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Interactions of Cyclooxygenase Inhibitors with Reactive Oxygen Species

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Abstract: Reactive oxygen species (ROS) are a natural means of defense in the body produced mainly by phagocytic and mast cells. However, their overproduction plays an important role in the pathogenesis of numerous diseases, such as atherosclerosis, cancer, myocardial infarction (MI) and other chronic inflammatory diseases. This review attempts to present our understanding in the interaction of anti-inflammatory drugs with ROS. It has been proposed that in addition to inhibition of prostaglandin synthesis, the anti-inflammatory actions of non-steroidal anti-inflammatory drugs (NSAIDs) may also be due to their free radical scavenging effect. The action of NSAIDs has been demonstrated against an array of ROS, including O^{-}_{2}, OH^{-}, HOCl, as well as various Reactive Nitrogen Species (RNS) such as nitric oxide (NO) and peroxynitrite $(ONOO)^{-}$. NSAIDs are thought to compete with chloride for the active site of myeloperoxidase (MPO), thereby inhibiting HOCl strong oxidant radical production.

Key words: ROS, NSAIDs, anti-inflammatory drugs, pathological conditions

Introduction

Free radicals are chemical moieties, which, by virtue of their instability, possess enormous reactivity. They are common in aerobic organisms, where they are formed usnally as by products of numerous oxygen-dependant metabolic processes. Normally these organisms have potent anti-oxidant defenses in the form of either enzymes (e.g., superoxide dismutase and glutathione peroxidase) or anti-oxidants like α-tocopherol (vitamin E), ascorbic acid (vitamin C) and β-carotene, glutathione, which act as radical scavengers (Weber *et al*., 2005). Ascorbate happens to be one of the most potent naturally occurring anti-oxidant since it acts directly by reaction with aqueous peroxyl radicals as well as indirectly by enhancing the anti-oxidant properties of vitamin E (Cotelle *et al*., 2003). However, the generation of Reactive Oxygen Species (ROS) in mammalian cells profoundly effects numerous critical cellular function and leads to various pathological events such as asthma, atherosclerosis, cancer, chronic granulomatous disease, diabetes, Myocardial Infarction (MI) and other inflammatory conditions (Meyer and Schmitt, 2000; Oz *et al*., 2005; Long *et al*., 2004; Storz, 2005; Rahimi *et al*., 2005). Pharmacologic agents with both scavenging activity and anti-inflammatory properties could prove to be beneficial in slowing down of the disease process.

Non Steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed drugs world wide. The anti-inflammatory and analgesic analgetic effect results primarily due to an efficient inhibition of prostaglandin synthesis (Bauer and Marker-Hermann *et al*., 2003). The most know

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NSAIDs e.g., piroxicam, diclofenac, indomethacin, paracetamol are non-selective inhibitor of cyclooxygenase (COX) (Saeed *et al*., 2001). However, it has been suggested that the anti-inflammatory activity of NSAIDs may also be due to their ability to scavenge ROS and RNS and to inhibit the respiratory burst of neutrophils triggered by various activating agents (Mouithys-Mickalad *et al*., 2000; Asanuma *et al*., 2001).

What Are The Reactive Oxygen Species

ROS is a collective term used for a oxygen derived species including oxygen bearing free radical, as well as certain non radicals. The free radicals are chemical species that are capable of independent existence and processes one or more unpaired electron that gives an immense reactivity which is inversely related to their stability (Froman and Boveris, 1992). ROS can be classified into oxygen centered radicals and oxygen centered non radicals. The earliers are superoxide (O_2^-), hydroxyl radical ($^{\cdot}OH$), alkoxyl radical ($RO^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$) and the latter comprises hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other reactive species are nitrogen species such as nitric oxide (NO^-), nitric dioxide (NO_2) and peroxy nitrite ($OONO^-$) (Imlay, 2003).

ROS Generation and Mechanism of Action

Upon activation of mast cells, macrophages, monocytes and neutrophils, superoxide is produced from oxygen by the membrane NADPH oxidase. The primary role of NADPH oxidase is as the transmembrane cytochrome *b*556 the central membranous component, which comprises two subunits, the glycosyleted protein gp91phox and a non glycosyleted p22phox. P47phox is the cytosolic component of the NADPH oxidase complex that translocates to the membrane and associates with cytochrome *b*556 to form the active complex that catalyses the reduction of oxygen to superoxide (vant Lent *et al*., 2005; El-Benna *et al*., 2005) (Fig. 1) (Dusting *et al*., 2005). This radical is rapidly converted to hydrogen peroxide by Superoxide dismutase. Hydrogen peroxide is subsequently used by the enzyme myeloperoxidase (MPO) to oxidize chloride to hypochlorous acid ($^{\cdot}HOCL$) (Costa *et al*., 2005).

The toxicity of ROS in tissues is attributable to many factors. Hydrogen peroxide being not very reactive and electrically neutral can easily penetrate the membranes of surrounding cells, whereas superoxide radical cannot (Holecek *et al*., 2004). Membrane lipids are particularly susceptible to oxidation by hydroxyl radical not only due to their high polyunsaturated fatty acid content, but also due to their association in the cell membrane with enzymatic and non-enzymatic systems able to generate ROS (Costa *et al*., 2005).

Steps of ROS Production

Neutrophils are part of innate immune response, which represent 50 to 70% of the total circnlating leukocytes. Upon activation they utilize molecular oxygen to produce ROS. O_2^-.produced by NADPH oxidase is the source of all ROS generated in the phagosome (El-Benna *et al*., 2005). It is produced by monovalent reduction of oxygen in the following reaction.

$$2\ O_2 + \text{NADPH-(NADPH oxidase)} \rightarrow 2\ O_2^-. + + \text{NADP}^+ + H^+$$

The O_2^-. is transformed into H_2O_2 by spontaneous dismutation (at acid pH in the phagosome) or enzymatic dismutation (by superoxide dismutase (SOD) in the cytosol):

$$O_2^-. + O_2^-. + 2H^+\text{-(SOD)} \rightarrow H_2O_2 + O_2$$

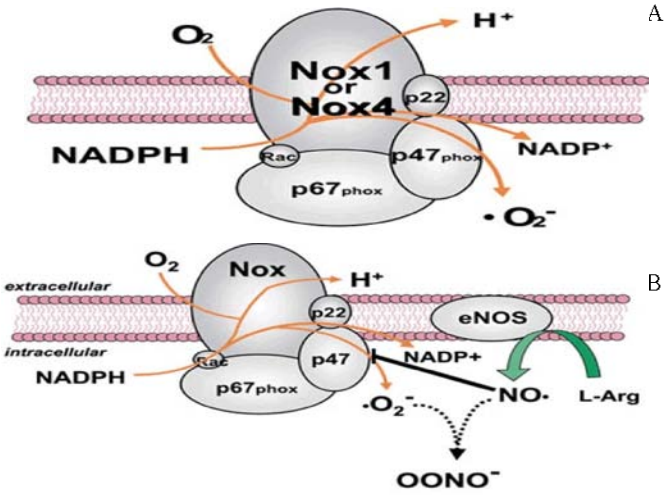

Fig. 1: (A) The vascular NADPH oxidase complex may contain Nox1 or Nox4 as substitutes for the catalytic gp91phox subunit of the phagocytic oxidase. Superoxide (lO_2^-) is produced intracellularly. The Nox subunit is bound to p22phox in the plasma membrane and they stabilise each other. The cytosolic subunits shown may also be required for full and sustained activation of the complex in vascular cells, although there is evidence that alternative subunits may be involved for Nox1 activation. (B) Interactions of nitric oxide (NO) with NADPH oxidase in vascular cells. NO produced by eNOS not only reacts with superoxide (lO_2^-) to produce the reactive species peroxynitrite ($OONO^-$), but it also may act to suppress NADPH oxidase activation

Interaction between H_2O_2 and O_2^-. can, in the presence of a transition metal (iron), give rise to the OH°:

$$O_2^-. + H_2O_2\text{-}(Fe^{++} \text{ or } Cu^{++}) \rightarrow OH° + OH^- + O_2$$

The, oxygen is reduced to form superoxide anion, followed by formation of hydrogen peroxide, which is used by the enzyme myeloperoxidase (MPO) released from azurophilic granules. The MPO catalyses the transformation of H_2O_2 in the presence of a halogen (Cl^-, Br^-, I^-) into highly toxic molecules: oxidized chloride to hypochlorous acid (HOCL) as illustrated below.

$$H_2O_2 + H^+Cl\text{-}(MPO) \rightarrow HOCl^- + HO$$

HOCL is highly oxidant chemical and has been proposed to be the main agent responsible for the antimicrobial and deleterious effects in chronic inflammation mediated by polymorphonuclear (PMN) cells (Dahlgren and Karlsson, 1999).

Other reactions between hypochlorous acid (OCl^-) and H_2O_2 can lead to the formation of singlet oxygen. Most of the OCl^- thus generated is converted into toxic chloramines:

$$H^+ + OCl^- + R\text{-}NH_2 \rightarrow R\text{-}NHCl + HO.$$

Physiological Significance of Free Radicals

Our body is equipped with a complete arsenal of defenses against the external and internal aggressions. ROS such as superoxide anion, hydroxyl radical, hydrogen peroxide and hypochlorous acid (HOCL) are extremely important in inflammatory and antibacterial responses where they participate in the pathophysiological processes mediated by Arachidonic Acid (AA) and phagocytosis (Kampf and Roomans, 2001). However, uncontrolled production of ROS can lead to the attack on many essential biomolecules like proteins, DNA, RNA, lipids and cell organelles causing oxidative damage see Fig. 3 (Galleron *et al*., 1999; Lee *et al*., 2004). We have recently reviewed the role of ROS in myocardial ischaemia and reperfusion injury: Oxygen species and the role of neutrophil (Saeed *et al*., 2005).

ROS as Signaling Molecules

It is widely known that ROS, at relatively low concentrations, serve as important second messengers mediating cellular responses to many physiological stimuli (Gonzalez *et al*., 2005). For example, T cells generate hydrogen peroxide and or superoxide anion in response to *in vitro* mitogenic stimuli, such as Con A, super antigens, anti-CD3 mAb, anti-CD28 mAb and TCR coupling (Williams and Henkar, 1996; Los *et al*., 1995; Weber and Abromson-Leeman, 1995; Hildeman *et al*., 1999). Recently Gulow and his coworkers show that it is hydrogen peroxide that function as essential second messenger in T-cell receptor signaling and they demonstrated that H_2O_2 induced by HIV-1 trans activator of transcription combines with CD4-dependant calcium flux causing interfere with TCR (T-cell receptor) signaling and causes massive T-cell apoptosis (Gulow *et al*., 2005). Other study demonstrates that it's H_2O_2 which can evoke marked changes in mitochondrial activity leading to cellular damages, cell dysfunction and generation of pathologies in the pancreas.

The role of ˙HOCL in inflammatory disease may be attributed to its function as a second messenger in signal transduction (Schoonbroodt *et al*., 1997) for instance in ˙HOCL induced tumor necrosis factor alpha (TNF-α) production in peripheral blood mononuclear cells through tyrosine kinase receptor pathway. Thus, the finding that therapeutic concentrations levels of NSAIDs are able to suppress the generation of ˙HOCL implies that the mechanism of the anti-inflammatory action of these drugs may be linked to this reaction (Schieven *et al*., 2002; Derevianko *et al*., 1998).

ROS Scavenging by NSAIDs

NSAIDs have been studied in terms of their effect on ROS production by phagocytic cells (Kast, 2000, 2000; Ju and Uetrecht, 1998). Paino *et al*. (2005) have studied the effects of NSAIDs on ROS generation in luminal-or lucigenin dependent chemiluminescence assays of stimulated rat neutrophils. It was demonstrated that the NSAIDs are both able to inhibit the formation of ˙HOCL and to scavenge it. They concluded that the major effect of NSAIDs was to impair the generation of ˙HOCL by competing with chloride for the active site of MPO without inhibiting the NADPH-oxidase enzymatic complex, except for indomethacin (Paino *et al*., 2005). In other studies, Kato *et al*. (2003) reported that phenol compounds are typical MPO substrates such as piroxicam and tenoxicam are more effective than non-phenolic structured compounds as diclofenac and naproxen. Moreover, in the other studies, they found indomethacin, which is also an MPO substrate, was as efficient as piroxicam and tenoxicam (Ju and Uetrecht, 1998).

In a recent study, the effects of COX-2 inhibitors on the oxidative activity of cells were investigated. It was found that the ROS production was significantly reduced by nimesulide, NS-398 and its metabolite, 40 OH-nimesulide. As far as the presently examined chemical structures are

concerned, it seems obvious that oxicams have more pronounced inhibitory effects against ROS than nimesulide and ibuprofen. More interesting is the scavenging against HOCL radical since there is a substantial difference between the oxicams and nimesulide. The oxidation by HOCL is carried out on the C-3 carbon of oxicam with a faster rate than the oxidation by nimesulide, which is a radical attached on the C-6 carbon giving rise to a chlorinated product (Van Antwerpen and Ne've, 2004; Warner *et al*., 1999).

ROS are also involved in a number of inflammatory processes due to activation of cyclooxygenase (COX) enzyme (Fernandes *et al*., 2004; Jaimes *et al*., 2005; Lu and Wahl, 2005). Some reactive species that may be produced in excess during the inflammatory reactions are, superoxide radical (O_2^-), peroxyl radical (ROO), hypochlorous acid (HOCL), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). This makes them potential targets for treatment and suppression of inflammation. NSAIDs are increasingly being recognized for their role as free radical scavengers in addition to their COX inhibition. In a recent study, the antioxidant potential of NSAIDs was investigated using endogenous antioxidants like catalase, melatonin and reduced glutathione (GSH). The hydrogen peroxide scavenging activity of the NSAIDs was measured using a chemiluminescence (CL) methodology. It was the first time that the scavenging activity of hydrogen peroxide by NSAIDs was assessed (Dusting *et al*., 2005). The scavenging activity of NSAIDs against an array of ROS (O_2^-,OH^-, .HOCL and ROO) and RNS (NO and $ONOO^-$) using noncellular *in vitro* systems has recently been evaluated (Van Antwerpen and Ne've, 2004). The results are indicative that tolmetin, ketorolac and oxaprozin were not active against O_2^-, while acemetacin and indomethacin exhibited concentration dependent effects. No scavenging effect for HOCL was reported for any of the NSAIDs tested. The ROO was effectively scavenged by etodolac, however, other drugs were found less active. On the other hand, NO and ONOO were scavenged by all the tested NSAIDs.

In a more recent study from our laboratory (Saeed *et al*., 2005 unpublished data) nine NSAIDs: A) diclofenac B) ibuprofen C) indomethacin D) etodolac E) naproxen F) piroxicam G) aspirin H) paracetamol I) nimesulide, were investigated for their inhibitory activity on ROS production using luminol-enhanced chemiluminescence assay as described by Helfand *et al*. (1982). The results given in Fig. 2 show that paracetamol (12.5-100 μg mL^{-1}) was a most potent drug in this assay whereas diclofenac and indomethacin produced significant inhibitory effects with nimesulide and or naproxen displayed much weaker activity.

Certain endogenous substances in the body, namely the indoleamines, melatonin, 5-hydroxytryptamine (serotonin), tryptophan and tryptamine, have already been shown to be potent antioxidant and free radical scavengers (Kast, 2000). These substances bear a chemical resemblance with the indole derived NSAIDs, indomethacin, acemetacin and etodolac. The indolic nitrogen appears to be the active redox center of indoles, due to its lone pair of electrons (Stolc, 1999; Westerlund *et al*., 1996). Delocalization of this electron pair over the aromatic systems seems to be critical for antioxidant activity, (Westerlund *et al*., 1996) which may explain the lack of effect found for tolmetin, ketorolac and oxaprozin against O_2^-. The lack of effect acemetacin, indomethacin and tolmetin was previously established against HOCL radical (Ne've *et al*., 2001).

This was extended to other indoleacetic, pyrroleacetic and oxazolacetic NSAID derivatives. Of the studied compounds, indomethacin and tolmetin were shown to be the most potent NO scavengers, followed by acemetacin, tolmetin and ketorolac, with a similar scavenging profile among them, oxaprozin being the least effective. Although the reasons for theses differences cannot be ascertained at present, previous studies with indoleamines, using computational approaches have reported the relative free energy of radical reactions indicating that the carbon atoms at positions 3, 4 and 6 and especially in the positions 2 and 7 of the indole heterocycle were the most reactive against RNS (Tan *et al*., 2002).

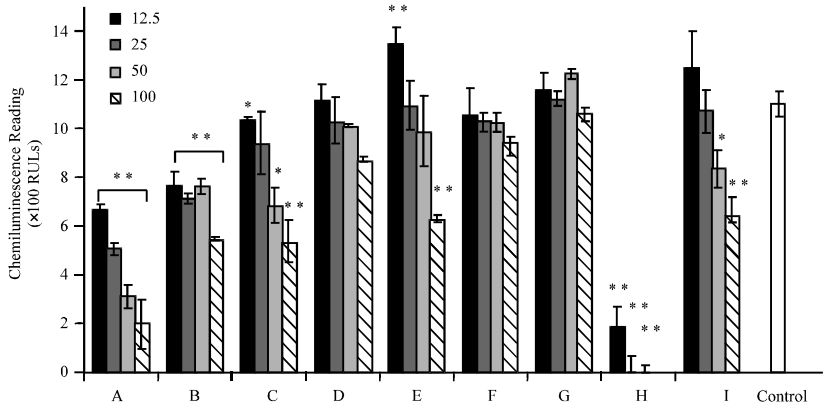

Fig. 2: Comparative effects of selective NSAIDs on ROS production as measured by luminol based chemiluminescence assay. The following NSAIDs: {a) diclofenac a) ibuprofen c) indomethacin d) etodolac e) naproxen f) piroxicam g) aspirin h) paracetamol I) nimesulide} were incubated at (12.5-100 μg mL^{-1}) concentrations for 30 minutes with whole blood (diluted 1:50). After addition of 50 μL (20 mg mL^{-1}) zymosan, followed by 50 μL (7×10^{5} M) luminol chemiluminescence peaks were recorded with the Luminometer (Luminoskan RS Labsystem, Finland) for 50 min. Results are expressed as means±SD of 3 replicates *p>0.05, **p>0.005 as compare to control (untreated samples)

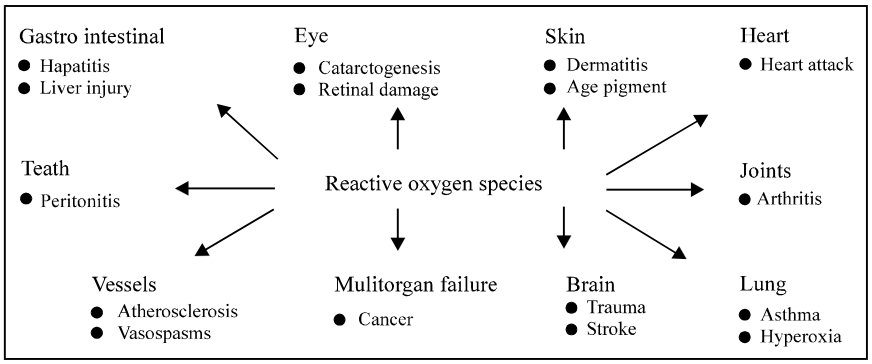

Fig. 3: Clinical conditions involving reactive oxygen species

ROS Scavenging by NSAIDS Demonstrated in Various Pathological Conditions

There are many diseases in which ROS are proposed to be important players in their damaging effects and pathogenesis (Fig. 3).

The expression of COX-2 enzyme in *atherosclerotic* lesions as well as the oxidative role of NADPH oxidase in macrophage cells is well known. It would therefore sound logical to use COX-2 inhibitors, since that would not ouly regulate COX-2 over expression pathologies, but would also modulate oxidative processes induced by cells.

NSAIDs, which readily cross the blood-brain barrier, confer protection against *Alzheimer's disease*. It has been reported that NSAIDs through their action on hydroxyl free radicals have been effective in delaying the onset and slow the progression of Alzheimer's disease. This effect was first reported in the Rotterdam study through clinical trials (Andersen *et al*., 1995). In the Alzheimer's diseased brain, microglial cells activate in respiratory burst pathways (Moore *et al*., 2005) This results in the formation of numerous hydroxyl radicals, contributing to overall radical accumulation caused by oxidative stress. Concurrently, the Ab peptide is generated from the amyloidal precursor protein (APP) and rapidly reacts with the hydroxyl radical. This reaction leads to the cross-linkage and polymerization of Ab, resulting in the formation of neurotic plaques, which are characteristic of AD. The plaques activate microglial cells, increasing hydroxyl radical production and producing a potential feedback mechanism of neuron destruction. The neutralization capability of NSAIDs prevents radical-mediated neurotoxin cell damage, inhibits neurotic plaque accumulation in the brain and accentuates the function of body's natural free-radical defense mechanisms to delay the onset and slow the progression of the disease (Basu *et al*., 2001). This effect poses new roles for the COX inhibiting drugs as effective treatments for Alzheimer's disease and other diseases progressed by hydroxyl radical-induced damage to the body.

The increase of plasma glucose concentration found in diabetes induces oxidative stress as a result of an imbalance between the production of ROS and the antioxidant defense mechanism (Bonnefont-Rousselot *et al*., 2001). The production of ROS has been reported to be increased in patients with diabetes (Sano *et al*., 1998) and more recent reports suggest the glucose-related ROS production has a central role in diabetic pathology (Lander, 1997; Sundaresan *et al*., 1995). In cultured vascular cells, high glucose concentration stimulates ROS production via protein kinase C (PKC) dependent activation of NADPH oxidase (Inoguchi *et al*., 2000). In addition to its various effects, aspirin has also been shown to be effective in the protection of endothelial cells and produced beneficial effects in diabetes mellitus (El Midaoui *et al*., 2002; Podhaisky *et al*., 1997; Hundal *et al*., 2002). Experiments have shown that aspirin exposure to human endothelial cells grown in high glucose concentration to induce a decrease in the control level of intracellular calcium concentration [Ca^{2+}]. The increase in free radical production in human endothelial cells due to hyperglycemia was also restored to normal by aspirin. Results indicate that aspirin inhibited the high glucose concentration-induced PKC activation by reducing translocation of cytosolic PKC to the membrane. Therefore, it is expected that human endothelial cells grown in high glucose concentration, aspirin restores the calcium homeostasis by inhibiting PKC mediated activation NADPH oxidase activity consequently reducing the production of oxygen radicals. Since oxygen radicals react with NO, aspirin may improve production and or bioavailability of NO by scavenging oxygen radicals produced under hyperglycemic conditions (Dragomir *et al*., 2002).

In recent years, numerous studies reported a close relationship between the increased ROS production and the incidence or development of pathological processes in the airways, such as *bronchial hyperactivity*, asthma, adult and newborn respiratory distress syndromes, etc. (Mátyás *et al*., 2002; Metnitz *et al*., 1999; Quinlan *et al*., 1997; Plaza *et al*., 1995). In these conditions, the use of drugs with both scavenging activity and anti-inflammatory properties may be of therapeutic benefit.

Conclusions

Alzheimer's disease, asthma, atherosclerosis, cancer, chronic granulomatous disease, diabetes and MI are few examples of a wide array of diseases where ROS are key players in pathology. Thus, any

means of either preventing production of or removing the fully formed ROS can play a key role in the curative therapy of these clinical conditions.

These findings point to new facts of pharmacological actions of NSAIDs. Owing to their potential benefits, NSAIDs deserve due consideration for their incorporation in the management of various diseases as well as studies aimed at their therapeutic uses.

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