ANTIOXIDANTS & REDOX SIGNALING
Volume 3, Number 3, 2001
Mary Ann Liebert, Inc.

Original Research Communication

Nitric Oxide and Superoxide Radical Production by Human Mononuclear Leukocytes

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ABSTRACT

Human mononuclear cells (90% lymphocytes, 9% monocytes, and 1% polymorphonuclear leukocytes) produced spontaneously in resting state 0.11 ± 0.01 nmol of nitric oxide (NO)/min/10^6 cells and 0.25 ± 0.02 nmol of superoxide anion (O_2^-)/min/10^6 cells, as primary products. When these cells were stimulated with phorbol 12-myristate 13-acetate (PMA), the NO and O_2^- production increased by 82% and 204% to 0.25 ± 0.02 nmol of NO/min/10^6 cells and 0.76 ± 0.12 nmol of O_2^-/min/10^6 cells, respectively. Oxygen uptake reasonably accounted for the sum of the rates of NO and hydrogen peroxide (H_2O_2), the latter calculated as 0.5 O_2^- production, in nonstimulated and in PMA-stimulated cells. H_2O_2 and peroxynitrite formation were detected and measured as secondary products of the primary products O_2^- and NO. An original assay to determine H_2O_2 steady-state concentration and production rates is described. The determined production rates of the involved reactive species are in good agreement with known chemical equations. It is apparent that NO and O_2^- production by human mononuclear cells may constitute the basis of intercellular signaling and cell toxicity. Antioxid. Redox Signal. 3, 505–513.

INTRODUCTION

THERE is a considerable interest in the role of nitric oxide (NO) in cell-mediated immune response and phagocytosis because NO is a mediator in several physiological responses (24). NO is formed as a product of the conversion of L-arginine to L-citrulline by the constitutive and inducible nitric oxide synthases (NOS) of macrophages (18, 28) and circulating and inflammatory human neutrophils (8–10). In the immune response mediated by monocytes and macrophages, NO controls the regulation of diverse processes, among them the release of cytokines (13) and the expression of HLA-DR on human monocytes (19). In addition, NO is a reactant for the production of the cytotoxic peroxynitrite ($ONOO^-$). The formation of $ONOO^-$ was reported to occur in many cell types, including macrophages (15), neutrophils (8), and Kupffer cells (33). NO reacts with superoxide anion (O_2^-) to produce $ONOO^-$ in a diffusion-controlled reaction ($k = 1.9 \times 10^{10}\ M^{-1}\ s^{-1}$) (17). Hence, the rate of $ONOO^-$ formation will be defined by the steady-state concentrations of both NO and O_2^- in the extracellular space surrounding the cytotoxic cells. The primary source of O_2^- is the membrane-bound NADPH oxidase of the immune cells. Phorbol esters and chemoattractants activate the NADPH oxidase complex to produce O_2^- (1), which either dismutates to hydrogen peroxide (H_2O_2) in a spontaneous or superoxide dismutase (SOD)-catalyzed reaction,

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or produces $ONOO^-$. NADPH oxidase activity was reported in "professional phagocytes" (neutrophils, eosinophils, and mononuclear phagocytes) and in B lymphocytes, where its role is unknown (2). In contrast to that of neutrophils and macrophages, the oxidative metabolism of monocytes and lymphocytes has not been extensively investigated. It has been reported that human B lymphocytes have also another enzymatic system that generates O_2^-, and this species would take part in the cross-linking of surface immunoglobulin in the antigen processing in B cells (21, 22, 32). However, neither O_2^- nor the H_2O_2 production nor the quantification or stoichiometry of the species involved during the "respiratory burst" of lymphocytes has been reported.

The aim of this work is to determine the production rates of a series of reactive species (O_2^-, H_2O_2, NO, and $ONOO^-$) in isolated human mononuclear cells, where the major proportion corresponds to lymphocytes (90%). NO production was measured by the oxidation of oxyhemoglobin to methemoglobin, and $ONOO^-$ formation was determined by the luminol-amplified chemiluminescence assay. The rates of O_2^- and H_2O_2 production were measured by conventional assays (3). This report recognizes human mononuclear cells as an active source of NO, O_2^-, $ONOO^-$, and H_2O_2 and advances the stoichiometry of the involved reactions. In addition, the results support the idea that NO and H_2O_2 could play a role as molecules involved in the signaling process in the immune system.

MATERIALS AND METHODS

Chemicals

Acetylated cytochrome *c*, catalase, hemoglobin-A_0 ferrous form, horseradish peroxidase (HRP), Hystopaque 1077, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), N^G-methyl-L-arginine (L-NMMA), *p*-hydroxyphenylacetic acid (*p*-HPA), phorbol 12-myristate 13-acetate (PMA), SOD, and Trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other reagents used were of analytical grade.

Isolation of human mononuclear cells and cell viability

Human blood samples were obtained from healthy human donors (20–50 years old) by venipuncture. Mononuclear cells were isolated by Ficoll–Hypaque gradient separation (4) and resuspended in phosphate-buffered saline solution (PBS; 138 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, and 1.47 m*M* KH_2PO_4 buffer, pH 7.4) at a concentration of 10^6–10^7 cells/ml (23). Cell viability was determined by total cell counting and determination of the cell fraction excluding trypan blue. The cells consisted of ~99% viable mononuclear cells with 90 ± 2% lymphocytes, 9.0 ± 0.3% monocytes, and 1.0 ± 0.3% polymorphonuclear leukocytes, and 10^6 cells of this cellular preparation corresponded to 0.10 mg of protein.

Stimuli and inhibitors

PMA was stored as a stock solution (2 mg/ml) in dimethyl sulfoxide at −20°C. This solution was diluted with ethanol to 20 μg/ml and used as the working solution, which was prepared fresh when required (23). The effect of L-NMMA was assayed in human mononuclear cells preincubated with the inhibitor for 45 min at 37°C.

Oxygen uptake

Oxygen consumption was determined polarographically with a Clark-type oxygen electrode thermostated at 37°C with human mononuclear cells suspended (2×10^6 cells/ml) in PBSG (PBS supplemented with 0.9 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, and 7.5 m*M* glucose) (16, 23). The rate of O_2 consumption was calculated from the initial linear traces in the recorder and expressed as nmol of O_2/min/10^6 cells.

NO production

NO production was determined spectrophotometrically through the oxidation of oxyhemoglobin to methemoglobin. The technique is based on the direct reaction between the NO molecule and the oxygenated, ferrous form of hemoglobin, which yields the ferric form, methemoglobin (9, 25). The reaction medium

consisted of mononuclear cells resuspended in PBSG, 3 μM SOD, 0.1 μM catalase, and 20 μM oxyhemoglobin. Determinations were made in cells preincubated for 1 min at 37°C in the absence or presence of 0.1 μg/ml PMA. Measurements were carried out using (a) a double-beam single-wavelength spectrophotometer (Jasco model 7850) at 577 nm ($\epsilon = 11.2\ mM^{-1}\ cm^{-1}$) with a reference cuvette, where all the reactants were added except the oxyhemoglobin; and (b) a double-beam double-wavelength spectrophotometer (Perkin Elmer model 356) at 577–591 nm ($\epsilon = 11.2\ mM^{-1}\ cm^{-1}$). The absorbance changes due to methemoglobin formation that were inhibited by 1 mM L-NMMA were expressed as nmol of NO/min/10^6 cells.

O_2^- production

The rates of O_2^- production were measured by the SOD-inhibitable reduction of acetylated cytochrome *c* (3, 27). Mononuclear cells were resuspended in PBSG supplemented with 1 mM L-NMMA (to avoid sequestering of O_2^- by NO) at 8×10^5 cells/ml and incubated 45 min at 37°C. The effect of 0.1 μg/ml PMA was assayed after incubation of 1 min (8, 9). Acetylated cytochrome *c* (20 μM) reduction was monitored spectrophotometrically at 550–540 nm in a double-beam double-wavelength spectrophotometer (Perkin Elmer model 356) ($\epsilon = 19\ mM^{-1}\ cm^{-1}$) with or without the addition of 3 μM SOD. Results are expressed as nmol of O_2^-/min/10^6 cells.

H_2O_2 steady-state concentration

The determination of H_2O_2 steady-state concentration in the extracellular space of mononuclear cell suspensions (Fig. 1) was afforded by an assay previously used with tissue slices (14) and with culture cells in monolayers (7). It is understood that the highly diffusible H_2O_2 reaches in the incubation medium a steady-state concentration in diffusion equilibrium with intracellular H_2O_2. Human mononuclear cells in PBSG (2×10^6 cells/ml) were incubated with or without 0.1 μg/ml PMA, in a shaking water bath at 37°C. Aliquots of 1.5 ml were collected after appropriate time intervals (2–10 min) and centrifuged for 5 min at 300 *g*. One-milliliter aliquots of the supernatant were added to a volume of 1.5 ml of 100 mM phosphate buffer (pH 7.4), containing 0.25 μM HRP and 40 μM *p*-HPA as hydrogen donor (7, 14). Fluorescence was measured at 317–414 nm in a Hitachi fluorometer model F-3010. The concentration of H_2O_2 was determined by subtracting the fluorescence of a 0.1 μM catalase-treated sample and compared with the standard solution tested spectrophotometrically at 240 nm ($\epsilon = 43.6\ M^{-1}\ cm^{-1}$).

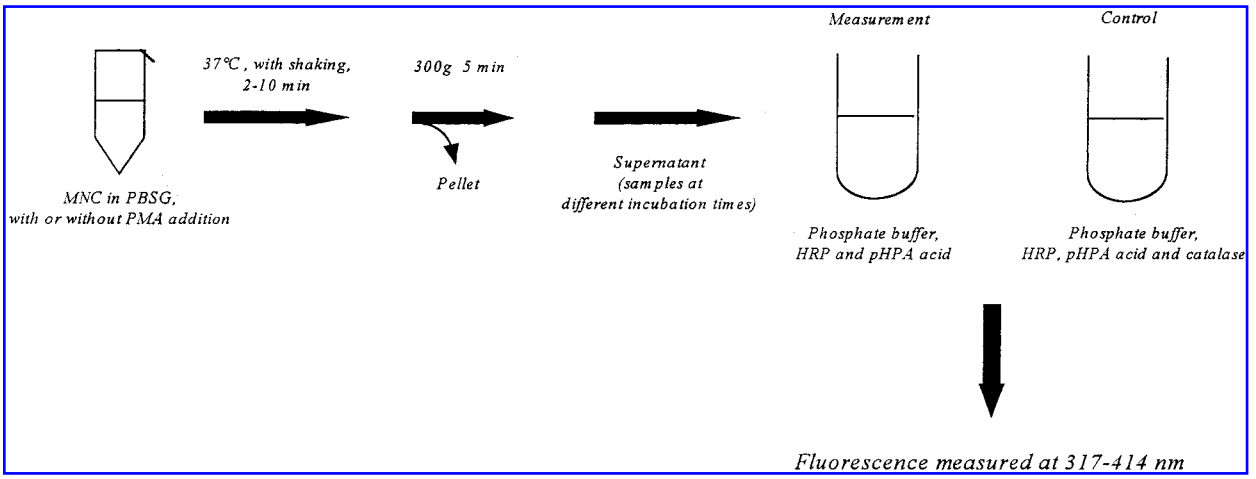

FIG. 1. Experimental design used to determine H_2O_2 steady-state concentration of human mononuclear cells (MNC) non-stimulated and stimulated with PMA.

H_2O_2 production

H_2O_2 production was determined by the formation of HRP enzyme-substrate complex II (HRP-H_2O_2) in the presence of a hydrogen donor (*p*-HPA) at 37°C (3). The fluorescent compound formed was measured spectrofluorometrically at 317–414 nm, excitation and emission wavelength, respectively. Human mononuclear cells suspended in PBSG (2×10^5 cells/ml), 16 μM *p*-HPA, and 1.2 U/ml HRP (with or without 0.1 μg/ml PMA) were added in the cuvette. H_2O_2 production was determined in the absence or presence of catalase (0.1 μM), and H_2O_2 generation was calculated from the catalase-sensitive fluorescence increase and a standard curve made with titrated H_2O_2 solutions. The results were expressed as nmol of H_2O_2/min/10^6 cells.

In addition, H_2O_2 production rates were also estimated using the experimental results of the determination of H_2O_2 steady-state concentrations, as described above, the catalase content 0.16 ± 0.01 pmol/10^6 cells, and the corresponding rate constant $k = 4.6 \times 10^7\ M^{-1}\ s^{-1}$ (11) neglecting the utilization of H_2O_2 by the glutathione peroxidases and using the following equation:

$$d[H_2O_2]/dt = k \times [H_2O_2] \times [\text{catalase}]$$

Luminol-amplified chemiluminescence

Luminol-amplified chemiluminescence was measured in a liquid scintillation counter in the out-of-coincidence mode using 1.5-ml glass vials. The cells (5×10^5 cells/ml) were added with 20 μM luminol in PBSG and incubated for 2 min at 37°C (20, 31). The photoemission of mononuclear cells was measured before and after the addition of 0.1 μg/ml PMA, as basal and poststimulation conditions. Counting intervals were 14 s, and the results are given in total counts. Controls in the absence of cells were carried out to discount nonspecific response.

Statistical analysis

Results are expressed as mean values ± SEM and are the averages of at least three independent experiments. Student's *t* test was applied for the statistical analysis.

RESULTS

NO production

There was a spontaneous production of NO in human mononuclear cells in the absence of PMA (0.11 ± 0.01 nmol/min/10^6 cells). This basal spontaneous production of NO was increased by ~82% in PMA-activated human mononuclear leukocytes (Table 1). The addition of L-NMMA inhibited by ~40% the rate of hemoglobin oxidation, indicating the existence of side reactions that also oxidize hemoglobin or an uncompleted inhibition of NOS. The results shown in Table 1 account for the L-NMMA inhibitable fraction of methemoglobin formation.

O_2^- production

O_2^- production by human mononuclear leukocytes in the absence of PMA was about twice the NO production (0.25 ± 0.02 nmol/min/10^6 cells). The supplementation with

TABLE 1. NO, O_2^-, H_2O_2 PRODUCTION, AND OXYGEN UPTAKE IN HUMAN MONONUCLEAR LEUKOCYTES

	− *PMA*	+ *PMA*
NO production (nmol/min/10^6 cells)	0.11 ± 0.01	0.20 ± 0.02*
O_2^- production (nmol/min/10^6 cells)	0.25 ± 0.02	0.76 ± 0.12*
H_2O_2 production (nmol/min/10^6 cells)		
Measured rate	0.06 ± 0.01	0.18 ± 0.02*
Calculated rate	0.12 ± 0.03	0.34 ± 0.03*
O_2 consumption (nmol/min/10^6 cells)	0.31 ± 0.03	0.78 ± 0.10

Values are means ± SEM of three to six independent experiments.
*Significantly different from the non–PMA-stimulated control cells ($p < 0.05$, Student's *t* test)

PMA produced a significant, about threefold, increase in O_2^- production (Table 1). In both conditions, with and without PMA supplementation, the rate of O_2^- production in mononuclear cells was higher than the rate of NO production by the factor of 2–4.

Oxygen uptake

The oxygen consumption by mononuclear cells in the resting state was 0.31 ± 0.03 nmol/min/10^6 cells; this rate of O_2 uptake accounts almost stoichiometrically for NO and O_2^- primary production, this latter expressed in H_2O_2 as stable product. The addition of PMA produced an activation of the mononuclear cells with a 2.5-fold increase in oxygen uptake (0.78 ± 0.10 nmol/min/10^6 cells). The production of H_2O_2, considered as the stable end-product of O_2^- dismutation, practically accounts for the total O_2^- production of human mononuclear cells (Table 1).

H_2O_2 steady-state concentration

The determination of the steady-state concentration of H_2O_2 in the extracellular space was carried out with human mononuclear cells that were either nonstimulated or incubated for 2–10 min after PMA addition. Nonstimulated mononuclear cells showed a H_2O_2 steady-state concentration in the reaction medium of 0.27 ± 0.07 μM. This concentration was significantly increased after PMA treatment to 0.78 ± 0.06 μM (Fig. 2). Therefore, the stimulation by PMA increased the H_2O_2 steady-state concentration in the extracellular space about three times.

H_2O_2 production

The addition of PMA produced a threefold increase in the fluorometrically measured rate of H_2O_2 production (0.06 ± 0.01 to 0.18 ± 0.02 nmol of H_2O_2/min/10^6 cells) in nonstimulated and stimulated cells, respectively (Table 1). When H_2O_2 production rates were calculated from H_2O_2 steady-state concentrations, the values obtained were 0.12 ± 0.03 and 0.34 ± 0.03 nmol/min/10^6 cells for nonstimulated and stimulated cells, respectively (Table 1). The H_2O_2 production rates measured fluorometrically were about one half lower than the calculated; the fact is explained by the release of hydrogen donors from the cells that interfere with the HRP-H_2O_2 assay.

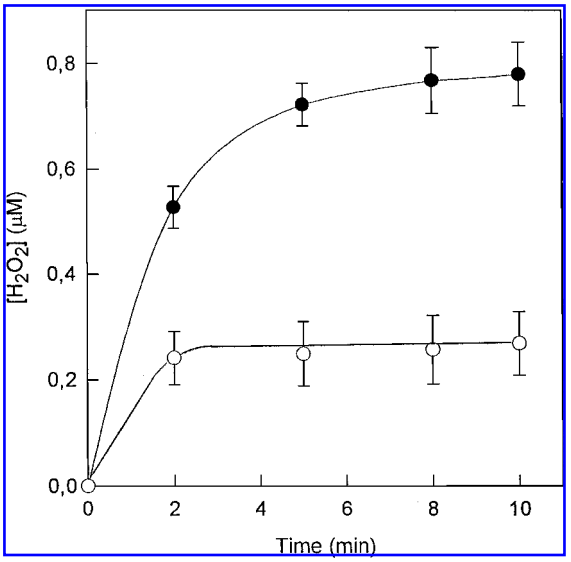

FIG. 2. H_2O_2 steady-state concentration in non-stimulated cells (○) and PMA-stimulated cells (●).

Luminol-amplified chemiluminescence

The luminol-amplified chemiluminescence of human mononuclear cells was 13-fold increased by PMA supplementation (Fig. 3A), in agreement with the increase in NO and O_2^- production reported above. To determine the participation of the involved species (O_2^-, H_2O_2, and NO), SOD, catalase and L-NMMA additions were assayed. Chemiluminescence was decreased 73% by SOD and 36% by catalase in PMA-treated cells (Fig. 3A). These results are in good agreement with the data reported by Davies and Edwards (12) in which the PMA-stimulated lucigenin chemiluminescence of monocytes was inhibited 76% by SOD and 52% by catalase. When both SOD and catalase were added, there was no further effect than the one observed after SOD addition. SOD was able to decrease chemiluminescence even added during the 3-min period of the response (data not shown). The competitive inhibitor of NOS, L-NMMA, decreased chemiluminescence by 43% when it was preincubated with the mononuclear cells before stimulation and by 83% and 71% when it was used in combination with SOD or catalase, respectively. The inhibi-

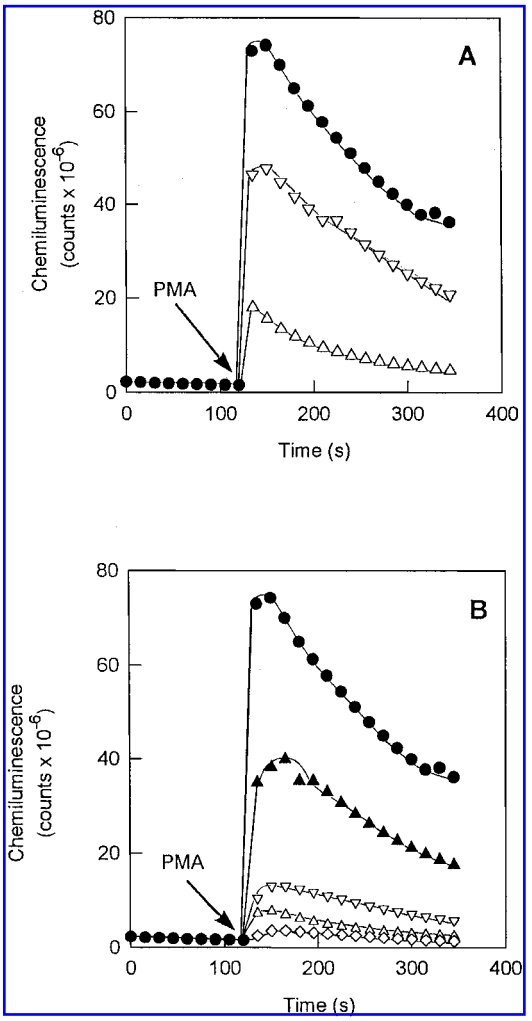

FIG. 3. (**A**) Effect of PMA addition on luminol-amplified chemiluminescence in human mononuclear cells. The light emission was recorded continuously in the absence (●) or presence of antioxidant enzymes: 3 μM SOD (△), 0.1 μM catalase (▽). Control in the absence of PMA is also shown. (**B**) Effect of L-NMMA addition on luminol-amplified chemiluminescence in human mononuclear cells. The cells were prewarmed in the presence of 1 mM L-NMMA for 45 min at 37°C. The light emission was recorded continuously in the absence (▲) or presence of antioxidant enzymes: 3 μM SOD (△), 0.1 μM catalase (▽), or SOD plus catalase (◇). Control in the absence of L-NMMA (●) is also shown.

tion was almost total when L-NMMA and both SOD and catalase were used simultaneously (Fig. 3B).

DISCUSSION

The production of signaling and cytotoxic chemical species by the phagocytic cells of the immune system, such as neutrophils and macrophages, is recognized as the essential physiological function of these cells. In contrast to neutrophils and macrophages, the oxidative metabolism of mononuclear cells, such as lymphocytes and monocytes, has not been extensively investigated. It is known that bloodstream monocytes possess an NADPH oxidase that appears to be structurally identical to that of neutrophils and is activated by analogous mechanisms (5, 6, 30), but monocytes contain lower levels of myeloperoxidase (12). In consequence, the reactive oxidants generated in neutrophils may follow chemical pathways different from the ones corresponding to mononuclear cells.

The results reported in this study show that human mononuclear cells display, after adequate stimulation, a metabolic change with a marked increase in oxygen uptake, which is similar to the respiratory burst of neutrophils and macrophages. The primary products of the stimulated oxygen uptake of mononuclear cells are NO and O_2^-, and the secondary products are $ONOO^-$ and H_2O_2. The primary products are the products of the activities of NOS and NADPH oxidase of mononuclear cells. This respiratory burst consists of about 26% NO and 97% O_2^- (Table 1), calculated as the fractions of the oxygen consumption of PMA-stimulated cells. The sum yields a number higher than 100% due to the back oxygen production of the O_2^- dismutation reaction. The relative contribution of lymphocytes and monocytes to the observed respiratory burst remains to be quantitatively established. For the moment, considering that the preparation used consisted of about 90% lymphocytes and 9% monocytes, lymphocytes are likely providing the observed response. Otherwise, monocytes should exhibit a quite high production of both NO and O_2^- (about 2.0 nmol of NO/min/10^6 cells and 7.6 nmol of O_2^-/min/10^6 cells), which is about three times higher than the production of these two species by PMA-activated neutrophils (8–10).

The simultaneous generation of NO and O_2^- to the extracellular space during the respiratory burst of human mononuclear cells leads to the formation of $ONOO^-$, as a product of the interaction of these two radicals, as evidenced by

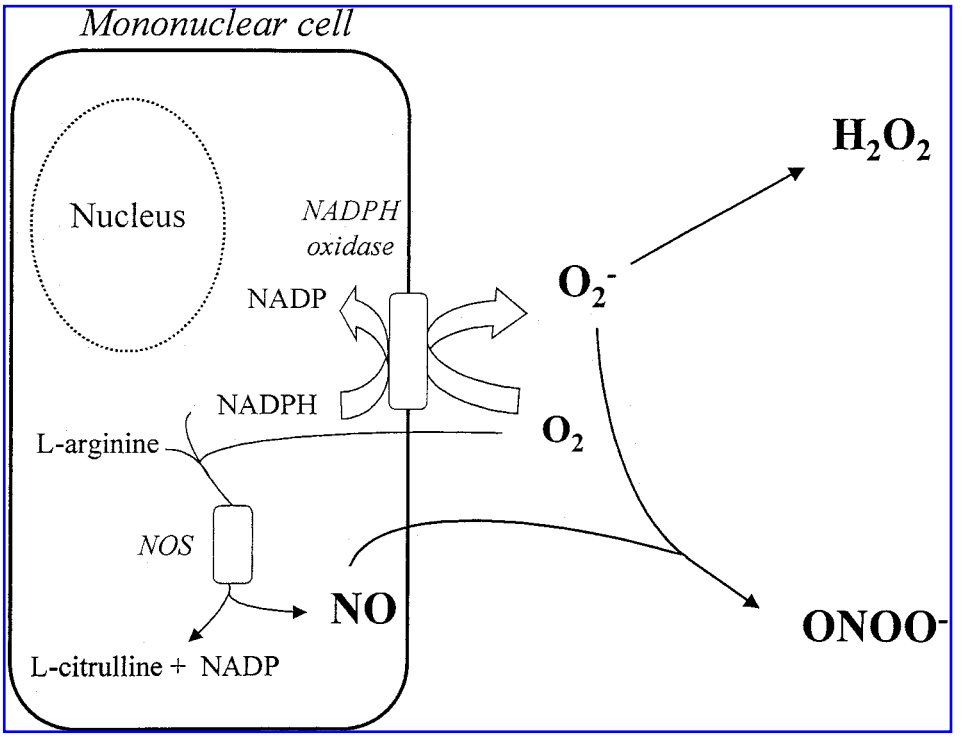

FIG. 4. The simultaneous generation of O_2^- and NO to the extracellular space, during the "respiratory burst" of human mononuclear cells, produced by the activities of NADPH oxidase and NOS, respectively, leads to the formation of $ONOO^-$ (by the direct reaction between O_2^- and NO) and H_2O_2 (by spontaneous dismutation of O_2^-).

the luminol-amplified chemiluminescence assay (9, 17). In agreement with the results reported by Ródenas *et al.* (29) for rat inflammatory polymorphonuclear leukocytes, both NOS and NADPH oxidase activities are required to elicit maximal luminol chemiluminescence in human mononuclear cells. The specificity of the assay is provided by the use of the inhibitor of NOS (L-NMMA), and of O_2^- and H_2O_2 scavengers (SOD and catalase).

Concerning H_2O_2, its steady-state concentration in the extracellular space was measured (Fig. 2) and the H_2O_2 production rate was calculated. The increase of ~2.5 times in the oxygen uptake observed in PMA-stimulated mononuclear cells was directly reflected in H_2O_2 production rate and steady-state concentration. The calculated rate of H_2O_2 production also agreed with the measured rate of O_2^- production and the dismutation stoichiometry.

The meaning of the spontaneous, non-induced by specific stimuli, generation of reactive species (NO and O_2^-) in isolated immune cells is a vexing question, and the usual answer is that this production is the consequence of an unspecific activation during the isolation procedure.

In conclusion, the stimulation of mononuclear cells with PMA produced a marked increase of NO, O_2^-, and H_2O_2 production, with a corresponding increase in oxygen uptake. The simultaneous generation of O_2^- by NADPH oxidase activation and of NO by NOS activation in human mononuclear cells is consistent with a common activation signal for both enzymes (Fig. 4). Protein kinase C is the major cellular target for activation by tumor-promoting phorbol esters (26); it is likely that this protein is the common step for NADPH oxidase and NOS stimulation (10).

ACKNOWLEDGMENTS

This research was supported by grants PIP 4110/97 from CONICET, PICT 97-01608 from ANPCYT, and TB11 from the University of Buenos Aires (Argentina).

ABBREVIATIONS

H_2O_2, hydrogen peroxide; *p*-HPA, *p*-hydroxyphenylacetic acid; HRP, horseradish peroxidase; L-NMMA, N^G-methyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; PBSG, phosphate-buffered saline plus glucose; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase.

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Received for publication December 1, 2000; accepted February 19, 2001.

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