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EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON HUMAN NEUTROPHIL NADPH OXIDASE IN BOTH WHOLE CELL AND CELL-FREE SYSTEMS

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Abstract—The effects of non-steroidal anti-inflammatory drugs (NSAIDs) on the respiratory burst oxidase (NADPH oxidase, EC 1.6.99.6) from human neutrophils in both whole cell and fully soluble (cell-free) systems were investigated. Three NSAIDs, indomethacin, salicylic acid and acetylsalicylic acid (aspirin), were found to inhibit the superoxide generation by human neutrophils exposed to phorbol myristate acetate in a whole cell system and the activation of superoxide-generating NADPH oxidase by sodium dodecyl sulfate in a cell-free system. Concentrations of these NSAIDs required for 50% inhibition of the oxidase (IC_{50}) were: indomethacin (180 μM in both systems), salicylic acid (1.30 mM in the cell-free system, and more than 3.0 mM in the whole cell system) and acetylsalicylic acid (1.35 mM in the cell-free system, and more than 3.0 mM in the whole cell system). In addition, in the cell-free system, these NSAIDs did not change the K_m values for NADPH of the oxidase. These results suggest that these NSAIDs, especially indomethacin, concentration-dependently inhibit the reconstitution of the solubilized membrane-bound enzyme by sodium dodecyl sulfate in the cell-free system.

Human neutrophils play a crucial role in host defense [1] and also cause tissue damage in the inflammatory loci [2] owing to their ability to migrate towards the injured and infected tissues [3], to adhere to microorganisms, to ingest them in phagosomes [4] and to kill them in various ways involving activation of their oxidative metabolism [5] and release of lysosomal enzymes [6]. Activation of the neutrophil oxidative metabolism is characterized by strong cyanide-insensitive oxygen consumption [7] and by concomitant production of active oxygen derivatives such as O_2^-, H_2O_2 and $\cdot OH$ [8]. Superoxide is produced primarily through the activation of plasma membrane-bound NADPH oxidase (EC 1.6.99.6) by stimulation with phagocytizable particles [9] or soluble agents [10]. The major contribution of superoxide and its dismutation products to the bactericidal capacity of the body is readily demonstrated by the susceptibility to infection shown by patients with chronic granulomatous disease, in which there is a defect in NADPH oxidase or its activating apparatus [8].

Non-steroidal anti-inflammatory drugs (NSAIDs†) have been shown to modify or inhibit the generation of superoxide by neutrophils exposed to stimulating agents [11–14] as well as the activity of cyclooxygenase [15]. Until now, the inhibition of the superoxide-producing NADPH oxidase by NSAIDs has mostly been tested using whole cell systems both *in vivo* and *in vitro*. However, the mechanisms by which NSAIDs inhibit the superoxide generation due to neutrophil NADPH oxidase are still unknown. This study is focused on the inhibition of activation of neutrophil NADPH oxidase in a fully soluble (cell-free) system by NSAIDs, which were chosen because of the encouraging results already obtained under some whole cell systems [11–14]. The inhibition of activation of the NADPH oxidase by NSAIDs obtained in the cell-free system was compared with that of superoxide generation of phorbol myristate acetate-stimulated neutrophils by NSAIDs in the whole cell system.

MATERIALS AND METHODS

The following chemicals were obtained from commercial sources: bovine erythrocyte superoxide dismutase, cytochrome *c* (type III), β-NADPH (type I), sodium deoxycholate, phorbol 12-myristate 13-acetate (PMA), PIPES, EGTA, ATP, flavin adenine dinucleotide (FAD), indomethacin, salicylic acid and acetylsalicylic acid (Sigma Chemical Co., St. Louis, MO); and Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY). Sodium deoxycholate was recrystallized from ethanol before use. Other chemicals were of the highest purity available from commercial sources.

Solubilized membranes and cytosolic fractions were prepared from resting human neutrophils as previously described [15]. Protein concentrations determined using the bicinchoninic acid protein assay reagent [16] (Pierce Chemical Co., Rockford, IL) were as follows: solubilized membranes, 32.6 ± 2.4 $\mu g/10^7$ cell Eq (means ± SD, N = 3); and cytosolic fractions, 189 ± 18 $\mu g/10^7$ cell Eq (means ± SD, N = 3).

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† Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PMA, phorbol myristate acetate; FAD, flavin adenine dinucleotide; and SDS, sodium dodecyl sulfate.

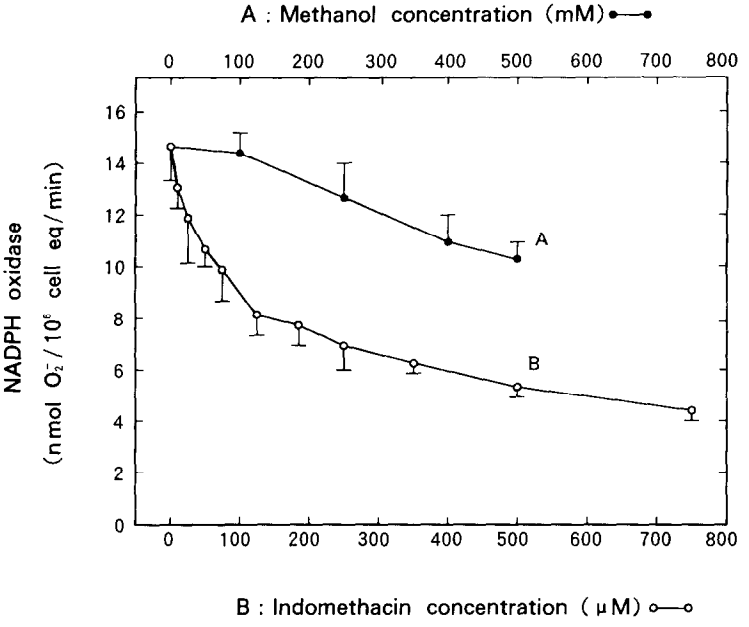

Fig. 1. Concentration-dependent changes due to indomethacin in superoxide generation of PMA-stimulated intact neutrophils. The assay method is described in Materials and Methods. Concentrations of 1–60 mM stock indomethacin–methanol solution (the final methanol concentration in each cuvette was less than 250 mM) were used in these assays. Results are the means ± SD of three different experiments. Key: (A) only methanol treatment; and (B) indomethacin treatment.

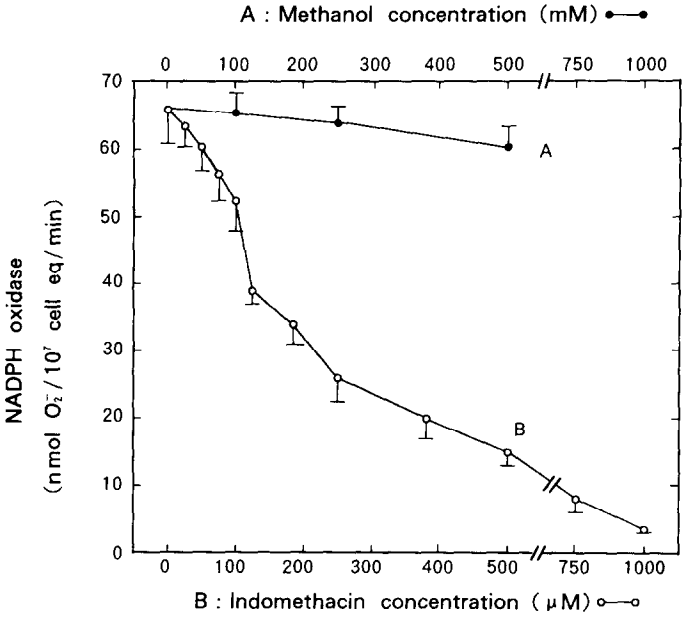

Fig. 2. Concentration-dependent changes due to indomethacin in the activation of NADPH oxidase in the cell-free system. The assay method is described in Materials and Methods. Concentrations of 1–60 mM stock indomethacin–methanol solution (the final methanol concentration in each cuvette was less than 250 mM) were used in these assays. Results are the means ± SD of three different experiments. Key: (A) only methanol treatment; and (B) indomethacin treatment.

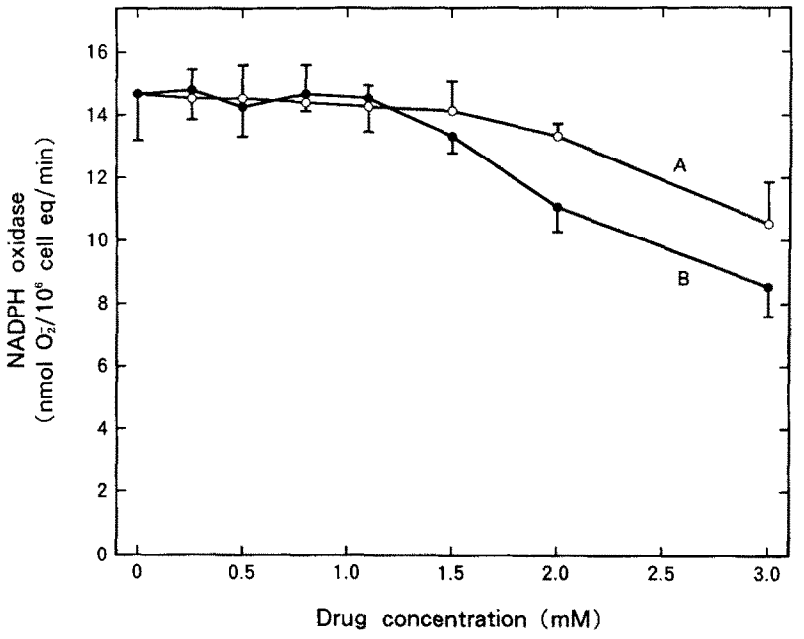

Fig. 3. Concentration-dependent changes due to acetylsalicylic acid (A) and salicylic acid (B) in superoxide generation of NADPH oxidase in the whole cell system. The assay method is described in Materials and Methods. Results are the means ± SD of three different experiments.

Superoxide production by intact stimulated neutrophils (whole cell system) was measured following the superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm by a modification [17] of the method of Markert *et al.* [18]. Neutrophils (2×10^5 cell Eq/cuvette) were incubated in an assay HBSS medium containing 0.12 mM cytochrome *c* and the desired concentrations of NSAIDs (indomethacin, salicylic acid and acetylsalicylic acid) for 2 min at 37° before the reactions were initiated by adding PMA (0.3 μg/cuvette). Assay mixtures were incubated for 4 min at 37°, in a total volume of 1.0 mL. The reference cuvette also received 20 μg of superoxide dismutase.

Superoxide production in cell-free systems was assayed by a modification [19] of the method of Curnutte *et al.* [15]. Assay mixtures contained 0.1 mM cytochrome *c*, 3.6 mM $MgCl_2$, 89 mM KCl, 2.7 mM NaCl, 0.5 mM PIPES (pH 7.3), 0.9 mM ATP, 1.2 mM EGTA, 0.5 μM FAD, 6×10^6 cell Eq of cytosolic fractions, 1.5×10^6 cell Eq of membranes solubilized in deoxycholate (0.94 mM), the desired concentrations of NSAIDs (indomethacin, salicylic acid and acetylsalicylic acid), 0.04 mM sodium dodecyl sulfate (SDS) and 0.16 mM NADPH, with alterations as noted in the figure and table legends, in a total volume of 0.75 mL. The reference cuvette contained 40 μg of superoxide dismutase. Basically, all of the constituents except NADPH were mixed in the cuvette and then were placed in the reference and sample cuvettes. Absorbance at 550 nm was followed for 3 min at room temperature (23–24°). Then the reactions were started by adding 25 μL of NADPH to each cuvette, and the change in absorbance at 550 nm was followed for 3–5 min on a Cary model 118 double-beam spectrophotometer. Superoxide production was calculated using an extinction coefficient of $E^{mM}_{550\,nm} = 19.6\ mM^{-1}\ cm^{-1}$ [20]. The concentration (IC_{50}) of NSAIDs required for 50% inhibition of oxidase was estimated by results from each concentration-dependent inhibition curve obtained.

In both whole cell and cell-free systems, a stock solution of 1–60 mM indomethacin–methanol, 5 mM salicylic acid–HBSS buffer, or 5 mM acetylsalicylic acid–HBSS buffer was used. In the experiments using indomethacin–methanol solution, final concentrations of methanol were all less than 250 mM.

RESULTS

Figure 1 shows concentration-dependent changes due to indomethacin in the superoxide generation of intact neutrophils stimulated by PMA (whole cell assay system). Indomethacin concentration-dependently inhibited NADPH oxidase, and the concentration of indomethacin required for 50% inhibition of oxidase (IC_{50}) was 180 μM. However, the IC_{50} value for methanol alone required a concentration of more than 500 mM. The methanol concentration in each cuvette was less than 250 mM.

Figure 2 shows the effects of adding indomethacin on the activation of superoxide-generating NADPH oxidase induced by SDS in the cell-free system. In this system, indomethacin concentration-dependently inhibited NADPH oxidase activation, and the concentration of the drug required for the IC_{50} value was 180 μM. No significant inhibition of SDS-induced activation of the NADPH oxidase by methanol alone at a concentration of less than 250 mM was observed in the cell-free system.

Figure 3 shows concentration-dependent changes

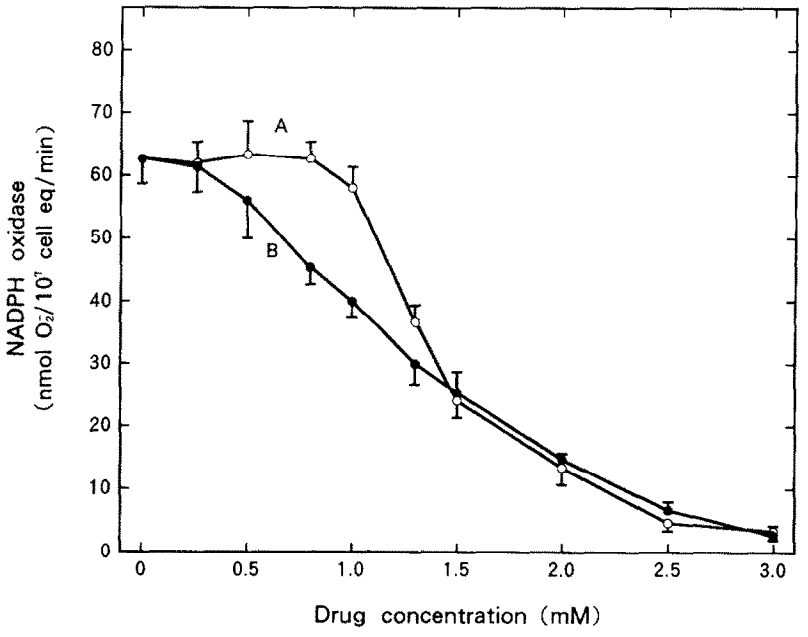

Fig. 4. Concentration-dependent changes due to acetylsalicylic acid (A) and salicylic acid (B) in superoxide generation of NADPH oxidase in the cell-free system. The assay method is described in Materials and Methods. Results are the means ± SD of three different experiments.

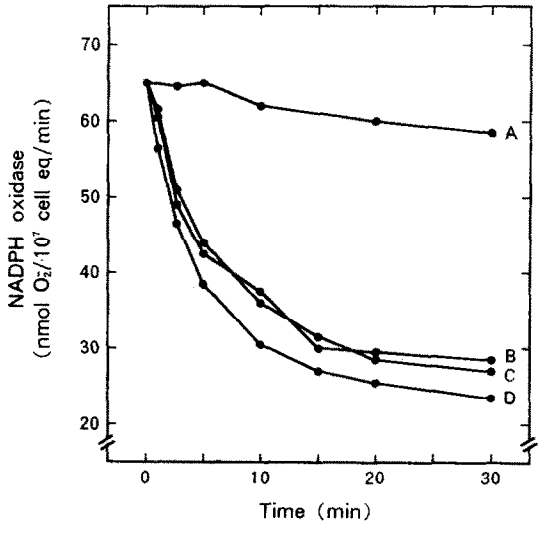

Fig. 5. Time-dependent changes in the activation of NADPH oxidase in the cell-free system after preincubation with NSAIDs but before the addition of SDS and NADPH. The assay method is described in Materials and Methods. In this system, 180 μM indomethacin, 1.30 mM salicylic acid, or 1.35 mM acetylsalicylic acid was employed. The results are the means of three different experiments. Key: (A) methanol (100 mM) alone; (B) salicylic acid; (C) acetylsalicylic acid; and (D) indomethacin.

due to salicylic acid and acetylsalicylic acid in the superoxide generation of PMA-stimulated neutrophils in whole cell systems. In whole cell systems, the concentrations of salicylic acid or of acetylsalicylic acid required to obtain an IC_{50} was more than 3.0 mM. There were no changes in the superoxide generation of PMA-stimulated neutrophils by salicylic acid or acetylsalicylic acid at concentrations of less than 3.0 mM.

Figure 4 shows concentration-dependent changes due to salicylic acid and acetylsalicylic acid in the SDS-induced activation of neutrophil NADPH oxidase in cell-free systems. Concentrations of salicylic acid and acetylsalicylic acid required to obtain IC_{50} values in the cell-free system were 1.30 and 1.35 mM respectively. There was no significant change in the SDS-induced activation of NADPH oxidase by an acetylsalicylic acid concentration of less than 1.0 mM.

Figure 5 shows time-dependent changes in the activation of NADPH oxidase in cell-free systems after preincubation with NSAIDs of concentrations required for IC_{50} and of methanol (100 mM). All the NSAIDs time-dependently inhibited the NADPH oxidase. There was, however, no significant change in the NADPH oxidase by methanol alone.

Table 1 shows the effects of NSAIDs on K_m and V_{max} values for NADPH of the NADPH oxidase in cell-free systems. Although the mean V_{max} values for NADPH of the oxidase after treatment with the three NSAIDs were about half that in the control assay, these NSAIDs did not change the K_m value for NADPH of the oxidase.

DISCUSSION

The results obtained here suggest that indomethacin, salicylic acid and acetylsalicylic acid, which are potent NSAIDs, inhibit the superoxide generation by PMA-stimulated neutrophils in whole

Table 1. Effects of NSAIDs on the K_m and V_{max} for NADPH in a cell-free system

Additions	N	K_m (μM)	V_{max} (nmol O_2^-/10^7 cell Eq/min)
None	3	37.6 ± 2.1	87 ± 12
Indomethacin (180 μM)	3	38.2 ± 1.9	41 ± 4*
Salicylic acid (1.2 mM)	3	36.1 ± 2.0	42 ± 7*
Acetylsalicylic acid (1.3 mM)	3	40.1 ± 2.7	45 ± 5*

The assay method is described in Materials and Methods, except that NADPH, at various concentrations, was used in these experiments. Kinetic constants were calculated by linear regression analysis of Lineweaver–Burk plots. Values are means ± SD; N = the number of experiments performed.

* $P < 0.001$, compared with the control (no additions).

cell systems and SDS-induced activation of neutrophil NADPH oxidase in cell-free systems. The rank of potencies (indomethacin ≫ salicylic acid ≒ acetylsalicylic acid) agrees with previously published data [11] which illustrated a correlation with the ability of the agent to suppress the inflammatory process in which oxygen active derivatives have been localized to the site of tissue injury. In the inhibition of superoxide generation of neutrophil NADPH oxidase, indomethacin was active at concentrations lower than 0.2 mM, which corresponds to the plasma levels currently reached when this NSAID is administered for therapeutic purposes [13]. However, neither salicylic acid nor acetylsalicylic acid inhibited superoxide generation of the NADPH oxidase at therapeutic doses.

The discovery of the inhibition of prostaglandin synthesis by NSAIDs, due to inhibition of cyclooxygenase, indicated an important mechanism of action of NSAIDs. At this moment, however, it seems very likely that, in addition, other mechanisms have a role in the beneficial effects of these drugs. Previous investigations have shown that NSAIDs like indomethacin, ibuprofen, phenylbutazone and piroxicam are able to decrease the release of superoxide from neutrophils exposed to some stimuli *in vitro* [21]. The production of superoxide by neutrophils is a consequence of two separate processes: activation (trigger) and enzyme activity. Since the enzymes and the activation mechanisms are known to be associated with the plasma membrane of the neutrophils, the molecular and functional changes in the plasma membrane may be affected by some drugs which are aimed for another membrane of the target cells. As demonstrated earlier, the addition of piroxicam to the isolated neutrophil membrane fragments has no effect on the superoxide production [14]. In that study [14], the superoxide production of NADPH oxidase of membrane fragments isolated from the PMA-stimulated neutrophils decreased when piroxicam was present during the activation of the NADPH oxidase in the intact neutrophils. These previous data indicate that NSAIDs do not directly inhibit the superoxide-generating NADPH oxidase but may affect the activation systems located in the plasma membrane. In the present study, indomethacin (more than 0.025 mM), salicylic acid (more than 0.5 mM) and acetylsalicylic acid (more than 1.0 mM) inhibited the SDS-induced activation of neutrophil NADPH oxidase in the cell-free system, suggesting that these NSAIDs may inhibit the reconstruction of the solubilized membrane-bound enzyme by SDS or that they may partially produce some conformational changes in the solubilized oxidase enzyme. However, indomethacin failed to achieve 100% inhibition of the superoxide generation of PMA-stimulated neutrophils in the whole cell system. To resolve the discrepancy between whole cell and cell-free systems, the effects of indomethacin on the SDS-induced activation system of NADPH oxidase were investigated. Although 180 μM indomethacin was able to achieve 50% inhibition of oxidase in the cell-free system when it was added to the cuvette before adding SDS (Fig. 2), the same concentration of the drug failed to achieve 50% inhibition when it was added after adding SDS (10–24% inhibition). These results suggest that indomethacin could not inhibit completely the NADPH oxidase previously activated by SDS, and that the drug may bring about modifications of the oxidase activation system rather than a direct effect on the oxidase enzyme. However, the latter may not be discounted.

In the results obtained in the cell-free system, three NSAIDs decreased the V_{max} for NADPH of the oxidase, but they did not change the K_m value for NADPH, suggesting that these drugs may not change the affinity of NADPH oxidase for NADPH. Further investigation, however, will be required to obtain a more detailed picture of the mechanisms for the inhibition due to NSAIDs of activation of the NADPH oxidase in cell-free systems.

Oxygen active derivatives derived from superoxide generated by neutrophils are essential to kill exogenous bacteria and maintain homeostasis of the body. In the inhibition of superoxide generation of neutrophil NADPH oxidase, indomethacin, unlike salicylic and acetylsalicylic acids, was active at therapeutic doses. The inhibitory effect of indomethacin on superoxide production may reduce the bactericidal action of neutrophils, i.e. the defence mechanism of the body against many kinds of pathogens.

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