DISCUSSION

Non-steroidal anti-inflammatory drugs (NSAIDs) are in clinical use for more than seven decades (c.f. Weissmann, 1991). In 1971, Vane demonstrated for the first time that aspirin, a NSAID, exerts its physiological action by inhibiting the production of prostaglandins. NSAIDs interfere with the biosynthesis of cyclic prostanoids derived from arachidonic acid (Awtry & Loscalzo, 2000). The major mechanism of action of NSAIDs involves lowering PGE$_2$ by directly inhibiting COX activity (Flower & Vane, 1972). In addition to direct inhibitory action, NSAIDs are also capable of reducing PGE$_2$ production by down regulating the expression of COX enzyme at transcription level induced by LPS and IL-1$\beta$ (Xu et al., 1999). In contrast, Yuan et al. (2000) have shown that in the presence of phospholipase PLA$_{2}$B, aspirin and sulindac upregulate the expression of COX-2, but inhibit the PGE$_2$ production, by suppressing PLA$_2$ mRNA, resulting in lowering of free AA concentration and depriving the enzyme COX-2 of its substrate (Yuan et al., 2000). These contradictory studies suggest that NSAIDs possess more than one effects produced by more than one mechanism.

NSAIDs have been reported to inhibit COX-2 expression by disabling NF$\kappa$B activation (Xu et al., 1999) via stabilization of I$\kappa$B by interfering with its phosphorylation (Pierce et al., 1996; Shackelford et al., 1997). NF$\kappa$B facilitates the transcription of genes encoding cytokines, chemokines, adhesion molecules and inflammatory enzymes (iNOS, COX-2) in certain cell types (Barnes & Karin, 1997; Kirtikara et al., 2000). NSAIDs like ibuprofen, sulindac and aspirin inhibit endotoxin stimulated NF$\kappa$B activation in human monocytes (Housby et al., 1999) as well as in endothelial cells and leukocytes (Kopp & Ghosh, 1994; Pierce et al., 1996; Yin et al., 1998). Ibuprofen, a NSAID, blocks NF$\kappa$B trafficking to nucleus in certain tumor cell lines (Palayoor et al., 1999) but does not do so in activated macrophages (Shackelford et al., 1997), whereas indomethacin failed to suppress NF$\kappa$B activation (Kopp & Ghosh, 1994; Yin et al., 1998; Ryu et al., 2000).
A COX-2 gene is not solely dependent on NFκB activation (Kirtikara et al., 2000), and thus working through IκB/NFκB is not the only pathway about anti-inflammatory effects of NSAIDs. Interestingly, clinical actions of NSAIDs may also be COX-2 independent (Cronstein et al., 1999). A variety of COX-2 independent functions of NSAIDs have been proposed which include diminishing leukocyte-endothelial cell interactions (Kapiotis et al., 1996; Housby et al., 1999) and reducing the cytokine production by inflammatory cells e.g. LPS exposed murine macrophages and human endothelial cells (Pierce et al., 1996; Sheckelford et al., 1997).

In the present work, we have tested the effects of nimesulide on the production of reactive oxygen species by primary rat alveolar macrophages on stimulation with various pro-inflammatory agents. Experiments were carried out to check the superoxide radical and DPPH radical scavenging activity of nimesulide. Superoxide anions were generated by chemical system, i.e. by autooxidation of hydroxylamine hydrochloride, and \( \text{O}_2^- \) anion scavenging activity of nimesulide was estimated through inhibition of NBT reduction by superoxide radicals. Nimesulide was able to scavenge \( \text{O}_2^- \) anions at concentrations of 250 μM or higher. The drug worked in synergism with biological scavenger and antioxidant, superoxide dismutase, in scavenging superoxide anions. Approximately, 850 nmols of nimesulide exhibited \( \text{O}_2^- \) scavenging activity equivalent to IU of SOD (amount of enzyme able to scavenge 50% of \( \text{O}_2^- \) anions being generated), which can be called as IC\(_{50}\) for nimesulide. In another chemical assay, it was found that nimesulide was not able to scavenge DPPH, a stable free radical. This was unlike to trolox, a water-soluble analogue of vitamin E, which scavenges DPPH effectively by donating its protons. The data suggested that nimesulide has some specificity towards superoxide radical but not towards DPPH.

The in vitro antioxidant potential of nimesulide was then studied by using biological assay, in which rat liver homogenate/postmitochondrial supernatant/microsomes were incubated with nimesulide as such or in the presence of some inducing system. Physical presence of nimesulide at various concentrations did not affect, either process of autooxidation or lipid peroxidation induced by non-
enzymatic means, i.e. Fe/tBHP in liver homogenate as well as in PMS. When microsomes were incubated with 100 and 250 μM of nimesulide for autooxidation, there was no effect on the process of lipid peroxidation, but in the presence of enzymatic inducing system i.e. NADPH/Fe, nimesulide inhibited the process of lipid peroxidation significantly. Presence of Fe in the inducing system i.e. Fe/tBHP or Fe/NADPH, leads to the generation of OH' radicals, which are able to enhance the process of lipid peroxidation. It seems that not nimesulide but some of its metabolite(s), produced by microsomes in the presence of NADPH, acts (act) as the scavenger of OH' radicals. Another conclusion drawn on the basis of the results of this set of experiments is that even during autooxidation, the process of lipid peroxidation seems to be initiated by hydroxyl radicals and not by superoxide anions.

The study was then extended towards the in vitro effects of nimesulide on superoxide production by intact cells, i.e. activated rat alveolar macrophages. Alveolar macrophages (AMs) were chosen as a model of the present work because of their importance in inflammation and injury caused to lung tissue. Primary rat AMs were isolated from bronchoalveolar lavage fluid aspirated from lung tissues of male Wistar rats. In different experiments, AMs (0.5x10^6 cells/ml RPMI-1640/well) were stimulated with various pro-inflammatory agents like, lipopolysaccharide (LPS), phorbol myristate acetate (PMA) and tumor necrosis factor-α (TNF-α) for 1.5 hr to study the effect of nimesulide on the production of superoxide anions by AMs in response to these agents. LPS activates macrophages to produce pro-inflammatory cytokines like TNF-α, IL-1β, GM-CSF and IL-6 (Berlato et al., 2002). In addition to cytokines, LPS stimulates host cells to produce prostaglandins, glucocorticoids, ROS and activated coagulation components (Schletter et al., 1995). TNF-α, production of which is elicited by LPS in macrophages, is one of the multifunctional primary cytokines of acute and chronic inflammatory processes (Adler & Fisher, 1994; Harada & Sekido, 1994) and this molecule is important mediator of pathophysiologic reactions in the lung (Hirano, 1992; Vanhee & Gossett, 1995). TNF-α production is tightly regulated at the transcriptional and translational levels. Gene expression is mainly
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regulated by binding of the transcription factor NFkB. The TNF-\(\alpha\) promoter contains 4 NFkB binding sites and its activation is dependent on NFkB binding (Collart et al., 1990). LPS induces TNF-\(\alpha\) transcription via NFkB dependent mechanism (Swantek et al., 1999; Liu et al., 2000). Most of the cells including macrophages exhibit receptors for TNF-\(\alpha\) as well as IL-1\(\beta\) (Aggarwal, 1991; Dower, 1991) which can subsequently activate macrophages in an autocrine way (Shparber & Nathan, 1986). IL-IR and p55 (CD120a molecule) have been reported to be the receptors for IL-1 and TNF-\(\alpha\) respectively (Taga & Kishimoto, 1993; Wallach, 1997). PMA is another well known stimulatory agent of oxidative burst in macrophages by enhancing NADPH oxidase activity through activation of protein kinase C (Cox et al., 1985; Wolfson et al., 1985). NADPH oxidases are a group of enzymes, associated with plasma membrane, found in a variety of cells of mesodermal origin. Superoxide anions generated here by macrophages in response to inflammatory agents, serve as a starting material for the production of vast assortment of reactive oxidants including oxidized halides, free radicals, singlet oxygen which are used by macrophages to kill microorganisms. But these oxidants also cause a lot of what the military would call "collateral damage" to nearby tissues. So their production has to be tightly regulated to make sure their quantity, time and site where required.

In the present set of experiments, when AMs were stimulated with various concentrations of LPS, LPS could not stimulate the cells to generate \(O_2^-\) anions during 1.5 hr. Longer incubations (4hr, 6hr, 8hr, 18hr) of cells with LPS did not yield anything. Even the control levels went off to zero due to inactivation of NADPH oxidase within 2-3 hr of isolation. PMA (0.1 \(\mu\)g/ml) was sufficient to stimulate AMs to generate more \(O_2^-\) anions, but 1 \(\mu\)g/ml concentration was selected as it gave maximum stimulation in our set up of experiments i.e. 0.5\(\times\)10\(^8\) cells/ml/well. Nimesulide only at 250 \(\mu\)M concentration was able to abrogate the effect of PMA to significant extent suggesting that nimesulide is effective in scavenging \(O_2^-\) anions at higher concentrations. TNF-\(\alpha\) in the present experiment could not stimulate \(O_2^-\) anion production by AMs, at concentration varying from 1 ng/ml to 100 ng/ml. To rule out any experimental
discrepancies, AMs from the same animals were together stimulated with LPS/PMA/TNF-α under same set up of experimental conditions for 1.5 hr, which gave clear indication that AMs respond only to PMA but not to LPS and TNF-α as far as O2⁻ anion production is concerned.

LPS has been known to activate transcription and subsequent release of TNF-α, which is mediated by NFκB (Ghosh et al., 1998; Liu et al., 2000). This LPS-induced NFκB activation in macrophages is mediated via ROS generation as ROS scavengers (catalase and sodium formate) inhibited the process (Dokka et al., 2001). Chandel et al., 2000 have shown that LPS did not enhance ROS production in J774 macrophages during the acute period of 2 hr required to elicit NFκB activation, but it led to higher ROS production during longer periods (12-18 hr). They suggested that LPS did not directly increase ROS production but did so via de novo synthesis of TNF-α. In their study, source of ROS has been shown not the NADPH oxidase, as inhibitor of mitochondrial NADPH oxidase, apocynin, did not abolish ROS production during longer periods. But, in our study, even longer periods of incubation with LPS, which might have led to release of endogenous TNF-α, did not enhance ROS production. Presence of exogenously added TNF-α did not affect the process of ROS generation by AMs, which might lead to the idea that neither endogenous TNF-α nor exogenously added TNF-α, act on primary alveolar macrophages. Comparison between the present study and the study carried out by Chandel et al. (2000) emphasizes on the fact that cell lines respond to stimuli in a way different than primary cells. Hence results obtained using cell lines should cautiously be extrapolated for the intact system.

In another set of experiments, effect of nimesulide on the release of nitric oxide (NO) radical by AMs in response to LPS/PMA/TNF-α was investigated. AMs were incubated with stimulants with or without nimesulide for 20 hr at 37°C, 5% CO₂ humid atmosphere in RPMI-1640. Although NO release was detectable at 6 hr in the presence as well as absence of 2 μg LPS, yet the level of stimulation was higher at 20 hr. Henceforth, 20 hr was considered to be the optimum duration for NO release. 1 μg PMA and 2 μg LPS were able to
stimulate AMs to produce higher levels of NO but 10 ng TNF-α did not do so. Nimesulide even in the absence of stimulants inhibited the release of NO by AMs, somewhat in a dose dependent manner, in all the three experiments. Surprisingly, nimesulide inhibited the LPS-stimulated NO release by AMs, but it did not inhibit PMA-stimulated NO release. When iNOS expression was studied by Western blotting, it was found that LPS could lead to induction in iNOS level, but PMA could not induce iNOS expression in rat primary AMs. Nimesulide was able to suppress this induction in iNOS in response to LPS, hence inhibited the NO release. PMA did not induce iNOS expression in AMs, but led to higher NO production. LPS has been shown to induce iNOS activity which is mediated by protein kinase C activation. Protein kinase C (PKC) mediates LPS induced iNOS activity and associated damage in rat colon epithelial cells (Tepperman et al., 2000), macrophages (Paul et al., 1997; Shapira et al., 1997), aortic smooth muscle cells (Paul et al., 1997) and microglial cells (Fiebich et al., 1998). Protein kinase C, a ubiquitous, phospholipid dependent enzyme (Nishizuka, 1992), is associated with cell proliferation, differentiation and apoptosis. The enzyme family now consists of 11 isoforms which differ in structure, lipid activation, phorbol ester sensitivity, cellular distribution and tissue expression (Hug & Sarre, 1993). PKC isoforms have been divided on the basis of the dependence of their enzyme activity on Ca²⁺ and their sensitivity to phorbol esters. The conventional PKCs (α,β1,β2, γ) are Ca²⁺ dependent and respond to phorbol esters. The novel PKCs (δ,ε,η,θ,μ) are Ca²⁺ independent but respond to phorbol esters, whereas atypical forms (ζ,λ) are independent of Ca²⁺ as well as phorbol esters. Studies directed at examining the isoforms that are activated in response to PMA have suggested that depending upon the tissue and cell type under investigation, the profile of PKC activation can vary. However, many of these studies have suggested that PMA mediated activation of PKC-ε may play an important role in iNOS induction and subsequent cellular injury (Fujihara et al., 1994; Keenan et al., 1997; Shapira et al., 1997). Tepperman et al., 2000, have shown that PMA could induce iNOS via PKC activation in rat colonic epithelial cells, whereas Paul
et al., 1997 have shown that PMA could not induce iNOS expression in RAW 264.7 macrophages and rat aortic smooth muscle cells.

In our study, PMA was unable to induce expression of iNOS in rat AMs, whereas LPS could do it. The results suggest to carry out further investigation on AMs to study the profile of PKC isozymes and their activation in response to PMA and LPS. And if PKC activation mediates LPS stimulated induction in iNOS, and nimesulide was able to attenuate it, then the results also implicate that the drug may act as a potent inhibitor of PKC induced by LPS, and hence inhibits the induction in iNOS expression. Further experimentation is required to explore the source of NO\(^{-}\) in case of PMA stimulated cells. Nimesulide could not affect the NO\(^{-}\) production by the cells in response to PMA, suggesting thereby, that nimesulide acts as an inhibitor of NO\(^{-}\) only by suppressing the expression of iNOS. As there was no induction in iNOS in case of PMA, nimesulide did not affect the release of NO\(^{-}\) radicals. TNF-\(\alpha\) again when added exogenously, did not stimulate AMs to generate higher levels of NO\(^{-}\).

Nimesulide in these \textit{in vitro} experiments was able to inhibit the elicited release of O\(_2^{-}\) anions and NO\(^{-}\) radical by AMs in response to inflammatory agents. In case of superoxide anions, nimesulide was effective at comparatively higher concentrations (>250 \(\mu\)M) whereas in case of NO\(^{-}\), it was effective at concentrations even as low as 10 \(\mu\)M in control AMs and 50 \(\mu\)M in case of LPS-stimulated AMs. On the basis of all these experiments, nimesulide seems to be a scavenger of O\(_2^{-}\) anions, rather than inhibitor of O\(_2^{-}\) release as it could scavenge O\(_2^{-}\) anions generated by the chemical system. Whereas, it seems to be an inhibitor of NO\(^{-}\) release as it down-regulated the LPS-induced iNOS expression.

The study was further extended to look into the antioxidant functions of nimesulide \textit{in vivo}. For this purpose, male Wistar rats were orally administered with 9mg/kg nimesulide, twice a day for 7 days. At the end of nimesulide treatment, acute inflammatory conditions in the lung tissue were simulated by intratracheal administration of 2 \(\mu\)g LPS directly into the lungs of animals, which were or were not pretreated with nimesulide. In our study, the intratracheal instillation of animals with 2 \(\mu\)g of LPS/animal led to higher production of
superoxide anions by AMs, 18 hr after instillation. Further, in vitro stimulation with 1μg PMA activated the cells to generate more O$_2^-$ anions, but the level of enhancement was maximum in AMs isolated from LPS challenged animals, showing thereby that after 18 hr of LPS instillation, AMs were still more sensitive to secondary challenge with PMA. This effect was significantly inhibited by nimesulide pretreatment. The mechanisms by which nimesulide administration reduces the formation of O$_2^-$ ions by LPS and PMA-stimulated macrophages in vivo, are not clear at the moment. It either scavenges the O$_2^-$ ions directly or inhibits the process of oxidative burst by inhibiting the activity of NADPH oxidase as PMA is known to stimulate the process of oxidative burst by activating NADPH oxidase enzyme through PKC activation (Cox et al., 1985; Wolfson et al., 1985). Bevilacqua et al. 1994, have earlier shown that stimulation of O$_2^-$ generation by PMA in neutrophils was inhibited by nimesulide by inhibiting polymorphonuclear phosphodiesterase type IV, resulting in an increase in cAMP and subsequent increase in protein kinase A, which by phosphorylation does not let NADPH oxidase to get assembled.

Phagocytes manufacture a stupendous battery of reactive oxidants that they use for the destruction of invading microorganisms by four enzymes: NADPH oxidase, SOD, NOS and myeloperoxidase which lead to the formation of four major products: O$_2^-$, H$_2$O$_2$, NO and HOCl (Babior, 2000). The superoxide free radical has occupied an amazingly central role in a wide variety of diseases. More than a dozen of important mammalian enzymes including catalase (Kono & Fridovich, 1982), glyceraldehyde-3-phosphate dehydrogenase (Armstrong & Buchanan, 1978), ornithine decarboxylase (Guarnieri et al., 1982), glutathione peroxidase (Blum & Fridovich, 1985), myofibrillar ATPase (Ventura et al., 1985), adenylate cyclase (Palmer, 1987), creatine phosphokinase (McCord & Russell, 1988) and glutamine synthase (Schor, 1988) have been shown to be inactivated by superoxide anions. Oxygen derived free radicals are believed to contribute to the cellular and tissue injury associated with endotoxin induced inflammation. It has been suggested that oxidative damage may be a major cause of organ failure and lethality associated with sepsis (Wiesel et al., 2000). ROS, proteolytic enzymes and other clinical mediators (TNF-α, IL-1) secreted by activated
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macrophages, are involved in the generation of immune response and inflammation, through cellular signaling for gene expression (Han et al., 2001). In the recent years, ROS have stimulated considerable interest as a major mechanism of LPS-induced acute lung injury. Oxidants produced by phagocytes have been found to be associated with a number of diseased conditions like emphysema, acute respiratory distress syndrome, atherosclerosis, reperfusion injury, rheumatoid arthritis and malignancy (Babior, 2000). The release of ROS and cytokines for inflammatory response is a vital component of host defense. Nonetheless, it can be highly harmful and potentially fatal if it is not tightly regulated. This lethal outcome of inflammation may be controlled to a certain degree by some antioxidants, which is important especially for phagocytes that use ROS to kill microorganisms and need antioxidants in order to support their functions (Victor et al., 2000; Victor & Fuente, 2002). Nimesulide exhibited its antioxidant efficiency by inhibiting the LPS and PMA stimulated enhanced production of superoxide anions by AMs. Similarly, inflammatory response to benzoylperoxide by AMs as studied by redox based luminol dependent chemiluminescence, was maximum in LPS treated animals. The response was limited to control levels by nimesulide pretreatment. Nimesulide feeding prior to LPS challenge could significantly abrogate the enhanced NO release by AMs and induced iNOS expression. Results altogether implicate antioxidant functions of nimesulide under inflammatory conditions of lungs, in vivo.

The fundamental importance of metabolic pathway of NO' production by iNOS in murine macrophages as a key defense element in various infections as well as its role in diverse settings of immunopathology are firmly established (Adler et al., 1997; Kroncke et al., 1995). The inducible isoform of NOS, i.e. iNOS, can be activated by bacterial lipopolysaccharides, cytokines, phorbol esters etc. (Kristof et al., 1998; Nakayama et al., 1992; Robbins et al., 1994). Once expressed, the iNOS enzyme generates significantly larger and sustained amounts of NO' than do the constitutive forms (Clancy & Abramson 1995). The excessive NO' formation may cause inappropriate vasodilation, which is a key feature in septic organ failure (Stewart et al., 1995; Ullrich et al., 1999). In LPS mediated inflammation, iNOS and NO' production regulate molecular functions
that determine the host inflammatory response. NO inhibits mitochondrial respiration by nitrosation of Fe-S center of aconitase, NADPH-ubiquinone oxidoreductase and cytochrome reductase (Brown, 2001; Stadler et al., 1991). iNOS has been reported to be constitutively expressed in respiratory tract epithelial cells (Gho et al., 1995). In our study too, very low levels of iNOS expression were detectable in AMs of control animals. In the present study, activation of AMs by LPS \textit{in vivo} as well \textit{in vitro} resulted in enhanced expression of iNOS, which has been found to be increased both at RNA and protein levels in many inflammatory diseases like bronchiectasis, ARDS and asthma (Vliet Vander & Cross, 2000). There are increasing number of reports showing that enhanced expression of iNOS is closely associated with the development of cancer (Ahn et al., 1999). Expression of iNOS, like many other pro-inflammatory genes is regulated by transcription factor and nuclear factor \textit{kB} (Fan et al., 2001). Almost all the stimuli (LPS, TNF-\textit{\alpha}, phorbol esters), which enhance the expression of iNOS (Kristof et al., 1998; Nakayama et al., 1992; Robbins et al., 1994) also increase NF\textit{kB} activation (Zhu et al., 1996; Barnes & Karin, 1997; Baeuerel & Baichwal, 1997; Blackwell & Christman, 1997). Nimesulide has been earlier reported to inhibit induced release of NO and iNOS expression in brain, colitis and hepatocellular carcinoma cell lines (Karmeli et al., 2000; Scali et al., 2000; Fantappie et al., 2002). For the first time we have found that nimesulide inhibits the iNOS expression in macrophages in response to LPS, \textit{in vivo} as well as \textit{in vitro}. Although the mechanism by which nimesulide suppresses the induction in iNOS expression can't be ascertained at the moment but it can be hypothesized that it may inhibit iNOS expression via inhibition in NF\textit{kB} activation, but the hypothesis still remains to be tested.

Nimesulide feeding prior to LPS challenge could attenuate the induction of MDA levels significantly. Fe/BHP involves the hydroxyl radicals (OH) to initiate the process of lipid peroxidation. As nimesulide feeding protected the cells from lipid peroxidation, the results suggest that \textit{in vivo}, nimesulide also acted as a scavenger of OH' radicals in AMs. Process of lipid peroxidation was analysed in AMs as a marker of oxidative stress. Levels of MDA formed by autooxidation were equal in all groups but \textit{in vitro} induction by Fe/BHP system led to maximum
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Enhancement in AMs of LPS-challenged rats. Superoxide radicals and other ROS derived from it are directly responsible for the oxidative stress associated with LPS-induced inflammatory response. Body responds to such oxidative stress by inducing enzymatic and non-enzymatic defense mechanisms. Levels of SOD in AMs were significantly enhanced in LPS-treated animals. The MnSOD is inducible under stress such as circumstances that elicit TNF-α production (Nelson et al., 1995) or under hyperoxic conditions (Ho et al., 1996), whereas CuZnSOD remains unaltered. Pretreatment with nimesulide inhibited the increase in SOD activity, as if oxidative stress caused by LPS after nimesulide treatment was under control and body defense mechanisms were not to be activated. The mechanisms by which nimesulide inhibits this induction in SOD in AMs in response to LPS may be via inhibition in NFκB activation caused by LPS in AMs. But the hypothesis has to be tested. In the absence of enhanced SOD in LPS-treated animals, we would have expected even higher levels of O₂⁻ anions production. Higher levels of SOD might have led to only marginal increase in O₂⁻ anions in LPS-treated animals as compared to control.

Another enzyme of antioxidant defense family, which was augmented by LPS treatment, was glutathione reductase; activity of which was decreased in response to LPS. However, levels of reduced glutathione (GSH) were significantly enhanced in bronchoalveolar lavage fluid of LPS-treated animals, which might have repressed GR activity by feed-back inhibition. GSH is one of the utmost important non-enzymatic low molecular weight antioxidants in the body. GSH in the lavage fluid is most likely involved in the protection of local functional constituents against oxidative damage by directly scavenging certain inflammatory oxidants (HOCl, ONOO⁻) or perhaps by working in concert with glutathione peroxidase (Vliet & Cross, 2000). A noteworthy point is that it is primarily the reduced forms of GSH and ascorbate that are found in epithelial lavage fluids, suggesting that respiratory tract epithelial cells possess the ability to maintain these antioxidants in reduced state by uptake of their oxidized forms, their intracellular reduction and secretion of reduced forms back. Presence of GSH in alveolar lavage should serve to maintain the primary gas exchange function of the lung (protection against oxidative damage) and to minimize the
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I oxidant injury by locally active immune cells such as AMs. Sources of GSH in lavage fluid are epithelial cells as well as macrophages (Vliet & Cross, 2000). In the present study, stress induced levels of GSH in bronchoalveolar lavage fluid (BALF) were restrained to a lower extent by nimesulide pretreatment, indicating the lower extent of stress induced by LPS in animals pretreated with nimesulide. As the levels of GSH decreased in LPS + nimesulide group, activities of GR were restored to normal.

Another part of this work consisted of studies on the effects of intratracheal instillation of LPS and oral administration of nimesulide on antioxidant defense system and oxidative stress (lipid peroxidation) in lungs and distant organs like liver and kidneys. In the present study, we tried to explore the inter-tissue interaction under acute lung inflammation and also to investigate the modulatory effects of nimesulide. Bacterial endotoxin (LPS) results in multiorgan NF\(_k\)B activation in the pathogenesis of systemic inflammation involving liver, lungs and spleen in a dose dependent manner (Blackwell et al., 2000). Lancaster et al. (2001) have suggested a liver-lung interaction in the pro-inflammatory response to systemic LPS treatment as NF\(_k\)B inhibition in liver could protect lungs from neutrophilic alveolitis.

To counteract the devastating effects of ROS produced under normal as well as inflammatory conditions, a net work of antioxidant enzymes have evolved in the body as primary defense. Certain factors present in diet or some synthetic drugs, may act as antioxidants either by directly scavenging free radicals and/or indirectly by enhancing the endogenous defense system in the body (Huang et al., 1994). The studies regarding the indirect antioxidant functions of nimesulide are totally missing. In the present work, efforts were made to explore whether nimesulide feeding augments the enzymatic antioxidant defense system in rats? Liver, being the major drug metabolising organ, drug metabolising system comprising of Cyt P450, glutathione-S-transferase and reduced glutathione in liver were also studied.

In our study, intratracheally instilled LPS could significantly affect the antioxidant enzymes and lipid peroxidation in remote organs, showing multiorgan involvement in acute local inflammation in lungs. It is generally accepted that
LPS stimulates inflammatory cells like neutrophils and macrophages that release a collection of inflammation mediators including cytokines, ROS, proteolytic enzymes and metabolic products of lipid metabolism (Goode & Webster, 1993; Boobies et al., 1989). These pro-inflammatory cytokines, have been referred to as "alarm cytokines" because in addition to their pro-inflammatory effects, they also induce the synthesis of second wave of cytokines by other cell types (Jordana et al., 1992). These cytokines also diffuse away and influence structure and functions of neighbouring cells and tissues. Intratracheal instillation with LPS, in our study, led to enhanced basal as well induced lipid peroxidation in liver, which was taken care by nimesulide pretreatment.

Process of lipid peroxidation was not affected by any of the treatments in kidneys, whereas, both LPS and nimesulide treatments enhanced the levels of MDA formed due to autooxidation in lungs as compared to control. When data of antioxidant defense system in various organs was examined carefully, it was found that in all the organs, nimesulide administration led to suppression in the activities of superoxide dismutase enzyme. Enzyme activities of catalase, glutathione peroxidase and glutathione reductase remained unaltered, except for that nimesulide treatment enhanced the activity of glutathione reductase enzyme significantly in kidneys, but not in lungs and liver. Increased autooxidation of lipids in lungs of nimesulide pretreated animals might be due to suppression in SOD activity, which might have led to the accumulation of superoxide anions which interact with NO to produce peroxynitrite, actively involved in oxidative stress.

In kidneys, where there was no difference in MDA formation in any of the groups, effects of suppressed SOD activity might be counteracted by enhanced glutathione reductase enzyme, which maintains cell glutathione (GSH) homeostasis and seems to be responsible for keeping the levels of MDA under control. Glutathione reductase, a flavoprotein (FAD-containing) enzyme, regenerates GSH from GSSG, with NADPH as the source of reducing power (Chance et al., 1979). GSH along with some other low molecular weight free radical scavengers has the property of acting as potent nucleophiles, competing with DNA in the chemical reaction with electrophilic mutagens and carcinogens.
(Wattenberg et al., 1987; Vanden-Goordbergh et al., 1987). GSH also serves as a reductant in the metabolism of various peroxides and free radicals. These reactions occur spontaneously but are usually catalysed by glutathione peroxidase and glutathione-S-transferases. Enhanced levels of MDA (basal as well as induced) in liver of LPS-treated animals might have been taken care of by enhanced levels of hepatic GST enzyme by direct scavenging of ROS and lipid hydroperoxides.

Superoxide dismutase enzyme is essential for life as its partial or total inhibition leads to increased susceptibility to oxidative stress and severe dysfunctions of mitochondria resulting from elevation of ROS. Several studies have reported a decline in MnSOD in cancer, aging, asthma, progeria and transplant rejection (MacMillan-Crow & Cruthirds 2001). It has been reported that inactivation of MnSOD leads to accumulation of superoxide anions and concomitant increase in peroxynitrite, which can lead to tyrosine nitration/oxidation of many key proteins, proving fatal to cell ultimately (MacMillan-Crow & Cruthirds, 2001). Mutation in CuZuSOD gene has been related to neurodegenerative disorder (e.g. amyotrophic lateral sclerosis) associated with enhanced oxidative stress and oxidative damage (Shibata et al., 2000). Over expression of extracellular SOD (ECSOD) has been found to exhibit protective effects against pulmonary fibrosis and lung injury (Bowler et al., 2001, 2002). Role of SOD in oxygen derived pulmonary injury has been evaluated in SOD knockout mice (Tsan, 2001), which has demonstrated that O$_2^\cdot$ produced in mitochondria or produced extracellularly by infiltrating neutrophils, and derivatives of O$_2^\cdot$ are important mediators of pathogenesis of pulmonary O$_2$ toxicity in SOD knock out mice, the effects of which were abrogated by restoration of SOD gene. All these studies together implicate the importance of superoxide dismutase enzyme in cellular homeostasis.

The inhibition of SOD in three major organs by nimesulide oral feeding can not be ignored. Several investigators have found that inflammatory cytokines such as IFN-γ and IL-1 can induce SOD in rat and human cell culture lines (Brady et al., 1997; Marklund, 1992). One pathway common to both of these pathways is through NFκB (Brady et al., 1997). NFκB controls the transcription
Discussion and regulation of extracellular SOD in alveolar type II cells (Brady et al., 1997). NFκB is also a putative regulatory element in human EC-SOD gene (Folz & Crapo, 1994). In the present work, it is possible that nimesulide also regulates the SOD expression via NFκB. As discussed earlier too, nimesulide inhibited the induced expression of iNOS (which is also under the control of NFκB), and the mechanism may be through inhibition of NFκB activation by a stimulant. In case of SOD, when there is no stimulant, even then its activity is inhibited. May be nimesulide inhibits NFκB activation even under physiological conditions. The process of inhibiting SOD activity by nimesulide may also be through feed back mechanism. As in the presence of antioxidant and anti-inflammatory drug requirement of SOD in the body may be lesser, hence the inhibition.

Besides looking into the antioxidant properties of nimesulide, some work on its effect on drug metabolizing enzymes was also done. Human body has adapted to react against xenobiotics, which are foreign to the body, as a protective factor to lower the active dose of deleterious agents. Two sets of enzymes carry out the metabolism of xenobiotics, termed as phase I and phase II metabolising enzymes (Williams, 1971), which include mono oxygenases, dehydrogenases, reductases, peroxidases, oxidases, hydrolases and conjugation catalytic transferases (Jakoby & Ziegler, 1990). These enzymes are very diverse in their chemical, physical and bioactive properties. Phase I enzymes, cytochrome P450s, metabolically activate xenobiotics to generate products, which are generally reactive electrophiles (i.e. epoxides and reactive oxygen species) where as phase II enzymes try to inhibit the formation of electrophiles by competing with phase I, and also catalyse the conjugation of these electrophiles with a variety of molecules to make them more water soluble for their rapid excretion. Nimesulide and LPS are xenobiotics to the body. Drug metabolizing system (Phase I, Phase II enzymes) is likely to get activated after their consumption. As liver is the major organ involved in metabolism of xenobiotics, we studied the drug metabolising system in liver comprising of cytochrome P450 as phase I and glutathione-S-transferase as phase II enzymes. Levels of GSH were also measured as. GSTs conjugate the
Discussion

electrophiles with glutathione. In our study, phase I enzymes (cytochrome P450) remained unaltered with any of the treatments (nimesulide and LPS), whereas, activities of GST were highly enhanced by nimesulide treatment. Even LPS could stimulate GST activities; whereas, GSH levels were similar. Induction of detoxification enzymes by either naturally occurring substances or synthetic agents (drugs) continues to be a promising chemopreventive strategy. Elevation of detoxification enzyme activity provides additional defense against carcinogens by inhibiting them from reaching their cellular targets. GST levels have been found to be lowered in a number of cancerous conditions (Szarka et al., 1995; Zhou et al. 1997; Lafuente et al., 1993). Detoxification enzyme inducers have been divided into two classes based upon their effects on phase I and phase II enzyme activity. Bifunctional inducers increase the activity of both classes of enzymes, while monofunctional inducers increase the activity of phase II enzymes without affecting the phase I enzyme activities. Monofunctional and bifunctional induction of enzymes occurs through distinct mechanisms (Procheska & Talalay, 1988). Nimesulide, as found in the present study, can be counted under monofunctional class of inducers. A monofunctional inducer can be considered as a chemopreventive agent if it is non-toxic and has compatible route of administration. Although many classic inducers of phase II enzymes have been identified, few are appropriate for long-term use as clinical chemopreventive agents. Corresponding induction of phase I enzymes by bifunctional agents can result in the metabolic activation of carcinogens. There are convincing data showing that phase I enzymes metabolise inactive carcinogens to active metabolites whereas there are hardly any instances of this event in phase II enzyme reactions, hence phase II monofunctional inducers possess the chemopreventive potential. Nimesulide has been reported to possess anticarcinogenic potential in carcinogenesis of colon, intestinal polyps, urinary bladder and mammary glands. (Nakatsugi et al., 1997; Okajima et al., 1998; Nakatsugi et al., 2000; Tardieu et al., 2000), but none of the study till date has reported that nimesulide may exhibit anticarcinogenic potential through induction of phase II detoxification system. An ideal agent should induce an entire battery of phase II detoxification enzymes and thus afford protection
against an array of structurally diverse compounds. Studying the effects of nimesulide feeding on the drug metabolising system in the rat was just a small part of the present thesis. Studies must be carried out further to investigate the effect of nimesulide on other phase II enzymes as well and also to look for the isozyme of GST family which is being induced by nimesulide.