Contrasting effects of NO and peroxynitrites on HSP70 expression and apoptosis in human monocytes

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Received 12 July 1999; accepted in final form 7 February 2000

Adrie, Christophe, Christoph Richter, Maria Bachelet, Nathalie Banzet, Dominique François, A. Tuan Dinh-Xuan, Jean François Dhainaut, Barbara S. Polla, and Marie-Jeanne Richard. Contrasting effects of NO and peroxynitrites on HSP70 expression and apoptosis in human monocytes. Am J Physiol Cell Physiol 279: C452–C460, 2000.—The free radicals nitric oxide (NO) and superoxide (O$_2^-$) react to form peroxynitrite (ONOO$^-$), a highly toxic oxidant species. In this study we investigated the respective effects of NO and ONOO$^-$ in monocytes from healthy human donors. Purified monocytes were incubated for 6 or 16 h with a pure NO donor (3-morpholinosydnonimine chlordhydrate, 0–2 mM), an NO/ONOO$^-$ donor (3-morpholinosydnonimine chlorhydrate, 0–2 mM) with and without superoxide dismutase (200 IU/ml), or pure ONOO$^-$. We provide evidence that 3-morpholinosydnonimine chlorhydrate alone represents a strong stress to human monocytes leading to a dose-dependent increase in heat shock protein-70 (HSP70) expression, mitochondrial membrane depolarization, and cell death by apoptosis and necrosis. These phenomena were abolished by superoxide dismutase, suggesting that ONOO$^-$, but not -NO, was responsible for the observed effects. This observation was further strengthened by the absence of a stress response in cells exposed to S-nitroso-N-acetyl-DL-penicillamine. Conversely, exposure of cells to ONOO$^-$ alone also induced mitochondrial membrane depolarization and cell death by apoptosis and necrosis. Thus ONOO$^-$ formation may well explain the toxic effect generally attributed to -NO.

Reactive oxygen species (ROS) are generated from molecular oxygen and include the free radicals superoxide (O$_2^-$), hydroxyl (·OH), and nitric oxide (·NO), as well as nonradical intermediates such as H$_2$O$_2$, peroxynitrite (ONOO$^-$), and singlet oxygen (¹O$_2$). During normal cellular respiration in the mitochondria, ROS are constantly produced at low rates. At these low concentrations, ROS can act as second messengers and mediators for cell activation. However, during infection or inflammation or on exposure to various environmen-
during infection and inflammation, as sources and as targets of mediators such as ROS. Using \( \cdot \)NO/ONOO\(^-\) donors or exogenous ONOO\(^-\), we investigated the respective contribution of \( \cdot \)NO and ONOO\(^-\) in the monocyte stress response (as markers of oxidative stress) and cytotoxicity. We analyzed HSP70 expression, mitochondrial membrane potential (\( \Delta \psi_m \)), and cell death by apoptosis or necrosis. Our results suggest that \( \cdot \)NO is not by itself stressful to human monocytes, whereas ONOO\(^-\) induces an oxidative stress response in these cells.

**MATERIALS AND METHODS**

**Reagents and media.** 3-Morpholinosydnonimine chlorhydrate (Sin-1) was kindly provided by Hoechst (Paris, France). S-nitroso-N-acetyl-DL-penicillamine (SNAP), superoxide dismutase (SOD) from bovine erythrocytes (EC 1.15.1.1), and saponin were purchased from Sigma Chemical (St. Louis, MO), antibiotic-free RPMI 1640 medium, fetal calf serum (FCS), HEPES, and glutamine from GIBCO (Paisley, Scotland), the monoclonal antibody SPA-810, specific for the inducible HSP70, from StressGen Biotechnologies (Victoria, Canada), the secondary anti-mouse antibody (IgG-FITC) from Dako (Carpinteria, CA), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) from Molecular Probes (Eugene, OR), and the annexin V-Fluos staining kit from Boehringer Mannheim (Mannheim, Germany).

ONOO\(^-\) was synthesized from sodium nitrite and \( \text{H}_2\text{O}_2 \) by use of a quenched flow reactor (8). Stock solutions (83 mM) were stored at \(-70^\circ\text{C}\) and pH 12. Because of the potential presence of \( \text{H}_2\text{O}_2 \) as contaminant in this solution, ONOO\(^-\) was applied to culture medium in the absence of cells at the highest concentration used in our study, and the solution was incubated for 30 min at \(37^\circ\text{C}\) to completely degrade ONOO\(^-\). The medium on the cells was replaced with the blank reagent obtained after complete degradation of ONOO\(^-\). This allowed us to distinguish the effects of ONOO\(^-\) from those of its by-products or contaminants (51).

**Cells.** Human peripheral mononuclear cells were isolated by Ficoll gradient centrifugation, and monocytes were purified by adherence in 60-mm-diameter petri dishes for 45 min and then washed with PBS. Cells were maintained in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, and 25 mM HEPES in a humidified atmosphere containing 95% air-5% \( \text{CO}_2 \) at \(37^\circ\text{C}\).

Cells were exposed to the pure \( \cdot \)NO donor SNAP (0.5, 0.75, 1, and 2 mM) or to Sin-1 (0.25, 0.5, 1, and 2 mM) for 6 or 16 h. Sin-1 releases \( \cdot \)NO and \( \text{O}_2\cdot \) in an equimolar manner, thus generating ONOO\(^-\), whereas SNAP releases only \( \cdot \)NO (20). Cells were incubated with Sin-1 or SNAP in the presence or absence of SOD (200 IU/ml, added 20 min before \( \cdot \)NO donors). SOD, by dismutating \( \text{O}_2\cdot \), prevents the formation of ONOO\(^-\) during Sin-1 decomposition.

**Nitrite/nitrate determination.** \( \cdot \)NO is oxidized in cell medium to form several nitrogen oxides (NO\(_x\)), in particular nitrate (NO\(_3^-\)) and nitrite (NO\(_2^-\)). NO\(_x\) can be measured in medium by conversion to \( \cdot \)NO by use of a strong reducing environment (3) consisting of vanadium and 1 N HCl at 90°C as follows: \( \text{NO}_3^- + 4\text{H}^- + 3\text{e}^- \rightarrow \cdot \text{NO} + 2\text{H}_2\text{O} \). The amount of \( \cdot \)NO produced was determined by chemiluminescence with...
use of a fast-responding analyzer (model NOA 280, Sievers Instruments) (3).

Analysis of HSP70 expression. The level of HSP70 expression in monocytes was quantified by flow cytometry analysis, as described previously (4). Briefly, a pellet of 10^6 cells was resuspended in 100 µl of 3% paraformaldehyde in PBS, kept for 10 min at room temperature, and then washed by addition of 1 ml of PBS with 1% BSA (PBS-BSA). For labeling, cells were incubated in 50 µl of 0.6% saponin and the antibody against the cytosolic inducible HSP70 at a dilution of 1:100 for 10 min at room temperature. Unbound antibodies were removed by washing twice in PBS-BSA. Bound antibodies were revealed with rabbit anti-mouse IgG-FITC conjugate diluted at 1:30 for 10 min at room temperature. Analysis was performed by flow cytometry (EPICS Elite flow cytometer; Coulter, Miami, FL). HS (44°C for 30 min, with 4 h of recovery) was used as positive control for HSP70 induction in monocytes. Data are expressed as the ratio of median fluorescence channels (data have been converted from logarithmic to linear scale) of cells incubated with an irrelevant isotypic matched antibody (i.e., the negative control) to that of cells incubated with the monoclonal anti-HSP70 antibody.

Determination of Δψm, ΔΨm was measured by using the lipophilic cation JC-1, which is able to selectively enter mitochondria. JC-1 exists in a monomeric form, emitting at 527 nm after excitation at 490 nm. Depending on the emission (590 nm) of JC-1, is able to form J-aggregates that are associated with a large shift in emission (590 nm). Dye color changes reversibly from green to greenish orange as mitochondrial membrane becomes more polarized. Cell staining was performed as follows: cell suspensions were adjusted to a density of 0.5 × 10^6 cells/ml and incubated in supplemented RPMI 1640 medium with JC-1 (10 µg/ml) for 10 min at room temperature in the dark. At the end of the incubation period, cells were washed in PBS, resuspended in a total volume of 400 µl, and immediately analyzed by flow cytometry using a EPICS Elite flow cytometer, as previously described (17, 47). H2O2 (4 mM, 4 h at 37°C) was used as positive control for mitochondrial depolarization (40).

Flow cytometry analysis of cell death by apoptosis and necrosis. Apoptosis was detected with annexin V, which has high affinity for negatively charged phospholipids such as phosphatidylserine. The simultaneous use of the DNA stain propidium iodide, which is excluded from intact and apoptotic cells, allows for the adequate detection of necrotic cells among the annexin V-positive cluster. Cells were washed, stained with annexin V and propidium iodide in HEPES buffer as described by the manufacturer, and analyzed by flow cytometry with an EPICS Elite flow cytometer equipped with a single 488-nm argon laser. In all cases, a total of 5,000 cells/sample were analyzed in list mode for green fluorescence through a 525-nm filter and for red fluorescence through a 575-nm filter. All data were analyzed with Elite software version 4.02. Cells exclusively positive for annexin V were considered to be undergoing apoptosis, whereas cells positive for propidium iodide and annexin V were considered necrotic (18, 52).

Electron microscopy. After Sin-1 treatment, monocytes were fixed in suspension (10^6 cells) with 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4, for 10 min at 4°C and as a pellet for 2 h. After they were washed in PBS, ultrathin sections were cut, counterstained, and then examined in a Philips EM-300 electron microscope operating at 60 kV.

Statistical analysis. Values are means ± SE. The data were analyzed using a one-way ANOVA for repeated measures followed by the Mann-Whitney test for post hoc comparison of the mean. The criterion for statistical significance was P < 0.05.

RESULTS

Effects of Sin-1 and SNAP on Hsp70 expression. Inasmuch as ROS were previously reported to induce a stress response in human monocytes, we tested whether NO itself, or its by-product ONOO−, induced HSP70 expression. Cells were exposed to Sin-1 with or without SOD to distinguish the respective effects of NO and ONOO−. Sin-1 (1 mM, 6 h) induced HSP70 expression (Fig. 1C) to levels similar to those induced by HS (Fig. 1B), although the distribution of HSP70-positive and HSP70-negative cells was different with HS (double peak) and with Sin-1 (single peak). In the presence of SOD, HSP70 expression induced by Sin-1 was abolished (Fig. 1D). The expression of HSP70 was detectable 3 h after exposure to Sin-1 and was no longer detectable after 16 h (data not shown).

![Image](http://apcell.physiology.org/Downloadedfrom/10.22032.247)
We then tested the dose dependence of HSP70 induction by Sin-1, in the presence or absence of SOD, and compared it with the pure NO donor SNAP. HSP70 expression increased gradually as a function of Sin-1 concentration up to 1 mM (Fig. 2A) but decreased at a higher concentration (2 mM, 6 h), an observation probably related to the toxicity of Sin-1 at this concentration. SOD completely prevented the increased expression of HSP70 at all concentrations tested, indicating that NO per se was unable to induce HSP70 expression in human monocytes, whereas ONOO⁻ generated during Sin-1 decomposition appeared as an efficient inducer of HSP70 expression. These conclusions were further supported by the lack of induction of HSP70 expression obtained with the pure NO donor SNAP (Fig. 2B) at any concentration tested (0.5–2 mM) after 6 or 16 h of exposure, despite dramatic increases in NO₂/NO₃ concentrations in the supernatant (Table 1). In addition, SOD had no effect in SNAP-treated cells (Fig. 2B).

Effects of Sin-1 and SNAP on ΔΨₘ. A close link between mitochondrial functions and apoptosis was previously proposed (33). We thus investigated the effects of NO/ONOO⁻ on ΔΨₘ (Figs. 3 and 4) and compared them with those obtained in cells treated with H₂O₂, an oxidant known to induce a marked mitochondrial depolarization (Fig. 3B). Sin-1 (1 mM, 6 h) induced a decrease in ΔΨₘ (Fig. 3C) that was prevented by SOD (Fig. 3D).

Exposure of human monocytes to Sin-1 induced dose-dependent (0.25–2 mM) disruption of ΔΨₘ, which was prevented by SOD (Fig. 4A). When cells were exposed to increasing doses of SNAP (0–2 mM) for 6 h (data not shown), no alterations in ΔΨₘ were observed with or without addition of SOD (Fig. 4B). These results indicate that the expression of HSP70 in cells exposed to Sin-1 is insufficient to afford protection from alterations of ΔΨₘ induced by ONOO⁻ in human monocytes. In addition, we observed that, parallel to the lack of induction of HSP70 expression, NO per se did not affect ΔΨₘ in human monocytes.

Effects of Sin-1 and SNAP on cell death. Inasmuch as mitochondrial depolarization is generally recognized as a prerequisite step in the pathways leading to ROS-dependent apoptosis, the effects of NO/ONOO⁻ on

Table 1. NO₂/NO₃ production after exposure of human monocytes to Sin-1 or SNAP

<table>
<thead>
<tr>
<th>NO₂/NO₃, μM</th>
<th>Sin-1, mM</th>
</tr>
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<tbody>
<tr>
<td>SOD⁻</td>
<td>0 0.25 0.5 1 2</td>
</tr>
<tr>
<td>37 ± 27</td>
<td>268 ± 96* 450 ± 88* 950 ± 241† 1,327 ± 349†</td>
</tr>
<tr>
<td>56 ± 59</td>
<td>315 ± 128 600 ± 127* 735 ± 198† 1,643 ± 538†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NO₂/NO₃, μM</th>
<th>SNAP, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD⁻</td>
<td>0 0.5 0.75 1 2</td>
</tr>
<tr>
<td>19 ± 23</td>
<td>175 ± 89 229 ± 40* 484 ± 47† 678 ± 311†</td>
</tr>
<tr>
<td>35 ± 43</td>
<td>187 ± 69 262 ± 52* 347 ± 117† 642 ± 330†</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 3. Nitrite and nitrate (NO₂/NO₃) production after 6 h of incubation with 3-morpholinosydnonimine chlorhydrate (Sin-1) or S-nitroso-N-acetyl-DL-penicillamine (SNAP) without (SOD⁻) or with superoxide dismutase (SOD +). Dose-dependent increase in NO₂/NO₃ monocyte exposure to SNAP or Sin-1 was not affected by SOD. *P < 0.05; †P < 0.01 vs. baseline.

Fig. 3. Cytofluorometric analysis of mitochondrial membrane potential (ΔΨₘ). Cytofluorometric analysis of ΔΨₘ was assessed by staining with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining in control cells (A), cells treated with 4 mM H₂O₂ for 4 h (B), and cells exposed to 1 mM Sin-1 for 6 h in the absence (C) and presence (D) of SOD (200 IU/ml). Results from 1 representative experiment are shown. Percentage of cells with depolarized mitochondria is shown.
monocyte death were analyzed. Monocytes exposed to Sin-1 for 16 h underwent cell death by apoptosis or necrosis depending on the concentration applied (Fig. 5A), with apoptosis reaching a maximum at 1 mM. At a higher concentration (2 mM), apoptosis decreased whereas necrosis increased up to 78 ± 8%. As observed for DCm and for HSP70 expression, SOD prevented Sin-1-induced cell death, indicating that ONOO⁻, but not ·NO, was responsible for cell death by apoptosis or by necrosis. High concentrations of SNAP for up to 16 h did not elicit any effect on human monocyte viability, further supporting the conclusions that ONOO⁻, but not ·NO, was cytotoxic in these cells (Fig. 5B).

**Effects of Sin-1 on ultrastructural morphology.** Functional criteria for Sin-1-induced cell death paralleled ultrastructural criteria of apoptosis or necrosis (Fig. 6). Electron microscopy showed that a low concentration of Sin-1 essentially led to an alteration of cytосolic organelles with “cytosolic boiling,” swollen mitochondria, and perinuclear condensation. At higher concentrations, cells exhibiting features of apoptosis coexisted with cells exhibiting necrosis features, such as plasma membrane disruption and pycnotic nuclei. These morphological alterations were prevented by preincubation with SOD.

**Effects of ONOO⁻ on ΔΨₘ and cell death.** Finally, to further confirm our results, we investigated the effects of exposure to increasing concentrations of ONOO⁻ (0–1,000 μM, 16 h) on human monocytes. ONOO⁻ induced ΔΨₘ in a dose-dependent manner (Table 2) as well as cell death by apoptosis or necrosis, depending on its concentration (Table 2). The blank reagent obtained after complete degradation of ONOO⁻ did not exert any cytotoxicity on human monocytes, excluding an effect due to contaminants or decomposition products of ONOO⁻.

**DISCUSSION**

Here, we report that ONOO⁻ induced a stress response in human monocytes that was characterized by
induction of HSP70 expression, mitochondrial membrane depolarization, and cell death by apoptosis or necrosis, whereas -NO per se, even at high concentrations, did not elicit such a stress response in these cells.

The HS/stress response is a conserved, physiological, and transient response to cellular injuries, including oxidative stress. The presence of abnormal, unfolded, or misfolded proteins, alterations in membrane physical state, classical second messengers, specific mitochondrial alterations, or ROS could represent the cellular sensors for HS induction (2, 13, 29, 38, 39). Among ROS, we previously showed that H₂O₂ and 'OH, but not the membrane-impermeant O₂−, are inducers of an HS response (6). Here we studied the expression of HSP70 in monocytes on exposure to -NO or ONOO−. Sin-1, which generates -NO and O₂−, induced the intracellular overexpression of HSP70, which was abolished by the addition of SOD, indicating that ONOO−, but not -NO, induced HSP70 accumulation. This conclusion was supported by the lack of induction of HSP70 expression that we observed with SNAP, a slow gener-
cell death). The latter represents an active process of cell “suicide,” resulting in the demise and subsequent removal of the affected cell by scavenger phagocytes without liberation of inflammatory mediators, as observed during necrosis. ONOO\(^-\) is well established as a very reactive species inducing cell lesions and cell death by apoptosis or necrosis, the latter at higher concentrations (10). Evidence for apoptosis induced by \(\cdot\)NO was provided by microscopic examination of chromatin condensation and by a specific pattern of internucleosomal fragmentation, i.e., DNA laddering detected by agarose gel electrophoresis (1, 46). In our experimental conditions, Sin-1 induced apoptosis and necrosis at low concentrations and necrosis only at higher concentrations. Our present data are consistent with other studies showing that the same biological, chemical, or physical stresses may induce apoptosis or necrosis depending on their concentration and/or the cell type (9, 25, 52). Apoptosis and necrosis observed in cells incubated with Sin-1 for 16 h were inhibited by preincubation with SOD (D).

Table 2. Effects of ONOO\(^-\) on \(\Delta\Psi_m\) and cell death

<table>
<thead>
<tr>
<th>ONOO(^-), (\mu)M</th>
<th>Cells With Depolarized Mitochondria, %</th>
<th>Apoptosis, %</th>
<th>Necrosis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.5 ± 1.05</td>
<td>9.5 ± 0.5</td>
<td>4.9 ± 3.6</td>
</tr>
<tr>
<td>50</td>
<td>15.8 ± 3.5</td>
<td>13 ± 1.5</td>
<td>3.3 ± 1.7</td>
</tr>
<tr>
<td>100</td>
<td>13.5 ± 2.8</td>
<td>10.7 ± 2.2</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>250</td>
<td>20.7 ± 6.9</td>
<td>10.8 ± 1.9</td>
<td>6 ± 3.2</td>
</tr>
<tr>
<td>500</td>
<td>23.3 ± 5.3(^*)</td>
<td>14.5 ± 2.6(^*)</td>
<td>11.2 ± 6</td>
</tr>
<tr>
<td>750</td>
<td>29.1 ± 4.6(^*)</td>
<td>20.9 ± 2.5(^*)</td>
<td>13.7 ± 5.9</td>
</tr>
<tr>
<td>1,000</td>
<td>33.8 ± 10.8(^*)</td>
<td>28.2 ± 7.3</td>
<td>21 ± 5.6(^*)</td>
</tr>
<tr>
<td>Blank reagent</td>
<td>10.2 ± 1.3</td>
<td>8.2 ± 0.9</td>
<td>4.4 ± 2.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n = 3\). Exposure to peroxynitrite (ONOO\(^-\)) for 16 h induced mitochondrial membrane depolarization and cell death by apoptosis or necrosis in human monocytes. Note lack of effect of the blank reagent after complete degradation of ONOO\(^-\), \(\Delta\Psi_m\), mitochondrial membrane potential. \(^*\) \(P < 0.05\) vs. baseline.

Fig. 6. Effects of Sin-1 on monocyte ultrastructure. Morphological analysis of the effects of Sin-1 (1 mM, 6 h) on human monocytes is shown. A: control monocyte displayed vacuoles, euchromatin, and heterochromatin. B: Sin-1 (0.5 mM, 16 h). Sin-1 essentially led to an alteration of cytosolic organelles with “cytosolic boiling,” swollen mitochondria, and perinuclear condensation. At higher concentrations, cells exhibiting features of apoptosis coexisted with cells exhibiting features of necrosis, such as plasma membrane disruption and pycnotic nuclei (C). Apoptosis and necrosis were completely prevented by preincubation with SOD (D).
apoptosis. They support the hypothesis that the oxidative injury associated with simultaneous production of ·NO and other ROS is mediated through ONOO− formation. DNA damage, once attributed to ·NO, is now believed to be the consequence of ONOO− formation (50, 51). Along these lines, inhibition of tissue factor expression circulating in human monocytes after stimulation by lipopolysaccharide (23) and rat pulmonary epithelial type II cell cytotoxicity (24), once believed to be induced by ·NO, appeared to be an indirect effect of ·NO by subsequent ONOO− formation. However, in our study, potential mitochondrial membrane disruption and cell death occurred despite HSP70 expression.

HSP accumulation has long been used as a marker of cell and tissue damage. This would seem to be in conflict with the previously described antiapoptotic properties of HSP. However, there is evidence that HSP could act to prevent and promote apoptosis. An overlap exists between the signals (the protein denaturation, the oxidation of protein thiols, or the increase in ceramide levels) that induce a protective stress response and those that initiate apoptosis (for review see Ref. 45). Although a number of signals are known to induce in parallel HSP expression, mitochondrial depolarization, and initiation of the program of cell destruction (6, 37, 53), there is a paucity of evidence that the cells that accumulate HSP are the cells that are destined to die. In this study, HSP70 expression was regarded as a marker of stressful conditions induced by ONOO− in human monocytes.

In conclusion, our data suggest that, in contrast to ONOO−, ·NO per se is not stressful or cytotoxic for human monocytes as assessed by HSP70 expression, ∆ψm, and cell death. ONOO− formation may well explain the toxic effect generally attributed to ·NO. Because of the widespread production of O2− by many cell types during inflammation, all subsequent studies in which ·NO is used as the donor should include selective scavengers such as SOD to distinguish the effect of ·NO from those secondary to ONOO− formation.

The authors thank Ewa Mariéthoz for technical assistance and Sarah Kreps for reviewing the manuscript.

This study was supported by Institut National de la Santé et de la Recherche Médicale.

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