EFFECT OF L(+)ASCORBIC ACID ON IN VITRO BIOSYNTHESIS OF NITRIC OXIDE IN HEPATOCYTES DURING OXIDANT HOMEOSTASIS CHANGED BY NITRATES

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Abstract

The effect of L(+)ascorbic acid and/or sodium nitrate in nitric oxide production by hepatocytes in vitro was studied. It was demonstrated that the compounds added into an incubation medium stimulated nitric oxide production in hepatocytes. The highest concentration of nitric oxide was assayed after the incubation of the cells with sodium nitrate and L(+)ascorbic acid. It was found that the addition of L(+)ascorbic acid to isolated hepatocytes supplemented with sodium nitrate resulted in time-dependent increase in nitric oxide production in the hepatocytes. We can suppose that the nitric oxide production in hepatocytes may be the result of defence against oxidative stress induced by L(+)ascorbic acid and sodium nitrate.

Key words: hepatocytes, ascorbic acid, nitric oxide, sodium nitrate.

Ascorbic acid is considered as a dietary supplement, partly because of its antioxidant properties. However, at the present time, it is known that ascorbic acid can be both a low molecular weight antioxidant and a prooxidant (4, 12, 14, 15, 46). Ascorbic acid is a water-soluble compound thought to be one of the least toxic antioxidants identified in mammalian systems (38). This acid is a major water-soluble antioxidant found in aqueous compartments of cells and extracellular fluids such as blood plasma. The compound is effective at scavenging the majority of soluble radicals and is involved in the subsequent regeneration of α-tocopherol by reducing the α-tocopheryl radical at the membrane surface. It plays a prominent role in the protection of biological systems such as the inhibition of oxidative modification of low-density lipoproteins in humans (22). Ascorbic acid prevents oxidation of variety of biomolecules too (18). Antioxidative activity of ascorbic acid was observed in the blood of cows affected with mastitis (23) and the antioxidant should be added to the feed of pregnant cows in order to prevent mastitis, metritis, and placenta retention (51). Ascorbic acid may be beneficial for rats with the septic syndrome (2). It may also ameliorate the biochemical parameters in diabetic rats (1) and limit pancreatic and hepatic damage produced during acute pancreatitis, via restoring tissue antioxidant enzyme activities (17). A megadose of ascorbic acid alleviates the severity of vitamin B12 deficiency in the rat (45). Ascorbic acid may also function as prooxidant, promoting oxidative damage to DNA (42). High doses of vitamin C can be effective in the therapy (as prooxidant) of cancer (31).

Nitrates are one of the chemical compound groups that human body is exposed to. Nitrates can come from water, food, and drugs (10, 48) and can be dangerous when they undergo chemical reactions in the organism, which can take place e.g. in the liver, during metabolic process (11, 27). Because the nitrogen can have different oxidation numbers and nitrates can undergo biotransformation process in human body, we can suppose that during this process nitrogen oxidation number can be changed. In this situation we assume, that nitrates can take part in redox reactions in cells, which can lead to the production of reactive oxygen species, or reaction with them. One of the reactive oxygen species is nitric oxide, which can be induced into various human tissues (29, 30). The nitric oxide has a variety of biological functions. Its presence can cause metabolic, physiological, and pathological modifications, among others, in hepatic cells (5, 20, 24, 26, 33, 44). The liver plays a central role in nitrogen metabolism. It is known that the nitric oxide is formed by different liver cell types, including hepatocytes, during endotoxaemia and inflammation (41). In the liver, the effect of nitric oxide on oxidative stress seems to be complex and paradoxical because both its prooxidant and antioxidant properties have been reported (8). As an antioxidant, nitric oxide could “scavenge” superoxide anion by removing it through the rapid formation of peroxynitrite (25, 37). On the other hand, the nitric oxide as a prooxidant could lead to secondary formation of highly oxidising molecules. Nitric oxide and superoxide anion react to...
form a peroxynitrite anion that can be protonated rapidly to yield two other oxidising molecules, i.e. nitrogen dioxide and hydroxyl radical-like (25). Peroxynitrite is capable of oxidising aminoacids and proteins (34), lipids (35), and deoxyribose (6).

During the nitric oxide biosynthesis, i.e. from nitrates, nitrogen oxidation number is changed. The changes are caused by redox reactions in cells. It is interesting to find out, whether and how much L(+)ascorbic acid can modify nitric oxide production from nitrates. Therefore, the aim of our studies was the attempt to find the intensity of nitric oxide production in hepatocytes incubated in vitro with sodium nitrate and/or with L(+)ascorbic acid.

Material and Methods

Hepatocytes were isolated from the liver of 3-month-old Wistar rats by the two-step collagenase perfusion method according to Berry and Friend (7), modified by Seglen (40) and Wagle (49). The cells were used if viability was greater than 95% (assessed by trypan blue exclusion) according to Page et al., (32).

The cells were resuspended in sterile Hepatocyte Medium (1 million cells per millilitre) and incubated for 6 h. After that time, the medium was replaced. The different media used were sterile solutions containing 0.9% saline and sodium nitrate (1 µmol/L) and/or L(+)ascorbic acid (1 mM). The samples were incubated in an incubator (type BB16 - Heraeus, Germany). The process was done at 37°C in humidified atmosphere containing 5% CO2. The times of hepatocytes incubation were: 30, 60, 90, 120, 180, 360, and 720 min. The viability of the cells and nitric oxide concentration were determined. The nitric oxide concentration was determined with the test sets “Total Nitric Oxide Assay” (R&D Systems, USA). The optical density of the samples was determined with the use of a microplate reader (ELX 800 – Biotek, USA) that was assayed during all the incubation times. The greatest increase in nitric oxide production was observed during the first 30 min after the onset of hepatocyte incubation. During the subsequent 90 min of incubation, the nitric oxide production slowly increased, reaching a maximal nitric oxide concentration of 310.9 ± 15.9 nmol/g protein at 120 min of incubation (Fig. 1).

Effect of sodium nitrate and L(+)ascorbic acid (used together) on nitric oxide production in hepatocytes. In hepatocytes, which were exposed to sodium nitrate (10-6 mol/dm³ concentration) and L(+)ascorbic acid (10-3 mol/dm³ concentration), significantly higher nitric oxide concentrations were assayed during all the incubation times. The greatest increase in nitric oxide production was observed during the first 30 min after the onset of hepatocyte incubation. During the subsequent 90 min of incubation, the nitric oxide production slowly increased, reaching a maximal nitric oxide concentration of 310.9 ± 15.9 nmol/g protein at 120 min of incubation (Fig. 1).

Statistically significant differences (P<0.001) were observed between control cells and cells incubated with L(+)ascorbic acid.

Effect of L(+)ascorbic acid on nitric oxide production in hepatocytes. The nitric oxide production by hepatocytes 30 min after exposure to L(+)ascorbic acid (10-3 mol/dm³ concentration) was only 2 times higher than in control cells (Fig. 1). A marked increase in nitric oxide production was observed during the first 30 min after hepatocyte incubation, and the concentrations increased during the subsequent 300 min. The highest (276.4 ± 9.62 nmol/g protein) concentration of nitric oxide in the cells was observed at 360 min of incubation (Fig. 1). The differences between nitric oxide concentration in hepatocytes incubated with sodium nitrate and the nitric oxide concentration in the control cells were statistically significant (P<0.001).

Results

Effect of sodium nitrate on nitric oxide production in hepatocytes. During the incubation of hepatocytes with sodium nitrate (10-6 mol/dm³ concentration), the nitric oxide concentrations were 10-20 times higher than those observed in the control cells (Fig. 1). A marked increase in nitric oxide concentration was observed during the first 30 min after hepatocyte incubation, and the concentrations increased during the subsequent 330 min. The highest (276.4 ± 9.62 nmol/g protein) concentration of nitric oxide in the cells was observed at 360 min of incubation (Fig. 1). The differences between nitric oxide concentration in hepatocytes incubated with sodium nitrate and the nitric oxide concentration in the control cells were statistically significant (P<0.001).

Estimation of relation between nitric oxide production at 30 min and at other times of hepatocyte incubation with the studied substances. During the estimation of changes between nitric oxide production by hepatocytes at 60, 90, 120, 180, 360, and 720 min after exposure to sodium nitrate and/or L(+)ascorbic acid, and its production by these cells at 30 min after exposure to the studied substances, it was observed that only L(+)ascorbic acid caused a significant increase in nitric oxide production during 60-720 min period of hepatocyte incubation. The highest (over 9-fold) increase in nitric oxide production at 180 min of incubation was observed. On the other hand, little differences of nitric oxide production by hepatocytes at 60, 90, 120, 180, 360, and 720 min after exposure to other substances in comparison with the nitric oxide production at 30 min of hepatocyte incubation were observed (Fig. 2).
Fig. 1. Effect of L(+)-ascorbic acid and/or sodium nitrate on nitric oxide production by hepatocytes. Control group – 0.9% NaCl (saline). Results are the mean ± SD of three experiments.

Fig. 2. Relation between nitric oxide production by hepatocytes 60, 90, 120, 180, 360, 720 min after exposure to sodium nitrate and/or L(+)-ascorbic acid. Nitric oxide production in 30 min of incubation we calculated as 100%. Results are the mean ± error of measurement.

Discussion

The aim of our study was to determine if and how much L(+)-ascorbic acid modifies the nitric oxide production in hepatocytes incubated in vitro with sodium nitrate. In this report, we demonstrated that both sodium nitrate and L(+)-ascorbic acid increased the nitric oxide synthesis in the incubated hepatocytes. The highest concentration of nitric oxide was observed after incubation of the cells with sodium nitrate and L(+)-ascorbic acid. We observed that addition of L(+)-ascorbic acid to isolated hepatocytes supplemented with sodium nitrate resulted in time-dependent increase in nitric oxide production. These results suggest that sodium nitrate and L(+)-ascorbic acid are possible exogenous sources of nitric oxide that may be formed either directly or indirectly. The indirect nitric oxide production may be through the induction of nitric oxide synthase, similarly to the induction of nitric oxide synthase in hepatocytes after the exposure to cytokines.
and lipopolysaccharide, as described by Geller et al., (19). The nitric oxide may be also produced when the cells are exposed to direct NO-donors such as 3-morpholinosydnoimine (SNAP) or indirect NO-donors such as glyceryl trinitrate (GTN), molsidomine (MSD) or sodium nitroprusside (NaNP) (36).

Nitric oxide is both an endogenously generated species and the compound actively released from a variety of important drugs. Due to its endogenous generation and use as a therapeutic agent, the metabolism and fate of nitric oxide is of interest and concern. To date, most attention regarding the metabolism and fate of NO has been paid to its oxidised metabolites. Due to the reducing environment of cells, it was considered that nitric oxide may also undergo reductive metabolism as well. Therefore, the reductive metabolism of nitric oxide by hepatocytes was examined. The generation of nitrous oxide (N₂O) was used as an indication of nitric oxide reduction. Indeed, we observed that NO could be reduced to N₂O by the cytosolic fraction of hepatocytes. The N₂O production was partially inhibited by the thiol-modifying agent, N-ethylmaleimide and thiol consumption was observed via a thiol-dependent process (21).

Moreover, Mizutani et al. (28) showed that ascorbate increased nitric oxide formation by approximately 40% in a mouse macrophage-like cell activated with lipopolysaccharide and γ-interferon. The authors suggested that ascorbate increased nitric oxide production by increasing the amount of iNOS in the activated macrophages.

The above information suggests that sodium nitrate may be directly or indirectly the source of nitric oxide, and L(+)-ascorbic acid was rather the indirect source of nitric oxide generation through induction of nitric oxide synthase.

Buettner and Jurkiewicz (12), showed that ascorbate is an excellent reducing agent that is able to reduce catalytic metals such as Fe³+ and Cu²+ to Fe²+ and Cu¹+. Additionally, van der Berg et al. (47) noted that vitamin C is only able to reduce metal ions (Fe³+, Cu²+) in the water phase, which results in acceleration of radical generation. Analogous to this data, these similar prooxidant properties of ascorbic acid may be present in our incubated hepatocytes, because they are the cells having iron and copper. Besides, Sedlak and Hoigne (39) in environmental research observed that iron and copper are co-conspirators in the oxidation of organics in atmospheric waters. They also determined that reduced copper transferred an electron to iron, which in turn participates in the oxidation process. These same processes may be in the studied hepatocytes that were incubated with L(+)-ascorbic acid. Zhao and Jung (52) investigated the kinetics of deoxyribose degradation by 'OH radicals generated by the Fenton reaction in the presence of ascorbic acid, and they observed that the inclusion of ascorbic acid in the Fenton system greatly increased the rate of 'OH radicals production by regenerating Fe²⁺ from Fe³+. Consequently, the oxidized ascorbic acid (i.e. dehydroascorbic acid) and the oxidising nature of hydroxyl radical ('OH) may cause oxidative reactions in hepatocytes. Additionally, Bleau et al., (9) noted that ascorbic acid can react with oxygen to generate H₂O₂, a reaction catalysed by ferrous ion, with superoxide ion as a reactive intermediate.

On the other hand, Deutsch (16) showed that dehydroascorbic acid undergoes several reactions and interactions in aqueous solution, making it a unique compound in the ascorbate degradation pathway. Dehydroascorbic acid can be reduced to ascorbic acid or hydrolysed to diketogulonic acid and oxidised to downstream product. These described reactions may be one of possible mechanisms of redox cycling generation and generating reactive oxygen species in the cells. Nitric oxide radicals are able to react with reactive oxygen species like superoxide anions. The reaction of nitric oxide with superoxide anions yields peroxynitritre (3). The authors suggested that formation of peroxynitrite most likely depended on the exact conditions of the microenvironment, in which peroxynitrite is generated, such as relative amounts of nitric oxide and superoxide anions, pH, and carbon dioxide concentration. On the one hand, peroxynitrite is a toxic and powerful oxidising agent able to induce DNA damage, modify tyrosine residues in proteins into nitrotyrosine altering their shape and function, and induce lipid peroxidation (3, 13). Darley-Usmar et al., (13) noted that nitric oxide removes superoxide anions, which are also highly reactive molecules able to induce lipid peroxidation. Segovia-Baroni et al. (43) has shown that nitric oxide acts as a reactive oxygen species scavenger in vitro. The above information suggests that nitric oxide may possess both cytoprotective and cytotoxic properties.

These properties probably are depending on the incubation conditions and on the amount and isoform of NOS by which nitric oxide is produced. The mechanisms by which these properties are regulated are important in the maintenance of whole body homeostasis. We can suppose that sodium nitrate and L(+)-ascorbic acid (in the studied concentrations) may cause oxidative stress in incubated hepatocytes, correspondent to the reports from Sergent et al. (41) who induced the oxidative stress in hepatocytes by iron or ethanol. On the other hand, increased nitric oxide production in rat hepatocytes incubated with sodium nitrate and L(+)-ascorbic acid could lead to an inhibition of oxidative stress induced by these substances. Protective functions of nitric oxide in the liver were also observed by Taylor et al. (44). In these experiments, nitric oxide exerted a protective effect both in vivo and in vitro by blocking TNF-α-induced apoptosis and hepatotoxicity. These studies demonstrated the cytoprotective effects of nitric oxide in the liver and suggested that hepatic iNOS expression functions as an adaptive response to minimise inflammatory injury. In addition, the authors showed that NO had anti-tumour effects as well as known mutagenic effects. According to the above information, we can suppose that the nitric oxide production in hepatocytes may be results of defence against oxidative stress induced by L(+)-ascorbic acid and sodium nitrate.
References