Modification of IL-6 by hypochlorous acid: effects on receptor binding and possible role in treatment of COVID-19

Lori I. Robins1*, Erika K. Keim2, Deborah B. Robins3, Scott Edgar4, John. S. Meschke2, Phil Gafken5, and Jeffrey F. Williams6

1Department of Physical Sciences, University of Washington Bothell, 18115 Campus Way NE Bothell, WA 98011, USA
2Department of Environmental and Occupational Health Sciences, University of Washington, 4225 Roosevelt Way NE, Suite 100, Seattle, WA 98195, USA
3Issaquah High School, 700 2nd Ave SE, Issaquah, WA 98027, USA
4Department of Medicinal Chemistry, University of Washington, H172 Health Science Building Seattle, WA 98195, USA
5Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, Seattle, WA 98109, USA.
6Briotech Inc., 14120 NE 200th St, Woodinville, WA 98072, USA

*1Department of Physical Sciences, University of Washington Bothell, 18115 Campus Way NE Bothell, WA 98011, USA, lrobins@uw.edu, +1(425)352-3208
Abstract

Interleukin-6 (IL-6) has been implicated in the pathogenesis of acute inflammatory events in COVID-19 patients. Positive clinical responses to monoclonal antibodies directed against the IL-6 receptor (IL-6R) suggest that interference with IL-6-dependent-activation of proinflammatory pathways offers a useful approach to therapy in SARS-CoV-2 infection. We exposed IL-6 to hypochlorous acid (HOCl) \textit{in vitro} at concentrations reported to develop \textit{in vivo}. After HOCl treatment the cytokine failed to bind to IL-6 receptors in a bioassay using human cells engineered to provide a luminescent response to signal transduction upon receptor activation. Similar results followed exposure of IL-6 to N-chlorotaurine (NCT) and hypobromous acid (HOBr). SDS-PAGE analysis of HOCl-treated IL-6 showed neither fragmentation nor aggregation, suggesting that the modifications induced by these agents occurred on the intact protein. Mass spectrometry of intact and trypsin-digested fragments identified oxidative changes limited to two amino acid residues, methionine 161 and tryptophan 157, both of which have been implicated in receptor binding of the cytokine. Our findings suggest that exogenous HOCl and NCT might affect the inflammatory cascade in the treatment of COVID-19. Further studies on the effects of hypohalous acids and their halogenated amine derivatives on IL-6 and related cytokines \textit{in vivo} may open up alternative therapeutic interventions with these compounds in COVID-19 and other hyperinflammatory diseases.

Introduction.

Interleukin 6 (IL-6) has long been associated with chronic inflammatory diseases such as rheumatoid arthritis where levels of IL-6 are elevated in the serum and synovial fluids. IL-6
has now been implicated in the pathogenesis of pulmonary and systemic lesions resulting from
the ‘cytokine storm’ in seriously afflicted COVID-19 patients. Elevated levels of IL-6 have
been identified as biomarkers for disease severity and are associated with hyperinflammation.
Monoclonal antibodies directed against the IL-6 receptor (IL-6R) are currently used to treat
inflammatory diseases. There is now compelling evidence of therapeutic benefits arising from
administration of these same monoclonal antibody products as part of the clinical management
of SARS-CoV-2 infection, particularly if the intervention is used in patients with severe disease
and early during treatment. These observations make a case for IL-6 mediated events being
causal in the most important and often terminal consequences of COVID-19. They point up the
need for mitigation of such processes in the treatment and resolution of SARS-CoV-2 infections.
While parenteral IL-6R monoclonal antibody products may provide proven clinical benefits
their routine use is not realistic for this purpose in many healthcare systems around the world.
They are expensive, their use requires hospitalization of patients, and they can cause adverse
reactions that need medical attention.

HOCl and other reactive oxygen species generated within phagocytic cells by the
myeloperoxidase system from intracellular Cl ions are known to affect the expression of
inflammatory cytokines, including IL-6. Used topically, exogenous HOCl brings about
beneficial changes that aid in the healing and resolution of injured and infected tissues,
probably by at least in part modifying proteins and other substrates that contribute to the
pathogenesis of lesions. HOCl readily reacts with endogenous taurine and other amines to
generate less reactive N-chloroamines, including N-chlorotaurine (NCT), which can also modify
proteins.
Here we demonstrate rapid modification of IL-6 in vitro upon exposure to HOCl, NCT and hypobromous acid (HOBr). Markedly impaired IL-6 receptor binding was demonstrated using engineered human cells that luminesce following IL-6 induced activation of its receptor. Mass spectrometry identified changes limited to oxidation of methionine and tryptophan residues that are involved with IL-6 receptor binding. The results suggest that hypohalous acids or NCT may have direct inhibitory effects on proinflammatory mediators such as IL-6. They also suggest it may be worthwhile exploring this mechanism in other systemic inflammatory pathological processes in which IL-6 has been implicated.

**Methods and Materials.**

Reagents for the iodometric titrations were purchased from Hach (Dissolved Oxygen 3 Powder Pills, Potassium Iodide Powder Pills, Sodium Thiosulfate Digital Titrator Cartridge, 0.113 N, and starch indicator solution). Sodium chloride (NaCl), sodium hydroxide (NaOH), ELISA Wash buffer (50-184-79), ELISA Stop buffer (SS03) and sodium thiosulfate (Na₂S₂O₃, STS) were purchased from Fisher Scientific. Sodium bromide and taurine were purchased from ACROS Organics. Water from MilliQ water purification system was used for all experiments. HOCl (pH 4) was provided by Briotech Inc. Human IL-6 was purchased from GenScript, 860 Centennial Ave. Piscataway NJ 0885 (Z03034). The IL-6 bioassay kit was purchased from Promega, 2800 Woods Hollow Road Madison, WI 53711 USA (JA2501).

**Conversion of HOCl to HOBr.** The conversion of HOCl to HOBr was achieved by the addition of NaBr (1.1 eq) to HOCl at pH 4. The conversion was monitored by UV-VIS (BioMate 3S).
absorbance of HOCl at 236 nm shifted to 260 nm. Test solutions were loaded in a 1 cm quartz cuvette (Sterna Cells).

**Synthesis of N-chlorotaurine.** The synthesis of NCT was done following literature procedures. Briefly, HOCl was diluted with 1M NaOH to convert HOCl to OCl. Taurine (10 molar equivalents) was added to the solution in four aliquots with 1 min between each addition. The absorbance was measured at 250 nm. The pH was adjusted to neutral using 5 M HCl prior to use.

**ELISA Assay.** The Invitrogen human IL6 ELISA kit (catalog # 88-7066) was purchased and used according to the provided protocol. Briefly, 96-well plates were coated with the capture antibody (anti human IL-6 antibody). IL-6 (150-200 pg/mL) was incubated with various concentrations of HOCl for 5 min and then quenched with 1% STS. The detection human IL-6 antibody conjugated with HRP was used to detect the antigen. All experiments were done a minimum of 3 times. Samples for each experiment were plated in triplicate. HOBr experiments were done following the same procedure, using fresh HOBr. NaBr (1.1 eq) was added to the stock solution of HOCl with thorough mixing for immediate use.

**Trypsin Digested IL-6 Peptide Analysis by Mass Spectrometry.** IL-6 and HOCl-treated IL-6 samples were prepared following the Rapid Digestion-Trypsin Protocol (Promega VA1060). Prior to digestion, the IL-6 treated samples were prepared by incubating 25 µL of 1 mg/mL with 25 µL of HOCl (3.43 mM) and HOBr (3.43 mM). After 5 min of incubation with the hypohalous acid, the solution was quenched with 5 µL of 1% STS. The control sample was prepared by adding 25 µL of 1 mg/mL with 25 µL of water. All samples were then digested following the
provided protocol. Tandem mass spectrometry was performed for the initial HOCl-treated
samples by using a Thermo Orbitrap mass spectrometer coupled to a Waters Acquity UPLC.
Separations were performed on a Waters CSH 1.0 x 100 mm column. All data analysis was
performed in PMI-Byonic. Additional mass spectrometry studies on HOCl- and HOBr-treated
samples was carried out on a ThermoScientific Orbitrap Fusion mass spectrometer coupled to a
ThermoScientific Easy nLC-1000 HPLC. Chromatographic separations were performed using a
home-built 75 um x 25 cm capillary column packed with ReproSil 3 um particle size C18
material. Data were analyzed with Proteome Discoverer v2.4.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). IL-6 (0.16 mg/mL) was incubated with
1.7 mM HOCl or 1.7 mM HOBr for 5 minutes. The samples were then quenched with 1 uL of
1% STS. The control sample contained IL-6 (0.16 mg/mL) in water. The samples were treated
with Laemmli Sample Buffer (Biorad 1610747) and heated at 95 °C for 10 min. The samples
were then electrophoresed on a 12% acrylamide gel (Biorad 4561043) and stained with
Coomassie (Biorad 1610786).

IL-6 Bioassay. The IL-6 bioassay kit from Promega (JA2501) was used according to the
provided protocols. HOCl-treated IL-6 was prepared by incubating 3 ug/ml IL-6 with 2.7 mM
(140 ppm) and 0.27 mM (14 ppm) HOCl for 5 min prior to incubation with bioassay cells. HOBr-
treated IL-6 was prepared by incubating 3ug/ml IL-6 with 1.34 mM (130 ppm) HOBr for 5 min.
NCT-treated IL-6 was prepared by treating 3 ug/ml IL-6 with NCT (2.2 mM; 351 ppm) for 15
min. All HOCl, HOBr, and NCT treated IL-6 samples and IL-6 control samples were serially
diluted by 3-fold in RPMI-1640 containing 10% FBS prior to application of cultured cells in IL-6
bioassay. This quenched remaining active halogen prior to the assay and was confirmed by negative tests for active chlorine (HACH 2745050). The starting concentration of IL-6 and HOCl-, HOBr- or NCT-treated HOCl was 0.33 ug/mL and was serially diluted 3-fold. The final concentrations in the presence of the cells ranged from 0.05-0.33 ug/mL. IL-6 inhibition was assayed by serially diluting 50 ug/ml anti-IL-6 receptor (ThermoFisher AHR0061) 3-fold and incubating it with the IL-6 bioassay cells 37 °C for 20 min. 12.5 ul untreated native IL-6 (1 ug/ml) was then added to the cells to test for inhibition. HOCl-treated IL-6 binding studies were accomplished by incubating serial dilutions of HOCl-, HOBr- and NCT-treated IL-6 with concentrations ranging from 1000-0.15 ng/ul for 30 min at 37 °C. 12.5 ul IL-6 (0.1 ug/ul) was then added to the cells to test for activity. Each experiment was incubated for 6 h at 37 °C prior to luminescence readings. All samples and combinations were run in at least two separate experiments with at least two replicates and read on a Molecular Devices SpectraMax iD3 multi-mode plate reader.

Results

IL-6 was incubated with various concentrations of HOCl to determine if monoclonal antibody binding was affected by HOCl treatment in an ELISA assay. A decrease in IL-6 binding was seen at all concentrations tested. >95% decrease in IL-6 detection was seen at HOCl concentrations greater than 1 ppm (0.02 mM). Similar results were seen with HOBr (Figure 1).
Figure 1. ELISA assay of HOCl- or HOBr-treated IL-6. A. IL-6 (150 ng/mL) was incubated with various concentrations of HOCl ranging from 0-3 ppm (0-0.058 mM) for 5 min. B. IL-6 (150ng/mL) was incubated with various concentrations of HOBr 0-3 ppm (0-0.031 mM) for 5 min. An in vitro assay with human cells engineered to express the IL-6 receptor with a luciferase reporter signal as an endpoint of receptor activation was used to determine if HOCl-induced modifications affected the response. Unmodified IL-6 induced signal transduction measured in relative luminescence units (RLU). All tested concentrations of HOCl-modified IL-6 produced little to no signal transduction (Figure 2A). NCT and HOBr exposure also markedly blunted signal transduction after incubation with IL-6 (Figure 2B). Binding of IL-6 to the IL-6 receptor decreased in a concentration-dependent manner after the engineered cells were preincubated with an anti-IL-6R antibody (Figure 2C). Modified IL-6 did not act as a receptor antagonist, as demonstrated in experiments in which authentic IL-6 was added to the cells subsequent to exposure to the modified protein (Figure 2D). SDS-PAGE analysis showed no fragmentation or aggregation of IL-6 modified by HOCl, although concentration-dependent aggregation of IL-6 modified by exposure to HOBr was observed (Figure 3).
Figure 2. In vitro IL-6 bioassay with engineered human cells. A. Signal transduction detection by luminescence of cells incubated with various concentrations of IL-6 and IL-6 treated with HOCl at 140 ppm (2.7 mM) and 14 ppm (0.27 mM). B. Signal transduction detection by luminescence for cells incubated with various concentrations of IL-6 and IL-6 treated with HOBr (213 ppm, 2.2 mM) and NCT (351 ppm, 2.2 mM). C. Inhibition of IL-6 binding to the IL-6R in the presence of various concentrations of anti-IL-6R antibody. D. Signal transduction detection for cells incubated with IL-6 pre-treated with HOCl, NCT or HOBr and then subsequently treated with native IL-6.
Figure 3. SDS-PAGE analysis of IL-6 treated with various concentrations of HOCl and HOBr for 5 minutes. Lane 1: Ladder; Lane 2: IL-6; Lane 3: IL-6 + HOCl (1.76 mM); Lane 4: IL-6 + HOCl (0.44 mM); Lane 5: IL-6 + HOBr (1.76 mM); and Lane 6: IL-6 + HOBr (0.44 mM).

Mass spectrometry was used to determine the location of modifications to IL-6. Trypsin digestion covering 77-97% of the protein sequence, revealed a single fragment that was oxidized in the presence of HOCl, HOBr and NCT (Figure 4). Met 161 and Trp 157 were oxidized in the presence of the hypohalous acids and NCT (Figure 4). The overall percent modification was similar for the HOCl-treated and untreated IL-6 digest samples (~87%). The percent oxidation increased to 95% for HOBr treated IL-6 and it was lower (78%) in presence of NCT. A single oxidation of Met 161 and dioxidation of Trp 157 was only seen in the presence of HOCl and NCT, indicating that, although the percent modification were similar to or less than the control, the products are different and not simply due to oxidations occurring during the digest. HOBr oxidation ratios were significantly higher than the control in 3/5 of the identified fragments (Figure 4C).
Figure 4. Il-6 sequence and digest fragments. A. The sequence of human Il-6. B. Trypsin digest fragment containing the two modified residues shown in blue. C. Ratio of specific oxidized fragments to the unmodified fragments. Oxidized fragments that co-elute are separated with a / and combined to calculate the ratio of the modified peptide fragment to the unmodified peptide fragment.

Discussion

IL-6 has emerged as a major participant in the cascade of inflammatory events that are critical to the pathogenesis of the ‘cytokine storm’ that is often causal for death in COVID-19 patients. In our ELISA experiments, exposure of IL-6 to HOCl or HOBr led to rapid decline in immunological reactivity of this protein with IL-6-specific monoclonal antibodies. The inhibition of binding in the ELISA assay by HOCl- and HOBr-treated IL-6 may have resulted from only minor modifications of the cytokine such as those seen by mass spectrometry.
analysis. SDS-PAGE analysis indicated no detectable fragmentation or aggregation of IL-6 as a consequence of HOCl treatment, suggesting that alterations in antigenicity were likely brought about by modifications to the intact protein.

Exposure to HOCl altered the ability of IL-6 to bind to the IL-6R and trigger receptor-mediated generation of luminescence; signal transduction induced by treated cytokine preparations was ablated at all concentrations tested. Since cells pre-incubated with HOCl-treated IL-6 responded normally following addition of native IL-6, we infer that HOCl inhibited the ability of the treated cytokine to bind to the receptor.

Cytokine modifications occurred after 5 min exposure to HOCl or HOBr concentrations of between 0.27 mM and 2.7 mM. These conditions are consistent with events that might occur in vivo. HOCl can be produced at a rate of 134 mM/min in phagosomes and at sites of inflammation, HOCl concentrations are estimated to be between 25-50 mM.28-30

HOCl has a short lifetime in vitro in the presence of biological molecules, estimated to be 0.1 μsec, which is attributable to its high reactivity and rapid modifications of substrates such as sulfur containing amino acids and amines.28, 31 Taurine, abundant in human plasma and tissues, is readily chlorinated by HOCl, and NCT is well recognized as a long-lived oxidant.32-35 HOBr, in contrast, is produced in vivo by eosinophil granulocytes through a myeloperoxidase pathway that depends upon availability of intracellular Br⁻ ions. The corresponding reaction product with taurine is N-bromotaurine (NBT).36 The relative molar concentrations of the two hypohalous acids (HOCl and HOBr) differ markedly due to differences in Cl⁻ and Br⁻ ion concentrations in vivo. Nonetheless, HOBr is clearly a significant factor in the reactive oxygen
species involved in host tissue responses to infection and injury; eosinophilia is associated with better clinical outcomes for COVID-19 patients. HOBr and NBT, are likely contributors.

Exposure of IL-6 to freshly produced HOBr and NCT led to similar changes in reactivity in regard to receptor-mediated transmembrane responses. Signal transduction was ablated by treatment of IL-6 with these reagents, and the modified cytokine did not interfere with subsequent native IL-6 activation of the receptor. These findings suggest that comparable modifications were effected by HOCl, HOBr and NCT. Mass spectrometry confirmed that the modifications after exposure to HOCl, HOBr and NCT were restricted to Met 161 and Trp 157. Met 161 has been implicated in IL-6 receptor binding, and was shown to oxidize in the presence of chloramine T (N-Chloro-p-toluenesulfonamide) in addition to other Met residues. Trp 157, a conserved residue near the receptor binding region, was modified by both HOCl and chloramine T. The structural integrity of IL-6 remained intact after treatment with chloramine T, which was also the case with HOCl.

HOBr modified a higher percentage of the digest fragments; this may be due to both the size and oxidation reduction potential differences between HOCl and HOBr. HOBr is 30-100 times more reactive than HOCl with Trp residues and is significantly less reactive with Met residues than HOCl. Both hypohalous acids are more reactive than the corresponding chlorinated taurine derivative of HOCl.

The susceptibility of IL-6 to HOCl, HOBr and NCT exposure is relevant to its involvement in pathological events in COVID-19 patients. Its causal role in a number of chronic inflammatory disorders such as rheumatoid arthritis, systemic lupus erythematosus and Castleman’s disease.
is well established.7-8 Therapeutic interventions in these diseases based upon interference with IL-6 binding to IL-6R clearly provide clinical improvements.40 Most treatments are monoclonal antibodies specific for IL-6R membrane-associated proteins (e.g., Tocilizumab), though one approved for treatment of Castleman’s disease, Siltuximab, is directed against the cytokine itself.7-8, 41 Those same monoclonal products are now being used or are in clinical trials aimed at relief of COVID-19 signs and symptoms.9 They appear to offer significant benefits, particularly if administered at specific time points early in the onset of disease.10-11, 42 The administration of monoclonal antibodies is costly and requires hospitalization of patients, and adverse reactions to these biological reagents occur in an important proportion of those treated.43

The selective chemical modifications of IL-6 that we have demonstrated after exposure to HOCl, HOBr and NCT suggest an alternative approach to mitigation of the cytokine storm. HOCl is widely used topically in clinical management of many infectious and inflammatory conditions, and both the hypohalous acid and NCT are known to affect events mediated by an array of cytokines, including IL-1B, IL-2, IL-4, IL-12 and IL-13.14-16, 44 Our data indicate that HOCl, HOBr and NCT are all capable of modifying IL-6 with comparable effects on IL-6R binding. The differences in the reactivity of these oxidants with specific amino acid residues may lead to important differential effects of these natural products on the diversity of biologically active polypeptides that participate in proinflammatory processes.29, 39, 45-47 However, HOCl is almost instantaneously consumed in the conversion of taurine into NCT so the likelihood is high that any beneficial effects of exogenous HOCl in vivo are due to generation of NCT.21, 27
Future studies may open up the potential for in vivo use of HOCl formulations for viral infections and inflammatory related diseases, including those caused by SARS-CoV-2. IL-6 is typically present at elevated levels in COVID-19 patients both systemically and locally in pulmonary tissues and exudates. A measure that leads to reduction of IL-6 interaction with the IL-6R in vivo could reasonably be expected to alleviate disease severity and progression. The advantages of exposure to formulations of these compounds via inhalation, in particular, arise from the potential for self-administration and avoidance of costly hospitalization. These are important considerations for the adoption of medical interventions in many countries where ready access to expensive resources and hospital facilities is unavailable to the great majority of the susceptible patient population.

Accession ID Code: IL-6_Human: P05231

Acknowledgements:

Funding: This work was supported by a gift to the University of Washington Bothell School of STEM Biochemistry Research Fund from Briotech Incorporated.

Disclosures: JFW is CSO of Briotech Incorporated and LIR is a consultant to the company.

References:


36. Marcinkiewicz, J.; Mak, M.; Bobek, M.; Biedroń, R.; Białecka, A.; Kprowski, M.; Kontny, E.; Maśliński, W., Is there a role of taurine bromamine in inflammation? Interactive effects with nitrite and...


