NITRIC OXIDE FORMATION IN ANIMAL TISSUES DURING INFLAMMATION

L. N. Kubrina, P. I. Mordvintsev, and A. F. Vanin

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Nitric oxide produced in the body from exogenous sources, such as from various nitro and nitroso compounds [1, 3], can activate a very important intracellular regulatory enzyme, namely guanylate cyclase [6, 10]. This activation also induces physiological reactions such as vasodilatation and a fall of blood pressure [4, 9], and weakening of platelet aggregation [5, 12]. The question accordingly arises: can nitric oxide be produced in the body from endogenous sources? Data in the literature suggest that one such source could be ammonia, formed in the course of various metabolic processes. We know that in the reaction with OH· and O_2^- radicals ammonia is oxidized to nitrite and nitrate [13]. This reaction can proceed both in an isolated enzyme system [13] and in vivo, especially during inflammation [15]. In the course of this oxidation of ammonia, nitric oxide may be formed as an intermediate product. The aim of the present investigation was to test this possibility.

EXPERIMENTAL METHOD

Complexes of iron with diethyldithiocarbamate (DETC) were used as nitric oxide traps, as in [3]. Complexes of this kind are formed in animal tissues after administration of DETC, with the participation of endogenous iron. The Fe-DETC groups bind nitric oxide with the formation of the corresponding paramagnetic mononitrosyl iron complexes (MNIC), which are characterized by an EPR signal with $g_\perp = 2.041$, $g_\parallel = 2.025$, with an allowed superfine triplet structure (STS) at $g_\perp$. Depending on the intensity of this signal it is possible to judge at least the lower level of nitric oxide concentration formed in animal tissues. Experiments were carried out on noninbred male albino mice aged 2-3 months and weighing 20-25 g. To initiate an inflammatory process the animals were given an intraperitoneal injection of 0.2 ml of an aqueous solution of lipopolysaccharide (LPS; 50-0.5 mg per animal), isolated from the cell walls of the bacterium Salmonella typhmurium (from "Difco," USA). After 2-3 h the temperature of these animals rose and they developed diarrhea. At this time the animals were given an intraperitoneal injection of 5 mg of $(NH_4)_2SO_4$ (as the source of ammonia) in 0.2 ml of water. Another group of animals received injections of the same substances by the same routes and in the same doses, but the ammonium sulfate was omitted. Control animals were given intraperitoneal injections of the same doses of $(NH_4)_2SO_4$ + Na-DETC or Na-DETC alone, in the same order, without any injection of LPS. The animals were killed 15-20 min after injection of the last agent (Na-DETC). Isolated tissues (liver, heart, spleen, and blood) were frozen in liquid nitrogen, after which their EPR spectra were recorded at 77 and 290 K on a "Rubin" radiospectrometer. In some experiments the animals were given an intraperitoneal injection of the water-soluble antioxidant Na-phenazan (the sodium salt of 3,5-di-tert-butyl-4-hydroxyphenylpropionic acid) in a dose of 2 mg in 0.2 ml per animal 1.5 h before the injection of Na-DETC.

EXPERIMENTAL RESULTS

In all the organs studied in both experimental and control animals, MNIC formation with DETC was observed. These complexes did not appear in the blood. They were found in the largest amount in the liver. In the experimental animals (treated with LPS) the content of MNIC with DETC and, consequently, the quantity of nitric oxide formed in the tissues, was 4-10 times greater than in the control animals.

The overwhelming majority of EPR measurements were made on the animals' liver. Typical EPR spectra recorded in this tissue within the range of values of the g-factor from 2.2

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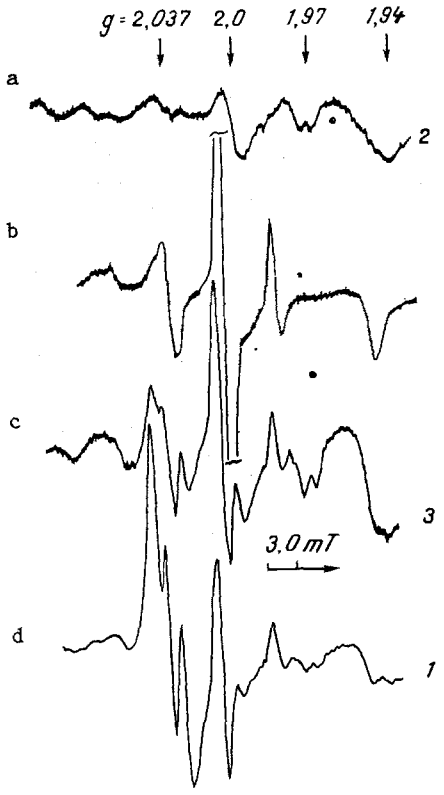

Fig.1. EPR spectra of liver tissue of original mice (a) and of mice after receiving an injection of DETC (c) or DETC + LPS (d). Recording at 77 K; relative amplification of spectrometer indicated on right. Signal at g ∿ 2.037 for c and d is due to MNIC + DETC; signal for c has STS component of Cu^{++}-DETC complex partly superposed on it; b) EPR signal of Cu^{++}-DETC complex in standard.

to 1.91, in animals of the control group and the experimental animals in the initial state, are given in Fig. 1. In the liver of the original animals (not treated with any agents, including Na-DETC) EPR signals of free radicals (g = 2.0), and of complexes of molybdenum (g = 1.97) and reduced ferrothioproteins (g = 1.94), and also the signal of Mn^{++} complexes, characterized by a 6-component STS, the three low-field components of which are located before the signal of free radicals , were recorded at 77 K. These same signals were observed in the liver of the control and experimental animals. EPR signals of complexes of DETC with Cu^{++} and also of MNIC with DETC also were recorded in the spectrum of this tissue. The content of the first of these varied sharply in different animals, and in some animals they were not found at all (Fig. 1b-d). According to data in [11], copper from the blood takes part in the formation of these complexes in vivo. The DETC-Cu^{++} complexes formed are insoluble in water and localized in the cell membranes. The EPR spectrum of these complexes was described previously [2]; it is characterized by an allowed STS at $g_{\perp}$. The EPR signal of MNIC with DETC is superposed on one of the components of ths STS, interfering with evaluation of its intensity (Fig. 1c). For evaluation of this kind we used the amplitude of the third (high-field) component of STS at $g_{\perp}$ of the EPR signal of MNIC with DETC, which is not masked by components of the signal of DETC-Cu^{++} complexes. Comparison of the third component of the EPR signal of MNIC with DETC in the tissues with the same component of the EPR signal of a standard specimen of a frozen solution of MNIC with DETC with known concentration enabled the concentration of these complexes in biological objects to be assessed. Measurements showed that on average 10^{-8} M MNIC with DETC is formed per gram wet weight of tissues in the liver of the control animals receiving or not receiving the ammonium salt (confidence interval $1.5 \cdot 10^{-9}$ M; $p < 0.05$). In a small proportion of the experimental animals (5%) this value reached $2 \cdot 10^{-7}$ M, and in the rest it averaged $5 \cdot 10^{-8}$ M/g wet weight of tissue (confidence interval $7.5 \cdot 10^{-9}$ M; $p < 0.05$). In the overwhelming majority of experimental animals it was virtually indistinguishable from the value characteristic of the liver of animals treated with LPS and DETC in the absence of the ammonium salt. Thus in the majority of animals the formation of nitric oxide, incorporated into MNIC with DETC, in the liver was virtually independent on administration of a large quantity of exogenous ammonium salt as the source of ammonia. Only in 10% of experimental animals did administration of this salt lead to an increase in the quantity of MNIC with DETC (at most up to $2 \cdot 10^{-7}$ mole/g tissue). Variation of the dose of LPS between 2 and 20 mg per animal did not affect the quantity of MNIC with DETC formed in the liver. If the dose of LPS was reduced to 0.5 mg or increased to 40-50 mg per animal, the level of these complexes fell.

In the modern view LPS of bacterial origin, initiating inflammation and diarrhea, on interacting with neutrophils, macrophages, and so on, cause the accumulation of free-radical forms of oxygen (O_2^- and OH· radicals) in the body [15]. Oxidative processes with the participation of these agents can induce the formation of nitric oxide in the body. It was therefore interesting to test the effect of inhibitors of free-radical reactions on this process. Naphenazan was chosen for this purpose. It completely inhibited the formation of MNIC with DETC in the liver of the mice. In none of the 16 experimental animals treated with phenazan could the EPR signal of MNIC with DETC be discovered against the background of the EPR signal of DETC-Cu^{++} complexes. There are thus grounds for considering a free-radical mechanism of nitric oxide formation in animal tissues in vivo.

From what compounds could this nitric oxide arise? It follows from the results that ammonia as a rule made no significant contribution to its formation. If ammonia had been actively involved in this process the yield of nitric oxide would have been increased as a result of injection of the ammonium salt into the mice, irrespective of whether the quantity of free radicals arising in vivo during inflammation limits or does not limit nitric oxide formation. In the first case, the validity of this statement will be evident. In the second case it becomes understandable if it is recalled that besides ammonia, various reducing agents present in sufficient amounts in the tissues, namely ascorbate, thiol-containing compounds, etc., may also take part in free-radical reactions as the substrate. If a large dose of ammonium salts is administered (they were injected in a dose only 5 times less than lethal), ammonia would have to compete successfully with the various reducing agents as a substrate for free-radical reactions. As a result, nitric oxide formation in the tissues would be increased. Since, as a rule, this was not observed, it remains to suggest that other agents than ammonia may perhaps serve as nitrogen-containing substrate of free-radical reactions in vivo. These could be, for example, various amino groups, and the ability of some of these to be oxidized in biological systems to nitroso groups was demonstrated in [7]. These groups may subsequently be liberated in the form of free nitric oxide [1, 3].

In this connection there may be another possible path of free-radical formation of nitric oxide in animal tissues, unrelated to O_2^- and OH· radicals arising under the influence of LPS on neutrophils, macrophages, and so on. We know that bacterial LPS can activate phosphorylase C, which hydrolyzes phosphoinositides [14], in membranes of various cells (including neutrophils and macrophages). Their breakdown products initiate a complex system of biochemical processes, leading to activation of Ca^{++}-dependent protein kinase C, to liberation of Ca^{++} from its intracellular depots, and finally, to prostaglandin formation. This last process can be coupled with oxidation of amino compounds, as was shown in [7]. This oxidation may lead to the formation of nitric oxide, which can affect various biochemical processes through activation of guanylate cyclase.

In conclusion, let us examine the possible cause of formation of small quantities of nitric oxide in the control animals, subjected only to the action of DETC. DETC is known to be a powerful inhibitor of superoxide dismutase [8], so that its administration could lead to accumulation of O_2^- radicals. The latter could initiate the same processes (although by a lesser degree), namely oxidation of nitrogen-containing compounds, as takes place more effectively on initiation of an inflammatory process in animals by injection of lipopolysaccharide.

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EFFECT OF PRELIMINARY FASTING ON DEVELOPMENT OF ACUTE LIVER DAMAGE BY D-GALACTOSAMINE IN RATS

D. Adzharov, N. Donchev, M. Kerimova, E. Naidenova, B. Borov, M. Beshevlieva, G. Savov, and E. Ivanov

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Parenteral administration of D-galactosamine (GalN) leads to the rapid development of hepatocellular damage, resembling virus hepatitis in its morphological, biochemical, and clinical features [4]. Fasting for 24 h before administration of galactosamine has been shown to diminish considerably elevation of the serum AsAT and AlAT levels and to maintain the liver glycogen at high values [12].

The mechanisms of galactosamine detoxication have not been fully studied. There is no information on excretion of D-glucaric acid with the urine, which is increased when binding with glucaronic acid in the liver is intensified [3]. There is likewise no information on the role of another essential detoxicating mechanism of binding with reduced glutathione (GSH). The role of mixed function microsomal oxygenase (MFMO) in the hepatotoxicity of galactosamine has not been fully explained despite research in this direction [1, 6, 9, 15].

The aim of this investigation was to study the effect of a longer period of preliminary fasting on the development of acute GalN poisoning and also to study changes in some parameters of the detoxicating function of the liver.

EXPERIMENTAL METHOD

Experiment were carried out on 48 male Wistar rats weighing 350-400 g. The animals were divided into four groups: two control groups consisted of intact rats which fasted for 24 and 72 h immediately before decapitation, and two experimental groups. Animals of the experimental groups received a single intraperitoneal injection of GalN in a dose of 1.855 mmole/kg body weight 24 h before decapitation. During this period the animals were given nothing to eat, and half of them fasted for a further period of 48 h before injection of GalN. Water was provided ad lib. None of the rats died. The rats were decapitated and equal volumes of serum from three animals were pooled for determination of AsAT and AlAT

TABLE 1. AsAT and AlAT Activity in Blood Serum

Parameter	Control group	Animals		
		fasting	poisoned with GalN	fasting and poisoned with GalN
AsAT, mmoles/(min·liter)	144 (126—150)	168 (114—216)	2100* (720—3720)	2778* (1380—4080)
AlAT, mmoles/(min·liter)	24 (12—36)	60 (30—72)	1200* (660—1800)	2730* (840—4260)

Legend. Here and in Table 2: four samples from three animals taken from all groups. Results shown as $\bar{x}$ and interval. *$p < 0.05$ compared with control group.

Research Institute of Gastroenterology and Nutrition, Medical Academy, Sofia, Bulgaria.
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