniquely potent phagocyte oxidant inducer of E-selectin, ICAM-1, and VCAM-1 expression in HUVEC as detected by Western blot and flow cytometry. EMSA and inhibitor studies show that HOSCN up-regulation of these adhesion molecules is transcriptionally mediated through a mechanism that is dependent upon activation of the NF-κB p65/p50 transcription factor and constitutively suppressed by PI3K-Akt pathway activity. HUVEC monolayers exposed to HOSCN bind 8-fold more neutrophils and 3- to 4-fold more Aml14.3D10 cells (a differentiated cell line model of mature eosinophils) than control monolayers. Blocking Ab studies confirm the involvement of E-selectin and ICAM-1 but not VCAM-1 in neutrophil adhesion and of all three in Aml14.3D10 adhesion. Intrapерitoneal injection of HOSCN evoked an 8-fold increase in neutrophil peritoneal extravasation. In addition to NF-κB, HOSCN also activates the potentially proinflammatory transcription factors Stat4, CDP, GRE, CBF, Ets-1/PEA3, and TFIID, a pattern easily distinguishable from that induced by LPS. These results suggest that phagocyte peroxidases function to amplify inflammation through a novel, HOSCN-specific oxidant mechanism.

Neutrophil myeloperoxidase (MPO) and eosinophil myeloperoxidase (MPO) are heme-containing oxidoreductases (EC1.7.1.11) that function in vivo primarily to catalyze the reaction: \(H_2O_2 + X^- + H^+ \rightarrow HOX + H_2O\), where \(X^-\) typically represents a halide or, alternatively, another suitable electron donor (1). MPO and EPO are abundant granule components of, respectively, neutrophils and eosinophils. All monocytes and some macrophages also contain appreciable amounts of MPO (2). The hydrogen peroxide substrate for these reactions is supplied by the phagocyte NADPH oxidase system that is highly active in monocytes and neutrophils and even more (up to 10-fold) active in activated eosinophils (3). The best-characterized phagocyte peroxidase system is the MPO/H\(_2\)O\(_2)/Cl\(^-\) system that generates HOCl, a potent bleaching oxidant that is orders of magnitude more microbical than the \(H_2O_2\) that fuels its generation (4, 5).

Although it is clear that the MPO system predominantly uses \(Cl^-\) to generate HOCl, the principal physiological substrates for EPO, a protein only 70% homologous to MPO (6), has remained less certain. In vivo three unusual substrates compete for oxidation by EPO under physiologic circumstances: bromide (Br\(^-\)) (7), nitrite (NO\(_2^-\)) (8), and the pseudo-halide thiocyanate (SCN\(^-\)) (9, 10). EPO-catalyzed oxidization of these three substrates yields three oxidants with strikingly different reactivities: HOBr, nitrogen dioxide radical (NO\(_2^*\)) (11), and hypochlorous acid (HOSCN) (12). Whereas HOBr is a potent bleaching oxidant with many similarities to HOCl and NO\(_2^*\) is a powerful lipid peroxidation-initiating agent, HOSCN is a weak, predominantly sulfhydril-reactive oxidant (13). Of note, whereas HOBr and NO\(_2^*\) appear to function as membrane-lytic oxidants (14), HOSCN has restricted reactivity that enables it to penetrate the membrane of mammalian cells and evoke intracellular oxidative modifications starting with depletion of glutathione (12, 15). At physiologic relevant concentrations of these three potential EPO substrates, SCN\(^-\) is by far the preferred substrate and therefore HOSCN is the predominant oxidant species produced by EPO (8, 10, 12).

Somewhat unexpectedly, SCN\(^-\) is a preferred substrate for MPO as well. Van Dalen et al. (16) found that MPO presented with physiologically relevant concentrations of Cl\(^-\) (100 mM) and thiocyanate (100 \(\mu\)M) generates approximately equimolar amounts of HOCl and HOSCN. Interestingly, unlike the case for EPO, where thiocyanate competitively inhibits oxidation of alternative substrates, MPO generates HOSCN in the presence of SCN\(^-\) without impairing generation of HOCl from Cl\(^-\) (16), suggesting that the MPO is far from saturated at physiologic concentrations of these anions. Thus, in physiologic conditions, the unusual oxidant HOSCN is a major phagocyte peroxidase product of both EPO and MPO.

We have recently examined the effect of HOSCN on mammalian host cells. We hypothesized that because HOSCN, unlike other phagocyte peroxidase products, penetrates intact mammalian cell membranes and induces intracellular oxidant stress, it might therefore induce the expression of genes through activation of various redox-sensitive kinase cascades and transcription factors, such as NF-κB (17–19). We found that HOSCN is a uniquely potent phagocyte oxidant inducer of endothelial cell (20) and monocyte (data not shown) tissue factor expression. This potent (up to 100-fold) induction of tissue factor depends upon activation of the ERK1/2 kinase pathway and the NF-κB-like “TF-κB” binding site in the TF promoter that predominantly binds the p65/c-Rel (21, 22) heterodimer of the NF-κB family.

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3 Abbreviations used in this paper: MPO, myeloperoxidase; EO, eosinophil; EPO, eosinophil peroxidase.

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Because their genes contain upstream p65/p50 NF-κB binding sites, we hypothesized that HOSCN, through activation of p65/p50, would induce the expression of the endothelial cell adhesion molecules E-selectin (23), ICAM-1 (24), and VCAM-1 (25) and thereby promote leukocyte-endothelium adhesion. To test this hypothesis we exposed cultured HUVEC to various phagocyte-derived oxidants and assayed whole cell lysate and cell surface expression of these adhesion molecules. We also assessed the functional significance of such exposure on the adhesion of both neutrophils and a highly differentiated eosinophil-like cell line, Aml14.3D10 (26), to endothelium as well as in an in vivo intra-peritoneal injection/extravasation model. Our results show that HOSCN is a singularly potent oxidant inducer of functional NF-κB-regulated endothelial cell adhesion molecules and suggest the possibility of a novel inflammation amplification mechanism based on the H2O2/phagocyte peroxidase/SCN– system.

Materials and Methods

Reagents

LPS (Escherichia coli 055:B5), andrographolide, lactoperoxidase, H2O2, NaOCl, NaSCN, NaBr, and protease inhibitor mixture (P8340) were from Sigma-Aldrich. Rabbit polyclonal Abs against human E-selectin (sc-14011), ICAM-1 (sc-7891), VCAM-1 (sc-9304), PECAM-1 (sc-1506), goat polyclonal Ab against actin (sc-1615), and alkaline phosphatase-conjugated goat anti-rabbit IgG and donkey anti-goat IgG were from Santa Cruz Biotechnology. Recombinant human TNF-α (210-1A/C), adhesion-blocking mAbs for E-selectin (BBA16), ICAM-1 (BBA3), and VCAM-1 (BBA5) were from R&D Systems. Recombinant mouse TNF-α was from ebioscience (no. 14-8321). Leukocyte adhesion-blocking mAb (B4) against CD18 subunit of Mac-1(CD11b/CD18) was from American Tissue Culture Collection. Rabbit polyclonal Abs against phospho-AKT (Ser473), no. 9271, AKT (no. 9272), U0126 (no. 9903), and wortmannin (no. 9951) were from Cell Signaling Technology. Nuclear Extraction kit (AY2002) and Protein/DNA array kit (MA1010) for analysis of transcription factor activation were from Panomics.

Cell culture

HUVEC were isolated from human umbilical cords and cultured as previously described (27) and used at passage ≤3. Human eosinophil cell line (Aml14.3D10) was cultured in RPMI 1640 medium supplemented with 8% FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate and at 37°C in the presence of 5% CO2 (26).

Oxidant preparation

Reagent HOSCN was synthesized and quantitated as previously described using lactoperoxidase (10, 12). HOCI was quantitated spectrophotometrically using its molar extinction coefficient 350 M–1 cm–1 at 292 nm and pH > 11 (28). Reagent HOBr was prepared by quantitative conversion of HOCI to HOBr by adding 20-fold excess sodium bromide (29).

Oxidant modulation of HUVEC whole cell E-selectin, ICAM-1, and VCAM-1 expression

HUVEC monolayers were exposed to growth medium and replaced with M199 (10% FBS and 1.5 mM L-glutamine) medium containing the indicated concentration (0–200 μM) of either HOCI, HOBr, HOSCN, H2O2, or a PBS buffer dilution control and incubated for the indicated time at 37°C. Whole cell lysates were prepared and western blots probed with specific Abs against human E-selectin, ICAM-1, VCAM-1, or PECAM-1 as described previously (20). Alternatively, HUVEC were exposed to a MPO/H2O2/SCN– enzymatic system. HUVEC monolayers were overlaid with M199 medium with 10% FBS, then supplemented with 5 μg/ml MPO, 100 μM H2O2, or 1 mM NaSCN as indicated or, alternatively, 10 ng/ml LPS. After 4 h, whole cell lysates were prepared and adhesion molecules detected by Western blots.

Flow cytometry

HUVEC monolayers were treated with buffer control, 150 μM HOSCN or 10 ng/ml LPS for 6 h and then detached with PBS containing 0.02% EDTA (Versene; Invitrogen Life Technologies). After washing once, they were resuspended in PBS containing 1% BSA (PBS/BSA) and incubated with 3 μg/ml mouse anti-human E-selectin (Ancell), mouse anti-human ICAM-1 (BBA3), or mouse anti-human VCAM-1 (BBA5) Abs, followed by 15 μg/ml FITC-conjugated anti-mouse IgG Ab at 22°C for 1 h with end-to-end rotation. HUVEC were spun (600 × g for 5 min), and supernatants were discarded. Each aliquot was then resuspended in 0.5 ml of PBS/BSA for immediate acquisition by flow cytometry (FACScan; BD Biosciences).

RT-PCR analysis of E-selectin, ICAM-1, and VCAM-1 mRNA

HUVEC monolayers were exposed in M199 medium supplemented with 10% FBS and 1.5 mM L-glutamine to either buffer control, 150 μM HOSCN, or 10 μg/ml LPS for the indicated time points (0–12 h). Total cellular RNA was isolated and RT-PCR was carried on as previously described (20). Specific primers purchased from Integrated DNA Technologies were listed as follow: E-selectin, sense, 5′-AGGTCTCTTCTGCTCAAGTGGTAA-3′, and antisense, 5′-TTCCGAAGCCACGACACAGC-3′; ICAM-1, sense, 5′-CAGTGACCATCTACAGCTTTCCGG-3′; and antisense, 5′-GCTGTCACAGTTGATGAGCAA-3′; and VCAM-1, sense, 5′-ACCTCCACAGGCACACACAG-3′; and antisense, 5′-GTAAGCTTACCTCCAGCTGTC-3′. Aliquots were analyzed by submarine electrophoresis by loading 10 μl of PCR products on 1.2% agarose gels. Ethophorosed gels were stained with 0.5 μg/ml ethidium bromide and imaged with UV transillumination.

Inhibitor studies and EMSA

For kinase pathway inhibition studies, 10 μM U0126 and 10 nM, 100 nM, and 1 μM wortmannin were used as specific inhibitors of the ERK1/2 and PI3K/Akt pathways, respectively. Andrographolide (10 μg/ml) was used as a specific inhibitor of the NF-κB pathway (30). Monolayers of HUVEC were pretreated 1 h with inhibitors, then exposed in 10% FBS-containing medium with fresh inhibitors to either buffer control or 150 μM HOSCN for 4 h. Whole cell lysates were prepared (20) and expression of E-selectin, ICAM-1, VCAM-1, phospho-AKT and total AKT was detected by Western blot analysis. Alternatively, HUVEC monolayers were treated as above for 1 h, and nuclear protein was prepared with a Nuclear Extract kit (PA-nomics) according to the manufacturer’s instructions and stored at –80°C until use. NF-κB consensus (5′-AGTGGAGGGAGTCTCCAGGGC-3′) and NF-κB mutant (5′-AGTGGAGGGAGTCTCCAGGGC-3′) oligonucleotides (italics represent the NF-κB-binding sequence) were purchased from Santa Cruz Biotechnology. Oligonucleotides were end-labeled with [γ–32P]ATP and T4 polynucleotide kinase (Amersham Biosciences). Five micrograms of nuclear extract was loaded per lane, and EMSA was performed as described previously (20).

Neutrophil preparation

Fresh human blood was collected from healthy, adult volunteers after their informed consent into acid-citrate-dextrose (38 mM citric acid, 75 mM trisodium citrate, and 100 mM dextrose; 1/7 acid-citrate-dextrose final concentration) (31). After removal of platelet-rich plasma by centrifugation of the whole blood at 100 × g at 22°C for 15 min, the cell pellet was diluted with ice-cold PBS to original blood volume and layered onto the same volume of Histopaque-1077 (1077; Sigma-Aldrich) and centrifuged at 400 × g at room temperature for 30 min. The supernatant and interface were removed, and ice-cold PBS was added to restore the original volume. The cell suspension was then mixed with one-sixth volume of 6% Hetastarch (Sigma-Aldrich, H2648), and erythrocytes were allowed to settle at 22°C for 30 min. The top layer was collected and was centrifuged at 500 × g at 4°C for 5 min. After hypotonic lysis of residual erythrocytes with cold H2O followed 30 s later by addition of 2× PBS, neutrophils were resuspended in ice-cold PBS at ~107/ml for immediate use. The purity of neutrophils was routinely >95%.

Static adhesion assay

Confluent monolayers of HUVEC in 24-well plates were exposed to either buffer control, 150 μM reagent oxidant (HOCI, HOBr, or HOSCN) or 10 μg/ml LPS at 37°C for 4 h. HUVEC monolayers were washed twice with PBS. Alternatively, HUVEC were treated by HOSCN (0–200 μM) or human TNF-α (0–10 ng/ml) for a dose-course analysis. Neutrophils or Aml14.3D10 cells were resuspended at 10 × 106/ml in HBSS (pH 7.4), 1.2 mM CaCl2, and 0.8 mM MgCl2 supplemented with 1% BSA (HBSS/Ca/Mg/BSA). The cells were laid onto HUVEC monolayers (0.25 ml/well). For Ab inhibition experiments, 10 μg/ml mouse IgG, anti-E-selectin (BBA16) or anti-VCAM-1 (BBA5) blocking Abs were incubated with HUVEC monolayer in 200 μl of HBSS/Ca/Mg/BSA at 37°C for 15 min. Alternatively, neutrophils were preincubated with 10 μg/ml mouse IgG or IgB (anti-CD18 Ab). After incubation at 37°C for 25 min in a 5% CO2 humidified atmosphere, nonadherent cells were carefully removed by washing three times with PBS. Adherent neutrophils were quantified using
a myeloperoxidase (MPO) assay (32). MPO activity was converted to neutrophil numbers using a standard curve, while adherent Aml14.3D10 cells were counted by using a hemocytometer.

Mouse peritonitis model

Male mice (C57BL/6J, 25–30 g body weight) were i.p. injected with PBS buffer control, 150 μM HOBr, 150 μM HOSCN (both 1.7–2.0 ml, 10 nmol/g body weight), or PBS containing mouse TNF-α (2 ng/g body weight). The mice were sacrificed 4 h later, and peritoneal cavities were lavaged and leukocytes were enumerated and identified as described previously (30, 33).

Protein/DNA array analysis of transcription factor activation

Transcriptional factor array assay was performed as recommended by the TranSignal Protein/DNA array kit maker (Panomics). Twenty-microgram aliquots of nuclear extracts (3–5 mg/ml) were analyzed and the resulting hybridized dots imaged using the IVIS Imaging System (Xenogen). The density of each signal was determined using NIH Image (version 1.1).

Results

HOSCN up-regulation of E-selectin, ICAM-1, and VCAM-1 expression in human endothelial cells

We tested whether HOSCN induces endothelial cell expression of the adhesion molecules E-selectin (CD62E), ICAM-1 (CD54), and VCAM-1 (CD106), all of which have 5′ binding sites for NF-κB. As shown in Fig. 1, A, pretreatment of HUVEC monolayers with 150 μM HOSCN causes pronounced up-regulation of expression of all three of these adhesion molecules with a time course similar to that of the potent physiological agonist LPS. Both agonists induced expression of VCAM-1 and ICAM-1 for 4 h that remained stable over 12 h. In contrast, E-selectin induction by HOSCN was detectable by 2 h, peaked at 4 h, then rapidly diminished toward baseline. PECA-1 (CD31), unlike E-selectin, ICAM-1, and VCAM-1, was constitutively expressed by HUVEC and unaltered by either HOSCN or LPS stimulation.

To define the mechanism underlying up-regulation of these adhesion molecules by HOSCN, we performed semiquantitative RT-PCR analysis on RNA extracted from HUVEC monolayers at various time points after exposure to both agonists. HOSCN, as does LPS, up-regulates E-selectin, ICAM-1, and VCAM-1 mRNA starting at 2 h and peaking at 3 h, compatible with a primarily transcriptional mechanism.

Comparative induction of E-selectin, ICAM-1, and VCAM-1 expression in endothelial cells by phagocyte-derived oxidants

The principal stable oxidants generated by neutrophils are H2O2, HOCl, and HOSCN (16) and by eosinophils H2O2 and HOBr, and HOSCN (7, 10). We compared the relative capacity of each of these oxidants to induce expression of cell adhesion molecules in HUVEC monolayers by Western blot analysis of cell lysates (Fig. 2A). In comparison with HOCl, HOBr, or H2O2, HOSCN is a uniquely effective oxidant inducer of all three adhesion molecules in HUVEC. Fifty micromolar HOSCN induced significant expression of VCAM-1, while the optimal induction occurs by 100 μM (Fig. 2B). A reagent MPO/H2O2 enzymatic system also induced SCN−-dependent adhesion molecule expression (Fig. 2C). Interestingly, MPO alone weakly induces adhesion molecule expression, perhaps related to its previously reported capacity to induce

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** HOSCN up-regulation of E-selectin, ICAM-1 and VCAM-1 expression in HUVEC. A, Western blot analysis of time course. HUVEC monolayers were stimulated by buffer control (left panel), 150 μM reagent HOSCN (a concentration chosen because it induces maximal tissue factor expression (20), middle panel), or 10 μg/ml LPS (right panel) in M199 medium containing 10% FBS for the indicated time, whole cell lysates were prepared, and Western blots probed with specific polyclonal Abs against human E-selectin, ICAM-1, VCAM-1, PECAM-1, and actin. B, RT-PCR analysis of time course. HUVEC were exposed as described for A for the indicated time. Total cellular RNA was extracted and analyzed by RT-PCR using either E-selectin, ICAM-1, VCAM-1, or 18S rRNA-specific primers. Gels were stained with ethidium bromide and imaged using UV transillumination.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Comparative induction of HUVEC E-selectin, ICAM-1, and VCAM-1 expression by phagocyte-generated oxidants. A, Comparative induction by phagocyte oxidants. One hundred fifty micromolar reagent HOCl, HOBr, HOSCN, or 100 μM H2O2 was added to HUVEC monolayers containing M199 medium with 10% FBS and incubated for 4 h. Whole cell lysates were prepared and Western blots probed for the indicated molecules. B, Dose-response analysis of HOSCN induction. HUVEC monolayers were treated with the indicated concentration of HOSCN in M199 medium with 10% FBS for 4 h. Western blots of HUVEC lysates were probed as for A. C, Induction by the MPO/H2O2/SCN− enzymatic system. HUVEC monolayers were overlaid with M199 medium with 10% FBS, then supplemented with 5 μg/ml MPO, 1 mM SCN−, or 100 μM H2O2, as indicated or, alternatively, 10 ng/ml LPS. After 4 h, Western blots of cell lysates were analyzed.
transcriptionally secretion of IL-6 and IL-8 in HUVEC (34). We also assessed the capacity of HOSCN to induce cell surface (as opposed to whole cell lysate) adhesion molecule expression by flow cytometry. As shown in the histograms in Fig. 3A, both 150 

\( \mu \)M HOSCN and 10 ng/ml LPS induce a significant up-regulation of these cell adhesion molecules. Analysis of fluorescence mean values shows a 5- to 6-fold induction by HOSCN, and an 8- to 16-fold increase for LPS (Fig. 3B).

**Influence of kinase pathway and NF-κB inhibitors on HOSCN adhesion molecule induction**

We have shown that the ERK1/2/Egr-1, PI3K/Akt, and NF-κB pathways all participate critically in HOSCN induction of endothelial cell tissue factor (20). To assess the functional importance of these pathways in HOSCN-induced adhesion molecule up-regulation, we assessed the influence on this phenomenon of wortmannin, a PI3K/Akt pathway inhibitor and andrographolide, a specific NF-κB pathway inhibitor (30) (Fig. 4A). HOSCN stimulation of all three adhesion molecules was ablated by 10 

\( \mu \)g/ml andrographolide. However, 10 nM, 100 nM, and 1 \( \mu \)M wortmannin all enhanced both baseline (Fig. 4A, left panel) and HOSCN-stimulated (Fig. 4A, right panel) adhesion molecule expression. All three concentrations of wortmannin suppressed Akt phosphorylation, 1 \( \mu \)M nearly completely. Unlike the case for tissue factor induction, the ERK 1/2/Egr-1 inhibitor U0126 (10 \( \mu \)M, a concentration we have previously shown blocks ERK 1/2 phosphorylation (20)) had no detectable effect on adhesion molecule expression.

To ascertain whether the enhancement of HOSCN-induced E-selectin, ICAM-1, and VCAM-1 expression by wortmannin was also mediated through activation of the p65/p50 NF-κB transcription factor, we assayed the effect of wortmannin upon p65/p50 activation. HUVEC were pretreated one hour with U0126, wortmannin, or andrographolide as in A, then exposed in to either buffer control or 150 \( \mu \)M HOSCN for 1 h before extraction of nuclear proteins. Five-microgram aliquots of the nuclear extracts were incubated with a radiolabeled NF-κB oligonucleotide probe and separated on 5% nondenaturing polyacrylamide gels. The mobility of the shifted NF-κB probe is shown by the arrow on the right; the slower migration of supershifted bands is shown in brackets. The lanes designated “C” show the effect of adding 50-fold excess unlabeled consensus probe; “M”, the effect of adding 50-fold excess unlabeled mutant NF-κB oligonucleotide; and “p65” and “p50,” the effects of adding polyclonal Abs to the designated proteins.
nM, but not 10 nM, wortmannin alone activated p65/p50 in unstimulated HUVEC; however, both 10 and 100 nM wortmannin significantly enhanced p65/p50 activation caused by HOSCN. Taken collectively, these results suggest that the p65/p50 NF-κB pathway plays a preeminent role in HOSCN regulation of adhesion molecule expression, unlike the case for tissue factor, where simultaneous activation of AP-1, Egr-1, and p65/c-Rel "TF-κB" appears to be required (20).

Enhancement of neutrophil and eosinophil adhesion to endothelium in vitro by HOSCN

Because neutrophil MPO generates equimolar amounts of HOSCN and HOCl as its principal physiologic products (16). HOSCN generated by neutrophils might activate endothelial cells and so promote neutrophil/endothelium adhesion at sites of extravasation/inflammation. We therefore tested whether HOSCN promotes neutrophil/endothelium adhesion in an in vitro static adhesion assay (Fig. 5). We compared adhesion to that induced by 10 μg/ml LPS, a concentration we previously showed induced maximal tissue factor expression (20). Neither HOCl nor HOBr significantly augmented neutrophil adhesion but HOSCN treatment stimulated an 8-fold increase (Fig. 5A). To define the mechanism of HOSCN-induced neutrophil adhesion, we preincubated HUVEC monolayers with blocking Abs against E-selectin and VCAM-1 or preincubated neutrophils with blocking Ab against the CD18 subunit of Mac-1, the ligand for ICAM-1 before neutrophil adhesion being assessed as in (Fig. 5B). Pretreatment of HUVEC with andrographolide severely attenuated (80%) the enhanced neutrophil adhesion induced by HOSCN, suggesting a critical role for NF-κB in this phenomenon.

Because HOSCN is the principal physiologic product of EPO, we assessed the effect of HOSCN on adhesion to endothelium using an eosinophil cell line (Aml14.3D10). These highly differentiated cells resemble mature eosinophils morphologically (26) and have been used widely as a surrogate model for studying several aspects of eosinophil function (35–37) including Mac-1-dependent adhesion (38). We found by flow cytometry analysis that Aml14.3D10 expresses P-selectin glycoprotein ligand 1, Mac-1, and VLA-4, the counterligands for, respectively, E-selectin, ICAM-1, and VCAM-1 (data not shown). As shown in Fig. 5C, HOSCN, but not HOCl and HOBr, induces a 3- to 4-fold increase of AML14.3D10/endothelium adhesion that was significantly inhibited by Abs blocking E-selectin, CD18 or VCAM-1 (Fig. 5D). HOSCN-dependent AML14.3D10/HUVEC adhesion was also substantially impaired by andrographolide pretreatment.

To compare the potency of HOSCN-induced PMN/HUVEC adhesion with that of a physiologically relevant and potent cytokine, we performed a dose-course experiment using TNF-α as a positive control (Fig. 6). Both HOSCN (0–200 μM) and TNF-α (0–10 ng/ml) increase of neutrophil/endothelium adhesion in a dose-dependent manner (ED50so of 100 μM and ~0.1 ng/ml, respectively). Maximal adhesion was ~14-fold baseline with 200 μM HOSCN and ~35-fold with 10 ng/ml TNF-α. Thus, although on a molar basis...
basis the potency of the oxidant HOSCN is, as might be expected, many orders of magnitude less than that of TNF-α, the maximal biological effect of HOSCN is roughly one-third to one-half of that of this powerful cytokine.

**HOSCN-induced i.p. PMN extravasation in vivo**

To assess whether HOSCN-induced enhancement of leukocyte/endothelium adhesion results in leukocyte extravasation and accumulation, mice were i.p. injected with control, 150 μM HOBr, 150 μM HOSCN, or murine TNF-α (2 ng/g). Four hours later, mice were sacrificed, peritoneal cavities lavaged and leukocytes enumerated. As shown in Fig. 7, HOSCN elicits an 8-fold baseline increase in neutrophil accumulation while HOBr effects a 3-fold increase. TNF-α induces an 18-fold increase. HOSCN also induces a 2.5-fold increase in monocyte/macrophage extravasation and the recruited leukocytes include ~20% lymphocytes and 20% monocytes (data not shown).

**HOSCN activation of other transcription factors**

Because redox signaling is widely implicated in regulation of a variety of kinase pathways upstream of several transcription factors (17–19), we hypothesized that HOSCN might activate other transcription factors in addition to NF-κB. We therefore assayed activation of 54 distinct transcription factors using a Panomics TransSignal Protein/DNA array I (Table I). In addition to confirming HOSCN activation of NF-κB, HOSCN, and LPS both also up-regulated significant expression of Stat4, CDP, GRE, CBF, Ets-1/PEA3, and TFIID. However, the vitamin D receptor, Pbx1, and thyroid hormone receptor are up-regulated by LPS but not HOSCN, indicating a certain level of specificity to HOSCN transcription factor up-regulation.

**Discussion**

Historically, the major focus of investigations into phagocyte peroxidase function was on their role in the killing of pathogens. These studies have shown that the H2O2/MPO and HOCl/EPO systems, using suitable substrates, can generate oxidants, such as HOCl, with cytotoxic activities as much as 1000-fold greater than that of the substrate H2O2 generated by the phagocyte NADPH oxidase system (4, 5). The cytotoxicity of both H2O2/MPO and HOCl/EPO systems is strongly influenced by the selection of substrate. For example, in both systems oxidation of substrates with high redox potential, such as chloride and bromide, yields oxidant products (i.e., HOCl and HOBr) with much higher cytotoxic capacity than oxidation of substrates with lower redox potential, such as SCN−, which generates HOSCN (14). Thus, both the H2O2/MPO and the HOCl/EPO systems evince substrate-dependent modulation of pathogen killing. More recent work has focused on the effects of sublethal concentrations of phagocyte peroxidase-generated oxidants upon a variety of intracellular signaling pathways in host tissue cells. Oxidants activate several kinase signaling pathways that in turn have the capacity to influence gene transcriptional expression through activation of a variety of transcription factors (17–19).

We now find that HOSCN is a uniquely potent phagocyte oxidant inducer of E-selection, ICAM-1 and VCAM-1 expression in HUVEC and that this up-regulation is transcriptionally mediated (Figs. 1–3). EMSA analysis shows that HOSCN strongly activates p65/p50 NF-κB and addition of andrographolide, a specific inhibitor of the NF-κB pathway, blocks both NF-κB activation (Fig. 4B) and cell adhesion molecule up-regulation (Fig. 4A). Wortmannin, an inhibitor of P13K phosphorylation of AKT, stimulated NF-κB activation (Fig. 4B) and increased expression of all three adhesion molecules in HUVEC (Fig. 4A). HOSCN induction of these adhesion molecules was markedly further enhanced in the presence of wortmannin. In aggregate, these experiments suggest that HOSCN induces the expression of endothelial adhesion molecule by activating of the NF-κB p-65/p50 transcription factor and that activation of NF-κB is suppressed by constitutive activity of the P13K/Akt pathway.

Although our data provide strong evidence for the importance of NF-κB activation in induction of adhesion molecules, they do not rule out the participation of other transcription factors, for example

**FIGURE 6.** Dose-response analysis of HOSCN and TNF-α enhancement of neutrophil adhesion to endothelium. HUVEC monolayers were exposed to 0–200 μM HOSCN (A) or 0–10 ng/ml human TNF-α (B) for 4 h, and static adhesion was assayed as in Fig. 4. Data shown ± SD.

**FIGURE 7.** HOSCN induction of i.p. neutrophil extravasation in vivo. C57BL/6 mice were i.p. injected with 2 ml of PBS buffer control (n = 5), 150 μM HOBr (n = 6), 150 μM HOSCN (n = 6), or 2 ng/g body weight mouse TNF-α (n = 5). The mice were sacrificed after 4 h, and their peritoneal cavities were lavaged. Lavage fluid leukocytes were enumerated with counting chambers and PMN identified by Wright-Giemsa staining. All data shown ± SD. * Difference from control significant by t test (p < 0.05).
AP-1. HOSCN activates JNK and promotes c-Jun phosphorylation, pathways upstream of AP-1 activation (data not shown), but this does not result in prominent activation of AP-1 as detected using a consensus AP-1 oligonucleotide probe in the Panomics transcription factor assay (Table I) or a tissue factor-specific AP-1 probe by EMSA (20). In these studies, we found HUVEC to exhibit constitutive activation of consensus AP-1 probe-binding proteins, including c-Jun but not c-Fos. However, activating transcription factor 2/c-Jun AP-1 heterodimers have been implicated in TNF-α up-regulation of E-selectin (39, 40), and we have not performed EMSA analysis using an E-selectin-specific AP-1 probe or attempted supershift analysis with an anti-activating transcription factor 2 Ab to test this possibility, so a role for AP-1 in HOSCN-induced adhesion molecules is possible.

H₂O₂ treatment of endothelial monolayers has been previously reported to stimulate neutrophil adhesion (41–43) and induce ICAM-1 expression (43–45) in vitro. However, these studies typically used nonphysiologic low (or e.g., 0.5%)-protein buffer conditions (41–45) or used lethal concentrations of H₂O₂ (41, 42). Moreover, some of these same studies report failing to find ICAM-1 expression (44), p65/p50 NF-κB activation (44, 45), or enhanced neutrophil adhesion (42) at sublethal H₂O₂ concentrations. In contrast, we have used arguably more physiologic conditions in media containing 10% serum and nonlethal concentrations of the various phagocyte oxidants. We have found that, in the conditions used for our current experiments, HUVEC monolayers 24 h after HOSCN exposure actually have enhanced viability and less apoptosis than do control, nonoxidant-treated monolayers (data not shown). In these conditions 100 μM H₂O₂ does not induce ICAM-1, VCAM-1, or E-selectin expression (Fig. 2, A and C) and does not activate p65/p50 NF-κB (20). Fig. 2C most clearly demonstrates SCN⁻-specific induction of adhesion molecule expression by the H₂O₂/MPO system and supports our conclusion that HOSCN is uniquely potent in this regard.

That HOSCN up-regulation of HUVEC endothelial cell adhesion molecule expression may likely be physiologically relevant is supported by our in vitro HUVEC adhesion assay in which HOSCN proved to be a singularly effective oxidant inducer of adhesion of both neutrophils (Fig. 5A) and an eosinophil cell line (Fig. 5C) to endothelium. Neutrophil adhesion was blocked by the NF-κB inhibitor androgrophildone and by blocking Abs to E-selec-
(50, 51) suggesting an important pathogenic role for MPO in atherogenesis. This association has been attributed to the capacity of MPO to oxidatively modify lipids such as low density lipoprotein and lipoproteins such as apolipoprotein A-I (52). Our data suggest the possibility that—especially in smokers that have up to 10-fold higher than normal serum thiocyanate levels (53)—MPO-catalyzed generation of HOSCN, whether from activated endothelium-attached neutrophils or from active extracellular MPO that accumulates on atheroma (54) or on endothelial cell surfaces, could provoke both endothelial tissue factor and adhesion molecule expression. This, in turn, would contribute to a proinflammatory and prothrombotic endothelial phenotype that promotes atherosclerosis and thrombotic infarction. Such a mechanism may, in part, contribute to explaining the well-described association of smoking, atherosclerosis, and thrombosis.

Because p65/p50 NF-κB binding sites are important transcriptional regulatory elements of genes such as IL-8 (55) and TNF-α (56), HOSCN might also induce expression of these proinflammatory signaling molecules in addition to tissue factor, E-selectin, ICAM-1, and VCAM-1. If so, elaboration of such chemokines and cytokines might function to further amplify inflammation through a secondary autocrine/paracrine mechanism. Supporting this hypothesis, we have found that HOSCN in combination with either TNF-α or IL-1β synergistically interact to induce adhesion molecule expression in HUVEC (data not shown). Moreover, HOSCN activates at least six other transcription factors, including Stat4, C/EBPα, CREB, Ets-1/PEA3, and TFIID. This pattern of transcription factor activation is distinctive and distinguishable from that induced by LPS (Table I). These latter transcription factors promote expression of important proinflammatory genes such as IL-6 (57), matrix metalloproteinase-1 (58), matrix metalloproteinase-9 (59), and MCP-1 (60), which predicts the generation of a complex proinflammatory, prothrombotic phenotype in endothelial, and possibly other mammalian cell types by HOSCN. We are currently using cDNA microarray transcriptome analysis to test this hypothesis.

We conclude that HOSCN, a major physiologic product of both the H2O2/MPO and H2O2/EPO systems, is a uniquely effective phagocyte oxidant inducer of biologically relevant E-selectin, ICAM-1 and VCAM-1 expression on endothelial cells. We propose that by this mechanism the H2O2/phagocyte peroxidase/SCN− system plays a hitherto unsuspected role to promote leukocyte adhesion and extravasation and so amplify inflammation.

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Disclosures

The authors have no financial conflict of interest.

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