AUMENTO DA SÍNTESE DE ÓXIDO NÍTRICO EM MACROFAGAS PERITONEAIS SOB A AÇÃO DO ÁCIDO ASCÓRBICO

INCREASED NITRIC OXIDE SYNTHESIS IN PERITONEAL MACROPHAGES UNDER THE ACTION OF ASCORBIC ACID

ПОВЫШЕНИЕ ПРОДУКЦИИ ОКСИДА АЗОТА В ПЕРИТОНЕАЛЬНЫХ МАКРОФАГАХ ПОД ДЕЙСТВИЕМ АСКОРБИНОВОЙ КИСЛОТЫ

KUROPTOVA, Zoia Veniaminovna^{1*}; BAYDER, Larisa Michailovna²; BELAYA, Olga Leonidovna³; ZUMABAEVA, Taasilkan Toktomamatovna⁴

^{1,2} Emanuel Institute of Biochemical Physics, Russian Academy of Sciences. Russia.

³ Moscow State medico-stomatological University, Department of Hospital Therapy No. 2. Russia.

⁴ Osh State University. Russia.

* Correspondence author
e-mail: zvk@sky.chph.ras.ru

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RESUMO

Foi estudado o efeito do ácido ascórbico (AA) na formação de óxido nítrico (NO) em macrófagos peritoneais de camundongos da linha SHK. A espectroscopia de RSE e UV revelou um aumento significativo na formação de óxido em uma suspensão de macrófagos com AA incubado à temperatura ambiente. Nos estudos de VHS, a hemoglobina adicionada a uma suspensão de macrófagos foi usada como uma armadilha de NO, e o óxido nítrico resultante foi detectado pela formação dos complexos de hemoglobina Heme-NO. A avaliação quantitativa mostrou que nessas condições, sob a ação de AA, $1,5 \times 10^9$ moléculas de NO por célula são produzidas em macrófagos. O aumento da síntese de óxido nítrico sob a ação do AA também foi estabelecido por espectroscopia UV no sobrenadante após a incubação do macrófago com AA. Considerando os dados conhecidos sobre o efeito citotóxico do NO em vírus e bactérias, pressupõe-se que o efeito do aumento da síntese de óxido nítrico nos tecidos peritoneal, MP tecidual e leucócitos sob a ação do AA seja o principal no efeito preventivo e terapêutico do AA no uma série de doenças, como um doente de frio.

Palavras-chave: *óxido nítrico, ácido ascórbico, macrófagos peritoneais, método ESR, complexos nitrosil Hb Hem-NO.*

ABSTRACT

The effect of ascorbic acid (AA) on the formation of nitric oxide (NO) in peritoneal macrophages of SHK line mice was studied. ESR and UV spectroscopy revealed a significant increase in oxide formation in a suspension of macrophages with AA incubated at room temperature. In ESR studies, hemoglobin added to a suspension of macrophages was used as a NO trap, and the resulting nitric oxide was detected by the formation of hemoglobin complexes Heme-NO. The quantitative assessment showed that under these conditions, under the action of AA, 1.5×10^9 molecules of NO per cell are produced in macrophages. The increase in nitric oxide synthesis under the action of AA was also established by UV spectroscopy in the supernatant after incubation of MP with AA. Considering the known data on the cytotoxic effect of NO on viruses and bacteria, it is assumed that the effect of increasing nitric oxide synthesis in peritoneal, tissue MP and leukocytes under the action of AA is the main in the preventive and therapeutic effect of AA in a number of diseases, such as a sick of cold.

Keywords: *nitric oxide, ascorbic acid, peritoneal macrophages, ESR method, nitrosyl complexes Hb Hem-NO.*

АННОТАЦИЯ

Исследовано влияние аскорбиновой кислоты (АК) на процесс образования оксида азота в перитонеальных макрофагах мышей линии SHK. Методами ЭПР- и УФ-спектроскопии было

зарегистрировано значительное повышение образования оксида в инкубированной при комнатной температуре суспензии макрофагов с АК. В исследованиях методом ЭПР качестве ловушки NO использовали гемоглобин, добавленный в суспензию макрофагов, и образующийся оксид азота регистрировали по образованию нитрозильных комплексов гемоглобина Гем-NO. Количественная оценка показала, что в этих условиях, под действием АК в макрофагах вырабатывается $1,5 \times 10^9$ молекул NO на клетку. Увеличение синтеза оксида азота под действием АК было установлено также методом УФ-спектроскопии в надосадочной жидкости после инкубации МФ с АК. Учитывая известные данные о цитотоксическом действии NO на вирусы и бактерии предполагается, что эффект увеличения синтеза оксида азота в перитонеальных, тканевых МФ и лейкоцитах под действием АК является основным в профилактическом и лечебном действии АК при ряде заболеваний, в частности, при простудных заболеваниях.

Ключевые слова: оксид азота, аскорбиновая кислота, перитонеальные макрофаги, метод ЭПР, нитрозильные комплексы Hb Гем-NO.

1. INTRODUCTION

In the 80s, it was found that activated MP, cultured together with target tumor cells, kill target cells (Krahenbuhl and Remington, 1974). And the agent responsible for the death of tumor cells is nitric oxide (NO) synthesized by macrophages. The participation of NO in the cytotoxic activity of macrophages to tumor cells was one of the first functional properties described for this molecule (Hibbs *et al.*, 1987a, 1987b; Stuehr and Marletta, 1987b; Stuehr and Nathan, 1989). It was shown that inhibition of NO synthesis in macrophages leads to a decrease in the antitumor resistance of the body, and the discovery of this basic fact made a great contribution to the increased number of studies devoted to the formation and function of NO in normal and pathological conditions. Although there are various, sometimes contradictory data on the role of NO in the development of tumors, it has definitely been established that NO synthesized by leukocytes and macrophages plays a main role in their ability to destroy tumor cells (Stuehr and Nathan, 1989; Hibbs *et al.*, 1987a; Stuehr and Marletta, 1987a; Keller, 1993; Farias-Eisner, *et al.*, 1994).

It was shown that sustained continuous production of NO in macrophages ensures their cytotoxic/cytostatic activity not only to tumor cells, but also to viruses, bacteria, fungi, protozoa, and helminths (Croen, 1993; Bi and Reiss, 1995; Vazquez-Torres *et al.*, 2000; Sibille and Reynolds, 1990; Nathan, 1995).

On cell lines and mouse, lines were found that resistance to microbes is often associated with the expression of the induced NO synthase (iNOS). More direct evidence of the iNOS role was obtained first by Hibbs J. and his colleagues (Hibbs *et al.*, 1987a, 1987b), and then by many

other researchers. They used iNOS inhibitors and showed that inhibitors worsen the course of diseases caused by many viruses and bacteria. The mechanisms of nitric oxide action were established. They included inhibition of mitochondrial respiration (MR) and DNA synthesis, iron loss by cells, and inhibition of enzymes involved in the Krebs cycle (Billiar *et al.*, 1989a, 1989b; Kwon *et al.*, 1991; MacMicking *et al.*, 1997; Beltran *et al.*, 2000; Fang, 1997).

NO is obviously not the only anti-pathogenic immune defense effector, but in most cases, it is either an inducer or an executor of a bactericidal program.

Therefore, the increased (NO) synthesis in the cells of the immune system is important in raising the body's resistance to infectious diseases, and, possibly, due to the available literary data, the prevention of tumor diseases.

There have been reports that in a number of cells, NO synthesis can be enhanced by the AA. Studies of the AA effect on the activity of NO synthesis in cells are few, and the data are quite contradictory. Thus, in (Nakai *et al.*, 2003), AA the role in iNOS induction and NO synthesis in macrophages of RAW 264.7 line mice was studied, and it was found that AA did not increase or decrease iNOS protein expression in these cells, in contrast to (Mizutani and Tsukagoishi, 1999) where AA increased protein induction in a macrophage-like cell line J 774.1. It has also been shown that AA inhibits iNOS induction in rat endothelial cells of the skeletal muscle (Wu *et al.*, 2002). Increased NO production in endothelial cells, animal and human leukocytes, and *E. Coli* cells were described in (Kuropteva *et al.*, 2000, Heller *et al.*, 1999; Huang *et al.*, 2000). Such inconsistency of data may depend on the type of cells. Therefore, the study of the AA effect on NO

synthesis in the cells that can express iNOS is still important.

2. MATERIALS AND METHODS

Peritoneal macrophages were isolated by the standard method of washing the contents of the abdominal cavity of SHK mice (The SHK male mice Swiss-H mice from nursery Kriukovo, Russia) with a 0.9% NaCl solution. The collected cells were washed twice with the same solution and centrifuged at 250 g for 10 min (Wasley *et al.*, 1972). The precipitate was suspended in saline. The cell concentration was 2×10^9 in 1 ml. To feed the cells, 2% serum solution of cattle was added (Biowest, France). The macrophage suspension was divided into 2 parts. AA solution (AA from Sigma) in Tris-HCl buffer with pH 7 was added to one part. AA concentration in the MP suspension was 9–10 mM. The second part to which Tris-HCl buffer was added served as a control.

MP suspensions with and without AA were incubated at room temperature. After 20 and 48 hours of incubation, samples of the supernatant were taken, and absorption was measured on the Shimadzu spectrophotometer (Japan).

To study ESR Hb solution in a concentration of 10^{-5} M was added as a NO trap. After certain periods of times of incubation, samples were taken, and specimens were prepared in the form of columns in Teflon tubes with a diameter of 3 mm and a height of 30 mm. They were frozen at liquid nitrogen temperature. Then, the specimens were freed from Teflon tubes, and macrophage suspension samples frozen at liquid nitrogen temperature were obtained in the form of columns with a diameter of 3 mm and a height of 30 mm. The ESR spectra of the prepared samples were recorded on ESR-300 spectrometer (Bruker-Analitishe-Messtechnik, Germany) at a temperature of liquid nitrogen.

3. RESULTS AND DISCUSSION

Rodent macrophages (MP) were one the first cell types for which nitric oxide (NO) formation from L-arginine was shown with the involvement of the inducible NO synthase enzyme (iNOS). In response to the administration of lipopolysaccharides (LPS) with or without gamma interferon, these cells synthesized iNOS mRNA and the enzyme itself. At the same time,

the accumulation of a significant number of nitrites and nitrates was registered (Krahenbuhl and Remington, 1974).

But it turned out that peritoneal MP isolated in a standard way, incubated at room temperature, can also synthesize a small amount of NO without additional stimulation, which could be detected by the presence of nitrites and nitrates, which are stable products of NO oxidation and are registered by UV spectroscopy upon absorption of 330–340 nm. This MP ability was used in this work to study the effect of AA on NO synthesis in these cells. UV and ESR spectroscopy methods were used for recording NO formed in MP suspension incubated at room temperature with and without AA. When studying the formation of NO by ESR, Hb was used as an NO trap, which has a high affinity to NO, and interacting with it, forms stable nitrosyl Heme-NO complexes (Heme-NO NCs), which determine the well-known ESR signal — a wide singlet with g-factor 2.02 and characteristic triplet splitting of 17 G with a g-factor of 2.01.

ESR spectra of suspension of peritoneal macrophages (MP) specimens of mice incubated with AA (10 mM) (Figure 1 (1)) and without it (Figure 1 (2)) within 48 hours are shown in Figure 1. Comparison of the spectra in Figure 1 (1) and Figure 1 (2) show that in the MP specimens incubated with AA for 48 hours, the intensive ESR signal is observed, in samples without AA, the signal is of low intensity.

Figure 1. ESR spectra of samples of a suspension of peritoneal macrophages incubated for 48 hours at room temperature: (1) with AA (10 mM in 0.01 M Tris-buffer); (2) without AA (0.01 M Tris buffer). Spectrum registration conditions: microwave power 20 mW, the amplitude of magnetic field modulation 5 G, measurement temperature 77 K

The ESR signal in MP samples incubated with AA is due to nitrosyl complexes of Heme-NO NC, and its appearance indicates the formation of nitric oxide in the studied cell systems. Figure 2 shows the change in the intensity of the ESR signal in the MP suspension incubated for 20 and 48 hours, both with and without AA.

Figure 2. Change in ESR intensity signals of peritoneal macrophages suspension incubated for 20 and 48 hours at room temperature: in with and without AA.

By 48 h, the intensity of the ESR signal of Heme-NO NC and, consequently, the amount of NO formed in the samples incubated with AA was more than three times higher than the intensity of this signal in samples without AA.

A quantitative assessment showed that in these conditions, 1.5×10^9 NO molecules are produced in macrophages per cell by 48 hours under the action of AA.

The optical absorption spectra of the supernatant of MP suspensions incubated at room temperature for 48 hours with and without AA were also analyzed. Figure 3 shows the optical absorption spectra of the MP supernatant in the region of 330-340 nm.

In samples incubated with AA (Figure 3 (b)), absorption was significantly higher (more than twice) than in supernatant samples of macrophages incubated without AA (Figure 3 (a)).

(a)

(b)

(c)

(d)

Figure 3. Spectra of optical absorption of the supernatant suspension of macrophages incubated: (a) - without AA (control); (b) - with AA; (c) - with AA + L-arginine; (d) - to samples of the supernatant (AA + arginine), the spectrum of which is shown in 3(c), immediately before the measurement, AA was added.

The incubation medium consisted of Tris buffer with the addition of 2% cattle serum solution in control samples. AA or AA + L-arginine were added in test samples, all in Tris buffer. It should be noted that the amount of nitrite formed in the samples in which the absorption spectrum is shown in Figure 3 (b) actually should really be more than registered since AA has not yet been used completely, part of the formed nitrite decomposes again under the action of AA with the formation of NO following the reactions in Equations 1 - 3 (Archer *et al.*, 1975):

And although nitrite can again be formed from the resulting NO, part of the NO is yet lost (leaves as a gas). Therefore, in experiments using the ESR method, when the formed NO molecules are accepted by hemoglobin, by 48 hours, there was a more than 3-fold increase in NO amount compared to control samples, in contrast to the data shown in Figure 3(b), when the increase was slightly more than 2 times. Figure 3(c) shows the absorption spectrum of the supernatant of macrophages incubated in the presence of AA with the addition of L-arginine (10^{-3} mM), NO- synthase substrate and an additional source of NO, and in this case the absorption value (and, therefore, the amount of nitrite formed) was higher than in samples of macrophages incubated only with AA. After addition to the supernatant of MP incubated with AA and L-arginine, the absorption spectra of which are shown in Figure 3(c), ascorbic acid at the concentration of $\geq 5 \times 10$ mM, this absorption completely disappeared (Figure 3(d)) within 2-3 minutes confirming that the absorption in the region of 330-340 nm was due to nitrite ions, which rapidly decompose under the action of AA by the decomposition reaction of nitrites under the action of AA according to the above equations (Eq. 1 – Eq. 3).

Figure 4 shows the absorption spectra of macrophage supernatant samples after incubation with AA (10 mM) recorded regarding a control cuvette containing MP supernatant incubated without AA. Absorption in the region of

330-340 nm, due to nitrite and nitrate ions, the products of NO oxidation can be observed. This is one more evidence of the increased production of NO in MF under the influence of AA.

Figure 4. The optical absorption spectrum of the supernatant suspension of macrophages incubated with AA for 48 hours, measured relative to a control cuvette containing a supernatant suspension of macrophages incubated without AA under the same conditions.

The studies performed have shown that in peritoneal MP of SHK mice, NO synthesis in small amounts proceeds continuously without additional stimulating factors. And this is not a unique case. In the 1980s of the last century before the discovery of NO synthases, the formation of nitrosyl Heme-NO complexes was always observed during ESR study of the biotransformation of various biologically active compounds in isolated mouse liver tissues, and their number increased with the time of tissue incubation (Kuropteva and Pastushenko, 1985; Zhumabaeva and Kuropteva, 1986). Different hypotheses were made on NO appearance in the studied systems, but this effect became clear only after the discovery of NO-synthases. Now it is already well known that almost all cells in the liver tissue are able to express iNOS -

hepatocytes, Kupffer cells, endothelial cells, Ito cells. The appearance of intense signals of Heme-NO NC during incubation of liver tissues indicated that NO synthesis in liver cells proceeds continuously without additional stimulating factors. It was also reported in (MacMicking *et al.*, 1997) that some rat kidney cells or rat ovarian follicles during certain phases of the cycle, just like airway epithelium, are able to express iNOS normally. But previously, there were no reports that iNOS is expressed in MP normally, without induction.

The data obtained showed that AA is able to increase the synthesis of nitric oxide in peritoneal macrophages significantly and, therefore, activate NO-synthase. Using ESR, it was shown that after 48 hours incubation of MP with AA, the amount of NO formed was more than 3 times higher than in the samples incubated without AA. A quantitative assessment showed that each MP cell could synthesize up to 1.5×10^9 NO molecules under the experimental conditions (in 48 hours). Due to the absorption spectra of nitrites / nitrates, less increase in NO synthesis was recorded than by ESR. This is due to the fact that in the ESR experiments, all formed NO molecules are accepted by Hb, leading to the appearance of stable Heme-NO NCs, whereas when recording absorption spectra, the formed nitrites can partially be decomposed under the action of AA until all AA in the supernatant is consumed according to scheme 1.

To answer the question of the mechanisms of AA action, further studies are necessary. However, according to the data obtained and those available in the literature, it is possible to suggest that the main role of AA is to restore iNOS cofactors. NO-synthase is a carefully regulated enzyme, and for its normal functioning, 6 cofactors are necessary: FMN, FAD, NADH, BH₄, NADPH, and calmodulin connected with the active center. Moreover, tetrahydrobiopterin (BH₄), which is considered a necessary participant in the synthesis of NO as an active redox cofactor, is a key cofactor of iNOS (Heller *et al.*, 1999; Huang *et al.*, 2000; Hebel and Marletta, 1992).

This suggestion is based on the literature data on the increase in the NADPH content under the action of AA and on its participation in the maintenance of BH₄ in the reduced state. AA is necessary to prevent the irreversible oxidation of the key cofactor of the NO-synthase of tetrahydrobiopterin BH₄ to dihydrobiopterin BH₂ and thereby maintain the iNOS activity.

Really, there are experimental data showing that the increased iNOS activity is associated with maintaining the iNOS key cofactor - BH₄ in the reduced state (Nakai *et al.*, 2003; Huang *et al.*, 2000; Hebel and Marletta, 1992; Heller *et al.*, 2001; Toth *et al.*, 2002; D'Uscio *et al.*, 2003). The data obtained in (Wu *et al.*, 2002) that AA does not affect iNOS expression also confirm the suggestion of its participation as a reducing agent of the iNOS cofactors.

This assumption is indirectly supported by data on the functioning of the cycle of the NO and arginine synthesis in MP, as in the active work of the cycle, the need in BH₄ and NADPH increases significantly (Kuropteva *et al.*, 2019).

Previously there were reports on stimulation of NO formation by ascorbic acid in immunocompetent cells - animal and human leukocytes and some types of macrophages (Nakai *et al.*, 2003; Mizutani and Tsukagoishi, 1999; Kuropteva *et al.*, 2000). We believe that this effect plays a main role in the stimulation of the human immune system by ascorbate. In bacterial and viral infections, both iNOS induction and induction of humoral factors, cytokines, occur (Nathan, 1995; Bogdan, 2001). Cytokines activate NO formation in white blood cells and macrophages. NO, inhibiting the respiratory system and DNA synthesis in target cells (pathogenic bacteria, tumor cells) slows their reproduction and contributes to the death of these cells. AA, the content of which is high in leukocytes and MP, supports the process of NO production. But at the same time, AA is consumed. Therefore, in order for leukocytes and MP to be able to synthesize NO in sufficient amounts, it is necessary to maintain a sufficient level of AA in the organism.

The connection of the animal immune system with AA is confirmed by the fact that under adverse conditions in animals able to synthesize AA themselves AA immediately increases, the need in this vitamin increases several times.

The fact that AA can support the human immune system has been known for many years. AA turned to be especially effective in the prevention and control of colds. But the mechanism of AA action and its involvement in antibacterial action was earlier unclear. We believe that established in the recent

two decades effect of nitric oxide synthesis increase under AA action is the main one in the prevention and therapeutic effect of

this compound, at least for colds. The fact observed in practice.

5. CONCLUSION

Thus, it was shown that AA increases NO synthesis in the peritoneal macrophages of SHK mice. The amount of synthesized NO depends on the duration of AA activity. Maintenance of NO production in the cells of the immune system is very important since it was established that NO produced by leukocytes and macrophages is their main means for the destruction of bacteria, viruses, tumor cells, and other pathogenic organisms (Farias-Eisner *et al.*, 1994; Croen, 1993; Bi and Reiss, 1995; Vazquez-Torres *et al.*, 2000; Sibille and Reynolds, 1990; Nathan, 1995).

In mice macrophages and other immunocompetent cells, iNOS can synthesize NO for a long time after cell stimulation - for several hours or even days if there is a sufficient amount of arginine substrate and enzyme cofactors (Nathan, 1995; MacMicking *et al.*, 1997). In (Kuropteva *et al.*, 2019), data were obtained that suggested that in cells capable of expressing iNOS, there is a special cyclic process for the synthesis of arginine and nitric oxide, in which citrulline formed with iNOS can again be used for the synthesis of L-arginine and NO formation. The existence of such a cycle in MP allows us to explain how immunocompetent cells provide high necessities for L-arginine, necessary for NO synthesis. Thus, macrophages provide themselves with arginine. But in addition to arginine, oxygen and 6 cofactors, including tetrahydrobiopterin and NADPH, are also required for the normal functioning of iNOS. With the depletion of reserves of compounds that ensure the operation of this molecular machine - iNOS, NO production decreases. This results in the decreased functional activity of leukocytes and macrophages and a decrease in their cytotoxic / cytostatic effect on pathogenic organisms. As suggested above, AA action is probably associated with maintaining iNOS cofactors (in particular, BH₄ and NADPH) in the reduced working condition.

Therefore, the possibility of increasing NO production in the cells of the immune system using AA is important for increasing the effectiveness of prevention and control of bacteria, viruses, tumor cells, and other pathogenic organisms.

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