METHODS

Chronic Hepatitis C: Quantitative EPR Analysis of Nitrogen Oxide and Copper in Patients' Blood

V. I. Petukhov, L. Kh. Baumane, I. Ya. Kalvinsh, A. P. Skesters*, A. A. Silova*, B. Rozentale, and L. A. Ivanchenko****

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Measurements of nitrogen oxide and copper in the blood of 57 patients with chronic viral hepatitis C was carried out before antiviral therapy by electron paramagnetic resonance on a Radiopan EPR spectrometer. The results indicate elevated levels of nitrogen oxide and copper in the blood of these patients in comparison with normal subjects. Comparison of these findings with the results of a previous analysis of redox status of patients with chronic viral hepatitis C indicate that this disease is characterized by a significant pro-oxidant shift in the realization of redox processes and disorders in the metal ligand homeostasis (at least as regards copper).

Key Words: *nitrogen oxide; copper; EPR analysis' element imbalance*

Our previous studies [2] have shown that high (by almost one order of magnitude above the norm, on average) activity of plasma ALT at the beginning of chronic viral hepatitis C is associated with increased basal luminol-dependent chemiluminescence of the plasma and plasma MDA, paralleled by hyperproduction of nitrogen oxide (NO) (according to quantitative EPR spectrometry). In addition, all patients exhibited low activity of Cu,Zn-dependent erythrocyte SOD (Cu,Zn-SOD1), indicating deterioration of intracellular antioxidant defense.

We tried to clear out whether reduced Cu activity and Cu,Zn-SOD depended on the blood Cu concentration in the patients and to evaluate the efficiency of quantitative approach to analysis of EPR findings. We previously used EPR (electron paramagnetic resonance) method for NO measurements [2].

Latvian Institute of Organic Synthesis; *Stradynya University of Riga; **Latvian Center of Infectology, Riga. ***Address for correspondence:*** vip-val@yandex.ru. V. I. Petukhov

MATERIALS AND METHODS

Blood concentrations of nitroxyl radicals in the form of paramagnetic mononitrosyl Fe complexes (MNIC) and Cu complexes with diethyldithiocarbamate (DETC) were measured by the EPR method. Venous blood (0.3 ml) was collected in the morning in an outpatient setting, directly put into an ampoule with an NO radical trap (10 mg DETC sodium), and incubated for 30 min at 22-24°C. The ampoule was then transferred into Dewar's vessel with liquid nitrogen and frozen at -196°C. A column of frozen blood (4 mm in diameter and ~15 mm long) in a quartz Dewar's vessel with liquid nitrogen was then placed into an EPR spectrometer (Radiopan) resonator for recording the EPR signals. A typical MNIC-DETC signal, recorded at 77 K, is characterized by the g-factor $g_{\parallel}=2.02$ and $g^{\perp}=2.035$ with a triplet superfine structure. The content of NO radicals in a sample was evaluated by the intensity of the third (high field) component. In addition to NO radicals,

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EPR signals of endogenous Cu-DETC complexes and reduced ferroserum proteins were present in the spectrum. The content of Cu complexes was evaluated by the intensity of EPR signal with g-factor of 1.985. In some cases EPR signals of α- and β-hemoglobin nitrosyl complexes were recorded. The results were expressed in arbitrary units (U). Arbitrary units were used for quantitative evaluation of NO radicals by the EPR analysis. The intensity of the third (high field) component of the EPR signal (g=2.03) on an EPR-gram, measured in mm length (or, to be more precise, height of this component), was expressed in arbitrary units (U). The content of Cu complexes in EPR analysis was evaluated by the intensity of non-overlapped EPR signal with a g-factor of 1.98. The results were expressed in arbitrary units (U), that is, in mm of this signal's height on the EPR-gram. Activities of Cu,Zn-SOD and ALT in standard tests were also expressed in arbitrary units (U) denoting the optical density of solution in spectrometry of the studied sample.

The study was carried out in 57 patients (30 males and 27 females) with newly diagnosed chronic viral hepatitis C (CVHC) aged 20-75 years (mean age 41 years). The diagnosis of CVHC was verified by clinical and laboratory findings: aminotransferase activities, data of PCR analysis of virus loading and virus (HCV) replication), HCV genotyping, puncture biopsy of the liver, blood bilirubin level, alkaline phosphatase activity, *etc.* ALT activity several fold surpassed the normal in virtually all patients, its mean level being 142.6±6.9 U/liter. The majority (67%) of patients presented with the 1b HCV genotype, 28.6% with 3a genotype, 2.2% with 1b+1a and 2.2% with 2a+2c genotypes. All the studies were carried out before antiviral therapy (α-IFN, ribavirin). Control group consisted of 43 normal subjects (10 males and 33 females) aged 18-66 years (mean age 42 years).

RESULTS

The data of measurements of NO and Cu in whole blood of CVHC patients and normal subjects and our previous data on the redox status of the same patients [2] are presented (Table 1).

The results indicate NO hyperproduction in patients with newly detected CVHC (Table 1) in comparison with the normal level (30.6±1.7 *vs.* 23.8±0.6 U; $p<0.05$) and elevated content of Cu (2.23±0.03 *vs.* 2.11±0.02 U; $p<0.05$) in the patients. Importantly that NO production and Cu levels were directly measured by the EPR method. Greater range of values of EPR analysis in CVHC patients in comparison with controls is worthy to note. Coefficient of variations (CV) for NO in the patients was 42.9% *vs.* 17.5% in normal subjects; for Cu these values were 12.1 and 7.5%, respectively. High blood levels of NO and Cu in CVHC patients have been detected by other methods as well [1,3-6].

In addition to high NO level and high activity of plasma ALT, the patients developed a clear-cut pro-oxidant shift of the redox status (Table 1). Though the origin of the detected pro-oxidant shift deserves a more precise study, we attribute it to activation of endogenous production of not only oxygen, but also of nitrogen radicals, development of free radical phagocyte-dependent inflammation and switch-over of hepatocyte program from apoptosis to necrosis in CVHC patients with high ALT levels. Previously detected [2] significant positive correlation between plasma ALT (criterion of necrotic destruction of hepatocytes) and basal luminol-dependent chemiluminescence of the plasma ($r=0.8$; $p<0.5$) supports our opinion.

On the other hand, we failed to detect a significant relationship between ALT activity and virus replication (virus loading). This can indicate that further progress of the disease is determined mainly by the hepatocyte and defense systems of virus-infected organism, capable to become destructive under certain conditions (for example, NO hyperproduction), but not by the virus. This restructuring can eventuate in well-known complications of CVHC (fibrosis, cirrhosis, cancer) and triggering of processes leading to the death of the virus carrier.

The role of NO in HCV infection attracted much attention of scientists just in recent years, and hence, NO significance in the initiation and development of CVHC is virtually not studied. This is partly caused by methodological problems, as direct measurements of NO are difficult because of very short (no more than 5-6 sec) life span of this molecule. That is why the production of NO in the few studies of its involvement in the pathogenesis of CVHC was evaluated mainly by the level of stable NO metabolites (NO_2^- and NO_3^-) [1,3]. The flaw of this approach is the need to reduce NO_3^- ions (mainly eliminated from the body) to NO_2^- ions by metal cadmium or nitrate reductase, in order to increase the sensitivity of the method. In addition, this approach is fraught with a high risk of false-high results at the expense of exogenous nitrites (received with food or drugs), which limits the possibility of analysis under conditions of a common drinking regimen.

High release of NO_2^- and NO_3^- with the urine in patients with all etiological variants of viral hepatitis has been reported [1]. Correlation between

TABLE 1. Blood NO and Cu Levels and Some Parameters of Redox Status of CVHC Patients (*M*±*m*)

Parameter	NO, U	Cu, U	Plasma MDA [2], nmol/ml	Plasma BLC [2]	Cu/Zn-SOD [2], U/ml
Control	23.8±0.6	2.11±0.02	2.5±0.05	h=30-80 u s=≤250 mm^2 tgα=≤4.5	164-240
CVHC patients	30.6±1.7	2.23±0.03	3.15±0.09	h=153.5±18.9 u s=447.7±44.5 mm^2 tgα=9.4±1.1	140.9±3.3

Note. All the values of CVHC patients differed significantly (p<0.05) from the control. Plasma BLC: basal luminol-dependent chemiluminescence of the plasma.

the activities of hepatic aminotransferases (ALT, AST) and bilirubinemia, on the one hand, and urinary levels of NO_2^- and NO_3^- was noted. Another study revealed high serum concentrations of nitrates and nitrites and liver biopsy specimens of CVHC patients. Positive correlation between NO_2^- and NO_3^- in these biological substrates and the index of histological activity of inflammatory process in the liver was noted.

It is assumed that NO in physiological (basal) concentrations is characterized by a cytoprotective effect towards the hepatocyte, while hyperproduction of NO with participation of iNOS causes an opposite effect, stimulating apoptosis (and even necrosis) of liver cells. In addition, NO molecules, emerging in excess, easily bind to superoxide anion radical (O_2^-), forming peroxinitrite ($ONOO^-$), one of the most aggressive radical molecules. This compound is characterized by not only high destructive potential towards all cell components (from membrane to nucleus), but, binding NH- and SH-groups of antioxidant enzymes, increases the probability of the hepatocyte death (by necrosis and/or apoptosis) under conditions of oxidative stress.

Hence, comparison of our results of NO and Cu measurements in the blood of CVHC patients with the findings of other studies, in which NO and Cu were measured by other than EPR methodological approaches [1,3-6], showed their coincidence: the levels of NO and CU in whole blood of CVHC patients was increased significantly in comparison with the normal level. This suggests the use of quantitative EPR analysis for direct measurements of nitroxide and copper in biological substrates.

Though the causes of high levels of copper in CVHC remain unclear, this phenomenon can explained by predominance of subjects with low activity of N-acetyltransferase ("slow acetylators") among CVHC patients, as in this patient population activation of fibrogenesis processes, characteristic of CVHC, can lead to accumulation of nonacetylated *D*-glucosamine and *D*-galactosamine — active Cu chelators, capable of strong retention of this metal, thus creating endogenous deficiency of Cu. This can explain the reduction of Cu/Zn-SOD1 activity in CVHC patients.

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