Inhibitory Effects of Non-steroidal Anti-inflammatory Drugs on Superoxide Generation

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Effects of non-steroidal anti-inflammatory drugs (NSAID: amfenac sodium, diclofenac sodium, indomethacin and ketoprofen) on the generation of superoxide anion (O$_2^-$) by isolated rat polymorphonuclear leukocytes (PMN) were studied spectrophotometrically using cytochrome c. The effects of these drugs were also studied on O$_2^-$ production by the xanthine-xanthine oxidase and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-NADPH oxidase systems.

Amfenac sodium, at 0.1 mM, inhibited significantly O$_2^-$ generation in rat PMN induced by opsonized zymosan. At 0.5 mM, diclofenac sodium and indomethacin inhibited the O$_2^-$ generation in rat PMN. All of the above drugs slightly inhibited O$_2^-$ production by the xanthine-xanthine oxidase system. On the other hand, O$_2^-$ production by the NADPH–NADPH oxidase system was significantly inhibited by the addition of amfenac sodium, ketoprofen or indomethacin. These results suggest that non-steroidal anti-inflammatory drugs do not work as an O$_2^-$ scavenger and block O$_2^-$ production by the NADPH–NADPH oxidase system of rat PMN. It is concluded that amfenac sodium and the other drugs are able to inhibit granulocyte O$_2^-$ production by blocking the activation of NADPH-oxidase.

Keywords superoxide generation; polymorphonuclear leukocyte; reduced nicotinamide adenine dinucleotide phosphate-oxidase; xanthine-xanthine oxidase; anti-inflammatory drugs

Introduction
Active oxygen species have been known to be implicated in a variety of pathophysiological phenomena such as inflammation, aging, hepatotoxicity and ischemic brain and myocardium damage. Such active oxygen species include superoxide radical (O$_2^-$), hydroxyl radical (-OH), singlet oxygen (¹O$_2$) and H$_2$O$_2$.

The importance of oxygen free radicals and related activated oxygen intermediates in the pathogenesis of rheumatoid arthritis (RA) is increasingly recognised. In RA and in other inflammatory diseases polymorphonuclear leucocytes (PMN) and macrophages are stimulated, which results in the secretion of inflammatory mediators, including large amounts of superoxide and hydrogen peroxide. These cells produce O$_2^-$ by the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, an enzyme complex located in the plasma membrane and phagosome.

Oyanagi demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) inhibited O$_2^-$ production of paraffin-oil-induced macrophages but not those of the xanthine oxidase system; this suggests that NSAIDs block O$_2^-$ production and do not work as O$_2^-$ scavengers.

We studied the inhibitory activity of NSAIDs (amfenac sodium, diclofenac sodium, indomethacin and ketoprofen) against the generation of O$_2^-$ by isolated rat PMN. The effects of these drugs on O$_2^-$ production by the xanthine-xanthine oxidase and NADPH–NADPH oxidase systems were also studied.

Experimental

Animals Male Wistar rats (200–250 g) were used. The animals obtained from Nippon SLC Inc., Hamamatsu, Japan.

Materials Xanthine oxidase solution was obtained from Boehringer Mannheim Yamanouchi, Tokyo. Xanthine, NADPH, zymosan and ferricytochrome c (horse heart type III) were obtained from Sigma, U.S.A. The other chemicals were of reagent grade and were used without purification.

Preparation of Cell Suspension Wistar rat peritoneal cells were obtained 18 to 20 h after the peritoneal injection of 0.15% sterile oyster glycogen solution in 0.9% NaCl. The cells were freed of erythrocytes by hypotonic lysis and washed twice in Krebs–Ringer phosphate buffer (KRP), pH 7.4.

The proportion of PMN obtained in this manner averaged 85%.

Measurements of O$_2^-$ Generation The generation of O$_2^-$ was measured by the reduction of ferricytochrome c (horse heart type III) as described by Goldberg and his coworkers. Leucocytes (1 × 10$^7$) and 0.1 mM ferricytochrome c were incubated in the presence of opsonized zymosan for 30 min at 37°C. The final volume of the reaction mixture was adjusted to 2.0 ml. Opsonized zymosan was prepared by incubating 50 mg zymosan in 1 ml of freshly prepared normal rat serum for 40 min at 37°C. After centrifuging this at 1700 × g for 15 min, the opsonized particles were suspended again at a concentration of 50 mg/ml in phosphate buffered saline. This was stored at −80°C before use.

Incubation was terminated by placing the tubes in an ice-water bath; they were then centrifuged at 700 × g for 10 min at 4°C. The absorbance of the supernatants was read at 550 nm with a spectrophotometer.

Preparation of NADPH Oxidase The oxidase was obtained from zymosan-activated neutrophils by a modification of the method of Hohn and Lehrer. Opsonized zymosan was suspended in Dulbecco’s phosphate buffered saline (pH 7.4) containing 2 mM Na$_2$PO$_4$ at a concentration of 25 mg/ml. Two ml of zymosan suspension prewarmed to 37°C was mixed with 2 ml of a prewarmed suspension of PMN in Ca-free KRP (2–5 × 10$^7$ cells/ml). The mixture was incubated for 10 min at 37°C with gentle shaking. The incubation was stopped with 4 ml of ice-cold KRP, and the cells were centrifuged at 250 × g for 5 min at 4°C. The cell pellet was resuspended in 6 ml of ice-cold 0.34 M sucrose containing 1 mM NaHCO$_3$ and the cells were disrupted at 0°C by sonication. After disrupting the cells, the preparation was centrifuged at 250 × g for 5 min at 4°C to remove zymosan, nuclei and unbroken cells. The supernatant was then centrifuged at 30000 × g for 30 min at 4°C. The pellet, which contains the O$_2^-$-forming activity, was resuspended in sucrose at 1 mg protein/ml.

Measurement of NADPH Oxidase The oxidase activity was assayed by measuring the rate of O$_2^-$ formation in the presence of NADPH. The method was based on the spectrophotometric determination of superoxide-mediated ferricytochrome c reduction as originally described by Babior et al.

To a tube containing 1.0 ml of 65 mM KRP (pH 7.4) added 0.2 mM flavin adenine dinucleotide (FAD) (0.2 ml), 1.0 mM NADPH (0.2 ml), 0.2% Triton X-100 solution (0.2 ml), 0.1 mM cytochrome c (0.2 ml) and 10 μl of a solution which contains a drug or superoxide dismutase (SOD) solution. This mixture was incubated for 5 min at 37°C. To this solution 0.2 ml of O$_2^-$-forming particles was added, and this was incubated for 10 min at 37°C. The reaction was stopped by adding 10 μl SOD solution. The amount of reduced cytochrome c was measured spectrophotometrically.

Measurement of SOD-like Activity The activity of O$_2^-$ scavengers was assayed according to the nitrite method described by Oyanagi. 0.5 mM hypoxanthine (0.2 ml), 10 mM hydroxylamine plus 8.85 mM

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hydroxylamine o-sulfonic acid solution (0.1 ml), 0.1 ml of the sample, and 0.2 ml of water were added to a tube containing 0.2 ml of buffer solution (final concentration of 13 mM KH2PO4 and 7 mM Na2HPO4 containing 0.5 mM ethylenediaminetetraacetic acid (EDTA)-2Na (pH 8.2). The reaction was started by adding 0.2 ml of 1 M/ml xanthine oxidase (XOD). This mixture (1.0 ml) was incubated for 30 min at 37°C. 2.0 ml of a color reagent which contained 30 μM N-1-naphthyl-ethylenediamine, 3 mM sulfuric acid and 25% acetic acid were added. The optical absorption was measured at 550 nm.

**Measurements of Protein** The amount of protein was determined by the method of Lowry et al.

**Statistical Method** The results were calculated as mean values±standard deviation (mean±S.D.) and the probability for significance of the differences was determined by analysis of the variance.

**Results**

The inhibition of the generation of superoxide anion (O2−) by NSAID (amfenac sodium, diclofenac sodium, indomethacin and ketoprofen) in isolated rat PMN is shown in Table I.

Amfenac sodium at 0.1 mM significantly inhibited the O2− generation in rat PMN induced by opsonized zymosan. At 0.5 mM, indomethacin and diclofenac sodium inhibited significantly the O2− generation. At 0.05 mM, all of the above drugs only slightly inhibited the O2− generation (data not shown).

Table II shows the inhibitory effect of the NSAID on the generation of O2− by NADPH-dependent oxidase. At 0.1 mM, amfenac sodium and indomethacin significantly inhibited the O2− generation. At 0.5 mM, amfenac sodium, ketoprofen and indomethacin caused significant inhibition. At 1 mM, diclofenac sodium slightly inhibited the O2− generation, while the other drugs did not inhibit it.

All of the above drugs, from 0.1 to 1 mM, only slightly inhibited the generation of O2− by the XOD system and did not inhibit the production of uric acid.

**Discussion**

There are a number of reports which deal with the effects of NSAID on O2− generation. However, the results regarding the effects of certain drugs vary depending upon cell types and stimuli used. In addition, the mechanism by which NSAID retard the chemotactic factor-induced PMN responses remains conjectural. With regard to the O2− generation of PMN, Kitagawa et al.13 proposed that chymotrypsin-like serine proteases are essential for these cells to initiate and maintain the O2− generation in response to the stimuli. The suggested also that, these proteases might be involved in the activation of NADPH oxidase, which has been known to be the primary enzyme for O2− production.

Here we have shown that the anti-inflammatory drugs such as amfenac sodium, diclofenac sodium and indomethacin significantly inhibit the generation in PMN induced by opsonized zymosan. However, the above drugs scarcely inhibit O2− generation by xanthine oxidase system. From this, it is evident that these drugs do not have O2− scavenger activity. Oyanagi suggested that NSAID did not inhibit O2− production of the xanthine oxidase system. This agrees with our results. On the other hand, it has been speculated that the inhibitory effect of NSAID on the generation of O2− is based on the inhibition of NADPH oxidase activity. In the present study, amfenac sodium and indomethacin at 0.1 mM significantly inhibit the generation of O2− induced by the NADPH oxidase system. However, concentrations of NSAID as high as 1 mM scarcely inhibit the generation of O2− by the NADPH oxidase system. This shows that amfenac sodium and indomethacin have two different actions; at low concentrations they inhibit the generation of O2−, while at high concentrations they activate it. Recently, Dale et al.12 suggested that indomethacin increased O2− production from human neutrophils when used in concentrations of 10−6−10−4 M. They further reported that some other NSAID have effects similar to indomethacin and that the same drugs increase O2− production by aggregated immunoglobulin G (IgG) and opsonized zymosan. And then, Mibu et al.13 or Oda et al.14 suggested that the NSAID had a weak effect on O2− generation and scarcely inhibited it. However, amfenac sodium and the other NSAID have the capacity to inhibit NADPH oxidase. This activity may be regarded as the anti-inflammatory mechanism of these NSAID. In addition, it may be considered that these NSAID decrease the phagocytic function of PMN by non-specifically stabilizing the biological membrane and inhibiting the generation of O2− from PMN by NADPH oxidase.
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References