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Arachidonic acid triggers an oxidative burst in leukocytes

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Abstract

The change in cellular reducing potential, most likely reflecting an oxidative burst, was investigated in arachidonic acid- (AA) stimulated leukocytes. The cells studied included the human leukemia cell lines HL-60 (undifferentiated and differentiated into macrophage-like and polymorphonuclear-like cells), Jurkat and Raji, and thymocytes and macrophages from rat primary cultures. The oxidative burst was assessed by nitroblue tetrazolium reduction. AA increased the oxidative burst until an optimum AA concentration was reached and the burst decreased thereafter. In the leukemia cell lines, optimum concentration ranged from 200 to 400 μM (up to 16-fold), whereas in rat cells it varied from 10 to 20 μM. Initial rates of superoxide generation were high, decreasing steadily and ceasing about 2 h post-treatment. The continuous presence of AA was not needed to stimulate superoxide generation. It seems that the NADPH oxidase system participates in AA-stimulated superoxide production in these cells since the oxidative burst was stimulated by NADPH and inhibited by N-ethylmaleimide, diphenyleneiodonium and superoxide dismutase. Some of the effects of AA on the oxidative burst may be due to its detergent action. There apparently was no contribution of other superoxide-generating systems such as xanthine-xanthine oxidase, cytochromes P-450 and mitochondrial electron transport chain, as assessed by the use of inhibitors. Eicosanoids and nitric oxide also do not seem to interfere with the AA-stimulated oxidative burst since there was no systematic effect of cyclooxygenase, lipoxygenase or nitric oxide synthase inhibitors, but lipid peroxides may play a role, as indicated by the inhibition of nitroblue tetrazolium reduction promoted by tocopherol.

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- Reactive oxygen species

Introduction

Arachidonic acid (AA) is an essential polyunsaturated fatty acid which is the precursor of eicosanoids. These products are important in many processes, such as inflammation and healing, labor and control of blood pressure, besides being necessary for the functioning of many organs and systems, such as the immune, digestive and reproductive systems. Nevertheless, it has been found that many of the effects of AA are not dependent on eicosanoid generation since AA can directly modulate phagocytosis, cytokine

production, surface molecule expression, leukocyte migration, and antigen presentation (1).

One of the known mechanisms of action of AA is stimulating the generation of superoxide, which acts as a microbicidal agent in phagocytes and as a second messenger in many cell types (2,3). Such AA-triggered superoxide production might also explain the cytotoxicity of AA (4,5).

Superoxide may be generated by various systems, among which the most common are: NADPH oxidase, mitochondrial electron transport chain, xanthine-xanthine oxidase, and cytochromes P-450. NADPH oxidase is a protein complex that is found to be dissociated when the enzyme is inactive. NADPH oxidase activation is promoted by the phosphorylation of a cytoplasmic protein, p47 phox, by protein kinase C (PKC, ATP phosphotransferase, EC 2.7.1.37). Upon phosphorylation, p47 phox promotes the association of the NADPH oxidase subunits on the membrane and the activation of superoxide generation. Most membrane-bound NADPH subunits are found in intracellular granules into which superoxide is driven. Alternatively, NADPH oxidase-containing granules join with phagocytic vacuoles, exposing their contents to superoxide. Finally, granules may migrate to the cell surface and release superoxide into the extracellular environment (6).

The mitochondrion is also considered to be an important site for superoxide generation, which occurs mostly by the univalent reduction of oxygen in complexes I and III of the electron transport chain (3). The mitochondrial generation of superoxide can be assessed by the addition of uncouplers, hyperpolarizing agents and inhibitors of the respiratory chain (3).

The xanthine-xanthine oxidase superoxide-generating system is activated in cells suffering oxidative stress, when xanthine dehydrogenase, which reduces NAD to NADH, is changed into xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.1.3.222) which, instead of NAD, reduces oxygen to superoxide (3,7). The cytochrome P-450 complex is a family of enzymes that mediate oxidation-reduction reactions, some of which involve AA oxidation and superoxide generation (8).

The AA-triggered increase in reducing power, which we attribute mostly to oxidative burst (superoxide production) in leukocytes, was assessed by the nitroblue tetrazolium (NBT) method. The following cell lines were studied: human promyelocytic leukemia (HL-60, undifferentiated and differentiated into macrophage-like and polymorphonuclear- (PMN) like cells), human T-cell leukemia (Jurkat), and human B-cell leukemia (Raji). Thymocytes and macrophages were obtained from rat primary cultures. The pathways of the oxidative burst triggered by AA were evaluated pharmacologically by pretreatment of cells with stimulators or inhibitors of superoxide-generating systems and of AA metabolism.

Material and Methods

Reagents

Superoxide dismutase (SOD) was obtained from Boehringer (Mannheim, Germany), and cell culture medium, antibiotics and fetal calf serum were obtained from Gibco-BRL Life Technologies (Grand Island, NY, USA). All other reagents and drugs were obtained from Sigma (St. Louis, MO, USA). All agents were dissolved in ethanol, unless otherwise indicated.

Cell culture

The cell lines used in this study were all of human origin. HL-60 and Jurkat were obtained from the cell bank of the Dunn School of Pathology (Oxford University, UK) and Raji was obtained from the Rio de Janeiro cell bank. Cells were grown in RPMI 1640 medium supplemented with 2 mM gluta-

mine, 20 mM HEPES, 10% fetal calf serum, 10 U/ml penicillin G and 10 µg/ml streptomycin. The cell population was maintained between 1 x 10^5 and 1 x 10^6 cells/ml (logarithmic phase of cell growth).

HL-60 cells were induced to differentiate into PMN upon treatment with 100 nM *all-trans* retinoic acid for 5 days. Differentiation of HL-60 cells into macrophage-like cells was induced by treatment with 100 nM phorbol 12-myristate 13-acid (PMA) for 48 h.

Rat thymocytes and macrophages were obtained from male albino rats weighing 200 g, bred in the Institute of Biomedical Sciences, University of São Paulo, and killed by decapitation. Macrophages were obtained from the peritoneal cavity upon injection of PBS (10 mM NaH_2PO_4/Na_2HPO_4, 0.138 mM NaCl, 268 mM KCl, pH 7.4). The rat thymuses were removed and sheared in PBS to obtain thymocytes. Cells were then filtered through lens cleaning paper, washed twice in PBS and collected by centrifugation. Animal manipulation followed a protocol approved by the Ethics Committee on the Use and Care of Animals of the Institute of Biomedical Sciences, University of São Paulo.

Oxidative burst measurement - nitroblue tetrazolium reduction assay

Oxidative burst was evaluated by reduction of NBT, a yellow water-soluble powder that becomes blue and insoluble upon reduction. Cells (1 x 10^7/ml - 150 µl) were incubated for 1 h in 0.05% NBT in PBSG solution: 0.13 mM NaCl, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4. The reaction was stopped by the addition of 1 volume glacial acetic acid. After cell centrifugation (1 min, 12,000 *g*), reduced NBT was solubilized in 150 µl 50% acetic acid by sonication (1 pulse of 5 s; Sonics and Materials, Newtown, CT, USA). Cell debris were pelleted and the absorbance of the supernatant was determined at 560 nm in a microtiter plate reader (Spectramax plus, Molecular Devices, Sunnyvalle, CA, USA).

Alternative protocols used for measurement of superoxide production were as follows: a) cells were preincubated for 30 min with various substances, ethanol or DMSO (controls) prior to the addition of NBT; b) kinetic studies were performed by collecting cells at different times after NBT addition; c) cells were preincubated for 15 min with AA or ethanol, centrifuged and then exposed to NBT; d) *in vitro* NBT reduction was assayed using 15 U/ml of xanthine oxidase and different concentrations of xanthine; e) assays carried out with phloretin were only incubated for 20 min with NBT. The reactions were developed in PBSG solution (150 µl) at room temperature for 10 min. Precipitated NBT was centrifuged and extracted with 50% acetic acid by sonication. After centrifugation, the absorbance of the supernatant was determined spectrophotometrically at 560 nm. The values indicated correspond to the absorbances.

Statistical analysis

Comparisons were performed by the Student *t*-test, with the level of significance set at 0.05. Results are reported as means ± SD.

Results

Nitroblue tetrazolium reduction by arachidonic acid in different cell lines

AA increased NBT reduction in the leukemia cell lines and in normal rat thymocytes (Figure 1). This effect was more pronounced on HL-60 cells, both undifferentiated and differentiated into PMN-like cells (maximum increases around 16-fold). The human leukemia cell lines, Raji and Jurkat, and normal rat thymocytes showed a less pronounced response (a 2-4-fold increase). Cell treatment with PMA only showed a significant

effect in rat thymocytes, undifferentiated and PMN-like HL-60 cells, although the response was most striking in PMN-like HL-60 cells (18-fold increase) compared to the more subtle 2-fold increase in HL-60 and thymus cells. The combined treatment of cells with AA and PMA tended to decrease the effect of these agents when added separately. This can be observed in undifferentiated HL-60 and Jurkat cells, in which PMA reduced the effect of AA, and in PMN-like differentiated HL-60 cells, in which combined treatment with AA and PMA reduced both the individual effect of AA and that of PMA. In contrast, the combination of AA and PMA increased NBT reduction in Raji and rat thymocytes.

Experiments were also performed with rat macrophages and macrophage-like differentiated HL-60 cells (data not shown). PMA tripled NBT reduction in rat macrophages, but had only a slight effect on macrophage-like HL-60 cells, which was not always reproducible, probably due to the fact that differentiation occurs by cell treatment with PMA, which may saturate or even down-regulate the cellular response to this agent after differentiation is completed.

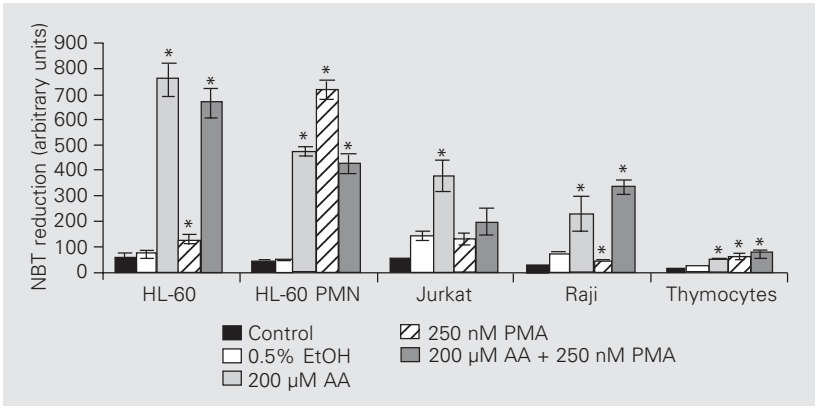

Figure 1. Nitroblue tetrazolium (NBT) reduction following cell exposure to arachidonic acid (AA) or phorbol 12-myristate 13-acid (PMA). Cells (1 x 10^7/ml, 150 µl; 6 samples each) were treated for 1 h with 0.05% NBT in PBSG (0.13 mM NaCl, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) and 0.5% ethanol (EtOH), 200 µM AA, 250 nM PMA or a combination of the last two agents, at 37ºC. A sample (control) was treated with acetic acid (0.5 volumes) prior to incubation. NBT reduction in the remaining samples was stopped with 0.5 volumes acetic acid after incubation. Cells were then pelleted and reduced NBT was extracted with 150 µl 50% acetic acid upon sonication. After debris sedimentation, reduced NBT absorption was determined at 560 nm. Results are reported as mean ± SD absorbance. Cell lines tested were: HL-60 (human promyelocytic leukemia), both in the undifferentiated form or after differentiation into polymorphonuclear- (PMN) like cells upon treatment with 100 nM *all-trans* retinoic acid for 5 days; Jurkat (human T-cell leukemia) and Raji (human B-cell leukemia). Thymocytes were obtained from male albino rats using 4 x 10^7 cells/ml. *P < 0.05 compared to ethanol-treated cells (Student *t*-test).

Control data (filled black columns) in Figure 1 correspond to absorbances of cells killed with acetic acid and therefore exclusively refer to the presence of the cells and the background reduction of NBT. The lowest value corresponded to thymocytes, even when they were added at four times the concentration of the cells used in the other assays. This probably reflects reduced membrane surface and cytoplasmic volume, where superoxide is generated. The white column in Figure 1 corresponds to the baseline reduction of NBT by live cells incubated only with 0.5% ethanol (EtOH). Although all values in the second column were increased when compared with the first, showing that a slight reduction of NBT was continuously occurring in live cells, this baseline reduction was far greater in Jurkat cells. In order to simplify the analysis in subsequent assays, an arbitrary value of 1 was attributed to all baseline values obtained by incubating cells only with ethanol and the corresponding values were expressed relative to this baseline.

Dose-effect plots for arachidonic acid-induced nitroblue tetrazolium reduction

NBT reduction promoted by AA peaked around 200-400 µM for leukemia cell lines and around 10-20 µM for normal rat cells (Figure 2). This pattern was also found in PMN-like differentiated HL-60 cells, with a peak value around 400 µM (data not shown), but differed greatly from that of macrophage-like differentiated HL-60 cells, which showed increasing NBT reduction up to the highest concentration tested - 800 µM (0 µM, 1; 100 µM, 2.0; 200 µM, 4.0; 400 µM, 8.0, and 800 µM, 18.2). These data were obtained with a mean of three replicates per AA concentration reported as arbitrary units.

Kinetics of arachidonic acid-induced nitroblue tetrazolium reduction

AA initially triggered NBT reduction at a high rate, which continuously decreased, reaching zero about 2 h after cell exposure to the acid (Figure 3). Baseline NBT reduction, on the other hand, still seemed to occur even after 2 h, although at much lower rates than for AA. Most experiments presented in this paper were performed after incubation with NBT for 1 h.

Cell pretreatment with AA triggered NBT reduction even after AA was washed out, particularly in Jurkat cells, although the presence of AA together with NBT usually led to higher NBT reduction levels (Figure 4).

Mechanisms of arachidonic acid-induced nitroblue tetrazolium reduction

The results presented in Tables 1 and 2 and Figures 5 and 6 correspond to cellular pretreatments with agents that interfere with the NADPH oxidase system. Two inhibitors of NADPH oxidase were used: N-ethylmaleimide (NEM), an agent that acts on protein sulfhydryl groups, and diphenyleneiodonium (DPI), a flavin antagonist (6). NEM showed dose-dependent inhibition of NBT reduction in all cell types tested, both in control and AA-treated cells, although inhibition was greater in the latter (data not shown).

DPI also prevented AA-triggered NBT reduction, but this effect was not as pronounced as that of NEM and occurred mostly in undifferentiated and PMN-like HL-60 cells (Table 1, lines 2 and 6). In addition to the data shown in Table 1, DPI also inhibited the oxidative burst in AA-stimulated rat thymocytes and macrophage-like differentiated HL-60 cells (control and AA-stimulated cells) (data not shown). The inhibitory effect of DPI was compared in cells subjected to different stimuli and was found to be more marked on NBT reduction promoted by PMA and Triton X-100 than by stimulation with AA (Table 1, lines 2, 4, 6, 7, 10, 11, 13, 14). The cell type most inhibited by DPI was PMN-differentiated HL-60, particularly upon stimulation with PMA (Table 1, line 7).

To determine whether superoxide was

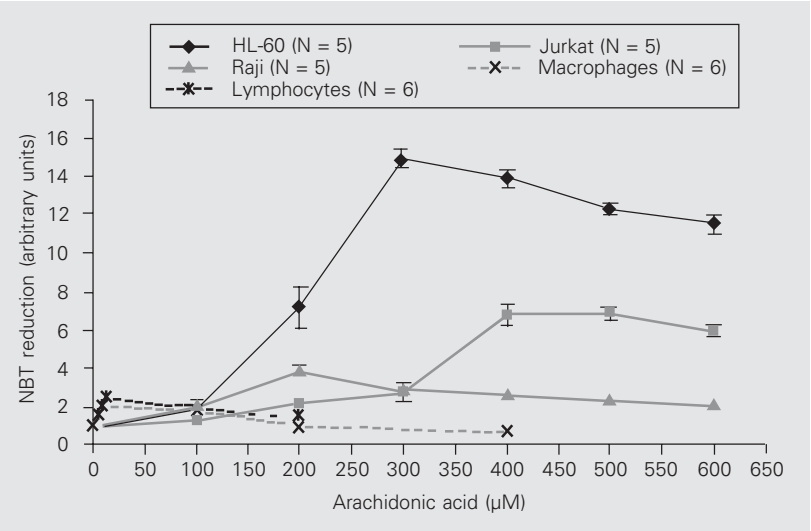

Figure 2. Nitroblue tetrazolium (NBT) reduction induced by different arachidonic acid concentrations. The experimental conditions are the same as those described in the legend to Figure 1. Results are reported as mean ± SD relative absorbance in relation to the value at time zero. The number of samples is indicated by N.

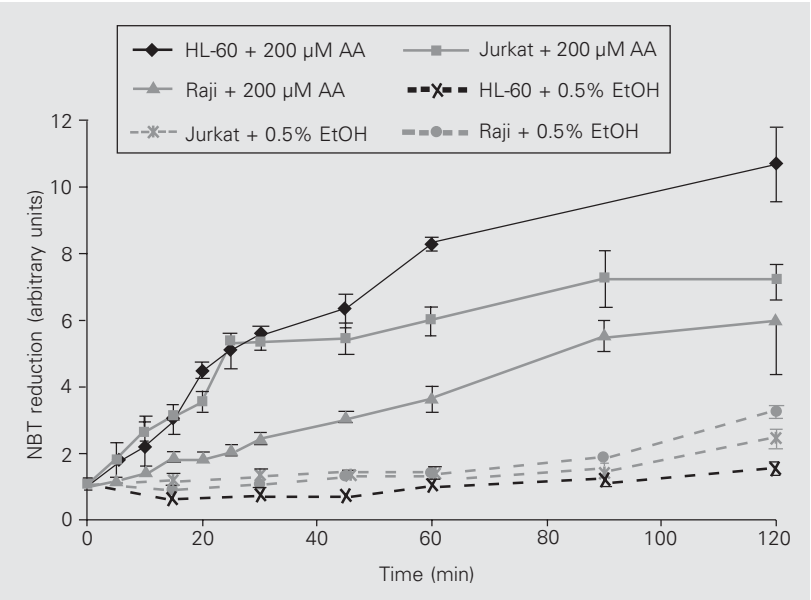

Figure 3. Kinetics of baseline nitroblue tetrazolium (NBT) reduction and of NBT reduction stimulated with arachidonic acid (AA) in leukemia cell lines. The experimental conditions are the same as those described in the legend to Figure 1. Cells were treated for different periods of time with 0.5% ethanol (EtOH) or 200 μM AA. Results are reported as mean ± SD relative absorbance in relation to the value at time zero. The number of samples was 4-5.

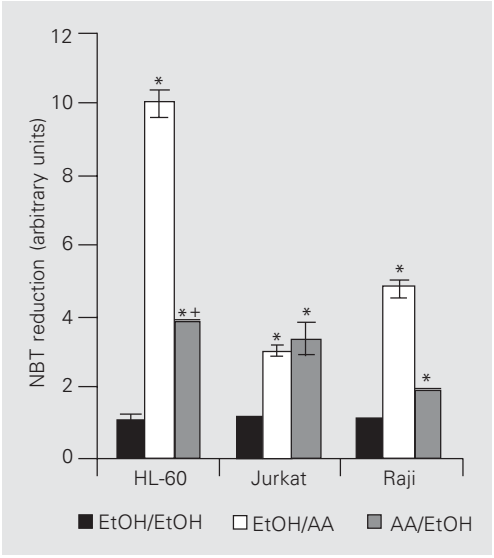

Figure 4. Nitroblue tetrazolium (NBT) reduction by ethanol- (EtOH) or arachidonic acid- (AA) pretreated cells. Cells were pretreated for 15 min with 0.5% EtOH or 200 µM AA in PBSG (0.13 mM NaCl, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) and centrifuged. The supernatant was discarded and the sediment was resuspended in 0.05% NBT in PBSG and 0.5% EtOH or 200 µM AA, and cells were further incubated for 1 h at 37ºC. NBT reduction was stopped with 0.5 volumes acetic acid and reduced NBT was then determined as described in the legend to Figure 1. Results are reported as mean ± SD absorbance in relation to control (cells pretreated and post-treated with EtOH). EtOH/EtOH denotes cells pre- and post-treated with 0.5% EtOH; EtOH/AA denotes cells pretreated with 0.5% EtOH and post-treated with 200 µM AA; AA/EtOH denotes cells pretreated with 200 µM AA and post-treated with 0.5% EtOH. The number of samples was 6. *P < 0.05 for the comparisons between EtOH/AA or AA/EtOH and EtOH/EtOH; +P < 0.05 for the comparison between EtOH/AA and AA/EtOH (Student *t*-test).

Table 1. Inhibition of nitroblue tetrazolium reduction by superoxide dismutase and diphenyleneiodonium.

	0.5% EtOH	100 µg/ml SOD	10 µM DPI
HL-60 + 0.5% EtOH	1.00 ± 0.17	0.77 ± 0.13*	0.69 ± 0.08*
HL-60 + 200 µM AA	3.01 ± 0.20	2.62 ± 0.18*	1.03 ± 0.12*
HL-60 + 250 nM PMA	1.87 ± 0.22	1.10 ± 0.09*	0.81 ± 0.08*
HL-60 + 160 µg/ml Triton X-100	3.94 ± 0.39	1.21 ± 0.09*	0.95 ± 0.10*
PMN + 0.5% EtOH	1.00 ± 0.23	0.73 ± 0.15*	0.44 ± 0.11*
PMN + 200 µM AA	10.90 ± 1.39	11.77 ± 0.75	3.05 ± 0.45*
PMN + 250 nM PMA	5.71 ± 0.35	3.97 ± 0.18*	1.11 ± 0.11*
PMN + 160 µg/ml Triton X-100	2.26 ± 0.38	1.51 ± 0.20*	0.75 ± 0.15*
Jurkat + 0.5% EtOH	1.00 ± 0.09	0.88 ± 0.08*	0.62 ± 0.03*
Jurkat + 200 µM AA	5.18 ± 0.33	5.70 ± 0.48*	4.78 ± 0.20*
Jurkat + 160 µg/ml Triton X-100	2.54 ± 0.30	2.21 ± 0.32	1.79 ± 0.11*
Raji + 0.5% EtOH	1.00 ± 0.14	0.83 ± 0.09*	0.79 ± 0.08*
Raji + 200 µM AA	4.03 ± 0.31	4.13 ± 0.21	3.30 ± 0.32*
Raji + 160 µg/ml Triton X-100	2.55 ± 0.47	2.38 ± 0.48	1.80 ± 0.13*

Cells (2 x 10^7/ml, 150 µl) in PBSG (0.13 mM NaCl, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) were treated for 30 min with 0.5% ethanol (EtOH), 200 µg/ml bovine CuZn-superoxide dismutase (SOD) or 20 µM diphenyleneiodonium chloride (DPI). One volume of PBSG containing 0.1% nitroblue tetrazolium (NBT) and 0.5% EtOH, 400 µM arachidonic acid (AA), 500 nM phorbol 12-myristate 13-acid (PMA) or 320 µg/ml Triton X-100 was added and the cells were further incubated for 1 h at 37ºC. NBT reduction was stopped with 0.5 volumes acetic acid and reduced NBT was then determined as described in the legend to Figure 1. Results are reported as mean ± SD relative absorbance in relation to the EtOH/EtOH-treated samples. The number of samples was 5. The Student *t*-test was used to compare pretreatment with 0.5% EtOH and pretreatment with SOD or DPI.
*P < 0.05 for NBT reduction by cells pretreated with EtOH and then treated with AA, Triton X-100 or PMA compared to cells pre- and post-treated with EtOH (Student *t*-test).

being released into the extracellular environment, the effect of SOD on AA-triggered NBT reduction was investigated. SOD had little effect on AA-induced NBT reduction in HL-60 cells and no effect on Raji and Jurkat oxidative burst (Table 1, column 2). In rat thymocytes, SOD inhibited control (0.5% EtOH, 1.00 ± 0.19; 0.5% EtOH + 100 µM SOD, 0.74 ± 0.11; N = 5) and AA-stimulated NBT reduction (20 µM AA, 1.77 ± 0.11; 20 µM AA + 100 µM SOD, 1.26 ± 0.07; N > 4). In macrophage-like differentiated HL-60 cells, SOD also inhibited both control (0.5% EtOH, 1.00 ± 0.07; 0.5% EtOH + 100 µM SOD, 0.71 ± 0.09; N = 5) and AA-stimulated NBT reduction (400 µM AA, 5.3 ± 0.4; 400 µM AA + 100 µM SOD, 4.6 ± 0.4; N = 5). In contrast to the results obtained upon cell stimulation with AA, SOD was particularly effective in inhibiting NBT reduction triggered by other stimuli, such as PMA or Triton X-100, although this effect could only be found in undifferentiated and PMN-differentiated HL-60 cells (Table 1, lines 3, 4, 7, 8).

Triton X-100 was used to determine whether AA was acting as a detergent. In this case, we expected that the effect of AA- and Triton X-100-treated cells would be the same. Triton X-100 stimulated the oxidative burst (Figure 5). The shapes of these dose-response plots are comparable to those obtained by cell stimulation with AA, i.e., they reach a peak at an optimum concentration and then decline, which suggests that AA might be acting as a detergent. Similar patterns of response were also found with other detergents, such as Nonidet, Tween 20 and Tween 80 (data not shown). Up to 200 µM, Triton X-100 treatment did not cause extensive lysis and cells maintained their shape, although they became permeable to Trypan blue and propidium iodide (data not shown). The detergent probably acts preferentially on some cell microenvironments, without affecting others. This could explain the fact that, although Triton X-100 can stimulate superoxide generation, this effect is not reversed by

the presence of SOD (Table 1, lines 11 and 14) in some cell types, such as Raji and Jurkat. Superoxide is possibly being generated in vesicles that are not permeable to this antioxidant enzyme even in the presence of the detergent.

Both NADPH and NADH increased NBT reduction in all three cell lines tested, although the first had a greater effect (Table 2 and Figure 6). PMA, a known stimulator of PKC, only increased NBT reduction in some cell types, showing no effect (or even an inhibitory effect) on Raji and Jurkat cells (Figure 1). Bisindolylmaleimide I, also known as GF 109203X (up to 10 µM), a specific inhibitor of some isoforms of PKC, did not reverse control or AA-induced NBT reduction in the cell lines studied (Raji, Jurkat, undifferentiated and PMN-differentiated HL-60 cells) (data not shown), although it completely reversed PMA-induced NBT reduction (control, 1.00 ± 0.07; 5 µM GF 109203X, 1.07 ± 0.02; 250 nM PMA, 14.2 ± 0.9; GF 109203X + PMA, 0.9 ± 0.1, 6 samples).

A percentage of electrons is known to cause univalent reduction of oxygen in complexes I and III of the mitochondria, generating superoxide. Rotenone (20 µM) is an inhibitor of complex I and oligomycin (5 µg/ml) is an inhibitor of ATP synthase that causes mitochondrial hyperpolarization, which in turn hinders electron transport. Neither of these agents showed a marked effect on AA-induced NBT reduction in the cell lines tested (undifferentiated and PMN-like HL-60 cells, Jurkat and Raji), although minor effects (differences below 30%) could be seen, particularly on control (ethanol-treated) cells, with NBT reduction being decreased in Raji and undifferentiated HL-60 cells by rotenone and in Jurkat and undifferentiated HL-60 cells by oligomycin (data not shown). Other agents tested without a great effect on AA-triggered NBT reduction were antimycin A (complex III inhibitor) and dinitrophenol (a mitochondrial uncoupler) (data not shown). All agents used apparently were active on cells since they were cytotoxic at the doses used. Similar results were also found in PMN-differentiated HL-60 cells (data not shown).

Allopurinol (100 µM), an inhibitor of the xanthine-xanthine oxidase system, had no general effect on NBT reduction in control or AA-treated cells. Of the cell lines studied (undifferentiated and PMN-like HL-60 cells, Jurkat and Raji), control HL-60 cells showed a 20% decrease in NBT reduction and Raji

Table 2. Nitroblue tetrazolium reduction by cells treated with NADPH or NADH.

	HL-60 + 0.5% EtOH (N = 5)	HL-60 + 200 µM AA (N = 5)	Jurkat + 0.5% EtOH (N = 5)	Jurkat + 200 µM AA (N = 5)	Raji + 0.5% EtOH (N = 3)	Raji + 200 µM AA (N = 4)
EtOH (0.5%)	1.00 ± 0.18	3.65 ± 0.13	1.00 ± 0.05	4.16 ± 0.11	1.00 ± 0.22	3.01 ± 0.28
NADH (100 µM)	0.87 ± 0.42	7.49 ± 0.23*	1.18 ± 0.11*	8.60 ± 0.78*	1.23 ± 0.09	5.51 ± 0.31*
NADPH (100 µM)	1.20 ± 0.11*	7.67 ± 0.53*	1.31 ± 0.25*	10.97 ± 0.41*	1.16 ± 0.22	8.48 ± 0.27*

Cells (2 x 10^7/ml, 150 µl) in PBSG (0.13 mM NaCl, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) were treated for 30 min with 0.5% ethanol (EtOH), 200 µM NADH or 200 µM NADPH. One volume of PBSG containing 0.1% nitroblue tetrazolium (NBT) and 0.5% EtOH or 400 µM arachidonic acid (AA) was added and the cells were further incubated for 1 h at 37ºC. NBT reduction was stopped with 0.5 volumes acetic acid and reduced NBT was then determined as described in the legend to Figure 1. Results are reported as mean ± SD relative absorbance in relation to the EtOH/EtOH-treated samples. The number of samples is indicated by N. Data obtained with pretreatment with 0.5% EtOH were compared to those obtained with NADH or NADPH treatment by the Student *t*-test.
*P < 0.05 for NBT reduction by cells pretreated with EtOH and then treated with AA compared to cells pre- and post-treated with EtOH (Student *t*-test).

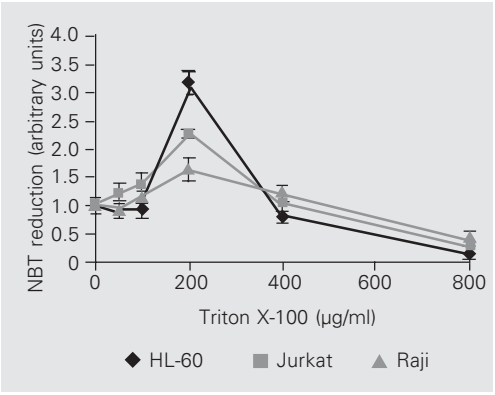

Figure 5. Nitroblue tetrazolium (NBT) reduction induced by Triton X-100. Cells were treated with increasing concentrations of Triton X-100 at 37ºC. Results are reported as mean ± SD relative absorbance. The number of samples was 6.

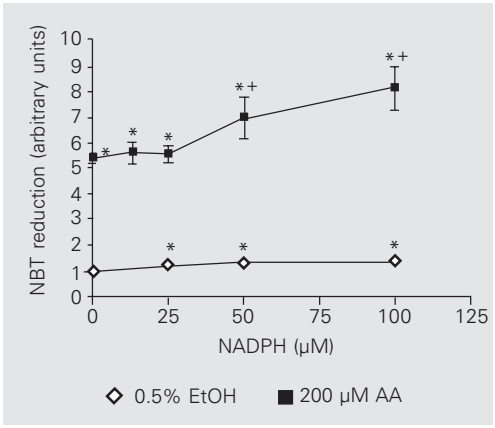

Figure 6. Nitroblue tetrazolium (NBT) reduction upon cell treatment with NADPH. HL-60 cells (2 x 10^7/ml, 150 µl) were treated for 30 min with 0.5% ethanol (EtOH, 0) and with twice the indicated concentrations of NADPH. One volume of PBSG (0.13 mM NaCl, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) containing 0.1% NBT and 0.5% EtOH or 400 µM arachidonic acid (AA) was added and the cells were further incubated for 1 h at 37ºC. Results are reported as mean ± SD relative absorbance in relation to the EtOH/EtOH-treated samples. The number of samples was 6. Data obtained with pretreatment with 0.5% EtOH were compared to those obtained with NADPH by the Student *t*-test. All samples treated with AA (squares) differed significantly from control samples (lozenges) ($P < 0.005$). Increasing concentrations of NADPH differed significantly from the value obtained with no NADPH: *0.05 > P > 0.005 and *+P < 0.005.

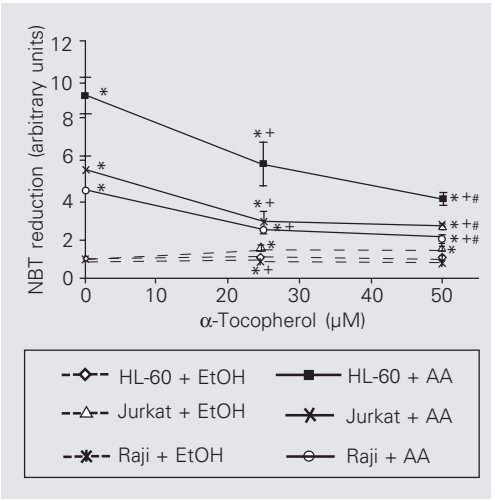

Figure 7. Tocopherol and arachidonic acid- (AA) induced nitroblue tetrazolium (NBT) reduction. Cells were treated for 30 min with ethanol or with twice the indicated concentrations of tocopherol. AA (400 µM) was added and the cells were further incubated for 1 h at 37ºC. NBT reduction was stopped with 0.5 volumes acetic acid and reduced NBT was determined as described in the legend to Figure 1. Results are reported as mean ± SD relative absorbance in relation to the EtOH/EtOH-treated samples. The number of samples was 6. *P < 0.05 for NBT reduction by cells pretreated with EtOH and then treated with AA; +P < 0.05 for pretreatment with 0.5% EtOH (0) compared to pretreatment with tocopherol; #P < 0.05 for comparison between treatments with 25 and 50 µM tocopherol (Student *t*-test).

cells showed a 20% increase in AA-triggered NBT reduction (data not shown). The effect of L-NAME (1 mM), an inhibitor of nitric oxide synthase was also evaluated. Nitric oxide and nitric oxide synthase had no effect on PMA- or Triton X-100-treated cells (data not shown).

In view of the fact that i) AA is known to be converted to a number of oxidized products - the eicosanoids - by oxidation-reduction reactions, ii) eicosanoids can modulate cell activity and metabolism through various pathways, and iii) eicosanoids are unstable products which can be broken down to other oxidized products, we investigated the role of AA metabolism in NBT reduction. Inhibitors of cyclooxygenase (prostaglandin synthase, 8,11,14-eicosatrienoate, hydrogen-donor:oxygen oxidoreductase, EC 1.14.99.1) (500 µM acetylsalicylic acid and 1 µM piroxicam) and of lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) (2 µM nordihydroguaiaretic acid (NDGA) and 2 µM AA861) had little effect on NBT reduction. The cell line most sensitive to these drugs was Raji, in which there was a decrease in AA-induced NBT-reduction upon treatment with cyclooxygenase inhibitors (19% for acetylsalicylic acid and 14% for piroxicam) (data not shown). This cell line also showed increased NBT reduction when control cells were pretreated with piroxicam (25%) and AA861 (19%) (data not shown). The basal NBT reduction of HL-60 cells was slightly decreased by acetylsalicylic acid (6%) and piroxicam (16%) but was more efficient after the AA stimulus, and pretreatment with NDGA (9%) (data not shown). None of these effects was pronounced or systematically found in different cell lines and therefore the effects were probably due to interactions independent of eicosanoid biosynthesis.

The effect of cytochromes P-450 on NBT reduction was studied using the following inhibitors: ketoconazole (100 µM), 8-methoxypsoralen (1 mM) and aminobenzotriazole (1 mM). There was no marked effect of any

of these agents on NBT reduction in the cell lines studied (undifferentiated and PMN-like HL-60 cells, Jurkat and Raji). There was a decrease in AA-triggered NBT reduction in Raji cells (11%) by ketoconazole, and in Jurkat cells by 8-methoxypsoralen (20%) and aminobenzotriazole (15%) and there was an increase in basal NBT reduction in HL-60 (20%) and Jurkat (10%) cells treated with 8-methoxypsoralen (data not shown).

To prevent lipid peroxidation, cells were pretreated with tocopherol, a lipid antioxidant. This substance prevented NBT reduction in AA-stimulated cells (HL-60, Jurkat and Raji) in a dose-dependent manner (Figure 7). A similar effect was also found in PMN-differentiated HL-60 cells and thymocytes (data not shown). Paradoxically, in Jurkat (Figure 7), thymocyte and macrophage-like differentiated HL-60 cells (data not shown) tocopherol increased baseline NBT reduction, which, as mentioned before, is usually high. This effect may be due to the prooxidant action of tocopheryl radicals or to the inhibition of the generation of 4-hydroxynonenal, a product of AA peroxidation that can inhibit NADPH oxidase (5).

Discussion

One of the greatest advantages of the NBT system, as mentioned before, is that it allows the detection of both intracellular and extracellular superoxide production. For this reason, significant results can be obtained with cell lines that are usually considered devoid of superoxide-generating systems (9,10), such as T lymphocytes (Jurkat) and undifferentiated HL-60 cells, as shown here. "Professional" superoxide-generating cells, such as macrophages and PMN-differentiated HL-60 cells, showed the greatest capacity for NBT reduction, but the production of superoxide was also clearly demonstrated in lymphocytes, even without cell stimulation.

AA had a stimulatory effect on NBT reduction in all cell lines evaluated, i.e., undifferentiated, PMN-like differentiated and macrophage-like differentiated HL-60, Jurkat and Raji cells, and rat thymocytes and rat macrophages. This action of AA has been shown by different investigators in various cell types, particularly phagocytes, endothelial cells, 3T3 fibroblasts, and cancer cell lines (11-23). Most published data indicate only a slight effect of AA on superoxide generation, probably because the superoxide assays normally used, such as the cytochrome *c* reduction system, can only determine extracellular superoxide. According to our results, most NBT reduction found in AA-stimulated cells occurred in the intracellular compartment since it could not be inhibited by exogenously added SOD. This enzyme, due to its large size, cannot penetrate the cell. These results bring us back to the possibility that the reduction of NBT did not indicate superoxide production but rather the presence of other agents, possibly AA itself. This last possibility cannot be completely excluded, but some evidence argues against it. Although different cells were treated with the same quantities of AA, the NBT reduction capacity varied considerably. Other evidence indicating that AA is not itself being reduced is presented in Figure 4, where it can be seen that cellular pretreatment with AA was sufficient to trigger NBT reduction after AA was washed away. This type of "priming" effect of AA has been shown by Huang et al. (12), Kweon et al. (21), Cherny et al. (10), and Corey and Rosoff (22).

The stimulatory effect of AA on NBT reduction is dose-dependent, with no effect at low concentrations, rising sharply at intermediate concentrations and then gradually falling, in an asymmetric pattern, sometimes even to values below baseline (for untreated cells). The low final values were probably due to cell necrosis, as visualized under the microscope. We have found that concentrations above 10 μM are apoptotic to these cell lines and those beyond 200 μM begin to

induce necrosis (4). The concentration of 200 μM AA was adopted for further studies to avoid the cell changes inherent to necrosis, when there is extensive activation of proteases, nucleases, esterases among other degrading enzymes, and pronounced changes in metabolism. This concentration was also adopted to permit a comparison between superoxide generation and the triggering of apoptosis (4). It is possible that the concentration that leads to an increase in NBT reduction correlates with the critical micellar concentration of AA, which has been found to be around 10 μM (22). For comparison, physiological plasma concentrations of free AA (non-esterified) in humans vary from 5.8 to 49.3 μM (24). In fact, the pattern of oxidative burst stimulated by AA mimics that of detergents, such as Triton X-100. AA is itself a detergent, having a long hydrophobic tail and a hydrophilic head, usually found in the dissociated form at physiological pH (arachidonate). The present paper shows various experiments run side by side in which NBT reduction was stimulated with AA or with detergents. It was found that many agents affected NBT reduction promoted by both stimuli in the same way. For instance, most inhibitors of mitochondrial respiration such as the xanthine-xanthine oxidase system, nitric oxide synthase and cytochromes P-450 had no effect on either forms of stimulation of NBT reduction. On the other hand, detergent-stimulated NBT reduction was more sensitive to the NADPH oxidase inhibitor DPI and, in HL-60 cells, particularly after differentiation to PMN-like cells, to SOD inhibition. Incidentally, in undifferentiated and PMN-like differentiated HL-60 cells, Triton X-100-stimulated NBT reduction showed the same pattern of inhibition as that of PMA-stimulated cells. AA-stimulated NBT reduction, on the other hand, was virtually not affected by SOD and was less sensitive to DPI and NEM inhibition. It seems that AA might act as a detergent, but its effect must involve other mechanisms as well, as suggested by Cherny et al. (10), Huang et al. (12), Corey and Rosoff (22) and Kadri-Hassani et al. (23), and will be discussed further on.

Many theories have been proposed to explain AA stimulation of the oxidative burst. In the PMA-stimulated NADPH system, it could cause dissociation of inhibitors of components of the NADPH complex, promote an ideal membrane environment for enzyme function, promote the phosphorylation or translocation of p47 phox (15,23,25), stimulate the NADPH oxidase proton pump (10,26), activate or promote PKC translocation (12,23,27-29), stimulate the cell, mimicking, when in the micellar state, particulate stimuli (14,22), or stimulating the cell via the generation of eicosanoids (6). Regarding the mitochondrial-derived superoxide production, AA could act by its known mitochondrial uncoupling ability (carrying protons across the inner mitochondrial membrane), stimulating the opening of the permeability transition pore or acting as a prooxidant, and affecting electron transport (10,30,31). AA is a substrate for many components of the cytochrome P-450 complex, known to produce superoxide (7). This polyunsaturated fatty acid is easily peroxidized, generating free radicals, which, in turn, could lead to superoxide production. Indeed, AA could be oxidized by peroxisomes, organelles known to generate superoxide. The prooxidant effect of AA could also lead to the conversion of xanthine dehydrogenase to xanthine oxidase, which produces superoxide (3). Finally, AA could act directly or upon conversion into eicosanoids on cell receptors, stimulating the production of superoxide as a second messenger (13,22). Some of these hypotheses were tested here and the following were ruled out: the role of mitochondria, cytochromes P-450, xanthine oxidase and eicosanoid-induced pathways of superoxide generation. The most promising hypothesis is that regarding NADPH oxidase: inhibitors of this system, such as NEM and DPI (a PKC

inhibitor) (10,31) may prevent AA-induced superoxide production, whereas NADPH, a substrate for the enzyme, promotes it. Indeed, NADH was also capable of promoting AA-stimulated NBT reduction. The following hypothesis may explain this effect of NADH: it could be consumed to convert NADP to NADPH; it could be a co-substrate for NADPH oxidase, or there may be a superoxide-generating NADH oxidase. Similar stimulating effects of NADPH and NADH on AA-induced superoxide generation were also found by O'Donnell and Azzi (13).

Although the present investigation was designed to study the effect of AA on superoxide production, we would like to mention some common results obtained with non-stimulated cells of different cell lines. The following agents prevented NBT reduction: DPI and NEM (as shown in results), SOD (100 μg/ml), which decreased control values by 23% ($0.05 > P > 0.005$) in HL-60 cells; 27% ($0.05 > P > 0.005$) in PMN-like HL-60 cells; 12% ($0.05 > P > 0.005$) in Jurkat and 17% ($0.05 > P > 0.005$) in Raji cells; L-NAME (1 mM), which decreased NBT reduction by 10% ($0.05 > P > 0.005$) in HL-60 cells and oligomycin (5 μg/ml), which decreased NBT reduction by 27% ($P < 0.005$) in HL-60 cells and by 19% ($0.05 < P < 0.005$) in Jurkat cells, implicating not only NADPH oxidase, but also the mitochondria and nitric oxide synthase in baseline superoxide production by the cell lines tested. This corroborates data obtained by Rothe and Valet (32), who found that baseline superoxide production by phagocytes could be inhibited by azide, a known inhibitor of mitochondrial oxygen consumption.

In conclusion, the results presented here implicate the NADPH oxidase system in AA-induced superoxide production in various forms of leukocytes, excluding the role of the mitochondrion and of xanthine oxidase, nitric oxide synthase, cytochromes P-450 and eicosanoid-stimulated systems.

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