Peroxyynitrile Is an Essential Component of Cytokines Production Mechanism in Human Monocytes through Modulation of Nuclear Factor-κB DNA Binding Activity*

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The mechanism of release of proinflammatory cytokines by peripheral blood monocytes is unknown. Peroxyynitrile (ONOO\(^{-}\)) formed by the reaction of nitric oxide (NO) and superoxide is released predominantly by inflammatory cells at the site of injury in several inflammatory pathologies. Here we show that human monocytes treated with ONOO\(^{-}\) at micromolar concentrations induce a dose-dependent release of proinflammatory cytokines. These effects were not antagonized by up to 100 \(\mu\)M epigallocatechin gallate, an inhibitor of protein nitration. However, the proteasome inhibitor Z-Ile-Glu(OkBu)-Ala-Leu-CHO and 5,10,15,20-tetrakis(4-sulfonatophenyl)prophyrinato iron (III) chloride, a cell-permeable scavenger of ONOO\(^{-}\), almost completely inhibited the release of cytokines and the nuclear translocation of the nuclear factor (NF)-κB transcription factor. SDS-PAGE electrophoresis separation with Western blotting of cell extracts also indicated that phosphorylation and nitration of tyrosine residues in IkB-α molecules correlated with NF-κB translocation and cytokine release. In addition, the DNA binding activity of the NF-κB from the nuclear extracts also correlated with its nuclear translocation. These findings indicate ONOO\(^{-}\) plays an essential role in the mechanism of proinflammatory cytokine release by monocytes and that Rel/NF-κB activation is the obligatory pathway.

Peroxyynitrile (ONOO\(^{-}\)) formed by the reaction of nitric oxide (NO), and superoxide is released predominantly by inflammatory cells at the site of injury in several inflammatory pathologies (1–3). ONOO\(^{-}\) is highly reactive toward all classes of biomolecules, including proteins, lipids, and nucleic acids and is a potential candidate for effecting the production of proinflammatory cytokines. In proteins, ONOO\(^{-}\) modifies residues such as cysteine, methionine, tryptophan, and tyrosine (4). The irreversible modification of protein residues by ONOO\(^{-}\), such as nitration of critical tyrosine amino acids exhibits functional consequences like inactivation of a wide variety of enzymes (5–7) and may also affect structural proteins (8,9). It has also been reported that tyrosine nitration may interfere with signal transduction pathways involving tyrosine phosphorylation (10). The formation of 3,3′-dityrosine is known to result in inter- or intramolecular cross-linking of proteins such as membrane receptors consequently affecting the cell signaling pathways (11). Accumulating evidence suggests that oxidants may play a role as signaling intermediates required for receptor tyrosine kinase function and downstream activation of mitogen-activated protein kinases (12). The aim of the present studies was to examine the role of ONOO\(^{-}\) in the regulation of cytokines production by human monocytes and to investigate the underlying mechanism.

EXPERIMENTAL PROCEDURES

Cell Preparations—Peripheral blood monocytes were isolated from whole blood taken from healthy donors by a density-gradient centrifugation method (Histopaque-1071® and 11199® mediums, Sigma, Poole, UK). Briefly, into 15ml conical Falcon centrifuge tubes (Falcon, Roskilde, Denmark) 3ml of Histopaque-1071® were layered on top of 3ml of Histopaque-11199® medium. 6ml of whole blood carefully layered onto the upper gradient of the tubes were then centrifuged at 700 × g for 30 min at room temperature (18–22 °C). Distinct opaque layers of mononuclear cells were recovered from the top band and twice washed in Tris-buffered saline, pH 7.4. The cells were suspended in serum-free Hanks’ balanced salt medium without phenol red, supplemented with Ham’s nutrients F-12 and bovine serum albumin (0.25% w/v) at pH 7.4. 1ml of cell suspension was added into 1.5-ml plastic tubes and incubated at 37 °C for 90 min. The culture medium was then removed from the tube leaving monocytes adhering to the plastic surface. The monocytes were further washed with phosphate-buffered saline to remove any non-adherent cells. Fresh 1 ml of medium was then added at the start of experiments.

Cell Treatments—Monocytes (1 × 10⁶) were treated with authentic ONOO\(^{-}\) (CN Biosciences, Nottingham, UK) at 4.7% NaOH at pH 12.0, in bolus doses. After 6 h of incubation at 37 °C in an air, 5% CO₂ incubator, the effect of various concentrations of ONOO\(^{-}\) on proinflammatory cytokine release was determined in the cell lysates. Cell lysates were prepared after rapid homogenization in the medium by using an Ultra-Turrax T25 homogenizer, with a dispersing tool (IKA Laboratories, Staufen, Germany) at 20,000 rpm. The crude lysates were then centrifuged for 5 min at 1,000 × g, and the supernatants were transferred into precooled tubes, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis. A control aliquot treated with decomposed ONOO\(^{-}\) (suspended in phosphate-buffered saline at pH 7.4) was also included. To study the effect of tyrosine nitration on proinflammatory cytokine release, cells were treated with the nitration inhibitor EGCG, at 0, 1, 10, and 100 μM, with or without 10 μM ONOO\(^{-}\) for 6 h. Furthermore, the distinct role of ONOO\(^{-}\) on cytokine release, was studied in monocytes by treatment with a ONOO\(^{-}\) scavenger, 5,10,15,20-tetrakis(4-sulfonatophenyl)prophyrinato iron (III) chloride (FeTPPS) at 200 μM in the presence and absence of EGCG (10 μM).

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1 The abbreviations used are: EGCG, epigallocatechin gallate; FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)prophyrinato iron (III) chloride; TNF, tumor necrosis factor; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; EBNA, Epstein-Barr virus nuclear extract; NF-κB, nuclear factor κB; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue.
Transcriptional regulation of proinflammatory cytokine release was investigated by treating the cells with 10 μM ONOO· in the presence and absence of proteasome inhibitor, Z-Ile-Glu(OtBu)-Ala-Leu-CHO, a known inhibitor of NF-κB activity. In addition, the role of ONOO·, FeTPPS, and EGCG in the induction of NF-κB activity in monocytes nuclei extracts was investigated.

Measurement of Cytokines—TNF-α, IL-8, and IL-6 levels in cell lysates were determined by standard ELISA assays (Becton Dickinson, San Diego, CA).

Determination of Tyrosine Nitration—Nitration of protein tyrosine residues was determined by a highly sensitive competitive ELISA assay described previously (13) using rabbit anti-nitrotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY).

Preparation of Cell Extracts—Nuclear and cytoplasmic extracts were prepared using a modification of a previously published method (14). Briefly, after experiments monocytes were washed in ice-cold phosphate-buffered saline and then 100 μl of Dignam buffer A, composed of 10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2% Nonidet P-40, 100 μl of inhibitor mixture (Sigma/ml of buffer A), was added, and the tubes were vortexed vigorously on the highest setting for 15 s. The tubes were then incubated on ice for 10 min. 11 μl of 10% Nonidet P-40 solution was added to each tube followed by vortexing for 5 s on the highest setting and incubated on ice for 1 min. The tubes were then centrifuged at 16,000 × g in a microcentrifuge for 5 min. The supernatant fractions were collected into clean prechilled tubes and stored on ice. The insoluble fractions (pellet), which contain nuclei, were then resuspended in 50 μl of ice-cold buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.2 mM EDTA, and 100 μl of inhibitor mixture/ml of buffer B), vortexed vigorously for 15 s, and incubated on ice for a total of 40 min with mixing at every 10 min. The tubes were then centrifuged at 16,000 × g in a microcentrifuge, for 10 min. The supernatant (nuclear extract) fraction was immediately transferred into clean prechilled tubes and placed on ice. 5 μl of all the extract fractions were used for protein quantitation by a standard Bradford assay (15), and the rest was stored at −80 °C until use.

SDS-PAGE and Western Blotting—Nuclear and cytoplasmic extracts, respectively, were separated for NF-κB p65/Rel A and IκB-α by electrophoresis on a 10% SDS-polyacrylamide gel. The presence of NF-κB p65/Rel A in nuclear extracts was determined by immunoblotting using rabbit polyclonal NF-κB p65 (Rel A) antiserum. IκB-α was determined in the corresponding cytoplasmic extracts after immunoblotting with a rabbit polyclonal antiserum or a monoclonal anti-phospho-IκB-α that recognizes phosphorylated IκB-α at amino acid residues serine 17 and serine 36 only. All the antibodies used in this part of the study were obtained from CN Biosciences, Nottingham, UK. The immunoblot signal was visualized through enhanced chemiluminescence and scanned with an Epson Expression 1600 Pro. The relative intensities of bands were determined using an NIH Image 1.62 package.

Electrophoretic Mobility Shift Assay (EMSA)—The assay is based on that DNA-protein complexes migrate slower than unbound DNA or double stranded oligonucleotides in a native polyacrylamide or agarose gel, resulting in a “shift” in migration of the labeled DNA band. The detection of bands was by “The LightShift™ Chemiluminescent EMSA kit” (Pierce) that uses a non-isotopic method to detect DNA-protein interactions. Biotin end-labeled DNA duplex of sequence 5’-AGT TGA GGG GAC TTT CCC AGG C-3’ and 3’-TCA ACT CCC CTG AAA GGG TCC G-5’ containing a putative binding site for nuclear factor κB was incubated with the nuclear extract. After the reaction the DNA-protein complexes were subjected to a 6% native polyacrylamide gel electrophoresis and transferred to a nylon membrane (Biodyne B membrane (Upstate Biotechnology, Lake Placid, NY). Double-stranded oligonucleotides in a native polyacrylamide or agarose gel complexes were subjected to a 6% native polyacrylamide gel electrophoresis on a 10% SDS-polyacrylamide gel. The presence of 3-nitrotyrosine was determined by Western blotting with the combination of FeTPPS and EGCG almost completely abolished the release of cytokines.

RESULTS

Dose-dependent Effect of ONOO· on Cytokine Production—ONOO· caused a bell-shaped dose-response curve on the production of TNF-α and IL-6 by monocytes (Fig. 1, A and B). Peak values were observed at ONOO· concentrations of 2–20 μM followed by a reduction at higher concentrations. The reduction in cytokine production at 200 μM ONOO· was not due to the loss of cell viability, since MTT values, an index of viability, were unaffected (data not shown). These results suggest that ONOO· is an important regulator of cytokine production by monocytes.

Role of Protein Tyrosine Nitration on Cytokine Production—The pattern of 3-nitrotyrosine formation resulting from treatment of monocytes with a range of ONOO· concentrations suggests that significant increase in protein nitration occur at 200 μM ONOO· (Fig. 2A). To study the effect of tyrosine nitration on cytokine response, monocytes were treated with EGCG, an inhibitor of nitration, in the presence and absence of 10 μM ONOO·. It was observed that a significant reduction in nitration of tyrosine residues occurs when mononuclear cells are treated with EGCG at 10–100 μM in the presence and absence of ONOO· (Fig. 2B). Fig. 3, A and B, show that ONOO· (10 μM) and EGCG (10 μM) increased TNF-α and IL-6 production and that the combination of the two did not have additive effect.

To further elucidate the molecular mechanisms for ONOO·-induced cytokine release, all spots of cells were treated with 200 μM FeTPPS, a ONOO· scavenger, with 10 μM EGCG and with the combination of FeTPPS and EGCG (Fig. 4, A and B). As shown before, treating the cells with EGCG significantly increased the release of TNF-α and IL-6; however, FeTPPS alone and in combination with EGCG almost completely abolished the release of cytokines.
**Transcriptional Modulation of Cytokine Production by ONOO⁻**—To determine whether ONOO⁻ modulates proinflammatory cytokines at the transcriptional level, mononuclear cells were stimulated with 0, 10, and 200 μM ONOO⁻ concentrations in the presence and absence of proteasome inhibitor (20 μM). The proteasome inhibitor blocks the ubiquitin-proteasome pathway and therefore suppresses activation of NF-κB by stabilizing IκB-α. The results show that the proteasome inhibitor blocks the release of proinflammatory cytokines induced by ONOO⁻ (Fig. 5, A and B), suggesting that this action is regulated via NF-κB activation.

Further evidence of transcriptional modulation of NF-κB by ONOO⁻ was obtained by investigating the nuclear translocation of p65/RelA subunits of NF-κB and DNA binding capacity. The proportion of phosphorylated versus unphosphorylated IκB-α molecules in cytoplasmic cell extracts was also determined. Monocytes were incubated with various doses of ONOO⁻ (0, 10, and 200 μM), EGCG (1, 10, and 100 μM), and FeTPPS (200 μM) for 30 min at 37 °C. Greater NF-κB p65/Rel A values were detected in cells treated with 10 μM ONOO⁻ and EGCG (1–100 μM) (Fig. 6, A and B), whereas they were decreased with 200 μM ONOO⁻ and with FeTPPS. EMSA shift assays also showed that the greatest DNA binding activity was with monocytes treated with 10 μM ONOO⁻ and EGCG (1–100 μM), while no binding was detected in those treated with 200 μM ONOO⁻ and FeTPPS (Fig. 7, A and B). As shown in Fig. 8, A–C, IκB-α was significantly dephosphorylated by ONOO⁻ at all concentrations and also significantly phosphorylated at all EGCG concentrations, but the greatest degree of IκB-α phosphorylation was induced by FeTPPS.

**DISCUSSION**

The detrimental effect of ONOO⁻ has been widely described; however, its role in the modulation of cell signaling pathways that modulate the inflammatory reaction has only recently begun to attract attention. In particular, the actions of ONOO⁻ on leukocyte migration and accumulation at the site of inflammation remains contradictory. On one hand, nanomolar concentrations of ONOO⁻ have been reported to inhibit adhesion of granulocytes to the endothelium of the rat mesenteric vascular bed, and this has been shown to be mediated via down-regulation of P-selectin expression on the endothelial cell surface (16). On the other hand, scavenging of ONOO⁻ by uric acid has been shown to decrease the invasion of inflammatory cells into the spinal cord of a mouse model of multiple sclerosis (17). Furthermore, recent studies (18) have shown that micromolar concentrations of ONOO⁻ activate Ras/Raf-1/MEK signal transduction pathway, which although leads to down-regulation of L-selectin, it also up-regulates surface expression of CD11b/CD18 and consequently increases neutrophil adhesion to endothelial cells. The demonstration that ONOO⁻ mediates IL-8 gene expression in lipopolysaccharide-stimulated human whole blood (19) has led to the notion that ONOO⁻ is involved in the regulation of inflammatory reaction by blood leukocytes; however, the mechanism of this action remains unclear.

The present studies have demonstrated that the production of cytokines TNF-α and IL-6 by human mononuclear cells is predominantly regulated by ONOO⁻ in a concentration-dependent manner. This action is mediated via the transcription factor NF-κB, by a mechanism that may involve nitration or dephosphorylation of its inhibitor IκB-α. We have shown that ONOO⁻ affects NF-κB activity in a concentration-dependent manner.
manner (Fig. 6, A and B) and completely dephosphorylates IκB-α at concentrations of 10–200 μM (Fig. 7, A and B). In addition, ONOO⁻ at high concentrations nitrates tyrosine residues in cell lysates (Fig. 2A) that in turn block phosphorylation of these amino acids in IκB-α molecules and possibly promotes IκB-α degradation. This thesis is supported by previous reports showing that tyrosine nitration interferes with the process of phosphorylation and that nitrated IκB-α molecules are themselves targets for rapid degradation by intracellular enzymes (10). Singh et al. (20) have shown that tyrosine residue at site 42 (Tyr-42) on IκB-α is the specific acceptor site for phosphorylation, which prevents IκB-α degradation. This implies that ONOO⁻ may activate NF-κB simply by nitrating critical residues in IκB-α that would normally be phosphorylated. Taken together, it is possible to speculate that ONOO⁻ at concentrations ≥20 μM may nitrate tyrosine residues, including the critical site Tyr-42, a reaction that blocks its phosphorylation. However, IκB-α molecules containing nitrated tyrosine residues are themselves targets for degradation that would result in increased NF-κB activity. In contrast, 200 μM ONOO⁻ inhibits NF-κB activation, because this concentration causes extensive nitration and dephosphorylation of amino acid residues in both NF-κB subunits and IκB-α. This extensive nitration step prevents NF-κB nuclear translocation probably in two ways. The first is by causing extensive nitration of tyrosine residues in NF-κB and IκB-α, which renders both molecules targets for degradation by proteases, thus resulting in very little nuclear translocation of NF-κB subunits (Fig. 6A). The second may involve inactivation of intracellular degrading enzymes induced by extensive nitration that in turn fails to degrade IκB-α. Candidate proteases that may be inactivated by high ONOO⁻ concentrations are the HOS-SCF E3 ubiquitin ligase complex, IκB-α kinases, other protein tyrosine kinases, and the 26 S proteasome.

Treatment of cells with ECGC (10 μM) induced increases in...
TNF-α and IL-6 release. Western blots of nuclear extracts separated by SDS-PAGE showed that EGCG at concentrations of 1–100 μM increases p65 nuclear translocation and DNA binding in the presence and absence of ONOO⁻, suggesting that this is the mechanism by which EGCG promotes NF-κB activity and cytokine release in mononuclear cells. From our results it may be possible to hypothesize that EGCG protects tyrosine residues from nitration (Fig. 2) without preventing phosphorylation. Thus, although EGCG may effectively protect tyrosine residues from nitration, it may not prevent the phosphorylation of serines 32 and 36, a phenomenon previously described as the signal for IκB-α degradation by the 26 S proteasome (10). Therefore, in the absence of nitration, phosphorylation of IκB-α leads to the observed NF-κB activation that in turn increases cytokine production.

The removal of ONOO⁻ by FeTPPS protects tyrosine residues from nitration but not phosphorylation. The absence of nitration resulted in an extensive phosphorylation of IκB-α molecule (Fig. 8A), probably of serine residues 32 and 36 and critical Tyr-42 residues. The phosphorylation of a large proportion of Tyr-42 residues would prevent the degradation of IκB-α molecules, as observed by Singh et al. (20), that remain trapped within the cytoplasm. However, it cannot be ruled out that an increase in protein synthesis may also play a part in the total elevation of cytoplasmic IκB-α (Fig. 8B).

It has been shown in the literature that ONOO⁻ exhibits diverse effects in vivo dependent on the tissue affected and its environment (16, 21, 22). Our studies have disclosed that ONOO⁻ modulates the production of proinflammatory cytokines by mononuclear leukocytes in a concentration-dependent manner via the transcription factor NF-κB activation. Furthermore, the interplay between nitration and phosphorylation of amino acid residues involved in the intracellular signaling pathways appears to be an important regulatory mechanism.

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