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BIOTRANSFORMATION OF SODIUM NITROPRUSSIDE INTO DINITROSYL IRON COMPLEXES IN TISSUE OF ASCITES TUMORS OF MICE

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SUMMARY: We have found that treatment of various types of murine ascites tumors with sodium nitroprusside (SNP) both in vitro (1,5 mM) and in vivo (25 mg/kg; i.p.) results in formation of EPR-detectable dinitrosyl nonheme iron complexes (DNIC) with RS^- groups of proteins ($g_\perp$=2,037; $g_\parallel$ =2,012). The DNIC were mainly localized in the ascitic extracellular fluid. The appearance of DNIC was unaffected by an inhibitor of nitric oxide synthase, N^ω-nitro-L-arginine (1 mM), but abolished with a SH-blockator, p-chloromercuribenzoate (0,1 mM). No DNIC formation was observed when ascitic fluid (after tumor cells separation) was incubated with SNP alone, but with SNP and L-cysteine (10 mM). Thus, ascites tumor cells contribute to the transformation of SNP into DNIC and thiols are essential in this process.

Sodium nitroprusside, $Na_2[Fe(CN)_5NO]$ (SNP) is widely used in clinical and experimental practice as a vasodilator and a nitric oxide (NO) donor (1, 2). However, precise mechanism of its biodegradation and NO liberation remains under discussion. Some of the investigators suggest spontaneous releasing of NO from SNP (2, 3). Others state that NO may be released only via reductive pathway of SNP (4-6). In model experiments, it was shown that nitroprusside-ion easely undergoes one-electron reduction, and then loses its cyanide ligand trans to the NO ligand forming paramagnetic "tetra" form of reduced SNP. This form in turn may transfer NO to heme (II) of globin(s) (6). But if low molecular weight thiols are present in excess SNP may be transformed into dinitrosyl iron complexes (DNIC) with thiolate anions $Fe(NO)_2(RS)_2$, which are characterized by distinguished axial EPR-signal with $g_\perp$=2,037; $g_\parallel$ =2,012 (7, 8). Here we report for the first

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Abbreviations: SNP, sodium nitroprusside; DNIC, dinitrosyl nonheme iron complexes; NO, nitric oxide; L-NNA, N^ω-nitro-L-arginine; p-CMB, p-chloromercuribenzoate; ATC, ascites tumor cells; AEF, ascitic extracellular fluid.

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time that the later route of SNP transformation occurs in a wide range of whole ascites tumors of mice but not in blood of healthy rats or mice.

MATERIALS AND METHODS

Reagents: Sodium nitroprusside, p-chloromercuribenzoate, (p-CMB) and trypan blue were obtained from Sigma (St. Louis, MO), L-arginine HCl was obtained from Reanal (Hungary), L-cysteine HCl was obtained from BDH Chemicals Ltd (England). N^{ω}-nitro-L-arginine (L-NNA) was synthesized according to the method of Hayakawa et al (9). DNIC with thiosulfate $[Fe^{2+}(S_2O_3^{2-})_2(NO)_2]$ was synthesized as was described previously (10). All inorganic reagents for synthesis of L-NNA and DNIC were of analytical grade from Reachem (Russia).

Animals, ascites tumors and blood: Studies were carried out on mongrel white mice weighing 18-20g. A wide range of transplantable ascites tumors of mice: Ehrlich, Krebs-2, Fisher, NKLy and ascites form of S-37 were used in the work. Ascites tumor cells (ATC) were grown as usual by weekly i.p. transplantation of 2×10^6 ATC in mice. Whole ascites tumor tissues without hemorragy were used for experiments 7-9 days after transplantation. The concentrations of ATC were about 1×10^8/ml; cells/extracellular fluid volume ratio were about 1/2; ATC viability were 94-98% as tested by trypan blue exclusion. Whole rat or murine blood was obtained with heparin after decapitation of concious animals. Tumor tissue and blood were used for experiments immediately after receiving.

Experimental procedure: In vitro experiments, whole blood or tumor tissue were incubated with 1,5 mM SNP for 10-40 min at room temperature. Then cells were sedimentated (1000g for 15 min) and samples of ascitic extracellular fluid (AEF) or unwashed ATC were frozen in liquid nitrogen (5mm×35mm cylinder) for EPR studies. In some cases ATC were washed three times with saline (1/10 volume ratio) before preparing samples. In some experiments tumor tissue were incubated with 1 mM L-NNA or 0,1 mM p-CMB, 5 min prior before adding of SNP. At separate studies SNP was added alone or with L -cysteine HCl (10 mM) to AEF. In vivo experiments SNP were given to tumor bearing mice by i.p. mode (25 mg/kg; in 0,5 ml saline). After 30 min the animals were sacrificed, tumor tissues were harvested and samples of AEF and ATC were prepared as described above.

EPR-spectrometry: EPR studies were carried out at 77K or 293K in an x-band spectrometer "Rubin" made in Russia. A microwave power was 10 mW, modulation amplitude, 5 gauss, scan rate, 100 gauss/min, time constante, 0,3 sec. EPR measurements at 77K were performed by commonly used technique (11). For recording EPR-spectra at room temperature the tissues were placed in 1 mm capillary tubes. Mn^{2+} in MgO crystal was used for field calibration . For quantitation of DNIC formed in tissues a stable paramagnetic $[Fe^{2+}(S_2O_3^{2-})(NO)_2]$ complex of known concentration was used.

RESULTS

Interaction of SNP with whole blood. After incubation of whole rat blood with SNP (1,5 mM) for 10 -30 min, erythrocytes exibited a singlet EPR signal with weakly defined triple structure and $g_{av.}$=2,03 (Fig.1A), the same was observed in various tissues of SNP subjected mice (12). According to (6-8, 12) this spectrum may be attributed to "tetra" form of reduced nitroprusside-ion. Higher concentrations of SNP resulted in formation of "penta" form of reduced nitroprusside-ion ($g_{\perp}$=2,00; $g_{\parallel}$ =1,93; $a_{\perp}(^{14}N)$=30 gauss), additionally to "tetra" (not shown). Plasma of SNP treated blood exibited an usual EPR-signal of serum ceruloplasmin without any qualitative changes (Fig.1B). Similar results were obtained on blood of healthy mice (not shown).

Interaction of SNP with whole ascites tumor tissue. After incubation of whole Ehrlich ascites tumor tissue with 1,5 mM SNP for 10-40 min an intensive axial EPR-signal ($g_{\perp}$= 2,037; $g_{\parallel}$=2,012; 77K) was found both in unwashed ATC (Fig.1C) and in AEF (Fig.1D). This signal was identical to that observed by several researchers in various tissues (13-18) and attributed to DNIC with RS^- ligands. When ATC were washed three times with saline, they exibited 5 fold decreased in amplitude and less anisotropic EPR-signal. It seems that EPR-spectrum of washed ATC is sum of spectra for "tetra" form of reduced SNP and DNIC (Fig.1E). In wash medium, DNIC was found in amounts which accout for the decrease of EPR-signal in ATC (not shown). So, the DNIC were mainly localized in AEF.

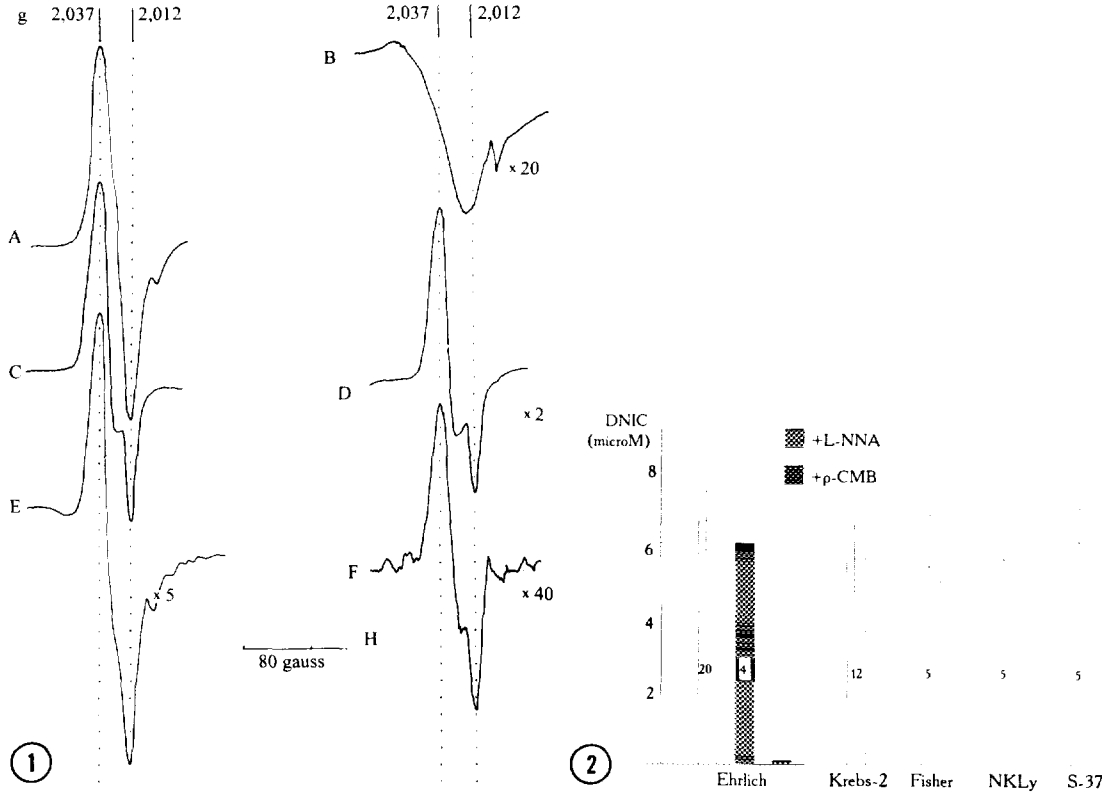

Fig 1. EPR-characteristic of sodium nitroprusside (SNP) reaction with blood of healthy rat and tissue of Ehrlich ascites tumor. **A.** Erythrocytes after incubation of rat blood with 1,5mM SNP for 10 min and sedimentation at 1000 g for 15 min. **B.** Plasma after the same procedure. **C.** Unwashed ascites tumor cells (ATC) after incubation of whole tumor tissue with 1,5 mM SNP for 30 min and sedimentation at 1000 g for 15 min. **E.** The same as C, but ATC were washed three times with saline. **D.F.** Ascitic extracellular fluid (AEF) after the same procedure. The spectra EPR were recorded in an x-band spectrometer, microwave power, 10mW, modulation amplitude, 5 gauss, time constant, 0,3 sec, T=77 K (**A-E**), T=293K (**F**).

Fig.2. Formation of dinitrosyl iron complexes (DNIC) in the extracellular fluid of various murine ascites tumors treated with 1,5 mM sodium nitroprusside for 30 min. Effect of 1mM N^{ω}-nitro-L-arginine (L-NNA) and 0,1 mM p-chloromercuribenzoate (p-CMB). Data are expressed as mean ±S.E.M.

We did not find any significant variations in amounts of DNIC formed in AEF of different types of ascites tumors treated with SNP. As a rule the concentrations of DNIC were about 4-8 microM (Fig.2). The appearance of DNIC in AEF was not effected by 1 mM L-NNA (Fig. 2). Figure 1F shows an EPR-spectrum for AEF recorded at room temperature. In this case we observed a clealy anisotropic EPR-signal identical to that recorded at 77K. Such observation indicates that DNIC were high molecular weight species more probably DNIC with RS^- groups of proteins. This conclusion comes from early studies wich showed that DNIC with low molecular weight ligands elicits at room temperature more narrow and isotropic EPR-signal (19). A known SH-blockator, p-CMB (0,1 mM) completely inhibited DNIC formation, proving that sulfhydryl groups are involved in the process (Fig. 2).

The formation of DNIC from SNP in AEF was confirmed in vivo. When SNP were given to Ehrlich tumor-bearing mice (25 mg/kg; i.p. n=4) the concentration of DNIC formed in AEF was 1,6±0,5 microM (not shown).

Interaction of SNP with AEF and blood plasma. Figure 3A shows a representative EPR-spectrum for AEF, treated with 1,5 mM SNP for 15 min. The same EPR-signal was found in SNP treated blood plasma of tumor-bearing mice (n=6).According to previous studies (6-8, 12) this spectrum may by attributed to "tetra" form of reduced nitroprusside ion. The concentrations of such species were ranged from 0,4 to 0,9 microM. No EPR-signals with g_{av}=2,03 were found in SNP untreated AEF and in SNP treated blood plasma of healhty rats or mice (not shown). Addition of L-cysteine (10 mM) to AEF-SNP incubates (n=6) changed their spectrum quantitatively (2,5 fold increase) and qualitatively (axial symmetry; $g_{\perp}$=2,037 and $g_{||}$=2,012) suggesting the formation of DNIC from SNP (Fig.3B).

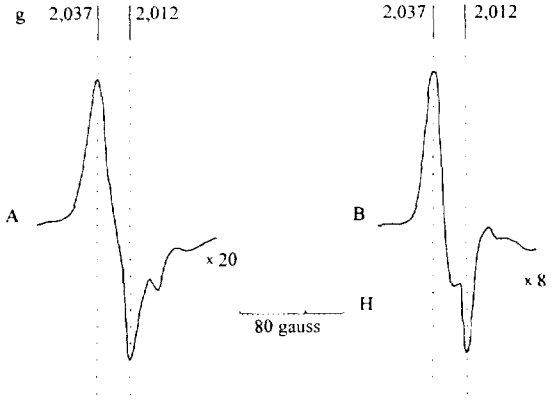

Fig.3. EPR-characteristic of sodium nitroprusside (SNP) reaction with ascitic extracellular fluid (AEF) of Ehrlich tumor. Effect of L-cysteine. **A.** AEF incubated with 1,5 mM SNP for 15 min. **B.** AEF incubated with 1,5 mM SNP and 10 mM L-cysteine for 15 min. EPR settings are the same as in Fig.1.

DISCUSSION

Here we have demonstrated by means of EPR method that SNP reaction with ascites tumor tissue has definite peculiarities, as compared with blood of healthy rats and mice. The most spectacular finding was the formation of protein-borne DNIC in AEF after challenge of whole ascites tumors (presence of ATC !) with SNP. Another finding was the particular reaction of SNP with extracellular fluids of tumor-bearing mice (without ATC !). Both AEF and plasma of tumor-bearing mice after incubation with SNP produced "tetra" form of reduced nitroprusside-ion discriminating them from the plasma of healthy animals.

The fact, that an inhibitor of NO synthase, L-NNA was unable to suppress DNIC formation moved us to the conclusion that both molecules of NO in DNIC were originally from SNP rather than from endogenous L-arginine. It might be suggested that the appearance of DNIC in AEF was resulted from ligand reorganisation of nitroprusside-ion. According to the model experiments (7, 8), such transformation of SNP into DNIC is feasible when low molecular weight thiols are present in excess. In our experiments the DNIC were mainly formed in the AEF and ATC were essential for it. L-cysteine mimics ATC contribution to DNIC formation from SNP. These results indicate that one of the possible explanation of the described phenomenon may be increased efflux of low molecular weight thiols from ATC upon challenge with SNP.

The reported data are of great importance for several reasons: 1. Users of SNP have to know that SNP biodegradation in whole tumor tissue may result in formation of DNIC, which are relatively stable and possess long lasting biological activity (20). 2. Many groups showed endogenous DNIC formation in tumor tissue (13,15), activated macrophages (16), hepatocytes (17) and islets of Langerhans (18). 3. Several studies demonstrated that some types of tumor cells (21-23) and patients with malignant deseases elicit features in thiol turnover (24). Further investigation is necesssary to understand the biochemical background and potential relation of our finding with above mentioned observations.

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