L-Arginine and nitric oxide synthesis in the cells with inducible NO synthase

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The effect of citrulline and ammonium chloride on the nitric oxide formation by peritoneal macrophages and liver tissue cells was studied using ESR spectroscopy. In *ex vivo* models, the incubation of cells capable of expressing inducible NO synthase (iNOS) with interferon-γ resulted in a moderate increase in the amount of hemoglobin—nitric oxide nitrosyl complexes (Heme—NO NCs), whereas incubation with L-citrulline and ammonium chloride increased the amount of Heme—NO NCs by an order of magnitude. It was assumed that a separate cycle of L-arginine and nitric oxide synthesis exists in the peritoneal macrophages and liver cells, with the major participants of the cycle being the inducible NO synthase enzyme (iNOS) and enzymes that synthesize L-arginine from L-citrulline and a nitrogen source. Functioning of this cycle makes immunocompetent cells with iNOS able to produce NO for a long time and in large amounts.

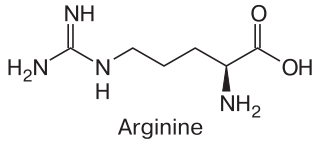
Key words: nitric oxide, arginine and nitric oxide cycle, liver cells, peritoneal macrophages, inducible NO synthase, citrulline, ammonium chloride, ESR spectroscopy.

During the last 25 years, it has been found that a simple chemical compound, nitric oxide (NO), which is formed *via* an enzymatic route in different cells, is involved in numerous biochemical processes in the human or animal body, including immune system functioning. According to recent studies, neurocirculatory dystonia (disbalance of the activities of nitric oxide synthase isoforms and other oxidative stress markers) may also lead to Alzheimer´s disease (AD),[1] apart from the "amyloid hypothesis" of AD origin. Nitric oxide is synthesized from L-arginine and oxygen by means of special enzymes, NO synthases. Three NO synthase isoforms are best known, two constitutive isoforms, that is, endothelial and neuronal NO synthases (eNOS and nNOS) and inducible NO synthase (iNOS). The former two nitric oxide synthases, eNOS and nNOS, are usually activated by increasing Ca^{2+} in the cytosol, which stimulates NO evolution within several minutes. These isoforms synthesize small amounts of NO up to tens of micromoles. The inducible NO synthase, which is expressed in macrophages (MPH) and other immunocompetent cells after mediated stimulation of immune or inflammatory response, generates millimolar NO concentrations in the cells.[2—5]

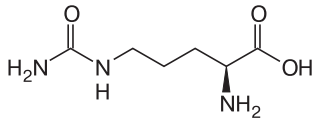
All three isoforms catalyze the formation of NO by one and the same pathway. The general scheme of nitric oxide biosynthesis can be depicted as follows:

L-Arginine ⟶ OH—L-Arginine ⟶ L-Citrulline + NO.

The transformation of L-arginine into NO and L-citrulline occurs in two stages. In the first stage, one L-arginine molecule is oxidized at the guanidine nitrogen to give L-*N*-hydroxyarginine. This stage requires participation of nicotineamide-adenine dinucleotide phosphate (NADPH) and oxygen. The second stage requires the presence of tetrahydrobiopterine (BH_4), oxygen, and NADPH and yields one NO molecule and one L-citrulline



Arginine



Citrulline

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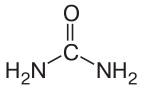
molecule. Thus, the major pathway to nitric oxide in the body is L-arginine oxidation catalyzed by NOS.

Since the reaction mechanisms are similar and the cofactor binding sites for NOS isoforms are retained, the dramatic difference between the NO production periods remains unexplained. In response to stimuli, eNOS and nNOS produce NO for several minutes, while iNOS can synthesize NO for a long period of time after cell stimulation, that is, for several hours or even days, which results in nitric oxide accumulation in a few orders of magnitude higher amounts than in the case of other isoforms.

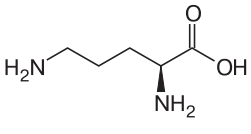
Even in the first studies that demonstrated the formation of NO, in rodent MPH, from L-arginine by means of inducible NO-synthase in response to the introduction of lipopolysacharides (LPS) with interferon-γ, these cells synthesized iNOS mRNA and the proper enzyme, which resulted in accumulation of considerable amounts of nitrites and nitrates.[2] In another study on experimental endotoxemia model,[3] the expression of iNOS mRNA was detected during 3 h after stimulation, and the maximum accumulation of iNOS and nitrites was observed by 12—16 h. In human hepatocytes[4] stimulated by cytokines and LPS, a single mRNA band was detected after 4 h. It reached a maximum within 8 h, and became almost indistinguishable after 48 h. The mRNA level was correlated with iNOS activity, which was estimated by NO_2^- and NO_3^- accumulation in the culture supernatant. The content of these anions increased 20—30-fold within 24—48 h after stimulation. To date, there are numerous studies indicating that supported long-term NO production in the MPH provides them with the cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths, and tumor cells.[6—12] In these cells, NO is used as the effector molecule.

The NO production in MPH, neutrophiles, and *E. coli* was shown to increase under the action of ascorbic acid.[13—15] It was ascertained that one peritoneal MPH cell is able to produce up to $1.5 \cdot 10^9$ NO molecules. The fact that the formation of one NO molecule requires one arginine molecule brings about the question of what is the source of arginine providing it in the required amount.

In the body of mammals, arginine is synthesized in the urea cycle of the liver from citrulline and ammonia. Arginine thus formed is rapidly decomposed by the arginase enzyme to give ornithine and urea.[16] It is believed that the synthesized arginine does not leave the cycle and cannot be used as the iNOS substrate.[17—18] In addition, arginine gets into the body with food. However, this arginine uptake route could hardly satisfy the high arginine requirement of immunocompenent cells during the fight against infection. This raises the question of how the body meets the increased arginine need of macrophages for NO synthesis?



Urea



Ornithine

The purpose of this study was to gain additional data about functioning of the cycle of arginine and nitric oxide synthesis in the cells with inducible NO-synthase under pathological conditions.

Experimental

Commercial ammonium chloride, citrulline, and L-arginine (Acros, USA) and recombinant interferon-γ (gammaferon) (Enzyme, Russia) were used in the study. Sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) (Fluka) were employed for the Griess assay.

The assays were performed using experimental animals received from the Unique Research Facility "Nursery and Vivarium" at the Institute of Problems of Chemical Physics, RAS. The animals were kept and tested in accordance with the rules adopted by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The experiments were carried out *ex vivo* on isolated mouse liver and peritoneal MPH.

Preparation of mouse liver samples. After dislocation of neck vertebrae of the animals, the liver was isolated and disintegrated into small pieces, physiological saline solution (0.9% NaCl solution) was added, and the tissue was divided into five portions. Interferon-γ in 100 IU dose was added to four portions of the tissue prepared in this way. After 30 min, arginine was added to the 1st portion, ammonium chloride was added to the 2nd portion, ammonium chloride and citrulline were added to the 3rd portion (concentration of the agents was $2 \cdot 10^{-3}$ mol L^{-1}); the 4th portion was incubated with interferon-γ only; and the 5th portion was used as the control (no additives). The samples were incubated at room temperature (~20 °C) for 19 h as described previously.[19,20] After 19 h of incubation, samples for ESR spectrometry were prepared. The pieces of liver prepared as described above were densely packed into Teflon tubes, frozen at liquid nitrogen temperature, then the contents was pushed out of the tubes by metallic rods; this gave columnar samples of 3 mm diameter and 30 mm height for ESR measurements.

Preparation of samples from peritoneal MPH. The peritoneal MPH were isolated by a standard procedure: washing out from the mouse peritoneal cavity with a 0.9% NaCl solution.[21] The collected cells were centrifuged at 250 *g* for 10 min and washed twice with a 0.9% NaCl solution. The precipitate was resuspended in a 0.9% NaCl solution to a cell concentration of $1 \cdot 10^8$ in 1 mL. The cell growth was promoted by adding 2% fetal calf serum (Biowest, France) into the incubation medium. The suspension of macrophages was divided into three portions. Interferon-γ and, after 30 min, ammonium chloride and citrulline were added to the first portion (concentration of the agents was $1 \cdot 10^{-3}$ mol L^{-1}), the second portion was incubated with interferon-γ (100 ME) only, and the third portion was used as the control (without additives). The incubation was carried out at room temperature. Pure hemoglobin, Hb ($1 \cdot 10^{-5}$ mol L^{-1}), or erythrocyte Hb was used as nitric oxide trap. In the latter case, 25 μL of freshly isolated heparinized whole blood was added to each portion of the suspension. After 20 and 40 h of incubation,

samples for ESR spectrometry were prepared from the suspension of macrophages similarly to the preparation of liver samples described above.

The ESR spectra of the samples were measured on an ESP-300 spectrometer (Bruker Analitische Messtechnik, Germany). The spectra were recorded under the following conditions: microwave power of 20 mW, magnetic field modulation amplitude of 4 G, and temperature of 77 K.

The NO concentration in the macrophage supernatant (culture medium) was determined by the Griess assay.[22] It is believed that the contents of nitrite and nitrate, which are stable products of NO oxidation, reflect the amount of the synthesized NO. The Griess reagent was prepared by mixing equal volumes of sulfanilamide (1.5% in 1 N HCl) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) (0.15% in 1 N HCl). A sample (1 mL) was mixed with the Griess reagent (1 mL). The mixture was incubated for 20 min and the chromophore absorbance at 540 nm was measured on a Shimadzu spectrophotometer (Japan).

Results and Discussion

In order to find out the possibility of provision of cells able to express iNOS and produce nitric oxide with high arginine concentrations for long periods of time, we studied the changes of metabolic paramagnetic centers in the samples of animal liver tissue prepared as described above. The liver tissue is convenient for this study, first, because the urea cycle producing arginine occurs in the liver and, second, because virtually all liver cells (hepatocytes, Kupffer cells, Ito cells, and endothelial cells) are able to induce iNOS and synthesize NO.

Figure 1 shows the ESR spectra of liver tissue samples after 30-min incubation with interferon-γ and with subsequent addition of the NO synthase substrate, L-arginine (curve *1*) or without arginine addition (curve *2*; control). In the normal state, the ESR spectra of liver samples exhibit signals for cytochrome P-450 with $g_1 = 2.42$ and $g_2 = 2.25$ and signals for components of the mitochondrial respiratory system: a singlet for flavosemiquinones in the free radical region and a signal for iron sulfur centers N-1b of NADH dehydrogenase complex (NADH is nicotinamide adenine dinucleotide) with $g = 1.94$ (see Fig. 1, curve *2*). The formation of nitric oxide was monitored by the appearance of signals for Heme—NO nitrosyl complexes (NCs) in the ESR spectra of liver samples. No additional NO traps were added, since natural liver and blood cell traps, heme proteins, were utilized. The heme groups of proteins (blood hemoglobin and cytochrome P-450 in the liver samples used) have high NO affinity and, upon binding to NO, give stable Heme—NO NCs, which are responsible for the well known characteristic broad ESR signal with $g = 2.02$ and a triplet splitting of 17 G at $g = 2.01$. While comparing curves *1* and *2* in Fig. 1, one can see that the signal for the Heme—NO NCs substantially grows after the addition of arginine. This increase in the ESR signal intensity is directly proportional to the increase in NO production in liver samples.

It follows from this experiment that if the addition of arginine induces an increase in the amount of synthesized NO, then the formation of NO in the incubation medium with interferon-γ as an iNOS inducer is limited by the substrate amount rather than by the efficiency of NO synthase. However, if the production of nitric oxide, which is highly needed for the efficient functioning of MPH, is limited by substrate depletion in the liver tissue in which arginine is directly synthesized in the urea cycle, then the cells probably cannot utilize arginine synthesized in the urea cycle for NO production, as has also been indicated in earlier publications.[17,18] We attempted to verify this conclusion by monitoring the formation of NO in liver tissue upon activation of the urea cycle. For this purpose, ammonium chloride was added to the liver tissue to activate the urea cycle. Figure 2 shows the ESR spectra of liver tissue samples incubated at room temperature in the presence of interferon-γ and ammonium chloride (curve *1*) and without ammonium chloride (curve *2*). The ESR spectra for these two cases barely differ in intensity. The subtraction of spectrum *2* from spectrum *1* resulted in a very weak Heme—NO NC signal (shown with twice higher amplification than other signals; curve *3* in Figs 2, 3, 5, and 6). This result also shows that even if a minor portion of arginine synthesized in the urea cycle can be utilized for NO synthesis, this is obviously insufficient for providing NO production in the cells with actively operating iNOS. The liver tissue cells can produce much more NO if the NO synthase substrate, arginine, is present, as can be seen by comparison of ESR spectra (see Fig. 1, curves *1* and *2*).

Further we studied the effects of citrulline as an urea cycle participant and ammonium chloride as a nitrogen

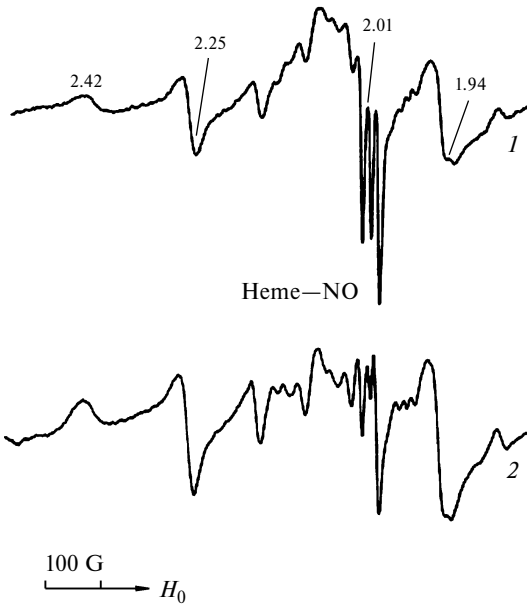


Fig. 1. ESR spectra of liver samples incubated for 30 min in the presence of interferon-γ after addition of arginine in concentration of $5 \cdot 10^{-4}$ mol L^{-1} (*1*) and without arginine (*2*).

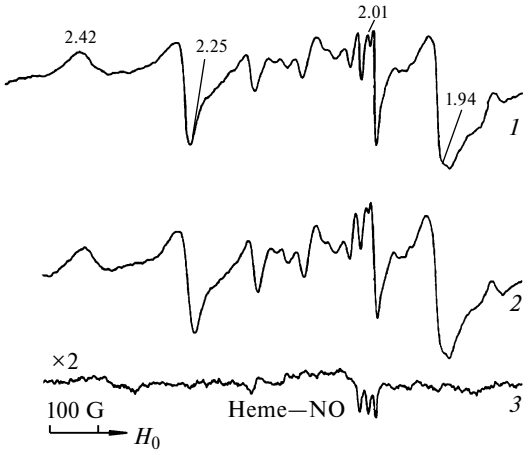


Fig. 2. ESR spectra of liver samples incubated with interferon-γ and ammonium chloride (*1*) and without additives (*2*) and difference spectrum (*3*) obtained by subtraction of spectrum *2* from spectrum *1*.

source and urea cycle activator on the NO formation. Figure 3 presents the ESR spectra of liver samples incubated at room temperature with the addition of interferon-γ, ammonium chloride, and citrulline (curve *1*) or with the addition of only interferon-γ (curve *2*). The ESR spectra of liver samples incubated in the presence of interferon-γ, ammonium chloride, and citrulline (see Fig. 3, curve *1*) exhibit an intense signal with a triplet splitting at $g = 2.01$ for the complexes of heme-containing proteins with nitric oxide, Heme—NO. This signal (see Fig. 3, curve *3*) was isolated by subtracting the ESR spectra of liver samples incubated in the presence of only interferon-γ (see Fig. 3, curve *2*) from the samples incubated in the presence of additives (see Fig. 3, curve *1*). The appearance of signals

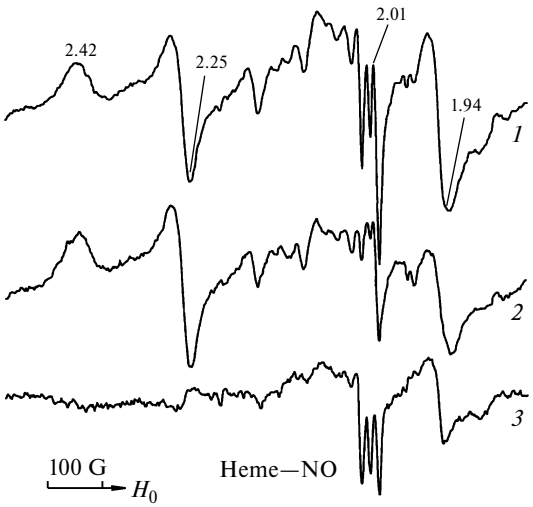
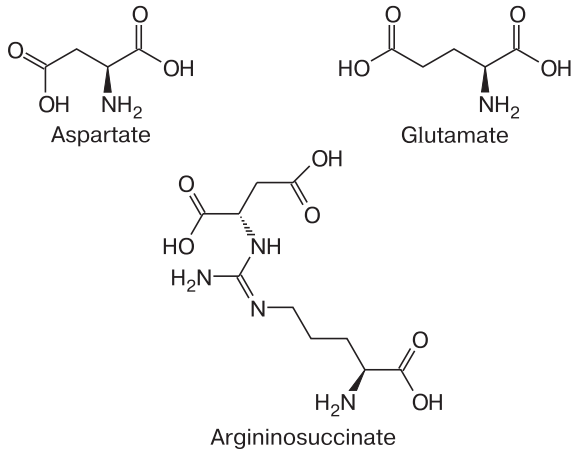


Fig. 3. ESR spectra of liver samples incubated at room temperature with interferon-γ, ammonium chloride, and citrulline (*1*) and with interferon-γ (*2*) and difference ESR spectrum (*3*) obtained by subtraction of spectrum *2* from spectrum *1*.

for the Heme—NO complexes (see Fig. 3, curve *1*) indicates that a considerable amount of nitric oxide is additionally formed in the liver tissue cells preactivated with interferon-γ in the presence of ammonium chloride and citrulline.

Thus, in the presence of a sufficient amount of a nitrogen source (in our case, ammonium ions), liver cells can directly utilize citrulline for the synthesis of arginine and NO. In other words, on the basis of our results, apart from the urea cycle, an arginine cycle functions in liver cells; it is shorter and more labile than the urea cycle and is probably coupled with the urea cycle, which is triggered only by iNOS operation. The cycle of arginine synthesis can be activated by substrates of enzymes synthesizing arginine from citrulline and a nitrogen source; in the animal body, possible nitrogen sources are ammonium, aspartate, and glutamine. A simplified scheme of the urea cycle and the hypothetical coupled arginine and nitric oxide cycle is shown in Fig. 4. The key participants of this cycle are three enzymes, namely, iNOS, which generates NO from arginine, argininosuccinate synthase (1), which forms argininosuccinate from citrulline and a nitrogen source (ammonium, aspartate, and glutamine), and argininosuccinate lyase (2), which forms arginine from argininosuccinate. This cycle is apparently triggered when iNOS is expressed and the cells require higher levels of NO. This small cycle is a sort of bypass in the urea cycle. It is quite probable that the use of arginine for NO synthesis is preferred over its use for the synthesis of urea, because nitric oxide is an important regulatory and effector molecule in the cell immunity system. The urea cycle (which is also called the ornithine cycle) provides its basic function when the body does not require large amounts of NO to suppress an infection and excretes excess nitrogen as urea.



Since the above data demonstrated the possible existence of small arginine and NO cycle in liver tissue cells, we checked the possibility of existence of a separate cycle of arginine and NO synthesis in MPH, as was reported by other researchers.[23—25] The influence of ammonium chloride and citrulline on the NO formation in peritoneal

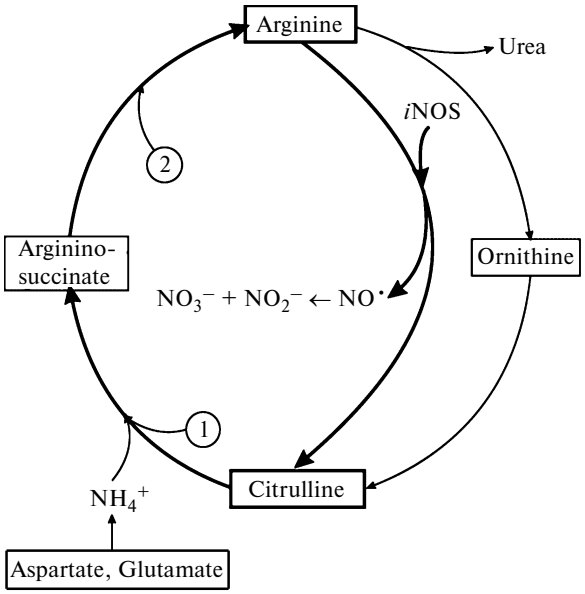


Fig. 4. Urea cycle and presumed coupled nitric oxide synthesis cycle in liver tissue with participation of inducible NO synthase (1 is argininosuccinate synthase, 2 is argininosuccinate lyase).

MPH was studied. As in the above-described experiments with liver issue, in this case, too, MPH were first stimulated by interferon-γ and then ammonium chloride and citrulline were added and the formation of nitric oxide was monitored.

Nitric oxide determination in the supernatant liquid of peritoneal MPH was performed by two methods, ESR spectroscopy and the Griess assay by the procedure described above. Figure 5 depicts the ESR spectra of MPH suspension samples incubated at room temperature for 40 h with the addition of interferon-γ: in one case, ammonium chloride and citrulline were added apart from interferon-γ (curve *1*), while in the other case, only interferon-γ was used (curve *2*, control). Figure 6 shows the ESR spectra of MPH suspension samples incubated at room temperature for 40 h with addition of whole blood and interferon-γ, *i.e.*, the same ESR spectra under the same conditions as shown in Fig. 5, but against the background of added whole blood.

The ESR spectra of MPH samples incubated in the presence of interferon-γ, ammonium chloride, and citrulline (see Fig. 5, curve *1*) exhibit an intense signal of the Heme—NO complexes. This signal differs in the spectral pattern from the Heme—NO NC spectra for the liver. This difference is attributable for the fact that, first, the signal in the liver is caused by the sum of Heme—NO and cytochrome P-450—NO NC signals. Second, the shape of the ESR spectra of Heme—NO NCs in Hb molecules depends on the conformational state of subunits.[26,27] The shape of the ESR signals of the nitrosyl complexes of Hb subunits in R- and T-conformations was described in our previous publication.[28] The ESR spectrum shown in Fig. 5 (curve *1*) mainly exhibits signals for the NCs of α- and β-subunits in the R-state (broad nonstructured signal) and the overlapping low-intensity signal of α-subunits in the T-state (signal with triplet structure at $g = 2.01$). In the liver spectra shown in Fig. 3, a large part belongs to the ESR signals of the cytochrome P-450—NO complexes and Hb conformers in the T-state (since oxygen in the liver tissue was consumed during incubation).

The appearance of an intense signal of the Heme—NO complexes in spectrum *1* of Fig. 5 indicates that nitric oxide is formed in MPH samples in the presence of ammonium chloride and citrulline. Macrophages activated by only interferon-γ also give off NO, as indicated by the appearance of an NC signal of much lower intensity in spectrum *2* in Fig. 5. In the presence of whole blood (see Fig. 6, curve *2*), the ESR signal is more intense than the signal with only interferon-γ, as blood contains both cytokines and enzyme substrates. However, the amount of these complexes in the samples incubated in the presence of citrulline and ammonia is several-fold higher (see Fig. 6, curve *1*). The increase in the amount of Heme—NO NCs indicates that NO synthesis is triggered in MPH in the

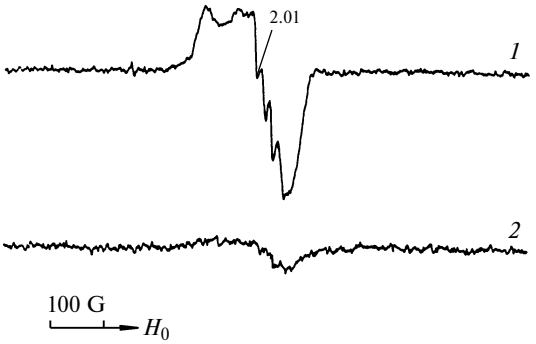


Fig. 5. ESR spectra of a suspension of peritoneal MPH after a 40-h incubation at room temperature: (*1*) in the presence of interferon-γ, citrulline, and ammonium chloride, (*2*) in the presence of interferon-γ.

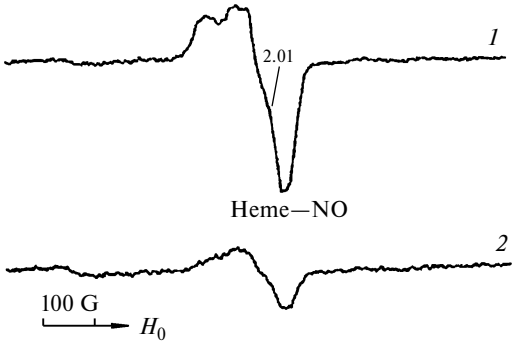


Fig. 6. ESR spectra of a suspension of peritoneal MPH after a 40-h incubation at room temperature upon the addition of whole blood: (*1*) in the presence of interferon-γ, citrulline, and ammonium chloride, (*2*) in the presence of interferon-γ.

presence of citrulline and ammonium chloride as the nitrogen source.

The nitric oxide level synthesized in MPH was also determined by the Griess assay. Macrophages were incubated in the presence of ammonium chloride, arginine, and citrulline. Figure 7 shows the results of measurement of nitrite content in the MPH supernatant. Nitrite is a stable product of NO oxidation reflecting the amount of NO synthesized by macrophages. Thus, the use of citrulline leads to nearly the same amount of MPH-based nitric oxide as the use of arginine. This implies that a special (separate) cycle of arginine and nitric oxide synthesis functions in MPH. This cycle is triggered upon iNOS induction and can be activated by enzyme substrates synthesizing arginine, *i.e.*, NO synthesis from L-arginine involving NO-synthase is probably not a linear process, but represents a closed cycle in which citrulline formed in the NO synthesis (and considered earlier to be a side product) is used again for arginine and then NO synthesis. The amount of resulting NO is still limited by the availability of substrates for arginine synthesis enzymes (other than citrulline) and by accessibility of co-enzymes of iNOS, which is a thoroughly regulated enzyme, like other NO synthases, and requires six cofactors for normal functioning: flavine mononucleotide (FMN), flavinadenine dinucleotide (FAD), NADH, BH_4, NADPH, and active site-bound calmodulin. Furthermore, effective functioning of iNOS requires oxygen. At a reduced oxygen content, enzyme operation efficiency decreases. The ability of ascorbic acid to promote the formation of NO in white blood cells and MPH, which we found previously, is probably also associated with occurrence of this cycle.[29] Ascorbic acid can participate in maintenance of iNOS cofactors (in particular, NADPH and BH_4) in the reduced state.

An increase in the NO production in the cells under the action of citrulline was also observed previously,[30] but the authors considered this cycle to be secondary and insignificant for NO synthesis. The assumption that the citrulline-involving cycle does not play a significant role in NO synthesis was mainly supported by detection of a certain (small) amount of citrulline in the cells during NO synthesis using arginine. Meanwhile, our results strongly suggest that citrulline is not completely consumed and is accumulated in the cells due to the deficiency of substrates (substrate depletion) of argininosuccinate and arginine synthesis enzymes.

Thus, the results indicate that NO synthesis from L-arginine involving inducible NO synthase in peritoneal MPH and liver cells is not a linear process, as was believed previously, but is a closed cycle in which L-citrulline formed in the NO synthesis and considered to be a side product is utilized again in the arginine and then nitric oxide synthesis. This biochemical cycle seems to function in all cells capable of iNOS expression. Functioning of a separate (small) cycle of arginine and nitric oxide synthesis in the peritoneal MPH and liver cells makes these cells able to produce NO for long periods of time in considerable amounts. The existence of arginine and NO synthesis cycles in the cells with inducible NO synthase may account for the difference between the amounts of NO formed by constitutive and inducible NO synthases.

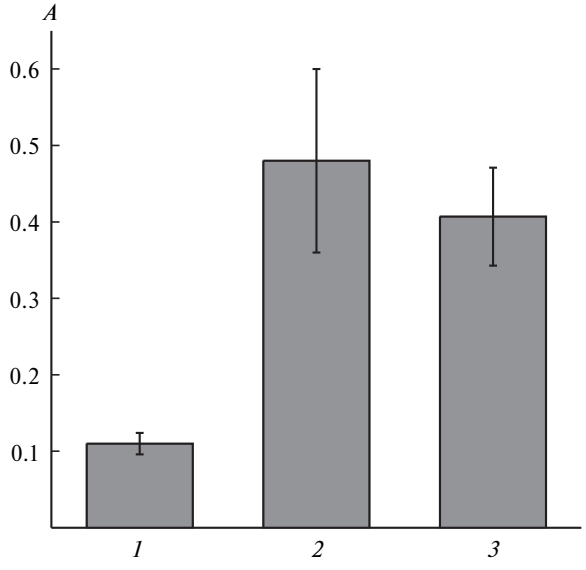


Fig. 7. Formation of nitric oxide by peritoneal MPH activated by interferon-γ during incubation at room temperature for 40 h: (*1*) control (interferon-γ); (*2*) with arginine; (*3*) with citrulline and ammonium chloride. The ordinate axis shows absorbance at 540 nm.

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