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INHIBITION OF LIPID PEROXIDATION INDUCED BY HYDROXYPROGESTERONE CAPROATE BY SOME CONVENTIONAL ANTIOXIDANTS IN GOAT LIVER HOMOGENATES

KAKALI DE, KUNAL ROY and CHANDANA SENGUPTA*

Division of Medicinal and Pharmaceutical Chemistry, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700 032 (INDIA)

Abstract: Among the cellular molecules, lipids containing unsaturated fatty acids with more than one double bond are particularly susceptible to action of free radicals. The resulting reaction, known as lipid peroxidation, has deleterious effect on biological membranes, leading sometimes even to disrupting them, or influencing their structure and function. Different toxic products are formed during this process. In this context, the present study was made to explore the suppressive actions of some conventional antioxidant compounds e.g., ascorbic acid, α-tocopherol and probucol on lipid peroxidation induced by hydroxyprogesterone caproate (HP), a progestogenic compound. The study has been performed using goat liver homogenate. It was found that HP increased thiobarbituric acid reactive substance i.e., malondialdehyde (MDA) and also other major toxic end product of lipid peroxidantion – 4-hydroxynonenal (4-HNE). HP decreased significantly the levels of reduced glutathione (GSH) and nitric oxide (NO) in the liver homogenates. This suggests that HP caused lipid peroxidation to a significant extent, which may be related to the toxic potential of the drug. It was further found that all of the above mentioned antioxidants could suppress HP-induced lipid peroxidation to the significant extent.

Keywords: hydroxyprogesterone caproate, malondialdehyde, 4-hydroxynonenal, glutathione, nitric oxide, α-tocopherol, probucol, ascorbic acid

Lipid peroxidation (LP) is a prominent manifestation of Reactive Oxygen Species (ROS) and/or free radical activity and oxidative stress in biological systems. LP can be defined as the oxidative deterioration of lipids containing a number of carbon-carbon double bonds (1). A large number of toxic by-products are formed during LP such as malondialdehyde (MDA), 4-hydroxynonenal, conjugated dienes, lipid hydroperoxides, isoprostanes etc. These compounds have effects at remote places from area of their generation. Hence they behave as toxic "second messengers". The oxidative modification of lipids and ROS has been implicated in the pathogenesis of many diseases (2), particularly atherosclerosis, coronary heart disease, neurodegenerative diseases, aging, inflammation, viral infections, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and gastric ulcer (3-5) etc. Oxidative lipid modifications occur through lipid peroxidation mechanisms in which free radicals or ROS withdraw a methylene hydrogen atom from polyunsaturated fatty acids, producing a carbon-centered lipid radical. Different methodologies have been developed that measure a variety of lipid peroxidation products used as markers of lipid peroxidation processes. Some of the products of LP are not overly toxic or are produced in negligible amounts (6). Major toxicological products are malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and various 2-alkenes. MDA is a small, three carbon aldehyde, generated from the hydrolysis of certain lipid hydroperoxides (LOOHs). MDA is cytotoxic and chemically reactive, but 4-HNE is more aggressive than MDA and leads to cell damage at nanomolar concentration (7). Oxidative free radicals are known to cause peroxidation of membrane polyunsaturated fatty acids. 4-HNE may contribute to the cytotoxic effects of oxidative stress (8-10). 4-HNE prevents NO production in cells by inhibiting nuclear factor-κB-dependent transcriptional activation of inducible NO synthase (11). Nitric oxide, a reactive radical, controls various vital physiological functions in the body. Determination of nitrite and nitrate, the stable end products of nitric oxide oxidation, is a common indirect method used to monitor nitric oxide (NO) levels in various body fluids and tissues (12).

*Corresponding author: E-mail: csgjupt@yahoo.com

Endogenous NO production is highly correlated with nitrite/nitrate levels in serum, plasma and urine. Hence determination of nitrite/nitrate may be used as a relative method of estimation of NO production.

Glutathione (GSH) is a tripeptide of glycine, glutamine and cysteine. It is the major redox compound of the aqueous phase in cells, scavenging reactive electrophilic substances, mainly ROS. Being the main antioxidant factor, it is also the chief reservoir of cellular SH groups (13, 14). GSH is a molecule which cysteinyl residue provides a nucleophilic thiol important for the detoxification of electrophilic metabolites and metabolically produced oxidizing agents (15). The importance of GSH conjugation in the detoxification of some xenobiotics depends on the extent to which the xenobiotic is metabolized to electrophiles.

A broader definition of an antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate (16). Elaborate and diversified antioxidant mechanisms protect tissues from oxidative damage in humans and other organisms (17), and susceptibility to benefit from exogenous antioxidants *in vitro* is conditioned by degree of their depletion (17). α-Tocopherol (TOC), ascorbic acid (AA), and probucol (PR) are known antioxidants (18, 19). Lipid peroxidation can explain the toxicity of various drugs and chemicals. The cell may protect cellular components from the deleterious effects of oxidative stress by: (i) direct interaction of oxidants and oxidizing agents with ascorbic acid, reduced glutathione and other reducing agents, (ii) scavenging free radicals with α-tocopherol (TOC), ascorbic acid (AA) etc., (iii) reducing hydroperoxides *via* the activities of glutathione peroxidase and catalase (20). Some of the antioxidants were found to produce significant increase in the levels of various endogenous antioxidant enzymes (21).

Hydroxyprogesterone caproate is a progestogen structurally related to progesterone that has been used for recurrent miscarriage and various menstrual disorders. It is several times more potent than progesterone and its duration of action is longer. It induces pregnancy, taking part in changes of uterus. It is also used in hormone replacement therapy, dysmenorrhea, and endometriosis. This steroidal drug has various toxic effects such as breast engorgement, headache, nausea, edema, esophageal reflux, acne, mental depression etc (22).

As part of our ongoing effort to explore drug induced lipid peroxidation and inhibitory actions of different antioxidants on drug induced lipid peroxidation (20, 23-27), our present study has been designed to explore induction potential of HP on the lipid peroxidation on goat liver homogenates. MDA, 4-HNE, GSH, and NO levels were used as markers of lipid peroxidation and suppressive actions of different antioxidants like AA, PR and TOC on HP-induced lipid peroxidation. As a whole, the aim of the study was to explore HP-induced lipid peroxidation and search some potential antioxidants that might have possible prospect and promise for reducing toxicity due to HP-induced lipid peroxidation. Thus, evaluation of antioxidants as supressors of the HP-induced lipid peroxidation provides a scope to select free radical scavengers, which on co-administration *in vivo*, may reduce toxic effects of the drug used for therapeutic purpose, in case of reduced endogenous antioxidants defense system.

MATERIALS AND METHODS

Chemicals

Hydroxyprogesterone caproate (HP) was gifted by Cipla Ltd., Mumbai, India. Trichloroacetic acid was purchased from Ranbaxy, S.A.S. Nagar; 1,1,3,3-tetraethoxypropane, reduced glutathione and DTNB (5,5'-dithio-bis(2-nitrobenzoic acid) were from Sigma Chemicals Co. St. Louis, MO, USA, 2,4-dinitrophenylhydrazine (DNPH) from SD Fine Chem. Ltd., Mumbai. The standard sample of 4-HNE was purchased from ICN Biomedicals Inc., Aurora, Ohio, USA. All other reagents were of analytical grade.

Liver collected from goat (*Capra capra*) was used as the lipid source. Goat liver was selected because of its availability and close similarity of its lipid constituents to that of human liver (28).

Preparation of tissue homogenate

Goat liver, perfused with normal saline through hepatic portal vein, was harvested and its lobes were briefly dried between filter papers to remove excess of blood and thin-cut with a heavy-duty blade. These small pieces were then transferred to the homogenizer to prepare homogenate (1 g, w/v) in phosphate buffer solution (0.1 M, pH 7.4) at room temperature. The work has been done according to the guidelines of the Institutional Animal Ethics Committee.

Incubation of tissue homogenate with drug and /or antioxidant

For each antioxidant (AA/PR/TOC), the tissue homogenate was divided into four parts of 50 mL each. The first portion was kept as the control (CL) which was not treated with drug or antioxidant,

whereas the second portion was treated with the drug – hydroxyprogesterone caproate at a concentration of 0.084 mg/g of liver homogenate. The third portion was treated with the drug along with antioxidant (AA/PR/TOC). The fourth portion was treated only with antioxidant (AA / PR / TOC). Ascorbic acid (AA), probucol (PR) and α-tocopherol (TOC) were used at effective concentrations of 0.12 mg/g , 0.1 mg/g and 0.1 mg/g of tissue, respectively. After treatment with drug and/or antioxidant, liver homogenates were stirred for 1 h below 20°C and then incubated at 15°C up to 24 h along with the control sample.

Estimation of lipid peroxidation breakdown products measured as malondialdehyde from tissue homogenate

The extent of lipid peroxidation was estimated in terms of malondialdehyde (MDA) content using thiobarbituric acid (TBA) method (29). Measurements were performed at 4, and 24 h of incubation. In each case three samples of 2.5 mL of incubation mixture were transferred to a tube containing 2.5 mL of 10% trichloroacetic acid (TCA). Then the tubes were centrifuged at 3000 rpm for 30 min to precipitate the protein. The TCA soluble fraction was separated and then a color reaction was developed as described by Yagi et al. (29) with slight modifications. In brief, to the tube containing TCA soluble fraction, 5 mL of 0.002 M TBA solution was added and a volume was made up to 10 mL with distilled water. The mixture was heated in boiling water bath for 30 min, and then tubes were cooled to room temperature and the absorbance was measured at 530 nm against a TBA blank (prepared from 5 mL of TBA solution and 5 mL of distilled water) using EC 5700B spectrocolorimeter. The values were determined from the standard curve obtained by using 1,1,3,3-tetraethoxypropane(TEP).

Estimation of lipid peroxidation end products measured as 4-HNE from tissue homogenate

The extent of lipid peroxidation was estimated in terms of 4-HNE content spectrophotometric method using 2,4-dinitrophenylhydrazine (7, 30). The estimation was done at 4 and 24 h of incubation during addition of drug and/or antioxidant and for the control portion of the liver sample. In each case three samples of 2.5 mL of incubation mixture were transferred to tubes containing 2.5 mL of 10% TCA. Then the tubes were centrifuged at 3000 rpm for 30 min to precipitate the protein part. The TCA soluble fraction was separated and then the supernatant was treated with DNPH solution (5 mM) and kept for 1 h at room temperature i.e. 25°C. Then the samples were extracted with hexane and after extraction the hexane layers were collected into graduated stoppered tubes and evaporated in a water bath at a temperature not exceeded 40°C. Then it was cooled to room temperature, 2 mL of methanol was added to it and shaken well. Then the absorbance was measured at 350 nm in Beckman DU-64 UV/VIS spectrophotometer. The values were determined from the standard curve obtained by using the standard – 4-hydroxynonenal.

Estimation of nitric oxide (NO) level from tissue homogenates

Nitric oxide was measured as the amount of nitrite (the stable metabolite of NO) present in tissue homogenates. Nitrite was measured colorimetrically by the Griess reaction method (31, 32). The Griess reagent was prepared by gentle mixing of 100 mL of 1.0% sulfanilamide (prepared in 3 M HCl) and 100 mL of 0.1% N-naphthylethylenediamine. The estimation was done at 4 and 24 h of incubation during addition of drug and/or antioxidant and for the control portion of the liver homogenate. In each case three samples of 3 mL of incubation mixture were transferred to tubes containing 3 mL of 10% trichloroacetic acid. Then the tubes were centrifuged at 3000 rpm for 30 min to precipitate the protein. The TCA soluble fraction was fully separated and then 5 mL of supernatant was treated with 0.5 mL of Griess regent and kept for 15 min at room temperature. After 15 min the absorbance was measured at 540 nm against a blank solution containing the same concentrations of ingredients but no biological sample. The nitric oxide levels were calculated from standard curve using sodium nitrite as the standard.

Estimation of reduced glutathione (GSH) level in tissue homogenates

Reduced glutathione was measured by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to give a compound that absorbs at 412 nm (the Ellman's method) (33). The determinations were done after 25 min and at 4 and 24 h of incubation. In brief, 0.5 mL of incubation mixture was transferred to a tube containing 1 mL of precipitating buffer (5% TCA in 1 mM EDTA) and then the sample was centrifuged at 2000 g for 10 min. The supernatant was saved and mixed with 5 mL of 0.1 M phosphate buffer (pH 8.0) and color was developed by adding 0.2 ml DTNB (0.01%). The absorbance was determined at 412 nm using EC 5700B spectrocolorimeter.

The percent changes in MDA, 4-HNE content and NO and GSH level of liver homogenates of different samples at different periods of incubation

were calculated with respect to the control of the corresponding duration of incubation. The change in MDA / 4-HNE level was considered as indicator of the extent of lipid peroxidation.

RESULTS AND DISCUSSION

In this study we analyzed the MDA, 4-HNE, NO and GSH levels after treatment of liver homogenates with HP. The percent changes in MDA and 4-HNE content with respect to control of the corresponding duration of incubation for different samples and their averages are shown along with statistical analysis in Tables 1 and 2 and Figures 1-4. They show the effect of AA, PR and TOC as free radical scavengers. Interpretation of the results is supported by the Student's 't' test (results not shown) and also by a statistical multiple comparison analysis using least significant different procedure (34, 35) (Tables 1 and 2).

Analysis of variance (ANOVA) and multiple comparisons were done to check statistical significance of the results. In multiple comparisons there are two possible sources of error: the random error associated with the replicate measurements and the other due to animal variations. The variation may be calculated and their effects estimated by a statistical method known as the analysis of variance (ANOVA) (34, 35), where the square of the standard deviation s^2 is called the variance V. Thus, $F = s_1^2/s_2^2$ where $s_1^2 > s_2^2$, and may be written as $F = V_1/V_2$. In our study, a degree of freedom (df) between samples is (2, 8) and that between animals is (4, 8). ANOVA is done to compare the means of more than two treatment groups.

If the F test (F = mean square between regimens/mean square within regimens) is significant and more than two treatments are included in the experiment, it may not be obvious immediately which treatments are different. Various multiple comparison procedures have been proposed to solve this problem. The general procedure of multiple comparison is to list the ranked means that are not statistically significantly different from each other are placed in a same parenthesis. The procedure is carried out by calculating a 5% allowance, which is defined as the critical difference between means which allows one to reject the null hypothesis and accept the alternative hypothesis for any two sample means at p = 0.05.

We have used the least significant difference procedure to solve multiple comparison analysis. Least significant difference procedure is the least conservative procedure, and this assures that the probability that any one comparison is judged to be significant by chance alone is 5%. However, the

Table 1. Analysis of variance and multiple comparisons of HP-induced lipid peroxidation (markers MDA and 4-HNE) by different antioxidants (AA, PR, TOC)

Markers	Antioxidant	Incubation period	F ratio		Pooled variance§	Critical difference# at 5% level with ranked sample means**	
			F_1	F_2	(s^2)	LSD	RM
MDA	AA	4 h	35.93[v]	0.51	44.37	9.71	(D) (DAA) (AA)
		24 h [1]	6.41[v2]	0.66	34.69	8.59	(D)(DAA, AA)
	PR	4 h	25.47[v1]	0.31	48.91	10.20	(D) (DPR) (PR)
		24 h	26.97 [v1]	1.05	17.04	6.02	
	TOC	4 h	18.79[v1]	0.62	72.81	12.44	(D, DTOC) (TOC)
		24 h	11.45[v1]	0.68	25.03	7.29	(D)(DTOC) (TOC)
4-HNE	AA	4 h	13.72[v1]	0.82	9629.16	143.11	(D, DAA) (AA)
		24 h	9.72[v1]	0.49	2883.38	78.31	
	PR	4 h	17.52[v1]	1.93	3567.50	87.10	(D, DPR) (PR)
		24 h	36.43[v1]	2.72	1094.46	48.24	
	TOC	4 h	13.05[v1]	0.42	2959.84	79.34	(D, DTOC) (TOC)
		24 h	9.25[v1]	0.85	4615.23	99.07	

D, DAA, and AA indicate HP-treated, HP & AA-treated and AA-treated samples respectively. D, DPR, and PR indicate HP-treated, HP & PR-treated and PR-treated samples respectively. D, DTOC, and TOC indicate HP-treated, HP & TOC-treated and TOC-treated samples respectively.

F_1 and F_2 correspond to variance ratios between samples [df = (2,8)] and between animals [df = (4,8)], respectively. [v1]F values are significant at $p < 0.01$; [v2]F values are significant at $p<0.05$. S^2 = pooled variance, LSD = Least squares difference (34, 35).

§Error mean square, # Critical difference according to least significant difference procedure (34, 35).

RM = ranked means. **Two means not included within the same parenthesis are statistically significantly different at $p< 0.05$.

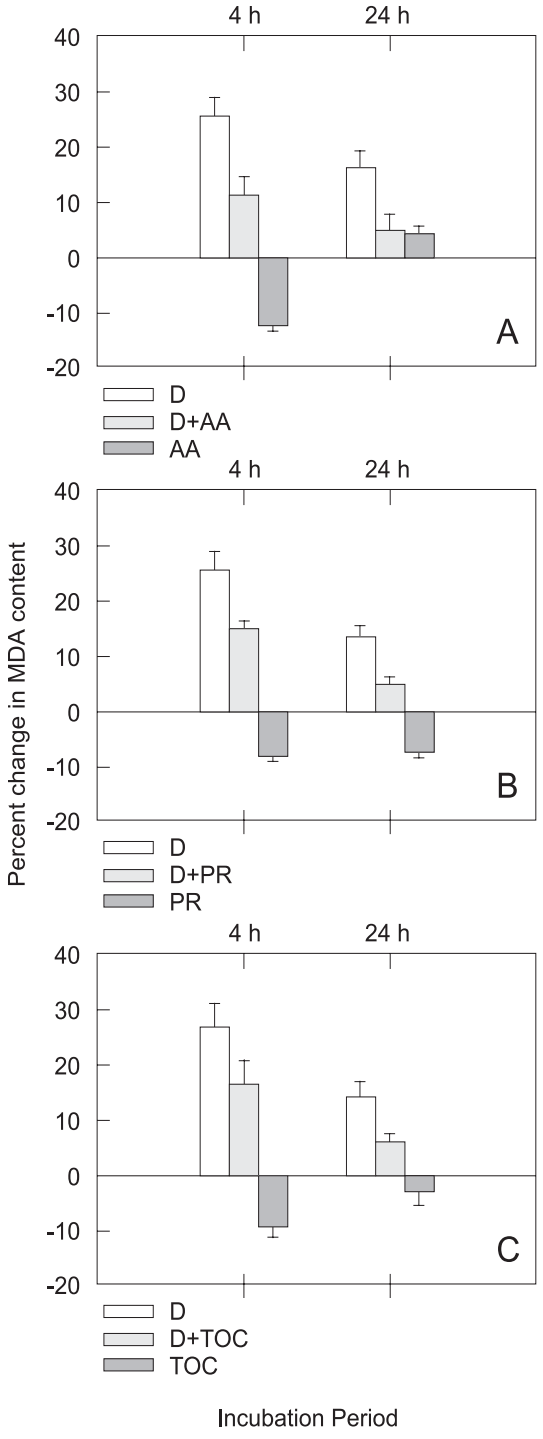

Figure 1. Effects of (A) ascorbic acid (AA), (B) probucol (PR) and (C) α-tocopherol (TOC) on hydroxyprogesterone caproate (HP)-induced lipid peroxidation: Average (mean of five animal sets) percent changes in malondialdehyde (MDA) with respect to control of the corresponding periods. D, (D+AA), and AA indicate only HP-treated, HP & AA-treated and only AA-treated samples, respectively. (D+PR), and PR indicate HP & PR-treated and only PR treated samples, respectively. (D+TOC), and TOC indicate HP & TOC-treated and only TOC-treated samples, respectively.

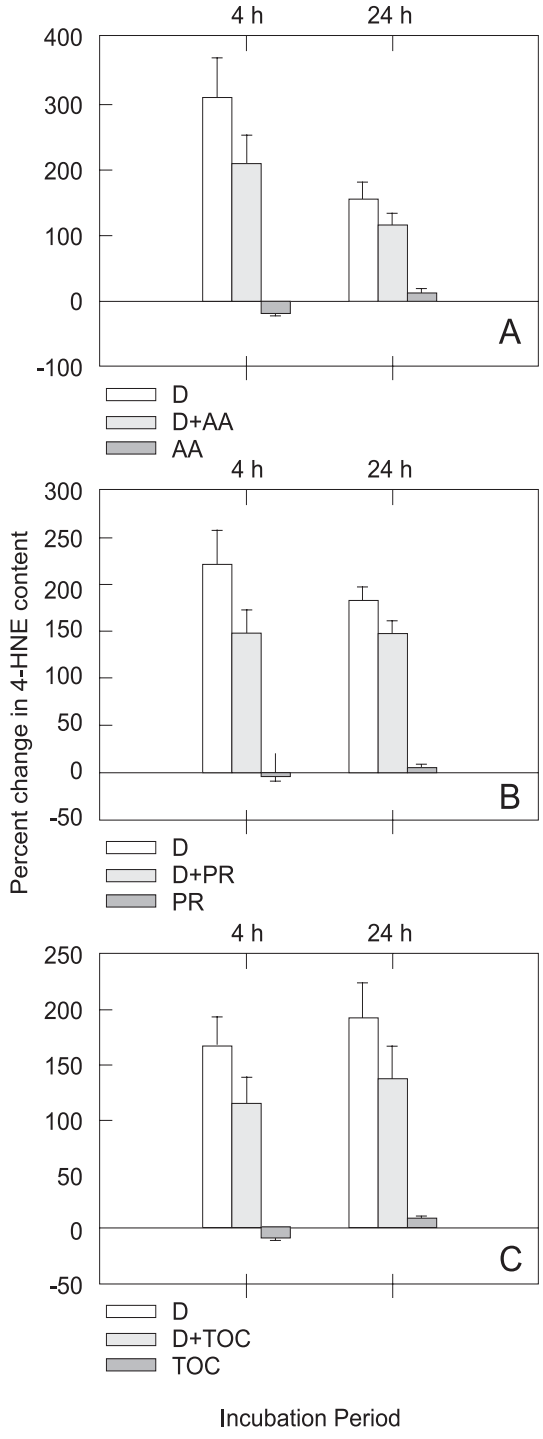

Figure 2. Effects of (A) ascorbic acid (AA), (B) probucol (PR) and (C) α-tocopherol (TOC) on hydroxyprogesterone caproate (HP) induced lipid peroxidation : Average (mean of five animal sets) percent changes in 4-HNE with respect to control of the corresponding periods. D, (D+AA), and AA indicate only HP-treated, HP & AA-treated and only AA-treated samples, respectively. (D+PR), and PR indicate HP & PR-treated and only PR treated samples, respectively. (D+TOC), and TOC indicate HP & TOC-treated and only TOC-treated samples, respectively.

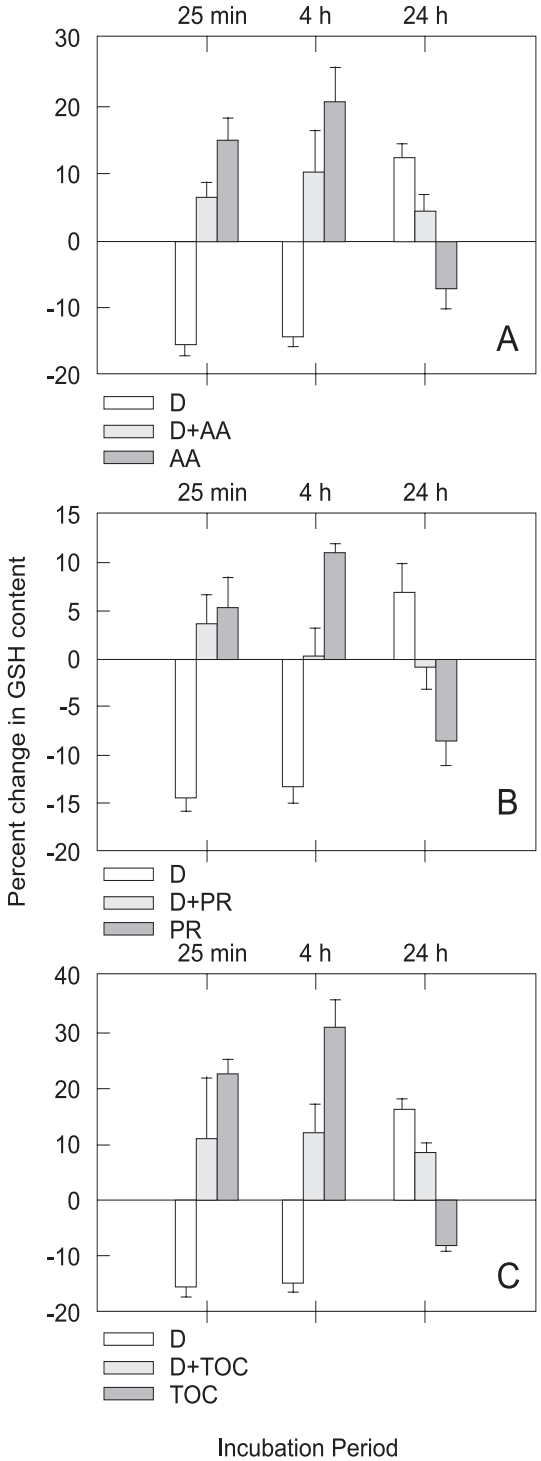

Figure 3. Effects of (A) ascorbic acid (AA) , (B) probucol (PR) and (C) α-tocopherol (TOC) on hydroxyprogesterone caproate (HP) induced lipid peroxidation : Average (mean of five animal sets) percent changes in GSH with respect to control of the corresponding periods. D, (D+AA), and AA indicate only HP-treated, HP & AA-treated and only AA-treated samples, respectively. (D+PR), and PR indicate HP & PR-treated and only PR treated samples, respectively. (D+TOC), and TOC indicate HP & TOC-treated and only TOC-treated samples, respectively.

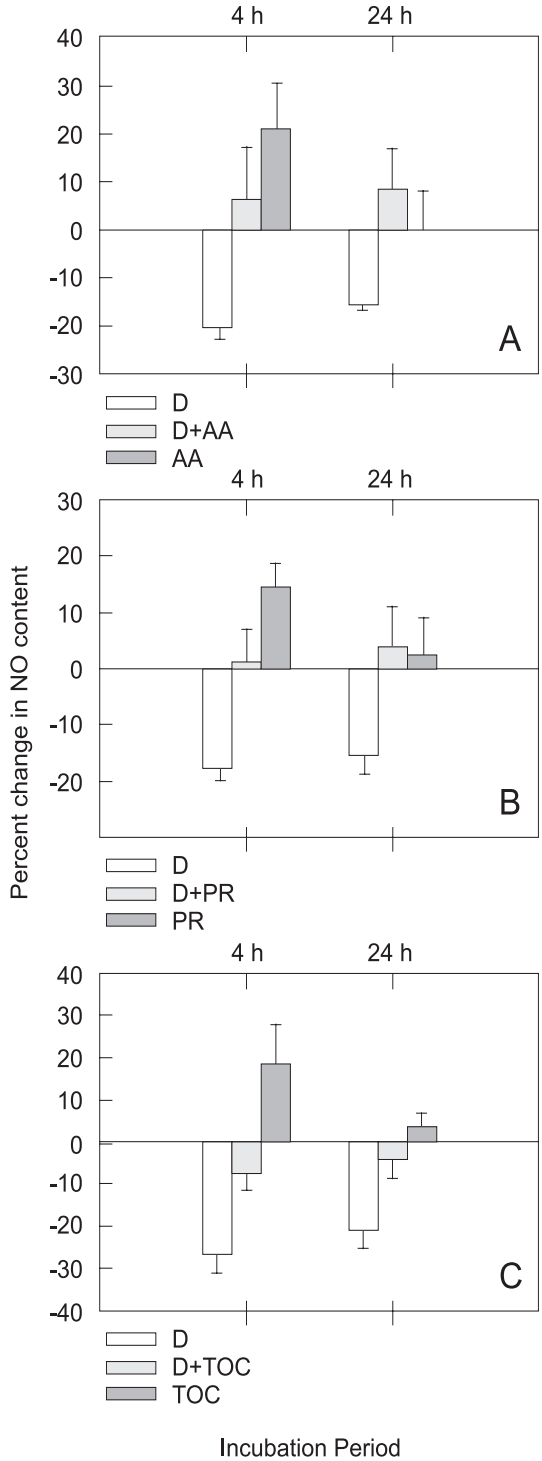

Figure 4. Effects of (A) ascorbic acid (AA) , (B) probucol (PR) and (C) α-tocopherol (TOC) on hydroxyprogesterone caproate (HP) induced lipid peroxidation : Average (mean of five animal sets) percent changes in NO with respect to control of the corresponding periods. D, (D+AA), and AA indicate only HP-treated, HP & AA-treated and only AA-treated samples, respectively.(D+PR), and PR indicate HP & PR-treated and only PR treated samples, respectively. (D+TOC), and TOC indicate HP & TOC-treated and only TOC-treated samples, respectively.

probability of one or more comparisons being judged significant would be greater than 5%. Any two means included in the same parenthesis do not differ significantly at p = 0.05. Any two means not included in same parenthesis are statistically significantly different at p = 0.05.

The obtained data indicate that treatment of liver homogenate with HP significantly increases MDA and 4-HNE content with respect to control of different periods of incubation (Figure 1, 2 and Table 1). This is possible due to enhanced lipid peroxidation due to the presence of HP. It was further found from the study that when the liver homogenates were incubated with drug (HP) in combination with antioxidants (AA / PR / TOC), an increase in MDA and 4-HNE content was reduced in comparison to the HP treated liver homogenates. This implies that the antioxidants (TOC, PR and AA) could reduce the extent of HP-induced oxidative stress. This is due to their protective effects against free radicals that may have been generated within the system due to the presence of the drug.

When the liver homogenates were treated only with antioxidant TOC / PR, then the MDA and 4-HNE content decreased in comparison with the control but in case of the antioxidant AA some increase in MDA and 4-HNE content was observed. Ascorbic acid may play a dual role with respect to free radical reactions (36). The ability of AA to release transition metals from a protein complex or to maintain transition metals, such as iron and copper, in a reduced state is the likely mechanism involved (37). Iron released from ferritin molecules by superoxide radicals is capable of initiating the Haber-Weiss reaction and generating oxygen radical species (38).

Glutathione, is an important intracellular antioxidant. It functions as an endogenous hydroxyl radical scavenger and as a substrate in the glutathione redox cycle as well as in other xenobiotic elimination reactions (39). The percent changes in the GSH with respect to the control of different periods of incubation for various samples collected from different animals and their averages are shown along with statistical analysis in Figure 3 and Table 2. The tables show the effects of AA, PR and TOC as free radical scavengers. Interpretation of the results is supported by Student's 't' test (results not shown) and also by the statistical multiple comparison analysis using the least significant different procedure (34, 35) (Table 2).

Incubation of the liver homogenate with HP decreased the GSH with respect to the corresponding controls. This is due to the fast oxidation of GSH probably induced by the steroidal drug HP. GSH plays a very important role in various biochemical processes including lipid peroxidation and it provides a defense mechanism for tissues against the reactive oxygen species (40). The importance of GSH conjugation in the detoxification of a xenobiotic depends on the extent to which the xenobiotic is metabolized to electrophiles. The decrease in GSH level in tissues may be a consequence of enhanced utilization of this compound by the antioxidant enzymes, glutathione peroxidase and glutathione-S-transferase.

Again, the liver homogenates were treated only with the antioxidant (TOC, PR and AA) then the GSH level was increased in comparison to the control and drug-treated samples of corresponding incubation periods. This increase in reduced GSH may be due to the free radical scavenging properties of these antioxidants. GSH plays an important role in the prevention of radical mediated injury to the tissue. It does so as a radical scavenger and by supplying GSH to the antioxidant enzymes (41).

Figure 3 shows that at 24 h of incubation the GSH content increased in the drug treated liver homogenates with respect to the control and when the liver homogenates were treated with the drug and antioxidants (TOC, PR and AA), or only with the antioxidants (TOC, PR, AA), the glutathione level decreased more than in the drug treated and control groups, respectively. This decrease may be due to the fast reaction of GSH with $OH^{\cdot}$ radical giving thiol radicals, which can also be formed when GSH is oxidized by peroxidase, or by O_2 in the presence of transition metal ions. This thiol radical, although less reactive than $OH^{\cdot}$ radical, in conjugation with superoxide dismutase (SOD) and GSH, converts superoxide anions into hydrogen peroxide (H_2O_2). Glutathione peroxidase converts H_2O_2 into water (36). As a result of the second conversion, GSH is oxidized to glutathione disulfide (GSSG). In this way, GSH acts as a cofactor in the removal of toxic radicals from the body. During oxidative stress, GSH levels decline and GSSG increases, which can influence signal transduction by stimulating NF-κB activation (42).

Thus it appears that the presence of lipid peroxidation induction capacity of HP may be related to the toxic potential of the drug. Lipid peroxidation, being a free radical chain reaction, is an attractive general mechanism, which could explain the toxicity of various drugs. The different antioxidants TOC, PR and AA prevent the rapid oxidation of GSH induced by the progesterone drug HP but the latter reduces MDA release. Due to free radical inducing

Table 2. Analysis of variance and multiple comparisons of HP-induced lipid peroxidation (markers GSH and NO) by different antioxidants (AA, PR, TOC)

Markers	Antioxidant	Incubation period	F ratio		Pooled variance§	Critical difference# at 5% level with ranked sample means**	
			F_1	F_2	(s^2)	LSD	RM
GSH	AA	25 min	69.84[v1]	2.53	16.63	5.94	(D) (DAA) (AA)
		4 h	16.01[v2]	1.74	70.17	12.21	(D) (DAA,AA)
		24 h	16.28[v1]	2.54	20.14	6.54	(D) (DAA) (AA)
	PR	25 min	11.93[v1]	0.92	38.61	9.06	(D) (DPR) (PR)
		4 h	30.09[v1]	1.00	22.82	6.96	
		24 h	4.84[v2]	0.76	50.16	10.32	(D, DPR) (DPR, PR)
	TOC	25 min	19.46[v1]	1.61	91.66	13.96	(D) (DTOC, TOC)
		4 h	22.21[v1]	2.07	102.55	14.76	
		24 h	28.19[v1]	0.54	21.91	6.82	(D) (DTOC) (TOC)
NO	AA	4 h	11.81[v1]	4.22[v2]	177.38	19.42	(D, DAA, AA)
		24 h	7.95[v2]	2.95	113.26	15.52	
	PR	4 h	18.10[v1]	2.15	60.76	11.36	(D, PR) (DPR)
		24 h	4.63[v1]	1.69	134.00	16.88	(D, DPR, PR)
	TOC	4 h	11.85[v1]	1.08	204.30	20.84	(D, DTOC, TOC)
		24 h	10.69[v1]	0.48	78.17	12.89	(D) (DTOC, TOC)

D, DAA, and AA indicate HP-treated, HP & AA-treated and AA-treated samples respectively. D, DPR, and PR indicate HP-treated, HP & PR-treated and PR-treated samples respectively. D, DTOC, and TOC indicate HP-treated, HP & TOC-treated and TOC-treated samples respectively.

F_1 and F_2 correspond to variance ratios between samples [df = (2,8)] and between animals [df = (4,8)], respectively. [v1]F values are significant at $p < 0.01$; [v2]F values are significant at $p<0.05$. S^2 = pooled variance, LSD = Least squares difference (34, 35).

§Error mean square, # Critical difference according to least significant difference procedure (34, 35).

RM = ranked means. **Two means not included within the same parenthesis are statistically significantly different at $p< 0.05$.

capacity of this drug, the increase in the formation of MDA is accompanied by the reduction in the reduced glutathione content.

The tissue levels of NO were also indirectly measured in the present study. NO is metabolized to its stable products nitrite and nitrate. Nitrite levels were measured as an index for NO generated in the tissues (43). The percent changes in the nitric oxide level (NO) with respect to the control values of different periods of incubation for various samples collected from different animals and their averages are shown along with statistical analysis of the data in Figure 4 and Table 2. The table also shows the effects of AA, PR and TOC as free radical scavengers. Analysis of results was done by Student's 't' test (results not shown), statistical multiple comparison analysis using least significant different procedure (34, 35) and has been shown in Table 2.

Incubation of the liver homogenate with only HP significantly decreased the NO level with respect to the control values. When the liver homogenates were treated with the drug HP along with antioxidants (AA, PR and TOC), then a decrease of NO level was less than that of drug treated samples of the same incubation period i.e., increased the NO level in comparison to only drug treated samples with respect to control values. Again, when the liver homogenates were treated only with the antioxidant (AA / PR / TOC), then the NO level was increased in comparison to the control values and drug-treated samples of corresponding incubation periods except in some cases with ascorbic acid. This increase in NO level may be due to the free radical scavenging properties of these antioxidants. This implies that the antioxidants could reduce the extent of HP-induced oxidative stress. This is due to their protective effects against free radicals that may have been generated within the system due to the presence of the drug (HP).

It is worth mentioning that when the liver homogenates were treated only with antioxidant ascorbic acid, in some samples a decrease in NO level with respect to control was observed. In the case of ascorbic acid it is due to its double role (antioxidant and pro-oxidant) with respect to free radical reactions (36). High 4-HNE / NO ratio indicates an increase in pro-oxidant status in the tissues. NO reacts with superoxide to form peroxynitrite (43, 44). NO, when produced in excess is capable of quenching superoxide anion (45). But if NO levels

are low, the increased amounts of peroxynitrite radical are formed which can cause an increase in the generation of products (43).

CONCLUSIONS

Based on the results of the present study, increased levels MDA/4-HNE content and decreased levels of GSH/NO content in drug hydroxyprogesterone caproate treated liver homogenates with respect to appropriate controls suggest–that HP has lipid peroxidation inducing capacity. The inhibitory actions of AA / PR / TOC on lipid peroxidation induced by some other drugs also were discussed in previous communications (20, 24-27, 46, 47). The present study also indicates the potential of different conventional antioxidants, *viz*., ascorbic acid, probucol and α-tocopherol, for suppression of lipid peroxidation induced by HP. These observations imply that the antioxidants merit further extensive studies to explore their possible potential in reducing drug-induced toxicity that may be mediated through free radical mediated processes. The concept of antioxidant co-therapy may also be exploited during future formulation design with an aim of reducing 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 a drug and necessary co-administration of antioxidants may be taken. However, further extensive study is required to prove such hypothesis.

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