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

CHRISTOPHE ADRIE,1,2 CHRISTOPH RICHTER,3 MARIA BACHELET,1 NATHALIE BANZET,1 DOMINIQUE FRANÇOIS,1 A. TUAN DINH-XUAN,1,3 JEAN FRANÇOIS DHAINAUT,2 BARBARA S. POLLA,1,5 AND MARIE-JEANNE RICHARD1,4
1Laboratory of Respiratory Physiology, Unité de Formation et de Recherche Cochin Port-Royal, Paris V University, and 2Medical Intensive Care Unit, Cochin Port-Royal Hospital, Paris, France; 3Institute of Biochemistry, Swiss Federal Institute of Technology, Zurich, Switzerland; and 4Laboratory of Biology of Oxidative Stress, Biochemistry C, A. Michallon Hospital, Grenoble, France

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 (O2·−) 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 chlorhydrate, 0–2 mM), an ·NO/ONOO− donor (3-morpholinosydnonimine chlorhydrol, 0–2 mM) with and without superoxide dismutase (200 IU/ml), or pure ONOO−. We provide evidence that 3-morpholinosydnonimine chlorhydrol 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.

nitric oxide; heat shock; cell stress; cell death.

REACTIVE OXYGEN SPECIES (ROS) are generated from molecular oxygen and include the free radicals superoxide (O2·−), hydroxyl (−OH), and nitric oxide (·NO), as well as nonradical intermediates such as H2O2, peroxynitrite (ONOO−), and singlet oxygen (1O2) (15, 41). 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 environmental toxic agents, ROS can accumulate to deleterious levels, leading to cell damage and subsequent adaptive responses (28).

NO is an endogenous mediator first characterized as an endothelium-derived relaxing factor (21). It is now recognized as a key mediator in many physiological processes. Although ·NO generated by activated macrophages plays a key role in the defense against tumor cells and pathogens, it has been reported to be protective or cytotoxic, according to its rate and amount of production (19, 54). ·NO-mediated cytotoxicity might underlie the pathogenesis of an organ in shock and inflammation.

The stress response is a marker of cellular injury and a conserved adaptive response to stressful conditions such as oxidative stress. It includes induction of the so-called heat shock (HS) proteins (HSP). The HSP and, in particular, the cytosolic, inducible, 72-kDa HSP70 protect human cells against the deleterious effects of ROS, including ·NO. These protective effects are exerted primarily at the mitochondrial level and are associated with an inhibition of apoptosis when HSP70 are induced before stresses (9, 39, 40, 52). However, the protective effects of HSP70 cannot be extrapolated to all cases of apoptosis (52, 53).

Several reports have suggested that ·NO is involved, on the one hand, in HSP70 induction (30, 35, 36) and, on the other hand, in mitochondrial dysfunction and cell death. ·NO and O2·−, however, are weak oxidants. Thus the question as to whether ·NO by itself or its by-products, such as ONOO−, are responsible for these various effects remains a matter of controversy. For example, toxic effects such as DNA single-strand breaks and activation of the enzyme poly(ADP-ribose) polymerase, once attributed to ·NO, are now believed to be mediated by ONOO− (50, 51).

We addressed this issue in human monocytes, since these cells play a key role in defense mechanisms.
during infection and inflammation, as sources and as targets of mediators such as ROS. Using \textsuperscript{·}NO/ONOO\textsuperscript{−} donors or exogenous ONOO\textsuperscript{−}, we investigated the respective contribution of \textsuperscript{·}NO and ONOO\textsuperscript{−} in the monocyte stress response (as markers of oxidative stress) and cytotoxicity. We analyzed HSP70 expression, mitochondrial membrane potential (Δψ\textsubscript{m}), and cell death by apoptosis or necrosis. Our results suggest that \textsuperscript{·}NO is not by itself stressful to human monocytes, whereas ONOO\textsuperscript{−} 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\textsuperscript{−} was synthesized from sodium nitrite and H\textsubscript{2}O\textsubscript{2} by use of a quenched flow reactor (8). Stock solutions (83 mM) were stored at −70°C and pH 12. Because of the potential presence of H\textsubscript{2}O\textsubscript{2} as contaminant in this solution, ONOO\textsuperscript{−} 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°C to completely degrade ONOO\textsuperscript{−}. The medium on the cells was replaced with the blank reagent obtained after complete degradation of ONOO\textsuperscript{−}. This allowed us to distinguish the effects of ONOO\textsuperscript{−} 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% CO\textsubscript{2} at 37°C.

Cells were exposed to the pure \textsuperscript{·}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 \textsuperscript{·}NO and O\textsubscript{2}\textsuperscript{−} in an equimolar manner, thus generating ONOO\textsuperscript{−}, whereas SNAP releases only \textsuperscript{·}NO (20). Cells were incubated with Sin-1 or SNAP in the presence or absence of SOD (200 IU/ml, added 20 min before \textsuperscript{·}NO donors). SOD, by dismutating O\textsubscript{2}\textsuperscript{−}, prevents the formation of ONOO\textsuperscript{−} during Sin-1 decomposition.

Nitrite/nitrate determination. \textsuperscript{·}NO is oxidized in cell medium to form several nitrogen oxides (NO\textsubscript{x}), in particular nitrate (NO\textsubscript{3}−) and nitrite (NO\textsubscript{2}−). NO\textsubscript{x} can be measured in medium by conversion to \textsuperscript{·}NO by use of a strong reducing environment (3) consisting of vanadium and 1 N HCl at 90°C as follows: NO\textsubscript{3}− + 4H\textsuperscript{+} + 3e\textsuperscript{−} → \textsuperscript{·}NO + 2H\textsubscript{2}O. The amount of \textsuperscript{·}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 ΔΨm, 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 an 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).
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 (0.25–2 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/NOO⁻ 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 (16 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/NOO⁻ on

![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.](http://ajpcell.physiology.org/)

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

<table>
<thead>
<tr>
<th>SNAP, mM</th>
<th>0</th>
<th>0.5</th>
<th>0.75</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₂/NO₃⁻, µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD⁻</td>
<td>19 ± 23</td>
<td>175 ± 89</td>
<td>229 ± 40*</td>
<td>484 ± 47†</td>
<td>678 ± 311†</td>
</tr>
<tr>
<td>SOD+</td>
<td>35 ± 43</td>
<td>187 ± 69</td>
<td>262 ± 52*</td>
<td>347 ± 117†</td>
<td>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-morpholinosydronimine 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.
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. 5), with apoptosis reaching a maximum at 1 mM. At a higher concentration (2 mM), apoptosis decreased whereas necrosis increased up to $78 \pm 8\%$. 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 cytosolic 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 ΔΨ$m$ 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 ΔΨ$m$ 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 HSP 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-

Fig. 5. Effects of Sin-1 on death of human monocytes. Incubation with the ·NO/ONOO⁻ donor Sin-1 for 16 h induced apoptosis (A) and necrosis (B) in human monocytes as assessed by annexin V and propidium iodide. Preincubation with SOD (200 IU/ml) prevented cell death induced by Sin-1. Values are means ± SE; n = 5. *P < 0.01 vs. respective controls; **P < 0.01 vs. controls with SOD.
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 ·NO was provided by microscopic examination of chromatin condensation and by a specific pattern of inter-nucleosomal 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 ΔΨₘ and cell death

<table>
<thead>
<tr>
<th>ONOO⁻, μM</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>32.8 ± 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⁻. ΔΨₘ, 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 \( \cdot \text{NO} \) and other ROS is mediated through \( \text{ONOO}^{-} \) formation. DNA damage, once attributed to \( \cdot \text{NO} \), is now believed to be the consequence of \( \text{ONOO}^{-} \) 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 \( \cdot \text{NO} \), appeared to be an indirect effect of \( \cdot \text{NO} \) by subsequent \( \text{ONOO}^{-} \) 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 \( \text{ONOO}^{-} \) in human monocytes.

In conclusion, our data suggest that, in contrast to \( \text{ONOO}^{-} \), \( \cdot \text{NO} \) per se is not stressful or cytotoxic for human monocytes as assessed by HSP70 expression, \( \Delta \psi_{\text{m}} \), and cell death. \( \text{ONOO}^{-} \) formation may well explain the toxic effect generally attributed to \( \cdot \text{NO} \). Because of the widespread production of \( \text{O}_{2}^{-} \) by many cell types during inflammation, all subsequent studies in which \( \cdot \text{NO} \) is used as the donor should include selective scavengers such as DOD to distinguish the effect of \( \cdot \text{NO} \) from those secondary to \( \text{ONOO}^{-} \) 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.

Present address of B. S. Polla: INSERM U326, Institut Cochin de Génétique Moléculaire, 75014 Paris, France.

REFERENCES


27. Lizasoain I, Moro MA, Knowles RG, Darley-Usmar VM, and Moncada S. Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. *Biochem J* 314: 877–880, 1996.


