

EXPLORING EFFECTS OF DIFFERENT NONSTEROIDAL ANTIINFLAMMATORY DRUGS ON LIPID PEROXIDATION. PART II. 4-HNE PROFILE

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Abstract: Non-steroidal anti-inflammatory drugs (NSAIDs) are common in alleviating pain, pyrexia and inflammation, in patients with rheumatoid arthritis and osteoarthritis. As these drugs are associated with high incidence of gastrointestinal ulceration, bleeding and kidney damage which may be linked with lipid peroxidation, our study was aimed to examine lipid peroxidation induction capacity of NSAIDs (diclofenac sodium, ibuprofen, flurbiprofen, paracetamol, nimesulide, celecoxib and indomethacin) by determining 4-hydroxy-2-nonenal (4-HNE) concentration as an index of lipid peroxidation and to see the suppressive potential of ascorbic acid on NSAID induced lipid peroxidation. The results suggest that diclofenac sodium, ibuprofen, flurbiprofen, paracetamol, nimesulide and celecoxib exerted mild antioxidant activity. Indomethacin exerted statistically significant increase in 4-HNE content, indicating statistically significant peroxidation activity. Ascorbic acid could significantly reduce indomethacin-induced lipid peroxidation.

Keywords: 4-HNE, lipid peroxidation, NSAID, diclofenac sodium, ibuprofen, flurbiprofen, paracetamol, nimesulide, celecoxib, indomethacin

Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids (1) resulting in the generation of stereospecific endoperoxides and hydroperoxides by enzymatic and non-enzymatic involvement of “reactive oxygen species” (ROS). These species resulting from the exposure of larger variety of drugs, chemicals and environmental agents or by normal metabolic process can easily initiate peroxidation of membrane lipids, leading to the accumulation of lipid peroxides and free radicals, which are believed to be a primary factor in various untoward effects of drugs and in various diseases (2-4). Different adverse reactions due to some drugs have been reported to be mediated through drug induced lipid peroxidation mechanism (1).

The non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in alleviating pain, pyrexia, inflammation, and in patients with rheumatoid arthritis and osteoarthritis. The drugs are associated with high incidence of gastrointestinal ulceration (5), bleeding (6), and kidney damage (7, 8).

(E)-(±)-4-hydroxy-2-nonenal (more commonly referred to as 4-hydroxynonenal, 4-HNE, or HNE) is found throughout animal tissues, and in higher quantities during oxidative stress due to the increase in the lipid peroxidation chain reaction

(9). 4-HNE shows a variety of cytotoxic effects such as inhibition of DNA, RNA and protein synthesis, cell cycle arrest, mitochondrial dysfunction, induction of cataracts of the lens and neuronal apoptosis (10-12). Intracellularly, 4-HNE reacts rapidly with thiol groups of GSH and cysteine and with lysine and histidine residues of proteins (12, 13). 4-HNE-modified proteins have been detected in pathological disorders such as chronic liver diseases (14-16), Alzheimer’s disease (17) and atherosclerotic lesions (18). Furthermore, plasma levels of 4-HNE increase in patients with rheumatoid arthritis (19).

Lipid peroxidation is known as a common mechanism of toxic manifestations of many drugs and attempt has been made to explore lipid peroxidation induction capacity of NSAIDs (20). In the ongoing effort of the authors in this direction, presently an attempt has been made to study the potential of ascorbic acid as possible suppressor of NSAID-induced lipid peroxidation (if any), taking 4-HNE as a model marker to provide a scope of further investigation on possible consideration of this as prospective chemoprotective supplement for co-therapy with drugs. The present study explores the effect of NSAIDs (diclofenac sodium, ibuprofen, flurbiprofen, paracetamol, nimesulide, celecoxib

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and indomethacin) on 4-HNE content of the goat liver tissue.

MATERIALS AND METHODS

The study had been performed on goat (*Capra capra*) liver using 4-HNE content as laboratory marker of lipid peroxidation. Goat liver was selected because of its easy availability and close similarity to the human liver, in its lipid profile (21). The work was carried out according to the guideline of the Institutional Animal Ethical Committee.

Preparation of tissue homogenate

Liver was perfused with normal saline through hepatic portal vein. Liver was harvested and its lobes were briefly dried between filter papers (to remove excess of blood) and were thin-cut with a sharp blade. These small pieces were then transferred to the glass-teflon homogenizing tube to prepare homogenate (1 g/mL) in phosphate buffer saline (PBS) (pH = 7.4) under cold conditions. It was centrifuged at 2000 rpm for 10 min and then the supernatant was collected and finally suspended in PBS to contain approximately 0.8-1.5 mg of protein in 0.1 mL of suspension to perform *in vitro* experiments.

Incubation of tissue homogenate with drug and/or ascorbic acid

For each drug, the tissue homogenate was divided into four different parts of 50 mL each in a glass stoppered 250 mL conical flasks. The first portion was kept as the control (C) while the second portion was treated with drug (D). The third portion was treated with both drug and ascorbic acid (A). After treatment with drug and/or antioxidant, liver homogenates were stirred for 1 h at 15°C on a mechanical shaker and then incubated at 15°C for 4 h along with the control sample.

Effective concentration of drugs and ascorbic acid

Considering therapeutic dose of drugs per average weight of human liver (i.e., 1500 g), diclofenac sodium (0.03 mg/g of liver homogenate), ibuprofen (0.267 mg/g of liver homogenate), flurbiprofen (0.03 mg/g of liver homogenate), paracetamol (0.33 mg/g of liver homogenate), nimesulide (0.067 mg/g of liver homogenate), celecoxib (0.067 mg/g of liver homogenate), indomethacin (0.016 mg/g of liver homogenate) and ascorbic acid (0.17 mg/g of liver homogenate) were taken to carry out the experiments.

Estimation of 4-HNE level from tissue homogenate

The estimation was done at 2 and 4 h of incubation, and it was repeated in replicates consisted of 5 animals. In each case, three samples of 2 mL of incubation mixture were treated with 1.5 mL of 10% TCA solution, and centrifuged at 3000 rpm for 30 min. Then 2 mL of the filtrate was treated with 1 mL of 2,4-dinitrophenylhydrazine (DNPH 100 mg/100 mL of 0.5 M HCl), and kept for 1 h at room temperature. Then, the samples were extracted with hexane, and the extract was evaporated at 40°C. After cooling to room temperature, 2 mL of methanol was added to each sample, and the absorbance was measured at 350 nm against methanol as blank (22). The 4-HNE content values were determined from the standard curve. The standard calibration curve was drawn based on the following procedure. A series of dilutions of 4-HNE in solvent (phosphate buffer, pH = 7.4) were prepared. Pure 4-HNE was used as primary standard. 4-HNE (1 mg; 6.4 µM) was dissolved in methanol (6.4 mL) to give a 1 mM solution. From this primary standard 4-HNE solution, 300 µL was diluted with 4.70 mL of methanol. Similarly, 400 µL was diluted with 4.60 mL of methanol, 500 µL of was diluted with 4.50 mL of methanol, and 600 µL was diluted with 4.40 mL of methanol. From each diluted solution of 4-HNE, 2 mL was pipetted out, and transferred into stoppered glass tube. 1 mL of DNPH solution was added to all the samples and kept at room temperature for 1 h. Each sample was extracted three times with 2 mL of hexane. All extracts were collected in a glass-stoppered test tubes. After that, the extract was evaporated to dryness under argon at 40°C and the residue was reconstituted in 1 mL of methanol. The absorbance was measured at 350 nm in a 1 cm quartz cuvette using methanol as a blank. The best fit equation is: nanomoles of 4-HNE = $(A_{350} - 0.005603185) / 0.003262215$, where A_{350} = absorbance at 350 nm, $r = 0.999$, $SEE = 0.007$

Statistical analysis

Analysis of variance (ANOVA) and multiple comparisons were done to check statistical significance of the results. Any two means not included in same parenthesis are statistically significantly different at $p = 0.05$. The interpretation of the results is supported by Student's 't' test and also by a statistical multiple comparison analysis using a least significant different procedure (23, 24).

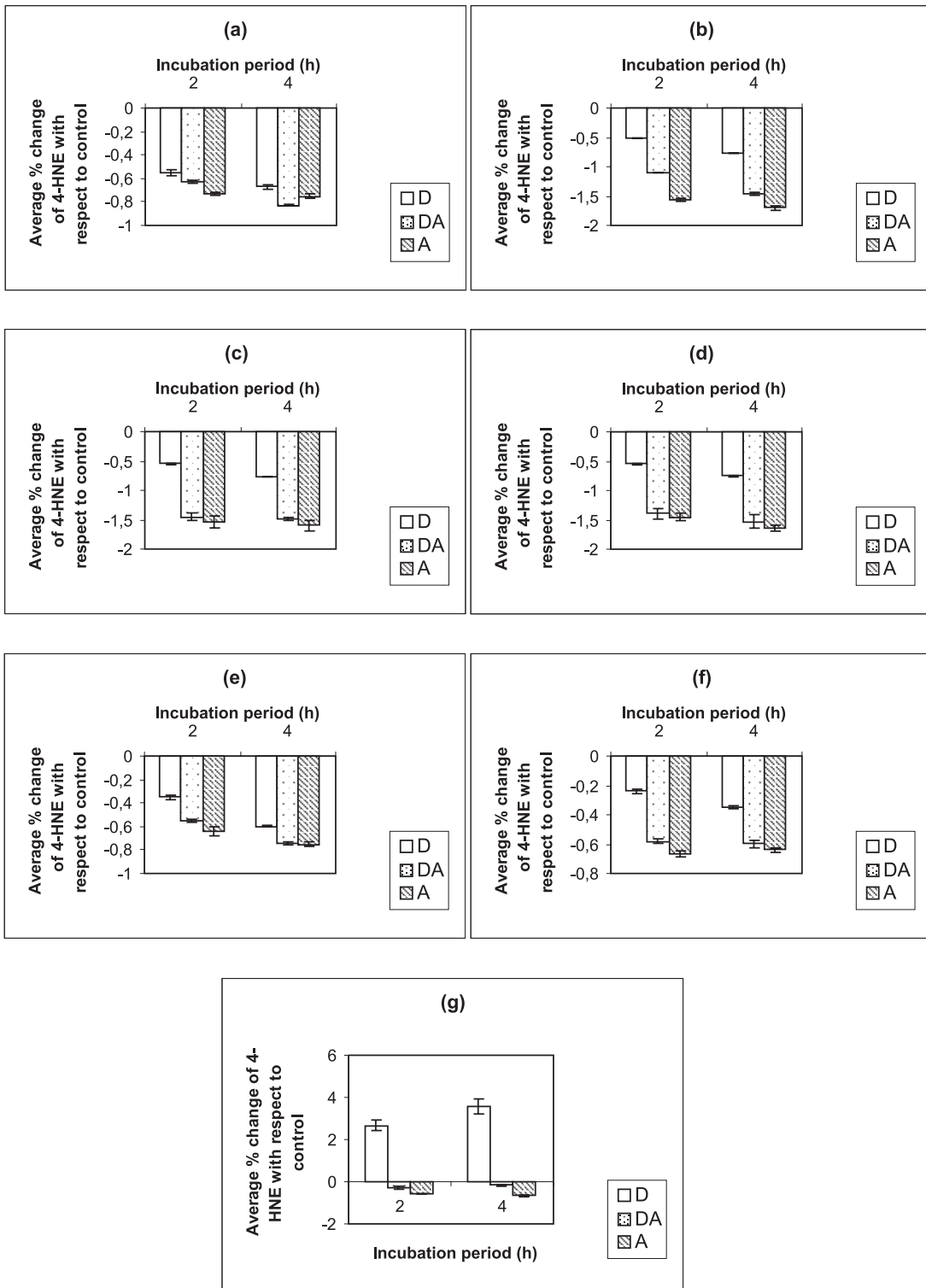


Figure 1. Average percent changes (\pm standard error) in 4-HNE profile after treatment with different NSAIDs [(a) diclofenac sodium, (b) ibuprofen, (c) flurbiprofen, (d) paracetamol, (e) nimesulide, (f) celecoxib, (g) indomethacin] and ascorbic acid (D, DA, and A indicate: drug-treated, drug and antioxidant-treated, and antioxidant-treated groups, respectively).

RESULTS

The average percent changes in 4-HNE content with respect to the control of the corresponding periods of incubation of different animal sets for different NSAIDs are shown in Figure 1. The results were analyzed using ANOVA and least significant difference procedure, which reveals that there was no statistically significant difference between the drug-treated group and the group treated with both drug and antioxidant except in the case of indomethacin.

DISCUSSION

From the obtained data shown in Figure 1, it is evident that 4-HNE levels decrease significantly with respect to control at both the periods of incubation (2 h and 4 h) after the treatment of the liver homogenates with diclofenac sodium, ibuprofen, flurbiprofen, paracetamol, nimesulide and celecoxib. This is due to the antioxidant action of the drugs. It was previously reported that diclofenac sodium has the ability to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (25), peroxyxynitrite (26) and has a significant cytoprotective effect (27).

Ibuprofen acts as an antioxidant, and thus makes a new therapeutic avenue for the treatment of Alzheimer's disease (28, 29). Flurbiprofen (30), paracetamol (31), nimesulide (32, 33) and celecoxib (34) have also antioxidative activity. In our earlier finding, we have suggested (20) that at 2 h and 4 h of incubation, celecoxib raises the malondialdehyde (MDA) level with respect to the control value, representing its significant lipid peroxidation activity. It may be due to the fact that celecoxib may have a prominent role for the formation of MDA in liver homogenate but not for the 4-HNE generation. The results of average percent changes of 4-HNE levels after treatment of liver homogenate with celecoxib are very close to the control value [-0.24(± 0.013) and -0.35(± 0.011) at 2 h and 4 h, respectively], suggesting its limited antioxidant activity.

An increase in 4-HNE levels of the drug-treated group suggests the occurrence of lipid peroxidation. 4-HNE is the end product produced by the ω -3 and ω -6 polyunsaturated fatty acids (35). This process of lipid peroxidation especially occurs in the presence of some metal ions like Fe^{2+} and other prooxidants (36). The increase in 4-HNE content with respect to control when the tissue homogenates were treated with indomethacin, indicates the prooxidant effect of

the indomethacin (37, 38). Lipid peroxidation mediated by oxygen radicals, especially hydroxyl radicals, plays a crucial role in the development of the gastric mucosal injury induced by indomethacin (38). Recent report suggests that indomethacin causes mitochondrial injury by dissipating the mitochondrial transmembrane potential (MTP) and inducing mitochondrial permeability transition pore (PTP), which liberates cytochrome C. This enzyme generates ROS, thereby triggering cellular lipid peroxidation, resulting in cellular apoptosis (39). Structural interpretation of prooxidant activity of indomethacin cannot be easily explained unless one carries out mechanistic study which is a scope of different paper.

At pH 7.4 (phosphate buffer), 99.95% of vitamin C remains present as ascorbic acid monoanion – AscH^- ; 0.05% as free acid – AscH_2 and 0.004% as dianion Asc^{2-} (40). Thus, the antioxidant chemistry of vitamin C is the chemistry of AscH^- . AscH^- reacts ($\text{AscH}^- + \text{R}\cdot \rightarrow \text{Asc}\cdot + \text{RH}$) rapidly with $\text{HO}\cdot$, $\text{RO}\cdot$ (*tert*-butyl alkoxy radical), $\text{ROO}\cdot$ (alkyl peroxy radical, e.g. $\text{CH}_3\text{OO}\cdot$), $\text{GS}\cdot$ (glutathionyl radical), $\text{UH}\cdot^-$ (urate radical), $\text{TO}\cdot$ (tocopheroxyl radical), $\text{O}_2\cdot^-/\text{HO}_2\cdot$ and similar oxidants making it an outstanding donor antioxidant. AscH^- donates a hydrogen atom ($\text{H}\cdot$ or $\text{H}^+ + \text{e}^-$) to an oxidizing radical to produce the resonance-stabilized tricarbonyl ascorbate free radical. $\text{AscH}\cdot$ is not protonated in biological system and is present as $\text{Asc}\cdot^-$. The dismutation reaction ($2\text{Asc}\cdot^- + \text{H}^+ \rightarrow \text{AscH}^- + \text{dihydroascorbate}$) is the principal route to the elimination of the $\text{Asc}\cdot^-$ *in vitro*. However, it is thought that reducing enzymes are involved in the removal of this radical, resulting in the recycling of ascorbate *in vivo* (41). Our study also reveals that ascorbic acid is capable of significantly minimizing the 4-HNE levels when used alone or together with the drugs.

CONCLUSION

From the present study it is suggested that diclofenac sodium, ibuprofen, flurbiprofen, paracetamol, nimesulide and celecoxib exert statistically significant mild antioxidant activity. By contrast, indomethacin is involved in oxidative processes. Ascorbic acid shows its antioxidant potential. These observations imply that the adverse effects of diclofenac sodium, ibuprofen, flurbiprofen, paracetamol, nimesulide and celecoxib may not be linked through free radical mediated processes, whereas adverse effects of indomethacin may be linked with its lipid peroxidation activity.

The concept of antioxidant co-therapy may also be exploited during future formulation design with an aim of reducing this drug-induced toxicity. Moreover, lipid peroxidation induction capacity of a drug may be tested at the individual level to determine the extent of risk from a drug in case of a particular individual in view of variable *in vivo* antioxidant defence and accordingly, the decision about safe use of drug and necessary co-administration of ascorbic acid may be taken. However, further extensive study is required to advance such hypothesis.

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