Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1753–1758, March 1996
Biochemistry

DNA strand breakage, activation of poly(ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity in macrophages and smooth muscle cells exposed to peroxynitrite

(nitric oxide/superoxide/nitric oxide synthase/endotoxin shock/inflammation)

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Communicated by Britton Chance, University of Pennsylvania, Philadelphia, PA, November 2, 1995 (received for review May 2, 1995)

ABSTRACT **The free radicals nitric oxide and superoxide anion react to form peroxynitrite ($ONOO^-$), a highly toxic oxidant species. *In vivo* formation of $ONOO^-$ has been demonstrated in shock and inflammation. Herein we provide evidence that cytotoxicity in cells exposed to $ONOO^-$ is mediated by DNA strand breakage and the subsequent activation of the DNA repair enzyme poly(ADP ribose) synthetase (PARS). Exposure to $ONOO^-$ (100 μM to 1 mM) inhibited mitochondrial respiration in cultured J774 macrophages and in rat aortic smooth muscle cells. The loss of cellular respiration was rapid, peaking 1–3 h after $ONOO^-$ exposure, and reversible, with recovery after a period of 6–24 h. The inhibition of mitochondrial respiration was paralleled by a dose-dependent increase in DNA strand breakage, reaching its maximum at 20–30 min after exposure to $ONOO^-$. We observed a dose-dependent increase in the activity of PARS in cells exposed to $ONOO^-$. Inhibitors of PARS such as 3-aminobenzamide (1 mM) prevented the inhibition of cellular respiration in cells exposed to $ONOO^-$. Activation of PARS by $ONOO^-$-mediated DNA strand breakage resulted in a significant decrease in intracellular energy stores, as reflected by a decline of intracellular NAD^+ and ATP content. 3-Aminobenzamide prevented the loss of NAD^+ and ATP in cells exposed to $ONOO^-$. In contrast, impairment of cellular respiration by the addition of the nitric oxide donors *S*-nitroso-*N*-acetyl-DL-penicillamine or diethyltriamine nitric oxide complex, was not associated with the development of DNA strand breaks, in concentrations up to 1 mM, and was largely refractory to PARS inhibition. Our results suggest that DNA damage and activation of PARS, an energy-consuming futile repair cycle, play a central role in $ONOO^-$-mediated cellular injury.**

Nitric oxide (NO) is synthesized from L-arginine by a family of isoenzymes termed NO synthases (1–4). Overproduction of NO has been demonstrated in a variety of inflammatory disorders, including arthritis and ileitis, and in circulatory shock (5–8). When present in high concentration, NO exhibits cytotoxic properties, resulting in the inhibition of cellular respiration and the lysis of cells. These effects may underlie the pathogenesis of organ failure in shock and inflammation (1, 2, 5–8).

NO reacts readily with the oxygen free radical, superoxide anion, to form the toxic oxidant species peroxynitrite ($ONOO^-$) (9, 10). The formation of $ONOO^-$ has been demonstrated in activated macrophages (11) and endothelial cells (12), in rheumatoid arthritis (13), and in endotoxic shock (14, 15). These data have raised the possibility that some of the previously ascribed cytotoxic actions of NO may not, in fact, result from the effect of NO *per se*, but rather via the production of $ONOO^-$.

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NO is known to interfere with cellular respiration and to deplete cellular ATP via direct inhibition of enzymes in the glycolytic pathway, Krebs cycle, and electron transport chain (2, 8). Recent evidence suggests, however, that NO may also lower cellular ATP via an indirect route: in pancreatic islet cells (16) and in neurons (17), high concentrations of NO have been shown to cause DNA injury and trigger a repair process by the nuclear enzyme poly(ADP ribose) synthetase (PARS), which consumes cellular energy stocks, resulting in the depletion of NAD^+ and ATP and culminating in irreversible cellular injury.

$ONOO^-$ is a toxic oxidant species that has greater cytotoxic potential than NO (9, 10). $ONOO^-$ directly inhibits mitochondrial respiratory enzymes (18, 19), reduces cellular oxygen consumption, and inhibits membrane sodium transport (20). Herein we investigate the potential contribution of DNA damage and PARS activation to the development of cellular injury in response to $ONOO^-$.

MATERIALS AND METHODS

Cell Culture. The mouse macrophage cell line J774 was cultured in Dulbecco's modified Eagle's medium (DMEM) and rat aortic smooth muscle cells were cultured in RPMI 1640 medium as described (21).

Measurement of Mitochondrial Respiration. Mitochondrial respiration was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (22).

Determination of DNA Single-Strand Breaks. The formation of strand breaks in double-stranded DNA was determined by the alkaline unwinding method as described (23).

Measurement of PARS Activity. Cells were treated with $ONOO^-$ and incubated for 10 min in 56 mM Hepes (pH 7.5) containing 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, and 125 nmol of NAD^+ containing 0.25 μCi of [^{3}H]NAD^+ (1 Ci - 37 GBq). PARS activity was then measured as described (24). Permeabilized cells were incubated for 5 min at 37°C, and the protein that was ribosylated with [^{3}H]NAD^+ was precipitated with 200 μl of 50% (wt/vol) trichloroacetic acid (TCA). After two washes with TCA, the protein pellet was solubilized in 2% (wt/vol) SDS in 0.1 M NaOH and incubated at 37°C overnight, and the radioactivity was determined by scintillation counting.

Measurement of ADP-Ribosylation in Soluble Extracts of J774 Cells. Cells were treated with $ONOO^-$ and incubated for 10 min in Hepes buffer containing 125 nM NAD and 2 μCi of [^{32}P]NAD. Proteins were precipitated with 200 μl of 50% TCA. The precipitates were centrifuged at 10,000 × *g* for 10

Abbreviations: 3-AB, 3-aminobenzamide; LPS, lipopolysaccharide; NO, nitric oxide; $ONOO^-$, peroxynitrite; PARS, poly(ADP-ribose) synthetase; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; TCA, trichloroacetic acid.

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min, and pellets were washed once with 0.5 ml of 5% TCA and once with 0.5 ml of water-saturated diethylether solution and resuspended in 200 μl of 62.5 mM Tris·HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% (vol/vol) 2-mercaptoethanol, and 0.00125% bromophenol blue. The resulting preparations were heated at 100°C for 5 min and loaded onto a 7.5% polyacrylamide gel containing 0.1% SDS. Gels were electrophoresed at 35 mA for 4.5 h in Laemmli buffer (0.3% Trizma, pH 8.3/1.44% glycine/0.1% SDS). Gels were dried and exposed to Fuji RX film for 24 h with intensifying screens. Radiolabeled polypeptides were visualized by autoradiography.

Measurement of Cellular NAD^+ Levels. Cells were exposed to $ONOO^-$ at 37°C for various periods in the presence or absence of PARS inhibitors. Cells were extracted in 0.25 ml of 0.5 N $HClO_4$, scraped, neutralized with 3 M KOH, and centrifuged for 2 min at 10,000 × *g*. The supernatant was assayed for NAD^+ by using a modification of the colorimetric method (25), in which NADH, produced by enzymatic cycling with alcohol dehydrogenase, reduces MTT to formazan through the intermediation of phenazine methosulfate. The rate of increase in the absorbance was read immediately after addition of the NAD samples and after 10- and 20-min incubations at 37°C against a blank at 560 nm in the Spectramax spectrophotometer.

Measurement of Cellular ATP Levels. ATP was extracted with 10% TCA containing 0.15% EDTA. The cell suspension was sonicated for 10 s and centrifuged for 10 min at 720 × *g* at 4°C. ATP levels were measured by using a detection kit (Analytical Luminescence Laboratory, San Diego) utilizing firefly luciferase (26) in a luminometer.

Materials. DMEM, RPMI 1640 medium, and fetal calf serum were from GIBCO. [^{3}H]NAD^+ and [^{32}P]NAD^+ were obtained from DuPont/NEN. Alcohol dehydrogenase and NAD^+ were obtained from Boehringer Mannheim. *S*-Nitroso-*N*-acetyl-DL-penicillamine (SNAP) was obtained from Calbiochem. Spermine–NO and diethyltriamine–NO were obtained from Research Biochemicals (Natick, MA). All other drugs were obtained from Sigma. SIN-1 was a gift from Cassella AG (Frankfurt, Germany). PD 128763 was a gift from J. Sebolt-Leopold (Parke–Davis).

$ONOO^-$ and Control Experiments. $ONOO^-$ was synthesized as described (27) and generously supplied by H. Ischiropoulos (University of Pennsylvania). The synthesis was performed by using nitrite and hydrogen peroxide in a quenched flow reactor (27). Stock solutions (160 mM) were stored at −70°C at pH 12.7. Under these conditions, $ONOO^-$ was stable for up to 1 month.

$ONOO^-$ synthesized with the above method contains hydrogen peroxide as a contaminant at 3.4 mM in the stock solution, as determined by the spectrophotometric method based on the reaction of hydrogen peroxide with 2,2′-azinobis(3-ethylbenztiazoline-6-sulfonic acid) in the presence of horseradish peroxidase. We have performed the following control experiments (see Fig. 1*c*) to exclude the effect of hydrogen peroxide: (*i*) To remove hydrogen peroxide, $ONOO^-$ stock solutions were passed through granular manganese dioxide columns and filtered. The effect of this $ONOO^-$ solution was compared to the effect of the $ONOO^-$ solution without this treatment. (*ii*) To investigate whether hydrogen peroxide, at the concentrations present in the $ONOO^-$ solution has effects on cellular respiration, a solution of hydrogen peroxide at pH 12.7 was prepared and diluted 1:160 (the dilution factor of the $ONOO^-$ solution to achieve 1 mM final $ONOO^-$ concentration), and its potential effects on cells were investigated. (*iii*) In reverse-order experiments, small volumes of the $ONOO^-$ were applied to culture medium in the absence of cells (e.g., 6.25 μl of the 160 mM $ONOO^-$ solution into 1 ml of medium to achieve 1 mM final concentration) and then incubated for 1 h at 37°C. After the 1-h incubation, medium on the cells was replaced with the medium that had been preincubated with $ONOO^-$, followed by a 1-h incubation at 37°C, and, finally, by the measurement of mitochondrial respiration. This protocol was chosen over the alternative method, which is neutralization of the pH of the $ONOO^-$ stock solutions by acid exposure, as the latter method would also decompose hydrogen peroxide, a potential contaminant of the $ONOO^-$ stock solution.

Statistical Evaluation. All values in the figures and text are expressed as mean ± SEM of *n* observations. Student's unpaired *t* test was used to compare means between groups. A *P* value of <0.05 was considered statistically significant.

RESULTS

$ONOO^-$ Causes a Decrease in Mitochondrial Respiration That Is Prevented by Inhibitors of PARS. There was a dose-dependent decrease in mitochondrial respiration in J774 cells and in RASM cells exposed to $ONOO^-$ (Figs. 1 and 2) in the concentration range of 100 μM to 1 mM. The decrease was maximal at 1 h after exposure of $ONOO^-$ and, at lower concentrations of $ONOO^-$, showed a gradual recovery over 24

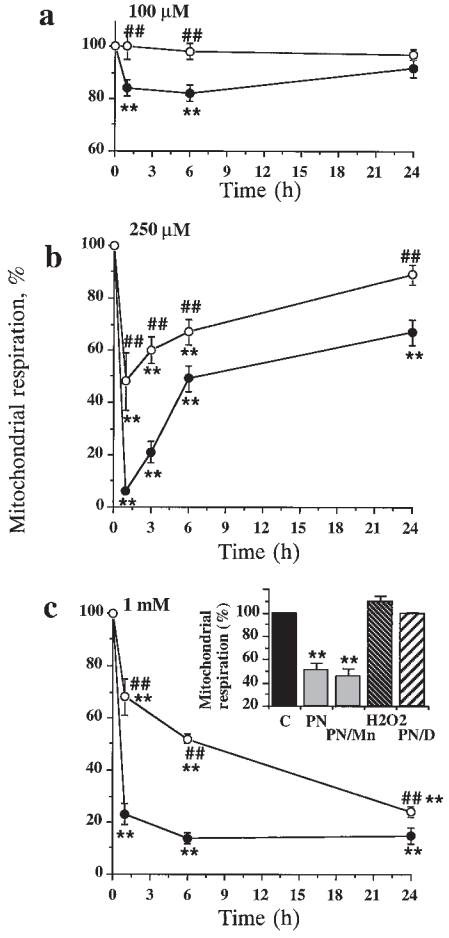

FIG. 1. Mitochondrial respiration at 1 h, 3 h, 6 h, and 24 h after exposure to various concentrations (*a*, 100 μM; *b*, 250 μM; *c*, 1 mM) of $ONOO^-$ in cultured J774 macrophages. Solid circles, respiration in the absence of inhibitors; open circles, respiration in the presence of 3-AB (1 mM). (*c Inset*) Effect of 1 mM $ONOO^-$ at 1 h (bar PN); the effect of 1 mM $ONOO^-$ after removal of hydrogen peroxide from the stock solutions by using MnO_2 columns (bar PN/Mn); the lack of effect of hydrogen peroxide at 21 μM concentration, which is equivalent to its concentration in the $ONOO^-$ solution (bar H2O2); and the lack of effect of the solvent applied after decomposition of $ONOO^-$ (bar PN/D). **, Significant decrease in the respiration when compared to controls ($P < 0.01$); ##, significant inhibition by 3-AB ($P < 0.01$); $n = 6$–12 wells.

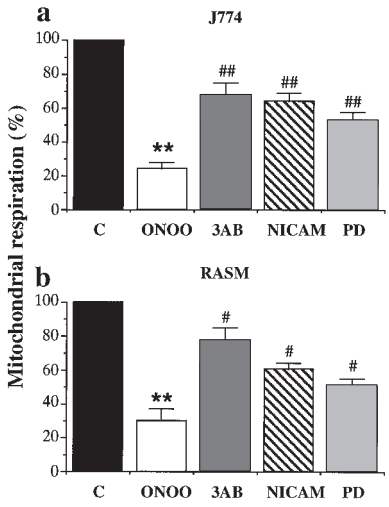

FIG. 2. Mitochondrial respiration at 6 h after exposure to 1 mM $ONOO^-$ in cultured J774 macrophages (*a*) and rat aortic smooth muscle cells (*b*); effect of the PARS inhibitors 3-AB (bars 3AB; 1 mM), nicotinamide (bars NICAM; 1 mM), and PD 128763 (bars PD; 100 μM). **, Significant decrease in the respiration when compared to controls ($P < 0.01$); #, ##, significant inhibition by the PARS inhibitors ($P < 0.05$ and $P < 0.01$, respectively); $n = 6$–12 wells.

h (Fig. 1). The decrease in mitochondrial respiration induced by $ONOO^-$ was significantly inhibited by the PARS inhibitor 3-aminobenzamide (3-AB). Similarly, other known inhibitors of PARS, such as nicotinamide (1 mM) and the dihydroisoquinolinone PD 128763 (100 μM), also inhibited the decrease in mitochondrial respiration in response to $ONOO^-$ exposure in J774 cells and in RASM cells (Fig. 2). The decrease in cellular respiration was, indeed, due to $ONOO^-$ and not due to hydrogen peroxide or other potential contaminants in the $ONOO^-$ solution, since (*i*) the potency of $ONOO^-$ in inhibiting mitochondrial respiration remained unaltered after its stock solutions passed through MnO_2 columns to remove hydrogen peroxide; (*ii*) hydrogen peroxide, at 21 μM, at which it is present in the 1 mM $ONOO^-$ solutions, did not inhibit mitochondrial respiration; and (*iii*) there were no significant decreases in cell respiration in cells that were exposed to solutions of $ONOO^-$ in which the $ONOO^-$ was left to decompose (Fig. 1*c*).

$ONOO^-$ Causes DNA Single-Strand Breaks. In cells exposed to $ONOO^-$, there was a dose-dependent increase in DNA single-strand breaks (Fig. 3). $ONOO^-$ caused DNA strand breaks in J774 cells (Fig. 3 *a* and *c*) and in RASM cells (Fig. 3 *b* and *d*) in the concentration range of 120 μM to 1 mM. The number of strand breaks within the first hour was unaffected by inhibition of the activity of PARS with 3-AB (1 mM) (Fig. 3 *a* and *b*).

In comparison, the NO donors SNAP, sodium nitroprusside, spermine–NO, or diethyltriamine–NO (up to 1 mM) did not cause any detectable DNA strand breaks in the cells at 1 h ($n = 3$–6). After 1 h of exposure to 10 mM sodium nitroprusside, however, we observed significant DNA strand breakage in both cell types studied (56 ± 8% and 42 ± 20% strand breaks in J774 cells and RASM cells, respectively; $P < 0.01$, $n = 3$). The superoxide generator pyrogallol (up to 1 mM) did not cause any detectable DNA strand breakage at 1 h ($n = 3$).

$ONOO^-$ Activates PARS. In parallel with the development of DNA strand breaks, there was a time- and dose-dependent increase in the activity of PARS in cells exposed to $ONOO^-$, as measured by the incorporation of radiolabeled NAD^+ into proteins (Fig. 4). The most pronounced ADP-ribosylation was observed for proteins of ≈110 kDa, possibly reflecting autoribosylation of PARS itself. We have also observed ADP-ribosylation for proteins of 35 kDa and of >200 kDa (Fig. 4*c*). ADP-ribosylation was inhibited by 3-AB. PARS activity and ADP-ribosylation were also detected in unstimulated cells and was inhibited by 3-AB (Fig. 4).

$ONOO^-$ Induces Cellular NAD^+ and ATP Depletion, Which Is Prevented by Inhibitors of PARS. Exposure to $ONOO^-$ (500 μM to 1 mM) resulted in a significant reduction of cellular

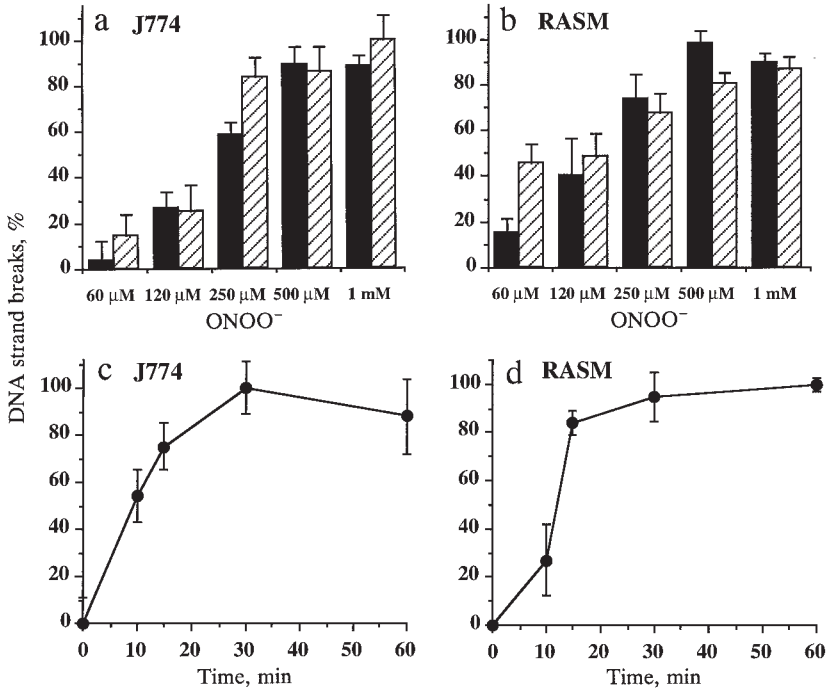

FIG. 3. DNA strand breakage in response to $ONOO^-$ in cultured J774 macrophages (*a* and *c*) and rat aortic smooth muscle cells (*b* and *d*). In *a* and *b*, solid bars represent the percent DNA strand breakage at 1 h after exposure to various concentrations (60 μM to 1 mM) of $ONOO^-$ in the absence of inhibitors (solid bars) and in the presence of the PARS inhibitor 3-AB (1 mM, hatched bars). In *c* and *d*, time course of DNA strand breakage is presented in cells exposed to $ONOO^-$ (1 mM) for various times (10–60 min); $n = 6$–12 wells.

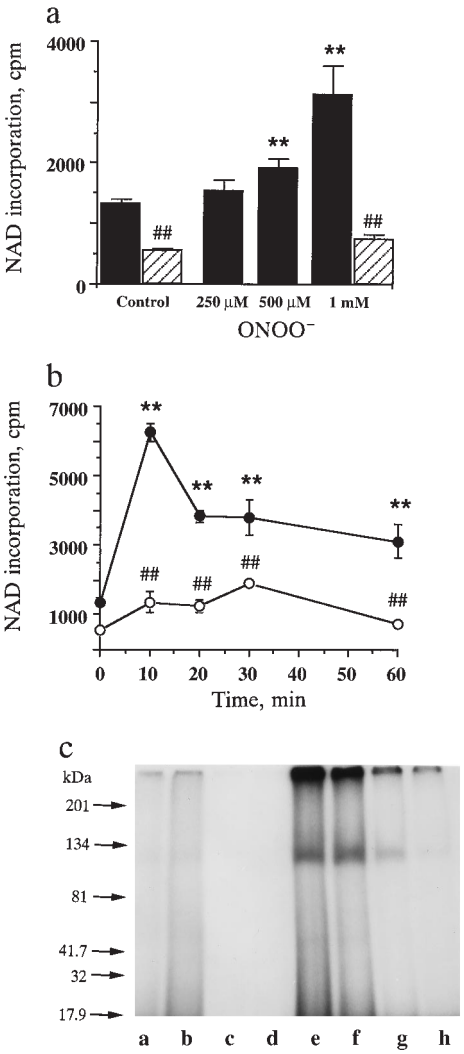

FIG. 4. [^{3}H]NAD$^+$ incorporation into proteins as an indicator of PARS activity in J774 macrophages in control conditions and after exposure to various concentrations of ONOO$^-$: (*a*) Dose–response results at 1-h exposure. (*b*) Time course. Solid bars or circles, effect of ONOO$^-$ on PARS activity in the absence of PARS inhibitors; hatched bars or open circles, same parameter in the presence of the PARS inhibitor 3-AB (1 mM). **, Significant increase in the PARS activity in response to ONOO$^-$; ##, significant decrease in PARS activity in response to 3-AB (1 mM) when compared to the respective PARS activity in the absence of 3-AB ($P < 0.01$); $n = 3$–6 wells. (*c*) Autoradiography of ADP-ribosylated polypeptides in J774 cell extracts in control conditions (lanes a and b), in control conditions in the presence of 3-AB (lanes c and d), at 10 min after exposure to 1 mM ONOO$^-$ (lanes e and f), and 10 min after 1 mM ONOO$^-$ in the presence of 1 mM 3-AB (lanes g and h).

NAD$^+$ level within at 1–6 h (Fig. 5), which was prevented by 3-AB (1 mM) (Fig. 5 *a* and *b*). Similarly, ATP concentration (control, 19.51 pmol per 10^6 cells) decreased at 1 h after ONOO$^-$ exposure (1 mM) to 32 ± 4% of control ($P < 0.01$, Fig. 5*c*). Inhibition of PARS by 3-AB caused an ≈50% protection against the ONOO$^-$-induced reduction in intracellular ATP levels ($P < 0.01$, Fig. 5*c*).

Comparison of the Effects of PARS Inhibition on the Decrease in Mitochondrial Respiration in Cells Exposed to Various Free Radical Generators or Immunostimulation. Exposure of J774 cells to the NO donor SNAP (2 mM), the superoxide generator pyrogallol (100 μM), hydrogen peroxide (1 mM), SIN-1 (1 mM), or to immunostimulation with bacterial lipopolysaccharide (LPS; 10 μg/ml) produced significant decreases in mitochondrial respiration. Inhibition of PARS

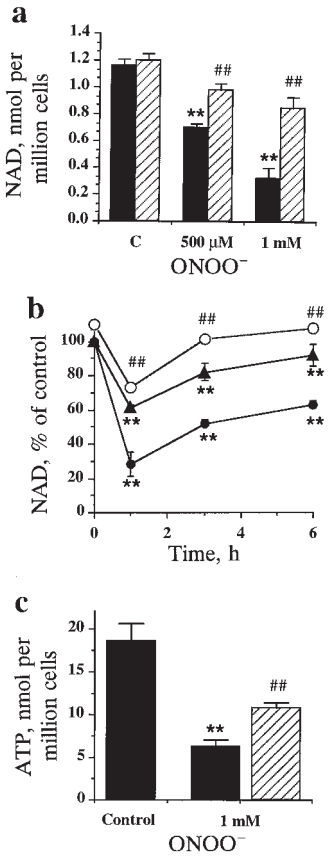

FIG. 5. NAD$^+$ (*a* and *b*) and ATP (*c*) levels in cells after exposure to ONOO$^-$ in cultured J774 macrophages; effect of the PARS inhibitor 3-AB (1 mM). (*a*) Effect of ONOO$^-$ (500 μM and 1 mM) on intracellular NAD$^+$ content at 1 h in the absence (solid bars) and in the presence (open bars) of the PARS inhibitor 3-AB (1 mM). (*b*) Change in NAD$^+$ content at 1–6 h in macrophages after exposure to ONOO$^-$ (500 μM, solid triangles) and 1 mM (solid circles); effect of 3-AB (1 mM) in cells exposed to 1 mM ONOO$^-$ (open circles). **, Significant decrease in the NAD$^+$ or ATP levels, as compared to control ($P < 0.01$); #, ##, a significant inhibition by 3-AB ($P < 0.05$ and $P < 0.01$, respectively); $n = 3$–6 wells.

afforded a protection of variable degrees (Fig. 6). 3-AB caused a significant protection against the decrease in response to SIN-1, an agent that releases NO and superoxide in a 1:1 ratio and can, therefore, be considered as a ONOO$^-$ generator (28). Consistent with previous data (24, 29, 30), 3-AB protected against the decrease in respiration in cells exposed to hydrogen peroxide. There was only a little, albeit significant, protection by 3-AB (Fig. 6) and other PARS inhibitors (not shown) against the decrease in viability in response to the NO donor SNAP. The PARS inhibitor, however, caused a significant protection against the decrease in respiration in cells stimulated with LPS.

DISCUSSION

Exposure to ONOO$^-$ Results in PARS Activation with a Consequent Decrease in Mitochondrial Respiration. The dose-dependent decrease in mitochondrial respiration in cells exposed to ONOO$^-$ was due to ONOO$^-$ and was not a result of residual contaminants (such as sodium chloride, hydrogen peroxide, or sodium hydroxide) that are present in the ONOO$^-$ solution, as demonstrated by (*i*) the effectiveness of ONOO$^-$ solutions after removal of hydrogen peroxide, (*ii*) the lack of effect of hydrogen peroxide at concentrations in which it is represented as contaminant, and (*iii*) the ineffectiveness of the decomposed solvent in the reverse-of-order control exper-

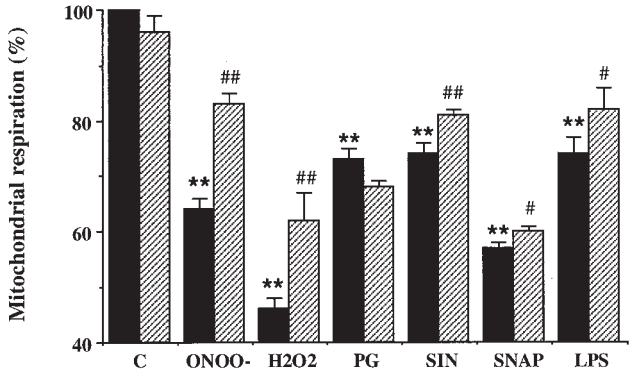

FIG. 6. Mitochondrial respiration after exposure to $ONOO^-$ (1 mM, 1 h), hydrogen peroxide (H2O2, 1 mM, 1 h), pyrogallol (PG, 100 μM, 24 h), SIN-1 (SIN, 1 mM, 24 h), SNAP (2 mM, 24 h), and bacterial endotoxin (10 μg/ml) (LPS, 24 h) in cultured J774 macrophages (solid bars); effect of the PARS inhibitor 3-AB (1 mM, hatched bars). **, Significant decrease in the respiration in response to the various agents when compared to controls ($P < 0.01$); #, ##, significant prevention of the decrease in mitochondrial respiration by 3-AB ($P < 0.05$ and $P < 0.01$, respectively); $n = 6$–12 wells.

iments (Fig. 1*c*). Although $ONOO^-$ was used in the high micromolar concentration range, one has also to consider the rapid decomposition of $ONOO^-$. Continuous production of much lower concentrations of $ONOO^-$ may well induce damage of similar extent (27).

The decrease in respiration in cells exposed to $ONOO^-$ was inhibited by 3-AB, nicotinamide, and PD 128763, which are inhibitors of PARS but belong to different structural classes (31, 32). PARS is a nuclear DNA repair enzyme that is activated by DNA single-strand breaks and that, in turn, catalyzes the transfer of the ADP-ribosyl moiety of NAD^+ to protein acceptors with the concomitant release of nicotinamide (33). Activation of PARS is also known to result in a rapid reduction of cellular NAD^+ and, subsequently, ATP levels (30, 33, 34). Consistent with this mechanism, we demonstrate (*i*) the rapid occurrence of DNA single-strand breaks in cells exposed to $ONOO^-$, an effect that is not affected by the PARS inhibitor 3-AB; (*ii*) a significant activation of PARS as measured by an increase in the levels of ribosylated proteins in cells after exposure to $ONOO^-$, an effect that is inhibited by 3-AB; and (*iii*) the depletion of cellular NAD^+ and ATP levels in cells exposed to $ONOO^-$, effects that were prevented by 3-AB.

A Decomposition Product of $ONOO^-$ Is Likely Responsible for PARS Activation. Given that the half-life of $ONOO^-$ in physiological solutions (at pH 7.4) is on the order of seconds, it seems improbable that such a reactive species could traverse the cell membrane, enter the nucleus, and damage DNA, thereby triggering PARS activation and the ensuing cascade of cellular energy depletion. A more stable decomposition product of $ONOO^-$ could, however, mediate DNA injury. The decomposition products of $ONOO^-$ are still a subject of debate and are dependent on the type of buffer and the presence of glutathione, scavenging tyrosine residues, and other aspects of the chemical environment (9, 10, 27, 35, 36). In the present study, similar to other investigators (20), we have added $ONOO^-$ to the cells present in standard DMEM culture medium with 10% fetal calf serum. DMEM contains, among others, $Fe(NO_3)_3$ (0.1 mg/ml). Metals may influence the actions and decomposition characteristics of $ONOO^-$ or its metabolites, while amino acids and proteins in the medium may scavenge $ONOO^-$, decreasing its apparent potency.

DNA damage, PARS activation, and inhibition of mitochondrial respiration have been observed after exposure to hydrogen peroxide, where the active metabolite is likely to be hydroxyl radical (24, 29, 30). One of the reactive breakdown products of $ONOO^-$ is a strongly oxidizing intermediate, peroxynitrous acid (HOONO) or its activated isomer, which is responsible for some of the hydroxyl radical-like oxidations caused by $ONOO^-$ (9, 10, 27, 35–37). It is likely that a species with hydroxyl radical-like properties (which is not hydroxyl radical itself) is responsible for the effects seen in the present study. (It is also noteworthy that hydroxyl radical, similarly to $ONOO^-$, is unlikely to cross cell membranes, due the short half-life at neutral pH.) Clearly, the exact nature of the reactive species that are produced from $ONOO^-$ under various biological conditions, and the nature of the species responsible for DNA damage and PARS activation in cells exposed to $ONOO^-$, remain to be further investigated. Under certain conditions, the decomposition of $ONOO^-$ may yield a NO-like species (38). NO, clearly, cannot be responsible for the DNA damage seen in cells exposed to $ONOO^-$, as (*i*) we found that NO donors are weak DNA strand-breaking agents when compared to $ONOO^-$ and (*ii*) the toxicity of $ONOO^-$ cannot be prevented by treatment of cells with the NO scavenger carboxy-2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (unpublished data).

Comparison of the Potencies of NO and $ONOO^-$ as Activators of PARS. NO, produced after the activation of *N*-methyl-D-aspartate receptors, has recently been proposed to activate PARS and induce cell killing in brain slices (17). It is noteworthy that sodium nitroprusside (SNP) alone did not cause detectable PARS activation in brain slices (at concentrations up to 10 mM) (17) and, at concentrations up to 1 mM, failed to induce DNA strand breakage in macrophages and smooth muscle cells (present study). Kolb and coworkers (16) have demonstrated that the decrease in cellular NAD^+ levels and cell viability can be prevented by inhibitors of PARS in pancreatic islet cells exposed to SNAP at the concentration of 1 mM. The DNA damage in the islet cells occurred over the course of several hours and was <50% (16), whereas, in our studies, the DNA damage in cells exposed to $ONOO^-$ developed more rapidly (within 20–30 min) and was near maximal at 250–500 μM.

In our study, inhibition of PARS caused only a minor prevention of the decrease in cell respiration in cells exposed to SNAP, whereas the protection was more pronounced in cells exposed to the $ONOO^-$ donor SIN-1 (28). There are multiple (direct and indirect) pathways that may lead to inhibition of the mitochondrial respiratory chain and thus reduce the formation of formazan from MTT. For instance, NO directly inhibits various enzymes of the mitochondrial respiratory chain (2). Recent data, however, indicate that $ONOO^-$ is a more potent inhibitor of aconitase than NO itself (39, 40). The residual inhibition of mitochondrial respiration by $ONOO^-$ in the presence of PARS inhibitors may be due to direct inhibition of the mitochondrial respiratory chain by $ONOO^-$ or its degradation products.

Thus, our data demonstrate that $ONOO^-$ is >100 times more potent than NO in causing DNA strand breaks and activating PARS. *N*-Methyl-D-aspartate receptor activation has been shown to activate PARS and cause cell death in brain slices that could be prevented by inhibitors of NO synthesis (17). In the same study, however, the "pure" NO donor SNP alone did not cause detectable PARS activation (at concentrations up to 10 mM), whereas the combination of SNP with SIN-1 (a compound that releases superoxide as well as NO) did (17). Based on the present and previous data, we propose that previous reports on NO-mediated PARS induction and consequent energy depletion (16, 17) have reflected an injury process triggered mainly by $ONOO^-$, rather than by NO *per se*. It is also noteworthy that combined application of NO donors and superoxide generators increases the inhibition of mitochondrial respiration in J774 macrophages (41). Moreover, uric acid, a scavenger of $ONOO^-$ (27), prevents the decrease in respiration in response to combined administration of NO

donors and superoxide generators, without inhibiting the decrease in respiration in response to either of these agents alone (41).

What is the biological relevance of the above findings? Although $ONOO^-$ was added to the cells in the high micromolar concentrations, continuous production of much lower concentrations of $ONOO^-$ induces damage of similar extent (ref. 27 and see above). Moreover, proteins, amino acids, and other constituents of the culture medium may well scavenge a portion of $ONOO^-$ before it can exert biological effects on the cells. Importantly, we have observed that 3-AB protects against the decrease in cellular viability in cells exposed to immunostimulants (Fig. 6). A potential explanation is that 3-AB inhibits the effects of endogenous $ONOO^-$, produced upon immunostimulation, since (*i*) exposure of J774 cells to LPS results in the production of both NO and superoxide (21, 42) and (*ii*) uric acid, a scavenger of $ONOO^-$, inhibits the decrease in cellular viability in LPS-stimulated J774 macrophages (41). Moreover, low levels of $ONOO^-$, produced continuously by these cells, decrease intracellular NAD and ATP levels. This energy depletion is inhibited by 3-AB (43).

Conclusion. The present study suggests that DNA strand breakage, PARS activation, and the consequent NAD^+ and ATP depletion significantly contribute to the decrease in cell respiration in macrophages and smooth muscle cells exposed to $ONOO^-$. An intermediate species, formed during the decomposition of $ONOO^-$, is likely responsible for these alterations. The mechanisms described herein may have relevance *in vivo*, in diseases that are associated with simultaneous overproduction of NO and oxygen free radicals, such as chronic inflammation and circulatory shock.

We thank Drs. H. Ischiropoulos and G. J. Southan for helpful discussions. The assistance of Ms. J. Giles with the tissue culture studies and the assistance of Mr. A. Denenberg with the ATP measurements is appreciated.

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