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ORIGINAL ARTICLE

Direct measurement of free radicals in the brain cortex and the blood serum after nociceptive stimulation in rats

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Runing title: Free radicals after nociceptive stimulation

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Abstract

The concentrations of ROS were measured in samples of the sensorimotor brain cortex and in the rat blood. We measured the following parameters: The six lines spectra, nitroxide radical, free hydroxyl radical and singleton oxygen. Their concentration was measured under physiological conditions, after the nociceptive stimulation and after the application of melatonin, both in normal and stimulated animals. In the brain cortex only the singleton oxygen decreased after the nociceptive stimulation, whereas the nitroxide radicals and six lines spectra increased. The free hydroxyl radicals did not change significantly. In the blood serum the six lines spectra and nitroxide radical increased, the concentration of the free hydroxyl radicals did not change. Melatonin increased both the hydroxyl and nitroxide radicals. There was a non-significant decrease in the six lines spectra. The estimation of ROS can be used as a tool for detecting metabolic changes and the consequences of different environmental influences, in our case the influence of nociception and melatonin.

Introduction

Reactive oxygen species ROS (hydroxyl radicals, peroxo radicals, superoxide radicals, singleton oxygen and others) as well as reactive nitroxide particles, RNS (nitroxide radicals) play an important role in physiological and pathological reactions. In some cases they are of use in immune reactions and in pathological conditions such as diabetes, cancers, ischemia, cardio-vascular disorders, reproductive cycles (free radicals on the surface of sperms), ophthalmologic disorders and others. Analogical situation is in physiology of plants' diseases. That is the reason why the research in medicine, biology and clinical practice needs to identify, determine and observe the process of changes in concentrations of free radicals in living organisms. For example in surgery of amputations of parts affected during diabetes it is important to know which tissues are irreversibly damaged and which ones not. Identification and determination of free radicals can be carried out by chemical (MDA, HPLC, TLC, UV spectroscopy), biological (observing of enzyme activities and measurement of antioxidative activities) and

spectroscopic methods. The most important method from these is the method of electronic paramagnetic spectroscopy, which enables the identification of free radicals, especially of their structure and reactivity, further it enables observation of disease progressions and treatment procedures as well as the control of treatment procedures' efficiency. The method enables to carry out measurements of withdrawn samples in vitro (ex vivo) such as tissues of brain, heart, lungs, livers, kidneys and blood. It also enables to carry out time dependent measurements in vivo of the abovementioned physiological reactions and pharmacokinetics. All of this is relevant for the observation of free radicals in an organism. For the short-term living radicals (hydroxyl radicals) the method of spin trapping is used, while for the study of long term changes in organism the method of spin label (spin marker) is used. The study of free radicals by the method of spin trapping requires the use of spin traps, which have to be tolerant towards the organism and its physiological reactions. These requirements meet Phenyl-tert-butyl-nitron (PBN) and 5,5-Dimethyl-1-Pyrroline N-oxide (DMPO), which enable the identification of various radicals with different chemical composition and structure. The study of EPR in vivo in these systems is used on laboratory animals (rats, mice), where free radicals in their tails are measured. Further step is the measurement of the whole laboratory animal with a space division of concentrations of particular radicals, which is an analogy of the computer NMR tomography in human medicine.

In our previous papers we described firstly the effects of nociceptive stimulation on the free radicals production, secondly the influence of the various antioxidants and analgesics [1,2].

We measured the production of TBARS, MDA, SOD, GSHPx (GPx), and the total antioxidant capacity (AOC). To measure free radicals directly, it is possible to use a method of electron paramagnetic resonance (EPR) [3–7], which can indicate the changes of reactive oxygen species (ROS) after the nociceptive stimulation.

We used this method to measure the influence of the nociceptive stimulation in the sensorimotor brain cortex and in the blood serum of the rat.

Materials and methods

Electron Paramagnetic (Spin) Resonance Spectroscopy (EPR/ESR). The estimation of free radicals by the EPR is based on the free radicals ability to absorb microwaves energy in strong magnetic fields [8]. The EPR spectra were recorded on an ERS-220 spectrometer (Academy of Sciences, Berlin, Germany), magnetic field was measured with an 1H-NMR magnetometer (Radiopan, Poznan, Poland) and microwave frequency with a frequency counter C3-54, all was done under the following conditions: microwave power 10 mW, modulation amplitude 0.02 mT, attenuation (gain) 12 dB, time constant 0.2sec., scan speed 0.3mT/min., calibration standard Cr(3+)/MgO, room temperature. The EPR spectra were recorded as a first derivation (in some cases as a second) and the main parameters such as g-factor values, hyperfine coupling constant A, line width Δ Hpp (peak-to-peak distance), and Δ App (peak-to-peak amplitude) were calculated according to Weil et al [8] . For spectra recording, handling and evaluation we used the CDAQ and CPRO (Galenuss GmbH, Berlin, Germany), and WINEPR (Bruker, Rheinstetten, Germany) programs. The Internet Databases from NIEHS, (Bethesda, Maryland, USA), and Univ. Bristol, UK were used.

An example of the measurement of nitroxide radicals (Fig. 1). Samples of the brain cortex and blood serum were transferred to the Eppendorf tubes with stoppers in which there was already 15 μl of DMPO (5,5´dimetyl-1-pyrroline N-oxide, Sigma Aldrich). The total volume was thus 200 μl. For the estimation of singleton oxygen 2,2,6,6 tetrametyl piperidine was used instead of DPMO.

Animals: adult male rats of the Wistar strain having the average weight 180–220 g were used. They were bred according to the principles of good laboratory

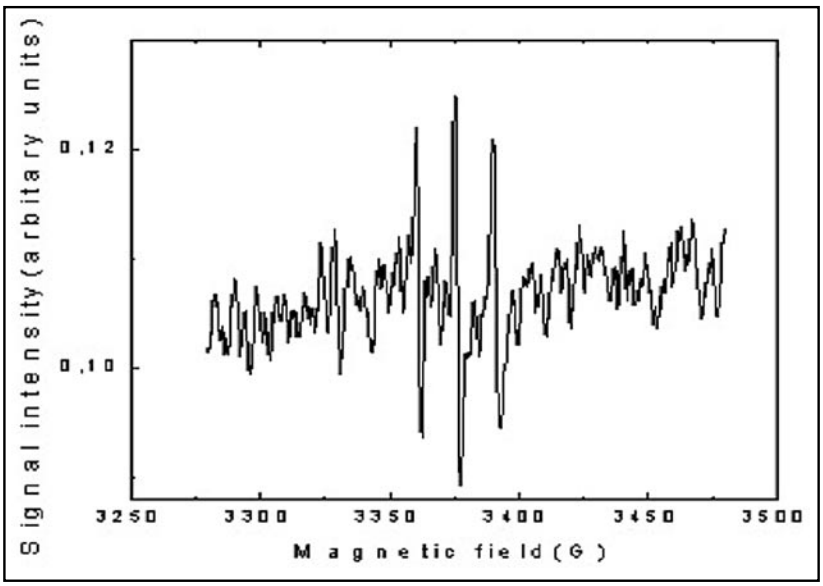

Figure 1: The example of EPR for three nitroxide radicals (1 NO, 2 NO, 3 NO) The spectrum of nitroxide radicals (NO). Spectral constants: g= 2.0046, a_N = 14.85 G = 1.485 mT, Signal width: ΔH_{PP} = 5.56 G = 0.55 mT, The interpretation: Triplet as a consequence of the interaction of 1 nonpaired electron with 1 nitrogen nucleus, Experimental conditions of the nitrogen radicals spectra:, X-Band, Field center: 3480 G, Field width: 200 G, Accumulation: 300 average scans, Resonator: hs 124 (Bruker), Microwave frequency: 9.76 GHz , Microwave Power: 0.02 mWatt, Time constant: 1 ms, Field modulation amplitude: H_m = 1 G (=0.1 mT).

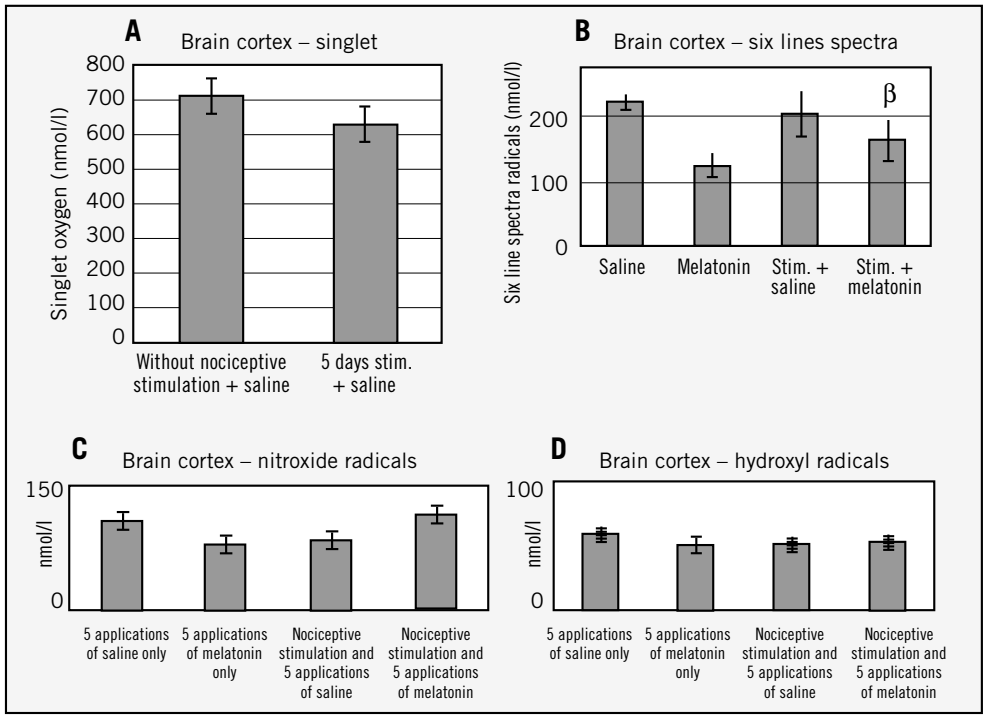

Figure 2: The concentration of singlet oxygen in the rat brain cortex (A)
The concentration of six lines spectra radicals in the rat brain cortex (B)
The concentration of nitroxide radical in the rat brain cortex (C)
The concentration of hydroxyl radical in the rat brain cortex (D)

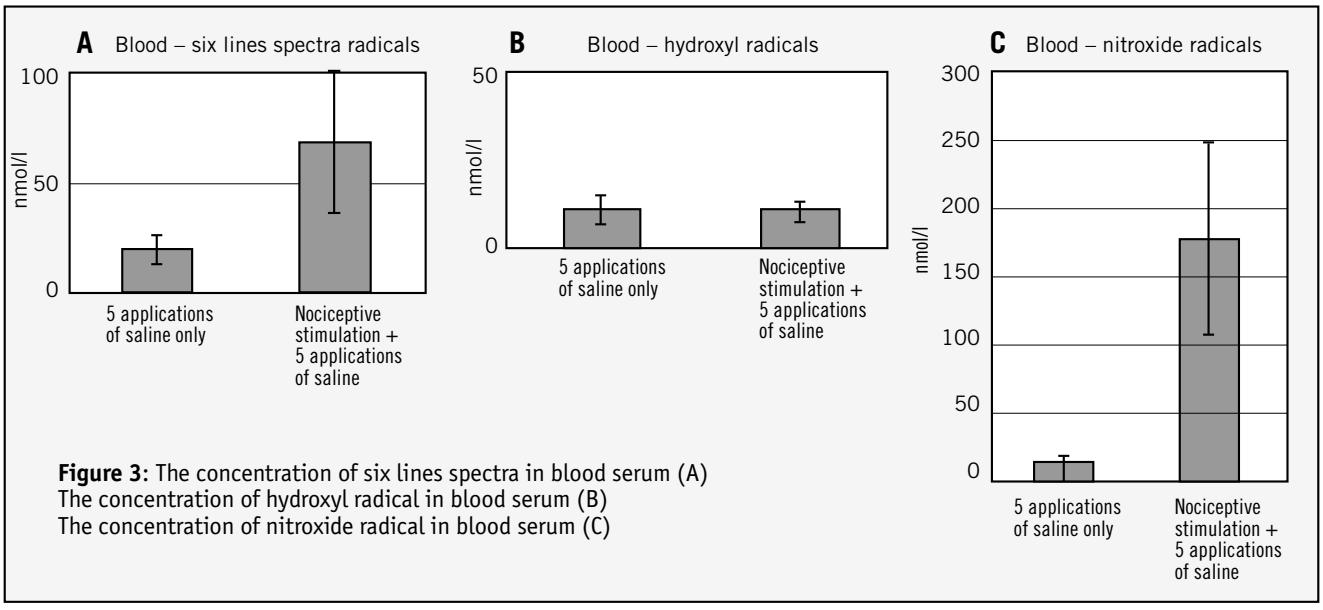

Figure 3: The concentration of six lines spectra in blood serum (A)
The concentration of hydroxyl radical in blood serum (B)
The concentration of nitroxide radical in blood serum (C)

practice (alternation of 12 hours light and 12 hours darkness, food ad libitum, constant light, 8 animals per cage. All animal procedures were in strict accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of the International Asssociation for the Study of Pain [9]. All experiments were approved by the Animal Care Committee of the 3rd Faculty of Medicine, Charles University, Prague. Suffering of the animals was reduced to the minimum.

Painful stimulation: our model of mechanical pain was used [10]. The clamping of both hind limbs was applied during 10 minutes for 5 consecutive days. Crocodile clamps were placed on the distal parts of hind limbs.

EPR determination: After the last 5th stimulation the sensorimotor brain cortex was removed under total anesthesia (Ketamin-Narkamon 90 mg/kg) and local anaesthesia (Procain 1%). The removed cortex was put in the DMPO Eppendorf tubes. The blood serum was taken simultaneously with the brain cortex.

Quantitative evaluation was performed by the integration of the measured spectra. The identification of individual spectra was performed on the basis of the spectral parameters and their comparison with the bibliographical data.

The application of melatonin: Animals were injected with melatonin (100 mg/kg, i.p. in 2 ml volume) or vehicle (2 ml of physiological saline i.p.) at 7:30 h. during 5 days of experiment [1].

Nitroxide radicals, six lines spectra, singlet oxygen and free hydroxyl radicals were evaluated.

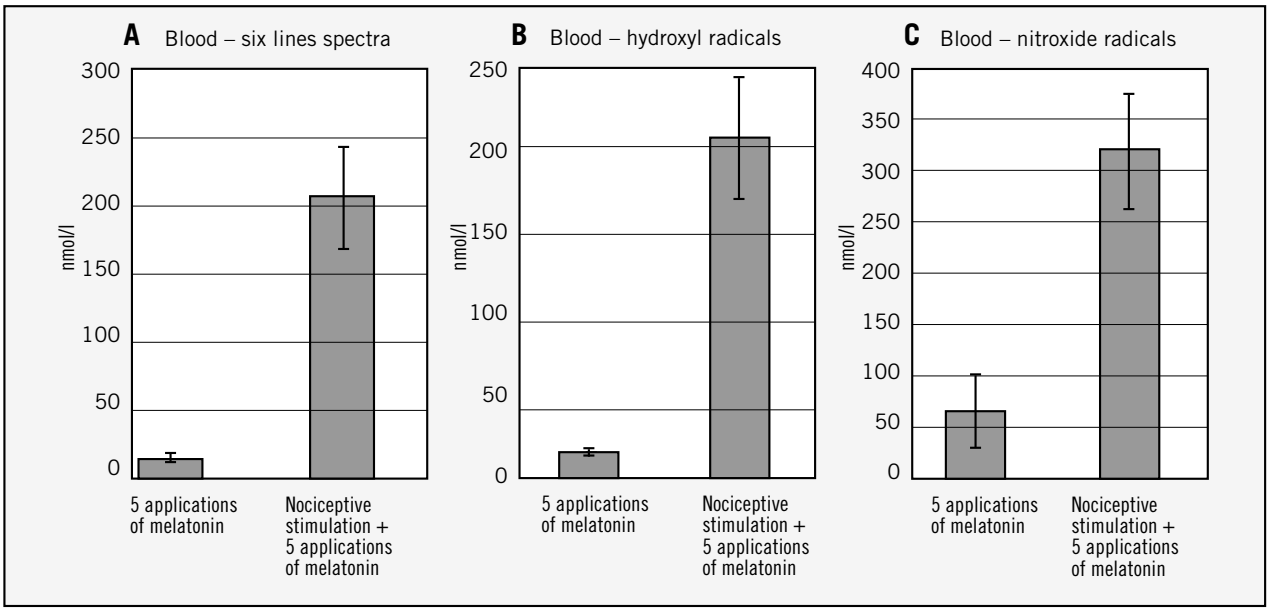

Figure 4: The concentration of six lines spectra in blood serum after the application of melatonin (A)
The concentration of hydroxyl radical in blood serum after the application of melatonin (B)
The concentration of nitroxide radical in blood serum after the application of melatonin (C)

Six Lines EPR spectra: Spin trapping technique used in biological reactions demonstrates the cases of partly oxidized proteins and amino acids or of semi products of oxidative reactions, which illustrate six lines spectra.

Results

Brain cortex (Fig. 2). The concentration of singlet oxygen decreased after 5 day stimulation. Six lines spectra : The nociceptive stimulation did not increase the six lines spectra, but melatonin decreased them. Nitroxid radicals: They increased after the nociceptive stimulation and melatonin decreased them. Free hydroxyl radicals: The level had been low and remained nearly (not significantly) changed.

Blood serum (Fig. 3). The six lines spectra were enhanced after nociceptive stimulation, There was no change in the hydroxyl radicals. Nitroxide radicals increased significantly.

The influence of melatonin (Fig. 4). Six lines spectra : melatonin slightly decreased the level. Hydroxyl radicals: they increased. Nitroxide radicals : they increased.

Statistical analysis

All data were evaluated using descriptive and ordinal statistics. According to the results obtained by this evaluation also the other hypothesis testing methods were chosen: ANOVA method represents the simple analysis of variance between groups, or, in the case of abnormal data distribution, variance F-test or mean value Z-test.

Discussion

Free radicals are eliminated from the body by several mechanisms. Radical reactions are strongly retarded by antioxidants. Free radicals are firmly fixed by other molecules and then detoxicated (quenching). A reaction between two free radicals results in their mutual inactivation. Free radicals can be eliminated from the body (for example by urine).

The brain is on one hand relatively badly protected by antioxidants and so the brain tissue is quickly attacked. On the other hand there is also the vasodilatation followed by the transfer of free radicals to the blood as a compensatory mechanism [11–13]. The decrease of singleton oxygen in the brain cortex could be explained by the generation of other free radicals that could react with the singleton oxygen. Singleton oxygen is not only highly reactive and easily oxidizes the neighboring substances, it also can be broken down by itself.

The nociceptive stimulation did not increase the six lines spectra because of their reaction with the surrounding biomolecules and the action of antioxidants, including melatonin. Some free radicals could also be inactivated by their reciprocal reaction. The six line spectra are relatively stable radicals and therefore their reaction with melatonin is slower [1, 14].

Nitroxide radicals [15]: The influence of melatonin was very interesting. Without the stimulation melatonin caused non-significant decrease. Nitroxyl radicals are not so dangerous and therefore it is possible that melatonin preferably reacts with more dangerous radicals. After the nociceptive stimulation which produced a higher amount of free extremely reactive hydroxyl radicals, melatonin was used mainly to elim-

inate them. The fact that it has no capacity for the nitroxyl radical elimination might be a possible explanation why the nitroxyl radicals increased after the stimulation. It is also possible that the superoxide was probably overproduced. It reacted promptly with the nitroxide radical to form peroxynitrite, which was followed by the nitration of the surrounding biomolecules. This process could explain the relatively low level of the nitroxide radicals in our experiments [16].

Free hydroxyl radicals have the half-time 10^{-9} s, they react very fast and consume the antioxidants. Consequently their level is low and stable.

BLOOD: The nociceptive stimulation increases the level of the six lines spectra because a higher amount of the newly formed hydroxyl radicals reacts with organic compounds and consequently increases the formation of six lines spectra. The free hydroxyl radicals react with nitrogen compounds and therefore also increase the formation of the nitroxide radicals. The used dose of melatonin is not able to protect either the six lines spectra formation or the formation of the nitroxide radicals. This might be the reason why the levels of these two less dangerous radicals are increased. Free hydroxyl radical and superoxide consume melatonin and they oxidize available biomolecules fast. The neutralization of the superoxide firmly increases nitroxide radical, which then reacts less with the superoxide. It is also possible that melatonin accelerates the transfer of free radicals from the brain into the blood.

However it is also necessary to mention that it is very difficult to distinguish strictly between nociception, pain and stress [17,18]

Acknowledgements

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