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PHARMACOLOGY AND TOXICOLOGY

Antioxidant Properties of a Pharmaceutical Substance Hypocard, a Potential Drug for Ischemic Disease

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Antioxidant activity of a pharmaceutical substance hypocard was compared with activity of nitromalic acid and well-known agents nicorandil and Mexidol. The ability of these substances to inhibit spontaneous and oxidant-induced LPO process in rat brain homogenate was analyzed. The mechanisms of these effects were studied. The antioxidant properties of hypocard manifested in the inhibition of Fe(II)-induced LPO were significantly more pronounced in comparison with Mexidol and nicorandil.

Key Words: *antioxidant; NO donor; lipid peroxidation; ischemic disease*

Oxidative stress is an important pathogenic mechanism of various human diseases including neurodegenerative, cardiovascular, and inflammatory disorders [1,4]. Ischemic brain and heart diseases over a long time remain the main cause of mortality in many developed countries. Drugs with antioxidant activity preventing or suppressing oxidative stress are used in the therapy of ischemia [6]. High efficiency of NO donors in the therapy of ischemic diseases of the brain and heart was demonstrated [9]. It was reported that introduction of NO donor fragments into drug structure increased their activity [14]. A pharmaceutical substance hypocard contains an NO donor fragment in its structure and potentially possesses antioxidant activity.

Here we compared antioxidant activity (inhibition of LPO in rat brain homogenate induced by different oxidants) of hypocard and well-known drugs nicorandil (contains NO donor fragment in its structure [11]), Mexidol (well-known drug with antioxidant activity widely used for treatment of the ischemic disease of the brain and heart [7,15]), and nitromalic acid (NO donor) and analyzed the mechanisms of this activity.

MATERIALS AND METHODS

The experiment was performed on male outbred rats weighing 200-220 g. All manipulations with animals were carried out in accordance with the decisions of the Bioethics Committee of the Institute of Physiologically Active Compounds. The investigation was performed using the equipment of the Common Use Centre of the Institute of Physiologically Active Substances.

Antiradical activity was measured using the DPPH test. A stable chromogen-radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) is widely used for the investigation of antiradical activity of antioxidants [10]. The amount of reduced DPPH was measured spectrophotometrically at λ=517 nm.

The effects of the test substances on spontaneous and Fe(II)-induced LPO in rat brain homogenate were evaluated using a modified TBA-test [13]. Optical density was measured at λ=540 nm.

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Fe(II)-chelating activity was evaluated by a modified method [12] based on the ability of potential iron chelators to compete with ferrozine for binding with iron ions. The generation of a Fe(II)—ferrozine complex was detected by the appearance of an absorption peak at 562 nm. EC_{50} of a concentration—effect curve corresponding to substance concentration inducing half-maximum effect was calculated using GraphPad Prism 7 software.

Control samples in all experiments contained distilled water.

Statistical analysis of the obtained data was performed using Microsoft Excel 2010 and GraphPad Prism 7 software. The data are presented as the *M*±*SE* by the results of at least 3 experiments. Student's *t* test for the estimation of statistically significant differences was chosen due to normality of data distribution.

RESULTS

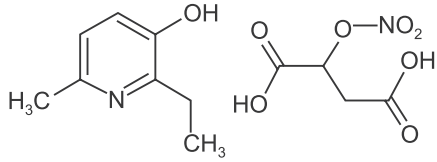
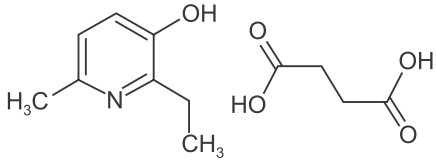
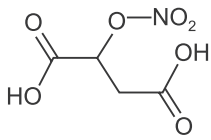
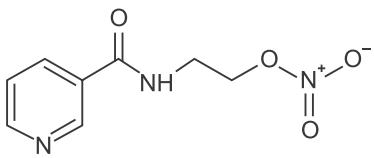
Antioxidant activity of a pharmaceutical substance hypocard, reference substances Mexidol and nicorandil, and nitromalic acid was compared (Table 1).

Mexidol and nicorandil are certified medicinal agents for the treatment of pathologies associated with ischemic damage to the brain and heart including acute cerebrovascular accident, dyscirculatory encephalopathy, vascular dystonia, atherosclerotic disorders of brain activity, neurotic and neurosis-like disorders with anxiety, as well as for the prevention and long-term treatment of angina [8]. These drugs have proven their high therapeutic effectiveness both in the treatment of various CNS and cardiovascular pathologies.

Under conditions of spontaneous LPO in rat brain homogenate, hypocard and reference agents (nicorandil, Mexidol, and nitromalic acid in a concentration of 100 μM) did not affect LPO intensity and exhibited no direct antioxidant effects (Fig. 1, *a*). The effects of the test substances on Fe(II)-initiated LPO were also studied. The concentration of the test substances of 100 μM (10^{-4} M) corresponded to the proposed therapeutic dose. Hypocard and nitromalic acid had pronounced antioxidant properties (malondialdehyde (MDA) content was 58.10±6.56 and 52.68±1.08% of the control, respectively; Fig. 1, *b*). These data suggest that the test substances exhibit antioxidant activity in case of Fe(II)-initiated LPO.

To elucidate the detailed mechanism of the effects of the test agents, antiradical and Fe(II)-chelating activities were assessed. Hypocard and reference agents did reveal no antiradical activity in the DPPH test, as its level remained unchanged (Fig. 2, *a*). Thus, taking into account inefficiency of hypocard and the reference substances towards spontaneous LPO and in the DPPH test, we can hypothesize that the mechanism of antioxidant properties of hypocard is not related to their ability to directly bind to free radicals. Preliminary experiments on the effects of the substances on

TABLE 1. Structure of the Test Substances

Test substance	Name	Structural formula
Pharmaceutical agent hypocard	2-Nitro succinate-3-oxy-6-methyl-2-ethylpyridine	
Mexidol	Ethylmethylhydroxypyridine succinate	
Nitromalic acid	2-(Nitrooxy)succinic acid	
Nicorandil	2-(pyridine-4-carbonylamino)ethyl nitrate	

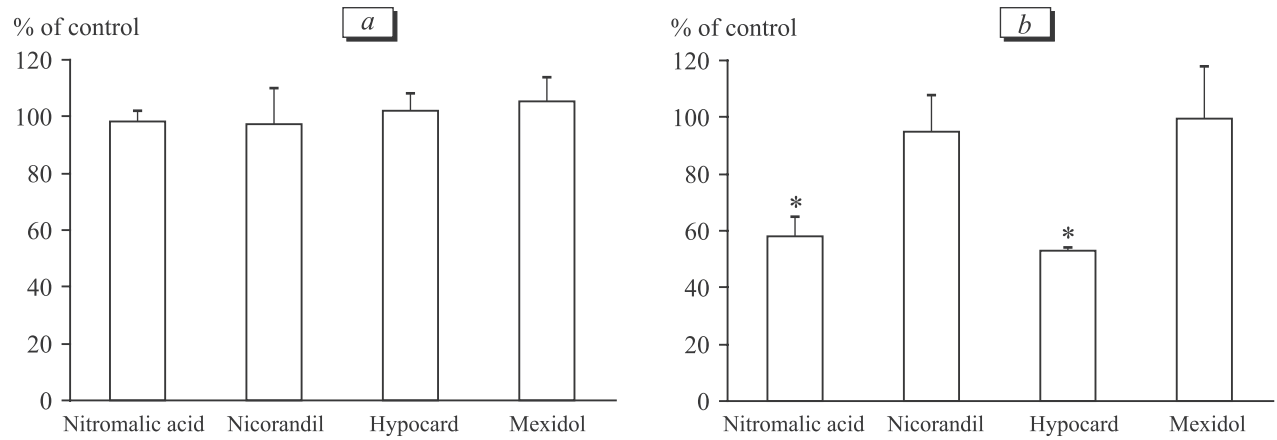


Fig. 1. Effects of hypocard and reference agents (100 μM) on MDA level during spontaneous (*a*) and iron-initiated LPO (*b*) in rat brain homogenates. *$p \leq 0.01$ in comparison with the control.

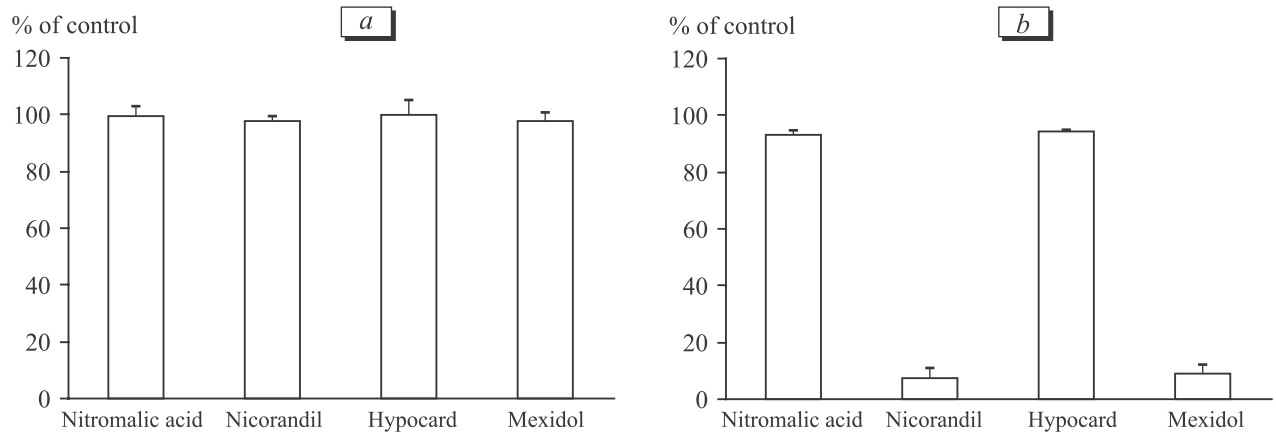


Fig. 2. Antiradical activity of hypocard and reference agents (100 μM) towards DPPH (*a*) and their Fe(II)-chelating activity (*b*).

Fe(II)-initiated LPO in rat brain homogenate revealed antiradical activity of hypocard and nitromalic acid, but not of reference substances Mexidol and nicorandil. Analysis of Fe(II)-chelating activity of hypocard and nitromalic acid was conducted.

Hypocard and nitromalic acid in a concentration of 100 μM, but no by Mexidol and nicorandil chelated practically 100% iron ions (Fig. 2, *b*).

For the substances with Fe(II)-chelating activity, EC_{50} was 8.21±4.8 μM for hypocard and 15.25± 3.46 μM for nitromalic acid. Hence, chelating process is dose-dependent for these substances.

The antioxidant effects of Mexidol were observed in various systems and concentrations (500 and 1000 μM). For example, Mexidol in a dose of 500 μM inhibited Fe(II)-induced LPO [3] and tert-butyl hydroxyperoxide-induced luminol oxidation [5]. An increase in Mexidol concentration to 0.8 mM and higher was followed by complete quenching of slow luminescence and disappearance of the latent period of Fe(II)-induced chemiluminescence in phospholipid liposome suspension [2]. In these and other studies of the antioxidant activity of Mexidol, its effect was manifested in concentrations >0.5 mM. Despite these concentrations significantly surpass the physiological (and therapeutic) range, we have analyzed antioxidant activity of hypocard, Mexidol, and nitromalic acid in concentrations of 0.5-1.0 mM in the reaction of Fe(II)-induced LPO in rat brain homogenates.

Mexidol exhibited antioxdant activity in doses >0.5 mM; 50% inhibition of LPO was attained at a concentration of 1 mM. On the contrary, hypocard and nitromalic acid induced 50% inhibition of LPO in a concentration of 100 μM (Fig. 3). Further increase in their concentration was followed by an interesting effect. Hypocard and nitromalic acid in a concentration of 1 mM exhibited pro-oxidant properties and increased MDA production to 175% of the control level. Both substances contain an NO donor fragment, and pro-oxidant activity can be explained by the fact that at concentrations <100 μM, the NO donor fragment of the molecule acts as a Fe(II) ion-chelating agent. When substance concentration in the model solution surpassed 100 μM, the amount of Fe(II) ions becomes

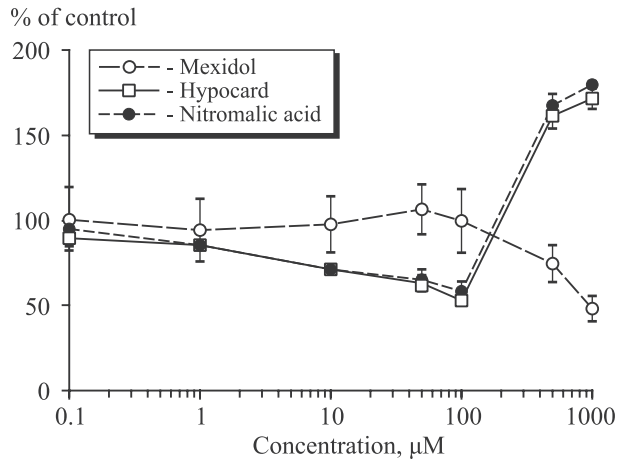


Fig. 3. Concentration dependence of the effects of hypocard and reference drugs on Fe(II)-induced LPO.

insufficient for binding with all hypocard and nitromalic acid molecules carrying NO donor fragment, which leads to the formation of nitroxyl radical, LPO intensification, and MDA accumulation. It should be noted that despite similar cheating activity towards Fe(II), hypocard exhibited antioxidant properties towards Fe(II)-induced LPO at significantly lower (by one order of magnitude) concentration than the reference substance Mexidol.

Thus, pharmaceutical substance hypocard by its antioxidant properties measured by inhibition of Fe(II)-induced LPO is superior to that the effects of the reference agent Mexidol and is of specific interest for further studies on *in vivo* models of ischemic damage to the brain and heart.

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