The relevance of circadian rhythms disruption on pulmonary SOD expression in rat

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Abstract
The light–dark cycle represents a significant component of the circadian system in most mammals. Any disturbance of this cycle is reflected in a large number of changes in the physiological and also behavioral status of the organism, together with considerable alterations of the redox balance. Increasing evidence suggests that reactive oxygen species (ROS) have their own function in the circadian system. Superoxide dismutases (SOD) family represents the first prompt antioxidant enzymatic system, identified in all aerobic organisms and able to counteract ROS toxicity; there are three distinct isoenzymes: CuZn-SOD (SOD1), Mn-SOD (SOD2), and extracellular EC-SOD (SOD3). In the case of circadian disruption, when ROS production is enhanced, the impact of the oxidative aggression on superoxide dismutases (SOD) rhythmicity and distribution is still unclear. To estimate the influence of circadian rhythms disruption on pulmonary SOD, we exposed male Wistar rats to continuous light stimuli for four weeks and then investigated the SOD immunohistochemical expression in lungs, which are among the most sensitive organs to oxygen. CuZn-SOD, Mn-SOD and EC-SOD presented a particular immunoreactivity in the investigated pulmonary tissues. These findings support our viewpoint that there is a direct correlation between the rhythmicity of circadian cycles and pulmonary SOD expression.

Keywords: circadian rhythms, light–dark cycle, SOD, redox balance, ROS, antioxidant.

Introduction
Recent years revealed new perspectives regarding the deciphering of circadian clocks mechanisms – genetic profile and molecular pathways. Chronobiology gains more and more ground because of its huge unexplored potential in a firm connection with health and diseases.

Hypotalamic suprachiasmatic nucleus (SCN) is unanimously recognized as the primary and the most powerful pacemaker, able to generate through its neurons’ coordinated activities a large variety of circadian rhythms, extending the rhythmicity to the peripheric organs. In order to synchronize all peripheric oscillations, the activity of SCN is also completed by the pineal gland and the eyes [1]. Biological clocks sustain the rhythmicity for an impressive number of processes – physiological, metabolic and behavioral, which collectively define the well-being of the organism. There are many signals, which can interact with the complex activity of SCN, inducing a direct or indirect regulatory action [2]. The synchronization of vital functions is essential for the organism to stay in resonance with external environmental conditions [3].

The SCN will modulate all the oscillators, at central and also at peripheric levels, generating a common well-defined rhythm for the entire organism. Molecular events develop in a particular arrangement of distinct transcriptional–translational feedback loops, which include genes that rule through their proteic profile [4, 5].

In normal conditions, circadian rhythms are usually aligned to exogenous time signals – among them, light and temperature are considered the most important for a great number of species. It is interesting to observe that mammalian circadian system is sensitive to light stimuli and that the daily light–dark cycle has an extremely impact on the biological functions [6]. Photoperiodicity is a strong link in a complex process of integration and adaptation, as a part of a continuous modulation of both timing of light exposure and light intensity [7].

Alternation of light and darkness, the so-called light–dark cycle, is crucial for the physiological response of the photosensitive structures from the body. Any disturbance of the light–dark cycle will be reflected in profound and substantial changes of physiological and behavioral processes; the amplitude of these changes is first revealed by specific photosensitive optic structures, specifically designed to react like light sensors [8]. This is not the
only response of the organism and the heterogeneity of involved mechanisms is far to be explained [9].

There is a consistent body of evidence supporting the fact that the circadian disturbance of light cycle triggers the production of a large amount of ROS in the whole body. Cytosolic SOD1, mitochondrial SOD2 and extracellular SOD3 are involved in a catalytic dismutation of superoxide radicals to hydrogen peroxide and oxygen. This experiment intends to investigate the adequate response of antioxidant defense systems, especially SODs in lungs, after a continuous exposure to light. The disturbance of circadian rhythms is related and reflected in SOD pulmonary expression [10, 11].

Materials and Methods

Animals

Twenty male Wistar rats (body weight 230–250 g) kept under standard laboratory conditions were used. The animals were allowed to acclimatize for three days before the experiment. The rats were housed in standard cages, at 22°C and 35–70% humidity and were fed with standard food and water ad libitum. Rats were divided into two groups: Group 1 – a control group (kept on a 12 hours light/day cycle) vs. Group 2 – kept in 24 hours of constant light, for six weeks. The experiment was performed in accordance with guidelines and all imposed ethical criteria, and under the permission of the Ethical Committee of “Grigore T. Popa” University of Medicine and Pharmacy, Iassy. All rats were weighted on a weekly basis. At the end of the experiment, lung and blood samples were collected.

Histologic exam

Paraffin-embedded pulmonary fragments were specifically processed; Hematoxylin and Eosin stain was performed on lung samples.

SOD assessment

To evaluate SOD activity, we used nitrotetrasolium blue chloride (NBT), as indicated by Csóvári S et al. (1991) [12]; the results were expressed as the activity units in mL of plasma.

Immunohistochemistry

This study intends to reveal the expression of each member of SOD family (CuZn-SOD, Mn-SOD, and EC-SOD) in the lung after continuous light exposure. The study was realized on sections cut from paraffin pulmonary embedded tissue, dewaxed in xylene and rehydrated in alcohol. Antigene retrieval was performed in citrate buffer (Dako-S1700) for 25 minutes at 96.5°C and washed in PBS. After blocking the endogenous peroxidase (3% hydrogen peroxide) and non-specific binding with Dako SO809 , the sections were incubated with adequate dilution of specific monoclonal antibodies: 1/200 – Rabbit Anti SOD1 Monoclonal Antibody, 1/200 – Mouse Anti SOD2 Monoclonal Antibody, and 1/100 – Mouse Anti SOD3 Monoclonal Antibody.

Statistical analysis

The data involving SOD activity was statistically analyzed using SPSS 17. All comparisons between the two explored groups were realized with ANOVA (analysis of variance) test, $\chi^2$-test and Student’s $t$-test (for differences between investigated groups). Differences were considered statistically significant if $p<0.05$.

Results

The assessment of SOD activity during the experiment revealed a significant increase of individual SOD values in Group 2 compared to the control group (Figure 1); SOD medium values for investigated groups present an ascendant trend, similar to the percentage difference of medium values (Figures 2 and 3).

The correlation SOD/weight for both groups of rats during the experiment is statistically significant for Group 2, where a decline of total body weight was observed during the experiment (Figures 4 and 5).
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The histopathological exam identifies the effects of oxidative pulmonary aggression: inflammation, destruction of the alveolar walls, incipient fibrosis; all of them are pathognomonic signs of oxidative injury for the second group of rats, reported to the control group (Figures 6 and 7). Because of its vast contact surface with oxygen, lungs are always extremely affected by oxidative damage, provided by an enhanced ROS release. The inflammatory processes are present along the main vascular branches, but also at bronchial and bronchiolar level, the amplitude of affected areas being considerable.

We correlated the resulted SOD immunolocations with the specific enzymatic distribution pattern in lungs. The study indicated a high CuZn-SOD immunoreactivity in the pulmonary tissue collected from Group 2, compared to Group 1 (Figures 8 and 9). Bronchiolar epithelium displayed an intense SOD1 immunoreactivity (Figure 10). The endothelial cells surrounding the lumen of large blood vessels presented an evident immune reactivity, instead of a moderate immunoreactivity of alveolar cells (Figure 11).

We observed an increase in the Mn-SOD immunoreactivity of bronchiolar epithelium, alveolar cells (Figure 12) and endothelial cells in Group 2 (Figure 13). Our study also indicates that EC-SOD immunoreactivity is moderately increased in bronchiolar epithelium and endothelium of large blood vessels in Group 2 (Figures 14 and 15).
Discussion

Under normal conditions, the circadian system is intimately related to the complex process of adaptation to the environmental conditions. A specific mechanism, centered on SCN, completed with peripheral tissues, was found able to coordinate a great number of biological functions, many of them with a vital impact on the body’s well-being status [13]. A triple functional axis controlled by circadian system is usually described, involving physiological, metabolic and behavioral reactions; this observation sustains the actual large interest for chronobiology and its implications.

The light–dark cycle is one of the most sensitive and influent components of circadian system. Any disorder or disturbance of its rhythmicity has distinct multiple negative consequences [14]. The central circadian pacemaker, SCN, together with many other secondary pacemakers, will produce altered signals, which interfere and generate altered phases of activity.

The disruption of periodicity and normal biological timing act as a trigger for a considerable ROS release, with a well-defined oxidative potential [15]. The organism has its own antioxidant defense system, in which SOD represents the first line of defense [16, 17].

Our study was realized starting from two long-standing questions:

- Can the circadian disruptions cause injuries in organs, which at first sight are not related to photoperiodicity?
- Can SOD determine an efficient antioxidant defense in these circumstances?

We have chosen the lungs, because their structure is very sensitive to oxygen and is deeply affected by ROS. At the same time, the lungs have developed their own antioxidant defense system, in which the SOD family has the dominant function [18].

The biochemical assessment of the rats under continuous light exposure revealed a clear increase of SOD values, as a principal parameter, which confirms the existence of an oxidative process.

The histopathologic exam revealed the pulmonary effect of oxidative aggression. It is known that the lung is vulnerable to superoxide anion radicals, which are excessively released during any imbalance of pro- and anti-oxidative factors [19, 20]. The result consists in the development of variable destructive injuries: inflammatory processes dispersed overall pulmonary surface, without a specific distribution, thickening of the alveolar walls and incipient fibrosis. The pulmonary affected tissue can no longer function as a normal respiratory structure.

The SOD immunohistochemistry assessment indicates the tissular distribution of SOD for each member of SOD family. There are certain SOD specific sites, which can generate antioxidant enzymes under distinct activation [21, 22].

In our experiment, SOD1 was expressed especially along bronchial and bronchiolar epithelium and endothelium of large blood vessels. Immunoreactivity for SOD2 was detected along the bronchiolar epithelium, alveolar cells and endothelium lining large blood vessels. SOD3 was expressed along the bronchi and bronchiolar epithelium and in a small degree in endothelial cells.

SOD posses a cyclic rhythmicity in lung, heart and skeletal muscle. The distribution of SOD is correlated with the most receptive sites to oxidative aggression. In fact, if we can specify the SOD immunolocaton, we can not exactly determine the mechanism, which allows a consistent release of SOD [23].

The most plausible mechanism suggests melatonin intervention. The so-called circadian clock is dependent on darkness, which ensures a normal melatonin production. During the disruption of circadian rhythms because of a long-term exposure to continuous light, a nocturnal suppression of melatonin production and secretion into the blood occurs [24–26].

Disturbed day/night cycle rhythm and cronobiologic periodicity of melatonin trigger the oxidative tissular impact. Melatonin can be considered a key factor – its falling level is usually related to a decrease of the antioxidant activity and an augmentation of tissular receptivity for oxidative aggression [27].

Conclusions

Our experimental study demonstrates that there is a complex interaction between the rhythmicity of circadian light–dark cycle and the tissular SOD immunoexpression
in lungs. Every disruption of circadian system components plays a major role in dysfunctions in the tight control of ROS production and release. As a significant consequence, pulmonary SOD activity and its immunodistribution reveal a direct enhanced antioxidant involvement.

References

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