Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 571-576

EPR EVIDENCE OF NITRIC OXIDE PRODUCTION BY THE REGENERATING RAT LIVER

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Received June 3, 1994

SUMMARY: Nitric oxide (NO) production in the regenerating liver was estimated from the intensity of the electron paramagnetic resonance (e.p.r.) signal of the mononitrosyl complexes of iron and diethylthiocarbamate (DETC). Preformed complexes of intracellular non-heme Fe^{2+} and added DETC served as a trap for endogenously produced NO. The time-dependent changes of NO production were connected with the periodicity of liver regeneration. The first increase in NO production occurred ca. 1 h after partial hepatectomy (PHE). The second and more pronounced peak of NO production was observed about 6 h after PHE, when the hepatocytes entered the first cell cycle; it originated mainly from these cells. The following minimum of NO synthesis coincided with the maximal rate of DNA synthesis. The third gradual rise of NO production was seen at the end of the investigated period that covered the G_2+M phases, the transit from the first to the second cell cycle of the hepatocytes and the entrance of the nonparenchymal cells into proliferation.

The strict temporal organization of liver regeneration facilitates the elucidation of physiological functions of participating molecules. This is particularly true for the most synchronized period between partial hepatectomy (PHE) and the end of the first cell cycle of hepatocytes. A role of NO during liver restoration is proposed, primarily during metabolic reorientation accompanying the transit from quiescence to proliferation and secondly during the cell cycle itself. Liver injury evoked a wide spectrum of regulators and possible NO targets: TNF-α, a powerful stimulator of NO synthesis in hepatocytes and Kupffer cells (1,2) is

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0006-291X/94 $5.00

upregulated after PHE (3); ribonucleotide reductase, the rate-limiting enzyme of DNA synthesis is strongly inhibited by NO (4); the activity of guanylate cyclase, regulated by NO, is dependent on the periodicity of liver restoration (5) as well as the regulation of vascular tonus, wound healing etc. (for review see 6).

NO production in the regenerating liver was estimated from the intensity of the specific e. p.r.signal of the mononitrosyl iron complex (MNIC) with DETC. Fe^{2+}-DETC complexes pre formed *in vivo* from endogenous iron ions and injected DETC served as a trap for the endogenously produced NO (7). The time course of NO production after PHE revealed a correlation with the previously established periodicity of liver regeneration (8), particularly an inverse correlation with DNA synthesis.

EXPERIMENTAL PROCEDURES

Treatment of the animals and preparation of the samples. Female Wistar rats (150-200 g) were used throughout. Partial (2/3) hepatectomy was performed by a standard procedure (9) on animals fed *ad libitum* and starved for 24 h respectively. Livers were removed at different times up to 37 h after PHE and processed for e.p.r. measurements (7). Thirty min prior to extirpation, 1.0 ml of 4% DETC in O.9% NaCl per 100 g of body weight was injected intraperitoneally. The origin of the NO trapped in the complex was controlled using N^G-mononitro-L-arginine (MNA), a competitive inhibitor of the enzymatic oxidation of L-arginine (10). Sixty and 45 min before sacrifice, the rats were injected intraperitoneally with 1.0 ml of 1% MNA in 0.9% NaCl per 100g body weight.

E.p.r. spectra were recorded on the radiospectrometer EPR Varian E9 at the microwave frequency 9.300 GHz, microwave power 10 mW and modulation amplitude 0.1 - 5 mT. The quantitative estimation of MNIC-DETC in the samples was accomplished by double integration using the stable nitroxyl radical, 2,2',6,6'-tetramethylpiperidol-1-oxyl, as a standard.

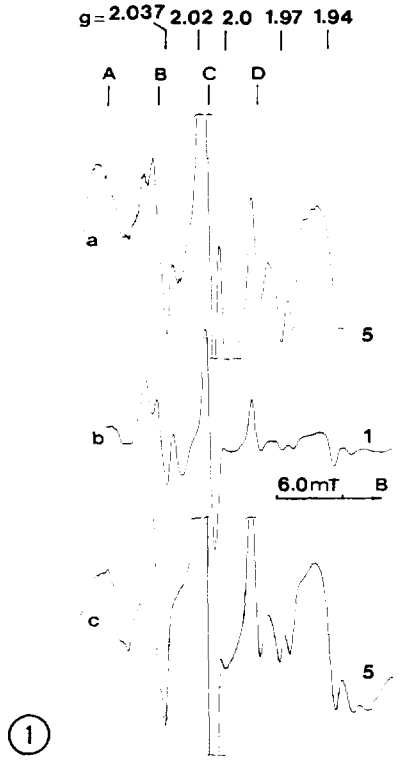
Control of trapping efficiency. After the e.p.r.spectroscopy, the liver samples were treated with gaseous NO at 300 mm Hg for 30 min to transform all Fe^{2+}-DETC complexes into MNIC-DETC and subjected once more to e.p.r. spectroscopy. The commercial NO (Messer-Griesheim, Rheinfelden, Germany) was purified by fractional sublimation in a high-vacuum system.

Isolation of hepatocytes and nonparenchymal cells was accomplished by differential centrifugation after *in situ* perfusion of the liver with O.05% collagenase (11). The amount of cells and their vitality were controlled with O.2% trypan blue in a hemocytometer. The aliquots of cell suspensions were prepared for e.p.r. spectroscopy by a standard procedure (7).

RESULTS AND DISCUSSION

E.p.r. spectra of liver samples from rats treated with DETC.

The typical e.p.r. spectra of rat livers producing NO are presented in Fig. 1a,b. A MNIC-DETC complex can be discriminated from other paramagnetic centers by its $g_\perp = 2.035$ and $g_\parallel = 2.012$ with triplet hyperfine structure (HFS) at $g_\perp$; Cu^{2+}-DETC complexes display typical four compounds of HFS from copper nuclei (A,B,C,D); free radicals possess signal at $g = 2.0$, complexes of Mo^{5+} at $g = 1.97$ and reduced iron-sulfur proteins at $g = 1.94$ (12). The second component (B) of the Cu^{2+}-DETC complex is superimposed on the signal of MNIC-DETC and



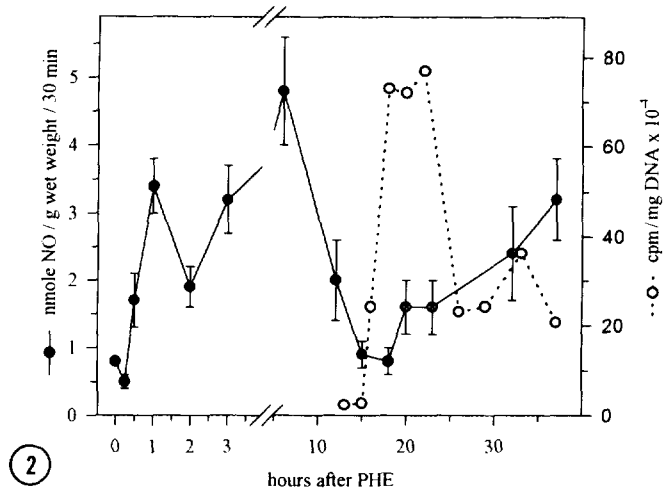


Fig. 1. E.p.r. spectra of samples from regenerating liver 12 h (a,c) and 6 h (b) after PHE with (c) and without (a,b) MNA pretreatment.
Numbers on the right side indicate the relative gain of spectrometer.

Fig. 2. NO production in regenerating liver in comparison with DNA synthesizing activity.
The data for the curve of DNA synthesizing activity is taken from (22) with the correction on the age of the animals (23).

partly masks it. The intensity of the MNIC-DETC signals was judged from the third (high-field) component of its HFS at $g_{\perp}$ that is not obscured by other components of e.p.r. spectra.

Fig. 1c represents the e.p.r. spectrum of liver samples from partially hepatectomized rats treated with MNA before DETC injection. The lack of a specific MNIC-DETC signal in comparison with the pronounced signal in corresponding rats without MNA treatment (Fig. 1a,b) supports L-arginine as the source of the NO signal.

Experiments performed to control the trapping capacity of preformed Fe^{2+}-DETC complexes showed that the amount of traps was not a limiting factor for the detection of endogenously produced NO (data not shown). Under NO-saturating conditions the content of MNIC-DETC exceeded that of endogenously generated MNIC-DETC approximately by a factor of 20.

NO production and the periodicity of liver restoration.

Though the precise targets for NO in the dynamics of liver regeneration remain to be elucidated, some clues can be obtained by comparison of the changes in NO production with the known periodicity of the overall process. The most relevant periodicity after PHE is connected with the period of the first cell cycle of the hepatocytes , i.e. up to 30 h (13). Kupffer and sinusoidal endothelial cells enter the cell cycles after parenchymal cells in that order (14).

The S phase of hepatocytes is preceeded by the prereplicative period (up to about 12 h after PHE) which is conveniently subdivided into the "situation check up" with early response reactions (ca. 0.5 -1 h after PHE), metabolic reorientation providing competence to proliferate (ca. 1 h to 4 h after PHE) and a G1-like period (about 4 to 12 h after PHE) (8,15).

An early and less pronounced peak of NO production was seen about 1 h after PHE (Fig.2) coinciding with the first stage of the prereplicative period. In this time several events take place. Some of them may elicit NO formation e.g. transcription factors with κB-binding activity (16,17), upregulated TNF-α (3), others may be cosequence of NO production such as transitorial changes of vascular tonus, chemotaxis and aggregation of blood cells, thromboresistance following thrombus formation, oxidative-stress response (6).

The second more pronounced increase in NO production (Fig.2) coincided with the transition of the hepatocytes from the phase of metabolic reorientation to the G1-like period: it originated mainly from parenchymal cells. Formation of 1.6 nmol NO by $1.3x10^8$ hepatocytes [correspon ding to one gram of liver (18)] was measured after 30 min of *in vivo* exposition with DETC and ca. 30 min of the procedure of hepatocytes isolation. This value is maximal among similar ones obtained at one and 30 h after PHE and comparable with the values from the whole tissue. E.p.r. spectroscopy did not detect signals in the samples representing the fraction of non-parenchymal cells from a single liver at this as at any other time point of maximal NO production. As shown previously the elevated expression of genes coding for TNF-α and its receptors (3) preceeded this second peak of NO generation suggesting the involvement of a Ca^{2+}-independent NO synthase that may be induced by TNF-α (1). The increase of blood flow (the liver acquires at this time intensively dark red colour) and of the content of cyclic guanosine 3': 5' monophosphate (cGMP) (19) stimulating RNA synthesis (20) may be considered as consequences of elevated NO. Recent data on macrophage-like cells revealed that NO may abrogate some check point during the G1 phase (21). Whether this takes place in the regenerating liver has yet to be elucidated.

The NO production decreased rapidly during the time corresponding to the end of the G1 phase of hepatocytes and reached a minimum when the rate of DNA synthesis was significantly elevated (Fig. 2). This time course is consistent with the reported (24) inhibitory role of NO on proliferation that is partly due to its reactivity with the non-heme iron. The latter is required for

full activity of ribonucleotide reductase (4) and $p34^{cdc2}$ regulating G1/S transition (25).

The third rise in NO production observed 30-37 h after PHE coincided with the end of the first and the beginning of the second cell cycle of hepatocytes and with the entrance of non-parenchymal cells into proliferation (13,14,23). The concurrent increased sensitivity of the liver to nitroso compounds (26), the reduced content of non-heme iron that is redistributed in the cells and bound more loosely within its complexes (27) and the increased amount of cGMP (19) are the potent consequences of elevated NO. Recently it was reported that the level of NO and intracellular distribution of NO synthase activity in non-hepatic cells appear to be strictly regulated at the end of cell cycle. Overproduction of NO keeps macrophage-like cells in G2 phase (21). The various phases of endothelial cells division reveal the elevated NO synthase activity with specific patterns of its intracellular localization (28).

It appears that the revealed changes in NO production are dominant during liver restoration. The observed time course was reproducible even with animals starved for 24 h prior to PHE, although the absolute values of NO production were significantly smaller. Sublethal injections of cycloheximide were followed by quasi regeneration with the periodicity of metabolic changes and of NO production similar to that observed after PHE (29).

It must be emphasized that the absolute amounts of NO production *in vivo* deduced from these e.p.r. measurements may be underestimated due to different reasons; for example, some part of NO through intracellularly generated nitrite and nitrate, would be able to bypass the traps specified for NO and localized in cellular membranes (30).

Despite these limitations, the e.p.r. method allowed to evaluate genuine NO production *in vivo* with minimal disturbance of the samples and revealed for the first time the correlation of NO production with the phases of liver restoration.

ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft, Bonn, through SFB 154, and from Fonds der Chemischen Industrie, Frankfurt-M, Germany.

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