cally decreases the characteristic relaxation time t_{30} and increases the relative rate of isometric relaxation. In the hypocalcium medium, PTH has no effect on relaxation for any L_{es}. In our experiments due to the use of the physiological loading regime the effects of PTH were studied at fixed L_{es} values, which allowed us to observe the pure loosening effect of PTH.

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CCl_4 as Inductor of L-Arginine-Dependent Synthesis of NO

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The effect of CCl_4 on the generation of NO in mouse liver cells is studied *in vivo*. Injection of CCl_4 is shown to modulate the synthesis of NO by activating the NO-synthetase system. The experimental data suggest that O_2^- plays an essential role in the regulation of NO-synthetase system.

Key Words: *nitric oxide; NO-synthetase; CCl_4; antioxidants*

Many types of animal cells — macrophages, neutrophils, endothelial and muscle cells, fibroblacts, neurons, and hepatocytes — synthesize nitric oxide (NO) which, first acts as a transmitter and second, possesses a cytotoxic activity. NO in the cells forms from L-arginine due to the action of NO-synthetase (NOS). There are two isoforms of NOS differing in structure and mode of regulation. The constitutive NO-synthetase (c-NOS) permanently present in cells is regulated by changes in the concentrations of Ca^{2+} ions induced by hormones and neurostimulators and synthesizes NO in picomole amounts. Another isoform of NOS (i-NOS) can be induced in macrophages, smooth-muscle cells, etc. by exotoxins and cytokinins and produces a large amount (nanomoles) of NO over a long period of time [3]. The regulation of i-NOS has been little studied. It is assumed that transcription factor NF-κB, which controls the induction of the expression of various genes in inflammation, the immune response, and stress, also participates in the initiation of i-NOS synthesis [6,9]. This assumption is based on the following observations. First, both the activation of NF-κB and the production of i-NOS can be inhibited by antioxidants [6,8-10]. Second, reactive intermediate oxygen species, including O_2^-, produced in various electron transfer reactions, activate NF-κB [7]. Moreover, O_2^- generation is necessary for the initiation of NO synthesis in the cells exposed to various stimulating agents. It should be noted that the generation of O_2^- and production of NO are related in a very complex manner: an excess of O_2^- reduces the amount of detectable NO [5].

The aim of the present study was to investigate *in vivo* NO production in mouse liver cells induced by the chemical toxin CCl_4 rather than the usual biological agents (lipopolysaccharides, γ-interferon, tumor necrosis factor, etc.). This toxin induses a

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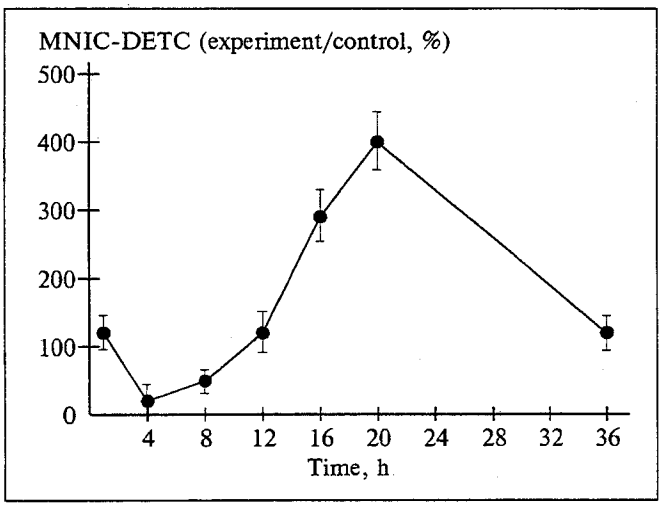

Fig. 1. Dynamics of the amount of NO detected in mouse liver after injection of CCl_4 in a dose of 0.05 ml/mouse.

number of pathological processes in liver cells, including damage to the cell membranes. Using our experimental model, we studied the effect of antioxidants on the process of NO generation.

MATERIALS AND METHODS

The experiments were carried out on random-bred male albino mice weighing 18 to 22 g. CCl_4 was injected intraperitoneally in a dose of 0.05 ml per mouse. The mice were killed by decapitation. Thirty minutes before sacrifice the animals were given an injection diethyldithiocarbamate (DETC) in a dose of 500 mg/kg, and, in some cases, N^G-nitro-L-arginine (L-NNA) in a dose of 350 mg/kg. DETC and L-NNA were dissolved in normal saline and injected intraperitoneally in a volume of 0.2 ml. Sunflower oil used as the antioxidant was administered daily *per os* in a dose of 0.1 ml/mouse during 2 weeks. After decapitation the liver was removed, washed in normal saline, and frozen in liquid nitrogen. The amount of NO was evaluated from its incorporation into the paramagnetic mononitrosyl iron complexes (MNIC) with DETC measured by electron paramagnetic resonance [4]. The EPR spectra were recorded at 77°K on an SE/x-2543 (Radiopan) radiospectrometer. The concentration of MNIC-DETC complexes were calculated as described previously [4]. The results were processed using Student's t test (p=0.95) for each group comprising 8-10 mice.

RESULTS

First of all it should be noted that the dose of 0.05 ml CCl_4 per animal was chosen on the basis of previous studies [1], in which this dose had proved to be nonlethal but did cause damage to the cell membranes, interfered with the production of cytokinins, and, in consequence, induced an inflammatory process.

It is important to note that the amount of NO in mouse liver cells differs markedly in different experimental groups (from 1 to 7 nmol MNIC-DETC per gram of wet tissue). These differences are probably derive from the fact that we used random-bred mice, whose physiological status varies in different groups of animals. For this reason the experimental data in Fig. 1 are presented as the percentage of the amount of NO in the liver of CCl_4-treated mice to that in the liver of intact mice of the same group.

Injection of CCl_4 was found to modulate NO production in mouse liver cells (Fig. 1). Four hours postinjection the amount of NO was somewhat decreased but after 12 h it returned to the control level and reached a maximum 24 hours postinjection, being 4 times as high as in the control.

TABLE 1. Formation of MNIC-DETC in Mouse Liver in Three Groups of Animals Treated with CCl_4, L-NNA, or Antioxidant (n=8-10, $M\pm m$)

Group of animals	Experimental conditions	Formation of MNIC-DETC 30 min after injection of DETC, nmol/g wet tissue
1	Control+DETC	4.3±0.9
	Control+DETC, L-NNA	2.0±0.5
	CCl_4 (24 h)+DETC	10.2±2.2
	CCl_4 (24 h)+DETC, L-NNA	0.4±0.1
2	Control+DETC	2.2±0.5
	Control+DETC, L-NNA	1.1±0.2
	CCl_4 (24 h)+DETC	7.1±1.5
	CCl_4 (24 h)+DETC, L-NNA	0.4±0.1
3	Control+DETC	2.7±0.5
	Control+antioxidant+DETC	1.1±0.2
	CCl_4 (24 h)+DETC	7.5±1.7
	CCl_4 (24 h)+antioxidant+DETC	0.9±0.2

To elucidate whether this CCl_4-induced rise in the production of NO was mediated through activation of the NOS system, we used L-NNA. An injection of L-NNA to experimental animals prevented the formation of MNIC-DETC in the liver, the amount of NO being reduced by one-half in the control group, and MNIC-DETC was practically absent in the CCl_4-treated group (24 h postinjection) (Table 1). Thus, the CCl_4-induced hyperproduction of NO in mouse liver is mediated through activation of the NO-synthetase system.

Recent data on a dual role of O_2^- in the regulation of NO synthesis [2,3,5,6,9] may help explain the above kinetics of the CCl_4-induced changes in NO production in liver cells. The CCl_4-induced damage to cell membranes results in a release of free iron from compartments of the endoplasmic reticulum [11]. Being a prooxidant, Fe^{2+} can initiate lipid peroxidation, leading to the generation of O_2^-. The maximal release of free iron was found to occur 1.5 to 2 h after the injection of CCl_4 to experimental animals (0.05 ml/mouse). This was followed by a drop of the content of NO in the liver. The O_2^- generated presumably first oxidizes NO synthesized by c-NOS and then activates the synthesis of the inducible NO-synthetase system. The activation of i-NOS induced by cytokines and other agents was previously found to occur after 8 to 12 h [11], which coincides with the observed CCl_4-induced rise of NO in mouse liver, reaching a maximum after 24 h.

To verify this assumption we studied the effect of antioxidants on NO production in mouse liver. The antioxidants used in our experiments were vegetable oil tocopherols, which have been shown to exhibit a pronounced antioxidant activity [2]. The experiments showed that a 2-week administration of the antioxidant to the control animals reduced the amount of NO in comparison with that in nontreated animals by about 50%. In experimental animals which had received the antioxidant during 2 weeks CCl_4 in a dose of 0.05 ml/mouse did not elevate NO in the liver 24 h postinjection; on the contrary, this parameter was even reduced in comparison with that in intact animals (Table 1).

Thus, the chemical toxin CCl_4 activates the NO-synthetase system and enhances the production of NO in mouse liver cells. The effects of antioxidants on NO synthesis noted here are in conformity with the data of other authorities obtained on cells cultures (neuronal, macrophagal), which suggests the important role of reactive intermediate oxygen species in the regulation of the NO-synthetase system.

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