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Ascorbic Acid Prevents Lipid Peroxidation by Sparing Nitric Oxide in Fibroblast Cells

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As an antioxidant, ascorbic acid's primary role is to neutralize free radicals and nitric oxide is an effective chain-breaking antioxidant in free radical-mediated lipid peroxidation. The goal of the present study was to determine the interaction between ascorbic acid and nitric oxide as cellular antioxidants especially where nitric oxide acted as an antioxidant for H_2O_2 induced lipid peroxidation in mouse fibroblast cells. All experiments were carried on mouse fibroblast cell line in five groups: (1) control (2) L-NAME (100 nM) (3) ascorbic acid (500 µM) + L-NAME (4) H_2O_2 (1mM) (5) ascorbic acid + H_2O_2. Nitrate and nitrite levels in the samples were determined spectrophotometrically, based on the reduction of nitrate to nitrite by VCl_3 and Griess reaction. Malondialdehyde and ascorbic acid levels were determined by HPLC. The present findings show that nitric oxide acts as an effective chain-breaking cellular antioxidant in free radical-mediated lipid peroxidation and ascorbic acid both directly and through sparing of nitric oxide protects cells against oxidant stress.

Key Words: Ascorbic acid, Lipid peroxidation, Nitric oxide, Mouse fibroblast cell.

INTRODUCTION

Ascorbic acid, commonly known as vitamin C plays significant functions in the human body, though its function at the cellular level is not very clear. Vitamin C acts as a potent water-soluble antioxidant in biological fluids by scavenging physiologically relevant reactive oxygen species and reactive nitrogen species and may thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids and proteins. Vitamin C is the most effective water-soluble antioxidant in human plasma against lipid peroxidation induced by aqueous peroxyl radicals. In addition to scavenging reactive oxygen species and reactive nitrogen species vitamin C can regenerate other small molecule antioxidants[1-3].

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There is great interest in the role of nitric oxide (NO) in biology because it can be a signalling molecule, a toxin, a pro-oxidant and a potential antioxidant. However, its diverse chemistry and its biological activity sometimes are seemingly contradictory. Nitric oxide has been proposed to act as a pro-oxidant at high concentrations or when it reacts with superoxide, forming the highly reactive peroxynitrite. On the other hand, nitric oxide can also inhibit oxidation, $NO^{\bullet}$ can terminate chain reactions during lipid peroxidation defined as the oxidative deterioration of lipids containing two or more carbon-carbon double bonds. Nitric oxide can serve as a chain-terminating antioxidant by reacting with chain-carrying peroxyl radicals. The possible reaction is:

$$LOO^{\bullet} + NO^{\bullet} \rightarrow LOON = O \ (k = 1\text{-}3 \times 10^{9} \ M^{-1} \ s^{-1})^{4\text{-}6}$$

The goal of the present study was to determine the interaction between ascorbic acid and nitric oxide as cellular antioxidants especially where nitric oxide acted as an antioxidant for H_2O_2 induced lipid peroxidation in mouse fibroblast cells.

EXPERIMENTAL

All the reagents and chemicals used in this experiment were of analytical grade of highest purity. All organic solvents were at HPLC grade. The chemicals were purchased from Merck or Sigma Chemical Co.

Cell cultures: The mouse fibroblast cell line L929 (American Type Culture Collection CCL-1) was cultured in RPMI 1640 medium (Biological Industries) supplemented with 2 mM L-glutamine (Biological Industries), 100 units/mL penicilin (Biological Industries), 100 mg/mL streptomycin (Biological Industries) and incubated 37 ºC in a humidified atmosphere of 95 % air and 5 % CO_2. After incubation, cells were diluted in fresh medium and seeded into 30 well plates (3 mL volume/well).

Incubation of fibroblast cell line with L-NAME and ascorbic acid: Six wells were used for 500 µM concentration of ascorbic acid. After incubation for 6 h, one concentration of L-NAME, 100 nM (final concentration) was added all wells and incubated at 37 ºC for 18 h[7,8].

Incubation of fibroblast cell line with H_2O_2 and ascorbic acid: Six wells were used for 500 µM concentration of ascorbic acid. After incubation 6 h, one concentration of H_2O_2, 1 mM (final concentration) was added all wells and incubated at 37 ºC for 18 h[9].

At the time of the experiments, culture media were removed and cells were detached with trypsin/EDTA for 1 h at 37 ºC. Each cell suspension was sonicated for 1 min and centrifuged at 18,000 g for 10 min at 4 ºC. The supernatants were stored at -20 ºC until further analysis.

Measurement of nitric oxide levels: Nitrate levels in the samples were determined spectrophotometrically, based on the reduction of nitrate to nitrite by VCl_3. Nitrite levels were measured by the Griess reaction. The absorbance of each sample was determined at 540 nm. Sodium nitrite and nitrate solutions (1, 10, 50, 100 µM) were used as standards[10,11].

Measurement of total malondialdehyde (MDA) levels

Sample preparation: The HPLC method used after some modifications of that described by Turkozkan *et al.*[12] for total MDA measurement. In brief, for alkaline hydrolysis of protein bound MDA, 200 µL 6 M NaOH were added to 1 mL supernatant and the sample was incubated at 60 ºC water bath for 45 min. An aliquot of 1 mL was diluted with an equal volume of acetonitrile to precipitate proteins. The resulting suspension was then vortex-mixed for 30 s and centrifuged at 15,000 g for 10 min. The upper clear supernatant (0.25 mL) was transferred to a Eppendorf tube, mixed with 25 µL DNPH solution (5 mM in 2 M HCl) and incubated for 10 min at room temperature and dark. After derivatization, the sample was filtered through a 0.2 µm filter. Aliquots of 20 µL were injected into the HPLC system.

Preparation of standard curve: A MDA standard was prepared by dissolving 25 µL of 1,1,3,3-tetraethoxypropane (TEP) in 100 mL of water to give a 1 mM stock solution. The working standard was prepared by hydrolysis of 1 mL of TEP stock solution in 50 mL 1 % sulfuric acid and incubated for 2 h at room temperature. The resulting MDA standard of 20 mmol/mL, was further diluted with 1 % sulfuric acid to yield the final concentrations of 5, 2.5, 1.25, 0.625 and 0.3125 nmol/mL to get the standard curve for the estimation of total malondialdehyde.

HPLC analysis: The samples were analyzed on an Thermo Finnigan HPLC apparatus. The analytical column was ODS 2 C18 (5 µm particle size, 125 × 4 mm). The mobile phase was acetonitrile:distilled water:acetic acid (38:62:0.2, v/v/v). The HPLC run apparatus was isocratic with a flow rate 1 mL/min and the UV detector was set at 310 nm. MDA peaks were determined according to its retention time and confirmed by spiking with added exogenous standard[12].

Measurement of ascorbic acid levels

Sample preparation: Measurement of ascorbic acid was accomplished by HPLC, using the method described by Emadi-Konjin *et al.*[13]. The supernatants were filtered through a 0.2 µm filter. Aliquots of 10 µL were injected into the HPLC system.

Preparation of standard curve: Ascorbic acid standard stock solutions (1000 µg/mL) were prepared fresh daily in 10 % *meta*-phosphoric acid and was further diluted with 10 % *meta*-phosphoric acid to yield the final concentrations of 1, 5, 10, 50 and 100 µmol/L to get the standard curve.

HPLC analysis: A Kromasil C18 analytical column (particle size 5 µm, 25 × 0.46 cm) was used with 0.2 M KH_2PO_4/H_3PO_4 (pH 3.0) containing 2 mM EDTA as the mobile phase. Detection was at 245 nm, the flow rate was 1.0 mL/min and autosampler was set at + 4 ºC[13].

Statistical analysis: Statistical analyses were performed using a software program (SPSS 12.0 for windows, Chicago, IL, USA). Basic parameters were compared among and between groups by Kruskal Wallis and Mann-Whitney U test as appropriate. Values of $p < 0.05$ were considered to be significant. Results were presented as means ± SD.

RESULTS AND DISCUSSION

Results of all experimental groups are given in Table-1.

TABLE-1
LEVELS OF NITRIC OXIDE, MALONDIALDEHYDE (MDA) AND ASCORBIC ACID IN ALL EXPERIMENTAL GROUPS (n = 6)

	Nitric oxide (μmol/10^5 cells)	MDA (μmol/10^5 cells)	Ascorbic acid (μmol/10^5 cells)
Control	12.500 ± 1.705	0.001 ± 0.001	0.130 ± 0.030
L-NAME	8.750 ± 1.640[a]	0.020 ± 0.009[a]	0.001 ± 0.001[a]
Vitamin C + L-NAME	7.200 ± 1.240[a]	0.001 ± 0.001[b]	0.001 ± 0.001[a]
H_2O_2	6.510 ± 1.428[a]	0.170 ± 0.028[a]	0.001 ± 0.001[a]
Vitamin C + H_2O_2	6.760 ± 1.523[a]	0.060 ± 0.034[a,c]	0.001 ± 0.001[a]

[a]$p < 0.05$, compared to the corresponding value of control group.
[b]$p < 0.05$, compared to the corresponding value of L-NAME (100 nM) group.
[c]$p < 0.05$, compared to the corresponding value of H_2O_2 (1 mM) group.

Nitric oxide is produced by a variety of human and animal cells including fibroblasts and is involved in a broad array of physiological and pathophysiological processes. In various studies it was demonstrated that fibroblasts derived from human and animal cells spontaneously produce nitric oxide. In present study production of significant amounts of nitric oxide by mouse fibroblast cells was demonstrated in control group. The small amount of ascorbic acid originated from culture medium was also measured in this group[14,15].

There is great interest in the role of nitric oxide in biology because it can be a signalling molecule, a toxin, a pro-oxidant at high concentrations or when it reacts with superoxide, forming the highly reactive peroxynitrite and a potential antioxidant. In this study, when L-NAME was added into the culture medium and synthesis of nitric oxide was inhibited malondialdehyde formation which is one of the end products of lipid peroxidation was observed. Affiliated to it's quantity, lessening the antioxidant effect of nitric oxide may be the major cause of malondialdehyde formation. On the other hand in this group, ascorbic acid level was decreased. Addition of ascorbic acid into this culture medium resulted in an obvious reduction of malondialdehyde. These findings show that nitric oxide really acted as an cellular antioxidant and ascorbic acid both directly and through sparing of nitric oxide protected cells against oxidant stress[4,16-18].

Recently, nitric oxide has been proposed to act as an effective chain-breaking antioxidant in free radical-mediated lipid peroxidation among its important biological functions and many studies showing the protective effect of nitric oxide on lipid peroxidation have been published.

In present study, when H_2O_2 was added into culture medium and lipid peroxidation was induced a decrease in the amount of nitric oxide was observed. Supporting other studies, this finding was interpreted as nitric oxide was an effective

chain-breaking antioxidant in free radical-mediated lipid peroxidation. Unfortunately effort of nitric oxide by itself was not sufficient to prevent the constitution of lipid peroxidation. After the addition of ascorbic acid into the culture medium nitric oxide reacted rapidly with peroxyl radicals as a sacrificial chain terminating antioxidant to a certain extend. At this point as mentioned above ascorbic acid both directly and through sparing of nitric oxide prevented lipid peroxidative damage due to oxidant stress in culture[4-6,19,20].

In conclusion these findings show that nitric oxide can act as an cellular antioxidant and ascorbic acid can spare nitric oxide in culture cells and ascorbic acid with nitric oxide can protect the cells from lipid peroxidation.

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