Cytochrome P450-mediated oxidative damage of nuclear membrane proteins and its prevention by vitamin C

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In this report, we present data to indicate that NADPH-cytochrome P450 reductase/cytochrome P450 system is present in the nuclear membrane. The reactive oxygen species generated in this free metal ion–independent P450 system oxidatively modifies and degrades the membrane proteins. The oxidative modification is evidenced by the formation of carbonyl, bityrosine and tryptophan loss. The degradation of membrane proteins is manifested using fluorescamine reactivity and SDS-PAGE. Ascorbic acid exclusively prevents the oxidative modification and degradation of the membrane proteins. Other antioxidants, such as superoxide dismutase, catalase, glutathione, α-tocopherol, probucol, β-carotene, mannitol, histidine and thiourea are found to be ineffective. The observation assumes significance, particularly in subclinical ascorbic acid deficiency, where oxidative damage of the nuclear membrane would occur. This, in turn, would affect the traffic of cytoplasmic enzymes and proteins required for DNA replication and repair, transcription and RNA processing, ultimately leading to disruption of gene regulation of the cell.

Keywords: nuclear membrane, cytochrome P450, NADPH-cytochrome P450 reductase, oxidative damage, ascorbic acid, antioxidants

Earlier, we demonstrated that NADPH-cytochrome P450 reductase/cytochrome P450/O$_2$ system produces oxidative modification of microsomal proteins in the absence of free metal ions, and that the oxidized proteins are rapidly hydrolyzed by proteases present in the microsomes. Also, the protein oxidation is exclusively prevented by ascorbic acid. Other scavengers of reactive oxygen species, including superoxide dismutase, catalase, and glutathione are ineffective. The mechanism of NADPH-initiated P450-mediated free metal ion-independent oxidative damage of proteins is different from metal ion catalyzed oxidation (MCO) system. All MCO systems, enzymatic or non-enzymatic, thus far described require the presence of free transitional metal ions in the incubation system$^{2,3}$. Perhaps, the most physiologically relevant of these is the NADPH/P450 reductase/P450/Fe (III)/O$_2$ (refs 2-4). However, in vitro studies with added iron salts have less relevance to in vivo situation, where in the normal physiological condition, most of the metals are not free and remain tightly bound with proteins$^1$. Nuclear membrane is a continuation of endoplasmic reticulum and contain mixed function oxidase$^5$. It would, therefore, be expected that NADPH should also initiate oxidative damage of nuclear membrane proteins. The damage of nuclear membrane would obviously affect the traffic of cytoplasmic enzymes and proteins required for DNA replication and repair, transcription and RNA processing. The objective of this study is to demonstrate that NADPH actually initiates oxidative damage of proteins in the nuclear membrane, which is mediated by P450. We also demonstrate that ascorbic acid exclusively inhibits this free metal ion-independent protein oxidation and thereby prevents subsequent proteolytic degradation.

Materials and Methods

Materials

NADPH, fluorescamine, glutathione (GSH), bovine erythrocyte superoxide dismutase (SOD), ascorbic acid and mannitol were purchased from Sigma Chemical Co. U.S.A. Catalase (free of SOD) was obtained from the Centre for Biochemical Technology (CSIR), New Delhi, India. α-Tocopherol was a gift from E. Merck (India). Phenylmethylsulfonylfluoride
with 50 to 70 ml of ice cold Tris-buffer (ether, the livers were perfused through the portal vein earlier liver were isolated following the methods described earlier. All the animal care procedures met the NIH guidelines. After sacrificing the animals by diethyl ether, the livers were perfused through the portal vein with 50 to 70 ml of ice cold Tris-buffer (pH 8.3), containing 0.025 mM KCl, 0.25 mM sucrose and 5 mM MgCl₂. Perfused liver (10 g) was homogenized in the same buffer and centrifuged at 120 g for 8 min. Supernatant containing the liver nuclei was again centrifuged at 2,500 g for 10 min. The pellet obtained (liver nuclei) was resuspended in 30 ml 2.3 M sucrose containing 3 mM CaCl₂ and centrifuged at 35,000 g for 50 min. The buff colored nuclei were washed by resuspending in 20 ml of 1 M sucrose containing 1 mM CaCl₂ and centrifuged at 2,500 g for 10 min. The isolated nuclei were resuspended in 20 ml Tris-Cl (pH 7.5) and incubated for 5 min at 4°C. The swollen nuclei were sedimented by centrifugation at 3,000 g for 10 min and sedimented nuclei were suspended in 20 ml Tris-Cl buffer containing 10 mM Tris-Cl (pH 7.4), 0.3 M sucrose, 0.2 mM MgCl₂ and 100 µg of DNase and incubated at 30°C for 20 min. After incubation, 8 vols (160 ml) of ice cold 1.5 M KCl were added. Nuclei were extracted for 1 hr at 4°C and then collected by centrifugation at 110,000 g for 60 min in a swing-out rotor. The pellet was resuspended in 5 ml of 0.44 M sucrose containing 2 mM MgCl₂ and 70 mM KCl and then finally adjusted to 2.1 M sucrose concentration. Nuclei in 2.1 M sucrose solution was overlaid by sucrose gradient ranging from 2.1 M to 1.3 M sucrose and was centrifuged in a swing-out rotor at 110,000 g for 16 hr. After the run, the nuclear membrane was collected as a band and again centrifuged at 100,000 g for 60 min. The pellet was finally suspended in 0.1 M potassium phosphate buffer (pH 7.4), at a concentration of approx. 10 mg of protein per ml of suspension.

Preparation of microsomes
Microsomal fractions were prepared from guinea pig liver homogenate in 1.15% KCl by centrifuging at 9,000 g for 20 min for two successive times, followed by centrifugation at 1,05,000 g for 1 hr as described earlier. Microsomes were washed three times with 1.15% KCl and suspended in 0.1 M potassium phosphate buffer (pH 7.4), at a concentration of approx. 10 mg of protein per ml of suspension.

Spectrofluorimetry
Fluorescamine reactivity, bitryosine production and tryptophan loss were measured with a Hitachi fluorescence Spectrophotometer model no. F3010.

Incubation system
Incubation mixture using nuclear membrane or microsomal suspension contained 1 mg of protein in a final vol. of 250 µl of 50 mM potassium phosphate buffer (pH 7.4). NADPH (0.24 mM or as indicated) was added to initiate the reaction.

Measurement of protein carbonyl
Protein carbonyl was measured by reaction with 2,4-dinitrophenyl hydrazine (DNPH). After incubation with NADPH in the presence of PMSF and EDTA to minimize proteolytic degradation and loss of protein carbonyl, the protein was precipitated with 20% trichloroacetic acid and the carbonyl content was measured in the precipitate. A blank preparation in 2 M HCl was kept. The difference in absorbance between DNPH treated sample vs. HCl control was measured at 390 nm. The results were expressed as nmol carbonyl groups/mg protein using a molar coefficient of 22,000 for the DNPH derivatives.

Measurement of NADPH oxidation
For measurement of NADPH oxidation, 1 mg protein equivalent of nuclear membrane was taken in 1 ml reaction mixture containing 20 µM DFO. The reaction was initiated by adding 240 nmole of NADPH and incubated at 37°C with occasional shaking. From the reaction system, 200 µl aliquots were withdrawn at different time intervals and added to 1 ml cold absolute ethanol. After 5 min, the mixture was centrifuged at 10,000 g for 10 min and absorption of the supernatant was measured at 340 nm. NADPH oxidation was calculated by subtracting this value from initial absorbance of NADPH at 340 nm, taking ε₃₄₀nm = 6.22 mM⁻¹ cm⁻¹.

Assay of P450
P450 was assayed by the method of Omura and Sato and P450 reductase was assayed by the method of Masters et al. SDS-PAGE was carried out according to the method of Laemmli.
Results
We have observed that like microsomal membranes, nuclear membrane also contains NADPH-P450 reductase and P450. The amount of P450 in the microsomal and nuclear membranes is found to be 1.3 and 0.623 nmole/mg protein, respectively, while the specific activity of NADPH-P450 reductase is found to be 0.48 unit and 0.35 unit/mg protein, respectively. Earlier, we reported that NADPH-initiated oxidative degradation of microsomal proteins occurs independently of added metal ions. Fig. 1 shows that addition of NADPH to the microsomal or nuclear membrane suspension in the absence of added metal ion results in rapid degradation of the membrane proteins in a time-dependent manner, as evidenced by the progressive release of fluorescamine reactive material in the trichloroacetic acid extract. Fluorescamine reacts with free amino groups and the production of fluorescamine reactive materials indicates production of new NH₂-terminus peptides. This oxidative degradation of membrane proteins i.e., the production of fluorescamine reactive material, was exclusively prevented by ascorbic acid. Other antioxidants, such as glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), histidine, α-tocopherol, β-carotene, probucol and thiourea were ineffective (Fig. 2). The possibility of the involvement of contaminated iron in the incubation mixture of NADPH-initiated protein degradation was eliminated, because use of desferrioxamine (20 µM), a strong chelator of Fe (III), did not prevent the oxidative damage.

Furthermore, we analyzed the degradation of the nuclear membrane proteins by SDS-PAGE (Fig. 3), which also shows that addition of NADPH results in a rapid degradation of nuclear membrane proteins and the degradation is exclusively prevented by ascorbic acid (20 µM). Higher concentrations of ascorbic acid (up to 100 µM) produced similar inhibition. Other antioxidants, such as SOD, CAT, GSH, α-tocopherol, mannitol and histidine were ineffective.

It is known that reactive oxygen species (ROS) interact with tyrosine to form several biphenolic compounds, of which bityrosoine appears to be the major product. We have observed that NADPH-initiated oxidative modification of nuclear membrane proteins leads to bityrosoine formation at an early stage, which is completely prevented by ascorbic acid (Fig. 4). No bityrosoine is formed in the absence of NADPH. Fig. 5 shows that NADPH-initiated oxidative modification of microsomal proteins in the...
absence of free iron also results in the loss of tryptophan, as evidenced by the loss of fluorescence emission at 340-350 nm (excitation at 280 nm). The loss of tryptophan is almost completely prevented by ascorbic acid.

Measurement of protein carbonyls has been used as a sensitive assay for oxidative damage to proteins. Table 1 shows that NADPH initiates carbonyl formation in the nuclear membrane proteins. Unincubated membranes or incubation of membranes in absence of NADPH gave low values of protein carbonyls (0.33±0.04 and 0.44±0.05 nmole/mg protein for nuclear and microsomal membranes, respectively). NADPH-initiated carbonyl formation in both is specifically prevented by ascorbic acid, while SOD, CAT, GSH, α-tocopherol, mannitol and histidine were ineffective (Table 1). Fig. 6 shows that carbonyl formation occurs at an early stage of oxidative modification. Maximum carbonyl formation takes place around 30 min of incubation period.

The protein oxidation in the nuclear membrane is accompanied by NADPH oxidation. Fig. 7 shows the time-dependent oxidation of NADPH. As reported earlier, NADPH oxidation leads to reduction of P450.Fe3+ to P450. Fe2+ through the NADPH-P450 reductase.

Table 2 shows that antibody to P450 prevents the NADPH-initiated protein oxidation in the nuclear membrane, indicating that P450 is involved in the protein oxidation.
Table 1—Effect of antioxidants on NADPH-initiated carbonyl formation in nuclear membrane and microsomes

[Conditions of incubation and estimation of carbonyl group are described under ‘Materials and Methods’. Incubation time was 30’. PMSF (25 µg) and EDTA (2.0 mM) were added to the incubation mixture to minimize the loss of peptides in the supernatant. Values are mean±S.D. of four independent determinations; P<0.05]

<table>
<thead>
<tr>
<th>Addition to incubation mixture</th>
<th>Carbonyl formed (nmole hydrazone/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>Nuclear membrane</td>
</tr>
<tr>
<td>None</td>
<td>0.33±0.04</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.86±0.07</td>
</tr>
<tr>
<td>NADPH + ascorbic acid (20 µM)</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>NADPH + SOD (100 units)</td>
<td>0.86±0.06</td>
</tr>
<tr>
<td>NADPH + catalase (40 µg)</td>
<td>0.86±0.07</td>
</tr>
<tr>
<td>NADPH + GSH (200 µM)</td>
<td>0.85±0.06</td>
</tr>
<tr>
<td>NADPH + α-tocopherol (20 µM)</td>
<td>0.87±0.08</td>
</tr>
<tr>
<td>NADPH + mannitol (20 mM)</td>
<td>0.85±0.07</td>
</tr>
<tr>
<td>NADPH + histidine (10 mM)</td>
<td>0.86±0.08</td>
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Fig. 6—NADPH-initiated time-dependent formation of protein carbonyl in nuclear membrane and microsomal proteins and its prevention by ascorbic acid [Conditions of incubations and estimation of carbonyl groups are described under ‘Materials and Methods’. Carbonyl values (control) for only nuclear membrane (0.33 ± 0.04 nmole/mg protein) and microsomes (0.44±0.05 nmole/mg protein) were subtracted from respective experimental data. (♦), nuclear membrane; (▲), microsomal membrane; (●), nuclear membrane+ascorbic acid; —, microsomal membrane+ascorbic acid. Values are mean of four independent determinations; S.D. <10% (P<0.05)]

Table 2—Inhibitory effect of antibody to cytochrome P450 on NADPH-initiated Fe (III)–independent protein carbonyl formation in nuclear membrane and microsomes

[Conditions of incubation and estimation of carbonyl group are described under ‘Materials and Methods’; 0.2 mg protein of γ-globulin from the immune/preimmune serum was used. Values are mean±SD of four independent determinations (P<0.05)]

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<td>NADPH</td>
<td>0.86±0.07</td>
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<tr>
<td>γ-Globulin from immune serum</td>
<td>0.33±0.04</td>
</tr>
<tr>
<td>γ-Globulin from preimmune serum</td>
<td>0.86±0.08</td>
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Fig. 7—Time-dependent oxidation of NADPH by nuclear membrane and microsomes in the absence of free iron [Incubation mixture (1 ml) contained nuclear membrane or microsomal suspension (1 mg of protein) in 100 mM Tris-Cl buffer (pH 7.5) and 20 µM desferrioxamine. Reaction was initiated by adding 240 nmoles of NADPH and incubated at 37°C; 200 µl of aliquots were withdrawn at different time intervals, added to 1 ml of spectroscopic grade cold ethanol, centrifuged and NADPH oxidation was measured in the supernatant by recording absorbance at 340 nm. A control was kept using NADPH in the absence of nuclear membrane or microsomal suspension. (♦), nuclear membrane; (●), microsomal membrane. Values are mean of four independent determinations; S.D. < 10% (P<0.05)]

Discussion

Proteins in a cell get oxidatively modified by reactive oxygen species such as hydroxyl radical (•OH), alkoxyl radical (RO•) and hydrogen peroxide (H2O2), that are formed in an aerobically respiring cell. The oxidatively modified proteins become highly susceptible to proteolytic degradation by various proteases. All the metal ion catalyzed...
oxidation (MCO) systems, thus far described, depend upon the presence of free transition metal ions in the incubation system, where ascorbic acid acts as a pro-oxidant. On the other hand, free metal ion-independent oxidative modifications of microsomal proteins produced by NADPH-P450 reductase/P450/O₂ system is exclusively prevented by ascorbic acid.

In the present study, we have demonstrated that besides microsomal membranes, nuclear membranes also contain P450 and NADPH-P450 reductase. Also, the free metal ion-independent protein oxidation is exclusively prevented by ascorbic acid. Other antioxidants such as glutathione, SOD, CAT, histidine, α-tocopherol, β-carotene, probucol and thiourea are ineffective. We have also shown that addition of antibody to P450 in the incubation mixture prevents protein oxidation. All these data clearly indicate that protein oxidation and proteolytic degradation in the nuclear membrane and their prevention by ascorbic acid take place by the mechanism similar to that proposed before. The initial step is reduction of P450.Fe³⁺ to P450.Fe²⁺ by NADPH-P450 reductase. Subsequently, P450.Fe³⁺ reacts with molecular oxygen to produce P450.Fe³⁺O₂⁻, which equilibrates to the perferryl radical P450.Fe³⁺O₂⁻, a reactive oxygen species, which abstracts hydrogen from amino acid residues, producing a carbon centered radical with subsequent conversion to imino derivatives. The latter undergo spontaneous hydrolysis, introducing carbonyl groups in the protein. The exclusive prevention of the P450-mediated free metal ion-independent protein oxidation by ascorbic acid may be explained by the consideration that the perferryl radical, P450.Fe³⁺O₂⁻ is reduced and inactivated by ascorbic acid and not by GSH, α-tocopherol and other antioxidants used.

NADPH-initiated oxidative damage of nuclear membrane proteins may have significant implication, as it will affect the traffic of cytoplasmic enzymes and proteins required in the nucleoplasm for DNA replication and repair, transcription and RNA processing. This would also affect the passing out of mRNA precursors through the nuclear pores. Moreover, the cell nucleus contains a host of different proteins including histones, which have important functions in genetic regulation. If the nuclear proteins come in contact with the nuclear membrane, they may be susceptible to oxidation by the NADPH-P450 reductase/P450 system. All these events may ultimately lead to disruption of the finely tuned machinery of the gene regulation of the cell.

The observation that ascorbate prevents P450-mediated oxidative damage of the nuclear membrane has important significance. Though frank scurvy is rare now a days, sub-clinical ascorbic acid deficiency is common in many parts of the world, particularly involving the nursing mother, infant and the elderly. Sub-clinical ascorbic acid deficiency relates to a state of low tissue level of ascorbic acid, which causes incipient biochemical changes, without showing any apparent clinical symptom. Sub-clinical ascorbate-deficient guinea pigs undergo lipid peroxidation and oxidative damage of proteins in different tissues, namely, liver, heart, lung and kidney. The results obtained with guinea pigs may be applicable to humans, as they lack L-gulonolactone oxidase activity and cannot synthesize ascorbic acid.

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References
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