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CYCLOOXYGENASE-INDEPENDENT EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON THE NEUTROPHIL RESPIRATORY BURST

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Abstract—A range of 12 non-steroidal anti-inflammatory drugs (NSAIDs), including members from each of the main chemical groups, were examined for their effects on the oxidative burst induced by the receptor stimulus, platelet-activating factor, and the two post-receptor stimuli, fluoride and dioctanoylglycerol. It was found that the NSAIDs fell into three categories: (1) those that increased the stimulated superoxide (O_2^-) response, (2) those that had no effect and (3) those that decreased O_2^- production. All the drugs were without effect in unstimulated cells. The mode of action of those drugs that caused enhancement of the O_2^- response is unlikely to be due to an inhibition of the cyclooxygenase pathway of arachidonate metabolism as not all NSAIDs caused the enhancement. This data could have clinical implications for the therapy of inflammatory disorders such as rheumatoid arthritis, in that those NSAIDs which cause an increased O_2^- response, while providing temporary relief of symptoms, could be exacerbating the underlying inflammatory condition and associated tissue damage.

When neutrophils are exposed to soluble chemotaxins or phagocytic stimuli they undergo an oxidative burst and generate superoxide (O_2^-), which gives rise to toxic oxygen metabolites. It has been demonstrated that toxic oxygen radicals can bring about extensive tissue damage [1–3] and there is evidence to suggest that they could be involved in the self-perpetuating mechanism of joint damage underlying rheumatoid arthritis [4].

A number of non-steroidal anti-inflammatory drugs (NSAIDs‡) have been shown to modulate neutrophil responses. In many *in vitro* studies, NSAIDs have been found to inhibit neutrophil aggregation, chemotaxis, lysosomal enzyme release and O_2^- generation [5–8]. The stimulus for the majority of these studies was formylmethionyl-leucyl-phenylalanine (fMLP) and the responses were inhibited with varying orders of potency depending on the NSAID. It was previously reported that the NSAID, indomethacin, could actually *increase* the neutrophil O_2^- burst activated with the post-receptor stimuli, OAG and A23187 [9]. Additionally, indomethacin has been reported to augment the neutrophil superoxide response stimulated with OZ [10] and to enhance O_2^- formation in chemically elicited guinea pig macrophages activated with a range of stimuli, as well as provoking an oxidative burst in its own right in the absence of any stimulation [11].

The present study followed on from these observations and involved examining the effect of a wide range of NSAIDs on stimulated O_2^- generation. Three stimuli were used, one (an inflammatory stimulus) acting on a receptor, namely PAF, and two acting at post-receptor sites, namely diC_8 (a direct PKC activator) and fluoride (a G-protein activator).

The NSAID, benoxaprofen, had been shown to cause a dose-related activation of the neutrophil respiratory burst, as measured by lucigenin-enhanced chemiluminescence, and to cause activation of purified PKC derived from both rat brain and human platelet [12]; the authors proposed that the direct stimulatory effect of benoxaprofen on the oxidative burst was mediated by PKC. In view of this report the possibility that our results were due to a direct action on PKC had to be addressed. Accordingly, the NSAIDs were also screened through a PKC activation assay.

MATERIALS AND METHODS

Preparation of neutrophils. Human blood was collected by venipuncture and neutrophils prepared by Ficoll-Isopaque separation as described [9]. Cell purity was greater than 97% and viability greater than 99% (Trypan Blue exclusion).

Superoxide assay. Neutrophils were suspended in a Ca^{2+}-free Tyrode solution, containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL glucose, 1 mg/mL BSA and buffered with 20 mM Hepes at pH 7.4, for those experiments with diC_8 and PAF. A modified Dulbecco's phosphate buffered saline was used for all fluoride experiments containing

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‡ Abbreviations: O_2^-, superoxide; NSAIDs, non-steroidal anti-inflammatory drugs; fMLP, formylmethionyl-leucyl-phenylalanine; OAG, 1-oleoyl, 2-acetylglycerol; OZ, opsonized zymosan; PAF, platelet-activating factor; diC_8, dioctanoylglycerol; PKC, protein kinase C; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; BSA, bovine serum albumin; DAG, diacylglycerol.

136.9 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mg/mL glucose and 1 mg/mL BSA in which the concentration of NaCl was reduced so that the final salt concentration was physiological after the addition of NaF.

The neutrophils suspended at 4×10^6 cells/mL were equilibrated for 20 min at 37°, the appropriate concentration of drug then added and preincubated with cells for 20 min. Cytochalasin B (5 μg/mL) (Sigma) was added to those cells which were to be stimulated with PAF and preincubation continued for a further 5 min. The reaction was initiated when 2×10^6 cells were aliquoted into assay tubes containing 1 mg ferricytochrome *c* (horse heart type III, Sigma), the particular stimulus at the appropriate dilution or Tyrode and either 75 units superoxide dismutase (bovine blood, Sigma) or Tyrode. The final Ca^{2+} concentration was 3 mM, except in fluoride experiments where it was 0.31 mM to avoid precipitation of CaF_2. The reaction was terminated after 30 min at 37° by the addition of 1 mM *N*-ethylmaleimide (Sigma). Following centrifugation at 1400 *g* for 10 min, at 4°, absorbance of the supernatant was read at 550 nm in a Beckman DU-50 spectrophotometer. The amount of O_2^- produced was calculated by dividing the difference in absorbance of the samples, with and without superoxide dismutase, by the extinction coefficient for the change between ferri- and ferrocytochrome *c* ($E_{550} = 15.5\ mM^{-1}\ cm^{-1}$) and the resulting value converted to nmol $O_2^-/5 \times 10^6$ cells. Results are expressed as a percentage of the maximum control response produced by stimulus alone.

PKC activation assay. PKC was partially purified from rat brains by modification of the extraction procedure reported by Niedel *et al.* [13]. PKC activity was determined using the assay described by Hannun *et al.* [14] and measures histone III phosphorylation using Triton X-100 mixed lipid micelles. The required amounts of phosphatidylserine (20 mole%), dihexanoylglycerol (0.001–10 mole%) and drug made up in chloroform/methanol were dried under a stream of nitrogen and solubilized in 3% Triton X-100 by sonication. A Tris/EGTA buffer was employed in the assay containing 25 mM Tris base and 6.25 mM EGTA, pH 7.5. The assay constituents given in final concentrations were added to the reaction tubes in the following order (1) 25 μL histone III at 1 mg/mL, (2) 25 μL Triton mixed micelles, (3) 25 μL PKC enzyme at 1–10 μg protein and (4) 175 μL ATP–Ca–Mg at 100 μM [γ-^{32}P]ATP (1.2 μCi/mL), 100 μM Ca^{2+} and 10 μM Mg^{2+}. The reaction was initiated by addition of the latter ATP solution and terminated after 10 min at 22° by addition of 1 mL ice-cold 25% trichloroacetic acid (w/v) and 1 mL ice-cold BSA (500 μg/mL). The resulting suspension was filtered through a GF/C filter and the precipitate then washed twice with 5 mL 25% trichloroacetic acid. Radioactivity retained on the filter was determined by liquid scintillation counting.

RESULTS

O_2^- *Response*

When a representative range of 12 NSAIDs was tested for the effect on stimulated O_2^- release it was found that four out of 12 caused an increase in PAF-stimulated O_2^- production and eight out of the 12 showed an increase in the O_2^- response stimulated with the post-receptor stimuli, diC_8 and fluoride (Table 1). Phenylbutazone and piroxicam caused a significant decrease of the O_2^- response induced by both PAF and fluoride while having no significant effect on diC_8-stimulated O_2^- production (Table 1, Fig. 1a–f). The effect of the drugs on PAF-stimulated O_2^- generation was chosen to classify the NSAIDs into categories: the O_2^- increasers, O_2^- decreasers and those that had no effect on O_2^-. Aspirin at 100 μM, a concentration that should inhibit cyclooxygenase, had no effect on the O_2^- response induced by all three stimuli (Fig. 1g–i).

Table 1. Effect of NSAIDs on the stimulated O_2^- response

NSAID	Stimulus: Fluoride	DiC_8	PAF
Aspirin	(−)	(−)	(−)
Benoxaprofen	↑ ↑ ↑	↑ ↑ ↑	↑ ↑
Diclofenac	↑ ↑	↑ ↑	(−)
Ibuprofen	↑	↑	(−)
Indomethacin	↑	↑	↑
Ketoprofen	(−)	↑	(−)
Meclofenamate	↑ ↑ ↑	↑ ↑ ↑	↑ ↑
Mefenamic acid	↑ ↑	↑ ↑ ↑	↑
Naproxen	↑	↑	(−)
Phenylbutazone	↓	(−)	↓
Piroxicam	↓	(−)	↓
Sulindac	↑ ↑	(−)	(−)

Key: (−) = no effect; ↑ = slight increase in response; ↑ ↑ = greater increase in response; ↑ ↑ ↑ = markedly increased response; ↓ = decrease in response.

Benoxaprofen, meclofenamate, mefenamic acid and indomethacin effected an enhancement of the O_2^- response induced by all three stimuli; the effects of the two most potent drugs, benoxaprofen and meclofenamate, are shown in Fig. 2a–f. It was noted that the order of increase obtained with the post-receptor stimuli was larger than that seen with PAF. In general, drugs that caused an enhancement, produced a marked left shift of the fluoride dose–response curve with a large increase in the maximum control response, whereas with the diC_8 dose–response curve they produced a marked left shift without a change in the maximum response. These four drugs consistently caused a leftward shift of the PAF curve at all concentrations.

The effect of meclofenamate on the fluoride-induced response was somewhat surprising in that at low fluoride concentrations (6 and 10 mM) meclofenamate caused a 7–8-fold increase of the O_2^- response whereas at higher fluoride concentrations meclofenamate effected an inhibition of the fluoride response back to control levels (Fig. 2d); a similar result was observed with mefenamic acid and fluoride (result not shown). It should also be noted that lower concentrations of meclofenamate, 1 and 10 μM, caused a leftward shift of the fluoride

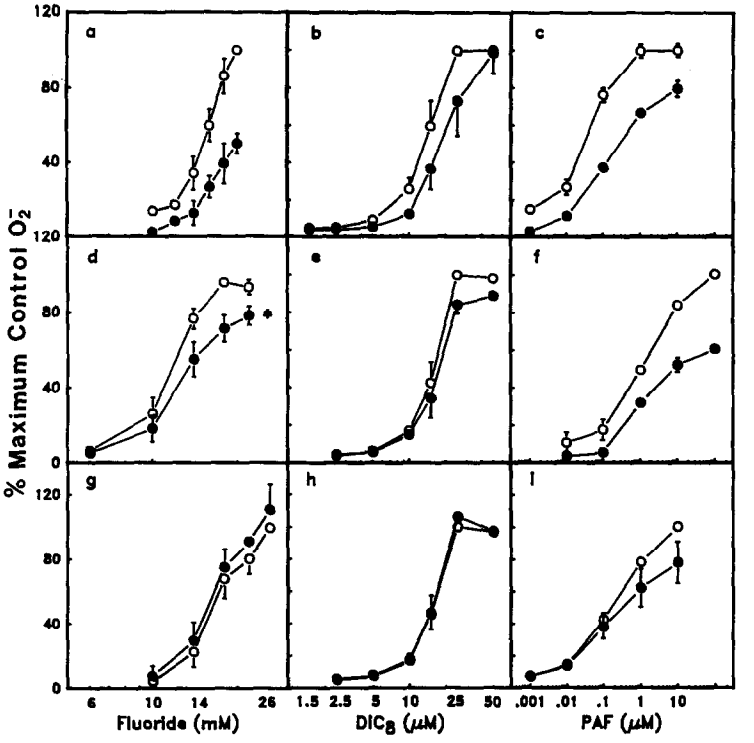

Fig. 1. The effect of phenylbutazone on the O_2^- response induced by (a) fluoride (N = 3), (b) diC_8 (N = 5) and (c) PAF (N = 1, representative of 3). The effect of piroxicam on the O_2^- response induced by (d) fluoride (N = 5), (e) diC_8 (N = 6) and (f) PAF (N = 3). The effect of aspirin on the O_2^- response induced by (g) fluoride (N = 10), (h) diC_8 (N = 5) and (i) PAF (N = 6). Responses were obtained with stimulus, alone (○) or in the presence of drug at 100 μM (●). Mean maximum control O_2^- release, as nmol/5 × 10^6 cells, for fluoride was 99.33 ± 20.39, 95.47 ± 15.99 and 76.94 ± 7.83 in (a), (d) and (g), respectively; for diC_8 was 146.88 ± 13.17, 133.37 ± 9.57 and 147.02 ± 10.67 in (b), (e) and (h), respectively; for PAF was 78.03 ± 2.88 (mean of sample duplicates), 58.79 ± 18.88 and 60.41 ± 10.55 in (c), (f) and (i), respectively. Error bars represent standard errors, except in (c) where they represent the range of sample duplicates.

dose–response curve and an increase in the maximum (result not shown).

The effect of sulindac on the O_2^- response was somewhat exceptional in that it had no effect on the O_2^- response induced by either PAF or diC_8 (Fig. 2h and i) but it effected a marked dose-dependent potentiation (10–100 μM) of the fluoride O_2^- response (Fig. 2g).

PKC assay

Benoxaprofen, meclofenamate, mefenamic acid and aspirin at concentrations ranging from 0.001 to 10 mole% (corresponding in molar terms to 0.06–600 μM) failed to cause an increase of PKC-induced histone III phosphorylation over and above that recorded in the presence of phosphatidylserine and dihexanoylglycerol. The same result was obtained if the concentration of phosphatidylserine, dihexanoylglycerol or Ca^{2+} were varied or if the drug was introduced to the assay system in the aqueous phase rather than in the Triton mixed micelle or lipid phase.

DISCUSSION

The results of the present study indicate that some NSAIDs can increase O_2^- production by the three stimuli employed. In addition to PAF it has also been found that some of the enhancing NSAIDs augmented O_2^- production induced by two other clinically relevant stimuli, namely OZ and IgG (Dale and Muid, unpublished results). These observations may be clinically significant in light of the possible *in vitro* effects of oxygen radicals. There is good evidence that toxic oxygen metabolites generated by activated phagocytic cells are implicated in general tissue damage [1–3]. More specifically, there is evidence that O_2^- produced by neutrophils can modify IgG molecules causing them to aggregate, these aggregates in turn acting as stimuli for further oxygen radical generation [4]; the IgG aggregates could also function as auto-antigens for T cells and B cells, thus recruiting the specific immune response. This could constitute a self-perpetuating mechanism for the production of tissue-damaging oxygen radicals. The IgG aggregates which are formed in the presence of O_2^- have a characteristic auto-fluorescence and aggregates with identical auto-fluorescence have been isolated from the serum and synovial fluid of rheumatoid patients. Superoxide production by phagocytic cells could, in fact, be a key event in the pathogenesis of chronic inflammatory

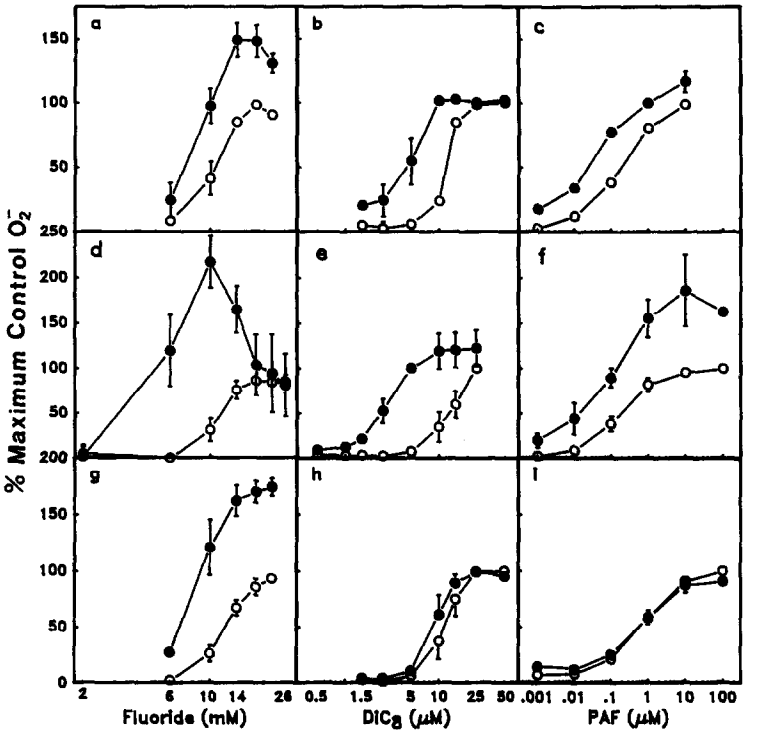

Fig. 2. The effect of benoxaprofen on the O_2^- response induced by (a) fluoride (N = 4), (b) diC_8 (N = 3) and (c) PAF (N = 6). The effect of sodium meclofenamate on the O_2^- response induced by (d) fluoride (N = 5), (e) diC_8 (N = 4) and (f) PAF (N = 4). The effect of sulindac on the O_2^- response induced by (g) fluoride (N = 4), (h) diC_8 (N = 4) and (i) PAF (N = 5). Responses were obtained with stimulus, alone (○) or in the presence of drug at 100 μM (●). Mean maximum control O_2^- release, as nmol/5 × 10^6 cells, for fluoride was 77.15 ± 19.22, 55.60 ± 16.76 and 60.94 ± 5.33 in (a), (d) and (g), respectively; for diC_8 was 166.89 ± 10.46, 157.21 ± 20.50 and 152.01 ± 15.65 in (b), (e) and (h), respectively; for PAF was 60.49 ± 10.66, 49.78 ± 10.85 and 63.61 ± 19.57 in (c), (f) and (i), respectively. Error bars represent standard errors.

conditions such as rheumatoid arthritis. Furthermore, there is a report that O_2^- can stimulate the production of an interleukin-1-like agent from human neutrophils [15]; interleukin-1 is another important potential mediator of inflammatory joint damage. Taking these factors into account it is possible that those NSAIDs that enhance the oxidative burst could, while alleviating symptoms, exacerbate the long-term pathological changes induced by toxic oxygen radicals. Those NSAIDs that did not affect the response would constitute safe drugs useful in symptomatic treatment while the NSAIDs that cause a decrease in response might have some degree of disease-modifying action.

In considering the clinical implications of the data obtained it is necessary to compare the concentration of NSAIDs used *in vitro* to the therapeutic plasma and synovial concentrations. The concentration of 100 μM NSAID was selected for presentation in this study to produce as definite an effect as possible in order to analyse the mechanism underlying the effects, but this is higher than the serum concentration of 1–8 μM indomethacin recorded in patients under treatment [16]. The potentiating effect observed, particularly for the more powerful enhancing NSAIDs (i.e. meclofenamate, mefenamic acid and benoxaprofen), was found to be dose-related over the range 1 to 100 μM, although the enhancement seen at the lower end of this concentration range was only marginal. Nevertheless, when the potentiation of the O_2^- response by NSAIDs is viewed in the context of the potential self-perpetuating cycles of O_2^- production and lymphocyte activation described by Lunec *et al.* [4], a clinical relevance for this phenomenon becomes a distinct possibility. Even a very small enhancement of O_2^- generation (such as that seen at clinically relevant NSAID concentrations) could, with each successive activating cycle, give rise to an amplified response and also an incremental increase in the accompanying tissue damage associated with production of toxic oxygen radicals. It may be of relevance that the NSAIDs found to increase O_2^-, and which are in clinical use, have a very similar profile in our assay to benoxaprofen—a drug known to be toxic and now withdrawn from general use. However, it has not been investigated whether the actions of benoxaprofen shown in this study are related to its clinical toxic effects.

What is the mechanism by which the potentiating NSAIDs act? Their potentiating effects are clearly independent of their effect on cyclooxygenase

because of the negative results obtained with aspirin on all three stimuli. The negative results obtained with the enhancing NSAIDs on isolated PKC, whether tested alone or in combination with a DAG activator of PKC, indicated that the drugs were unlikely to be increasing the O_2^- response by a direct effect on PKC. The difference between these results and those of Lukey *et al.* [12] is unexplained. It should also be noted that, in contrast to the latter study, none of the NSAIDs caused any activation of the O_2^- response in their own right.

The question of other post-receptor mechanisms by which some NSAIDs could increase the oxidative burst needs to be considered further. It has been proposed that transmembrane signalling in the neutrophil, as well as many other cell types, involves the breakdown of phosphatidylinositol bisphosphate to give inositol trisphosphate (which increases intracellular Ca^{2+}) and DAG (which activates PKC) and that the two pathways function synergistically [17–20]. Other phospholipids such as phosphatidylcholine and phosphatidylinositol have also been proposed as important sources of DAG [21–23]. Evidence has been put forward that the DAG/PKC pathway is of primary importance in mediating the neutrophil respiratory burst [24, 25]. It is thus proposed that the potentiating NSAID effect on the O_2^- response may be due to inhibition of the DAG metabolizing enzyme, DAG kinase, thus increasing DAG levels, PKC activity and the resultant O_2^- response. This proposal was based on the experimental findings that a specific inhibitor of DAG kinase, R59022, was first shown to augment the oxidative burst induced by the post-receptor stimuli, OAG and A23187 [26], the receptor stimuli, IgG, OZ and fMLP [24] and also by the post-receptor stimuli used in the current study, diC_8 and fluoride (unpublished result); on the other hand an inhibitor of the DAG lipase metabolizing enzyme, RHC80267, had no consistent effect with the range of stimuli. The profile of effects obtained with the DAG kinase inhibitor and the "enhancing" NSAIDs on the fluoride- and diC_8-stimulated responses were remarkably similar. The DAG kinase inhibitor and the "enhancing" NSAIDs both produced a marked leftward shift of the fluoride dose–response curve and an increase in the maximum control response; both also caused a marked leftward shift of the diC_8–O_2^- dose–response curve but left the maximum control response unchanged. The DAG kinase inhibitor, like the NSAIDs, produced no neutrophil activation in unstimulated neutrophils. Thus, it is suggested that the mechanism of action of the NSAIDs which increase the fluoride- and diC_8-induced response could be by inhibiting DAG kinase. It is interesting to note that indomethacin has been reported to cause an accumulation of DAG in thrombin-stimulated human platelets, with an inhibition of DAG lipase demonstrated as the locus of action of indomethacin [27].

The most potent enhancing NSAIDs display a biphasic effect with fluoride, a very marked potentiation at low fluoride concentrations and inhibition at high fluoride concentrations; this same profile of effects was also observed at high concentrations of the DAG kinase inhibitor, dioctanoylethylene glycol (unpublished result). This observation supports the proposal that the enhancing NSAIDs, like high concentrations of the DAG kinase inhibitor, are potently inhibiting DAG kinase, thus increasing DAG levels and bringing about marked PKC activation. Potent activation of PKC can lead to a negative-feedback mechanism, as has been investigated in a number of cell types by exposure to prolonged PMA treatment [28, 29] which could give rise to the decreased response at high concentrations of dioctanoylethylene glycol or NSAID.

However, in contrast to the other receptor stimuli it should be noted that PAF-mediated O_2^- generation was unaffected by inhibiting either the DAG kinase or lipase pathways of metabolism (unpublished data). R59022 has been shown to be a weak dopamine D_2, adrenaline α_1 and histamine H_1 receptor antagonist and a potent serotonin S_2 antagonist [30]; in addition R59022 has been found to possess anti-muscarinic properties [31]. It is possible that R59022 could also have antagonist effects at the PAF receptor, meaning that a DAG kinase inhibitory action might still account for the NSAID-mediated potentiating effect. Alternatively, a different mechanism may be involved in mediating the effect of NSAIDs on the PAF-induced O_2^- response.

The potentiating effect of sulindac on fluoride-mediated O_2^- release is unlikely to be due to inhibition of DAG kinase as it had no effect on the diC_8- or PAF-mediated O_2^- responses.

It has previously been suggested that the reduction of prostanoid synthesis itself by NSAIDs may have some pro-inflammatory effects by inhibiting the beneficiary role of prostaglandins in modulating inflammation [32–34]. It has also been demonstrated that PGE_2 and PGI_2 inhibit the oxidative burst [35, 36]. The potentially damaging actions of those NSAIDs which may augment toxic oxygen radical-mediated tissue injury, by a cyclooxygenase-independent mechanism, could well be additive with these pro-inflammatory effects. It could be important for clinicians to take these factors into account in prescribing for arthritic conditions in order to limit potential drug-induced adverse effects on joint tissues. It would appear to be important for long-term trials to be carried out comparing NSAIDs shown to decrease O_2^- or to have no effect with those NSAIDs shown, in this and other studies, to increase the O_2^- response.

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