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NITRIC OXIDE DETECTION IN AIR TREATED WITH TCHIJEVSKY IONIZER

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INTRODUCTION

Numerous investigations in the course of this century have provided evidence that the presence of a certain amount of negatively charged ionized gases in inhaled air is necessary for normal vital activity (Charry & Kvet, 1987). Their presence is detected by measurement of the amount of negative charge, and they are called "negative air ions" (NAI). Thus NAI are natural components of air and breath (Tchijevsky, 1960; Soyka & Edmonds, 1978). Their amount is high in clean natural air. It increases under the influence of such factors as electrical discharges, movement of water and water drops, and radioactive decay. In some health resorts negative air ions are particularly numerous, reaching several thousands and tens of thousands per cm^3. On the other hand, the amount of negative air ions falls dramatically to several tens per cm^3 or even to a complete absence of ions in polluted city air, in closed and conditioned rooms, in moving cars and aircraft, and near television sets and computers. The exhaustion of NAI leads to disturbance of health. Tchijevsky discovered the biological and physiological action of unipolar air ions in 1919. In the 1930's, Tchijevsky advanced the concept of artificial ionization of air for medical treatment and for the prevention of disease. He designed a high-voltage air ionizer called "Lustre". Tchijevsky demonstrated beneficial medical/biological effects of NAI inhalation in animals and later in patients (Tchijevsky, 1960). In particular, hypertension and bronchial asthma were cured in one to three weeks by daily half-hour sessions of negative air ion inhalation. In some cases, bronchial asthma attacks stopped immediately when inhalation was started. Many cases of successful treatment with negative air ions were observed in different countries. In particular, Kornblueh effectively used negative air ions for burn treatment (Minhart, David & Kornbluch, 1958). Krueger investigates the biochemical mechanisms of the beneficial, biological effects of negative air ion inhalation (Krueger & Smith, 1960). He found that inhalation of negative air ions decreases excessive levels of the neurotransmitter serotonin. Serotonin level elevates under unfavorable conditions and causes various pathological symptoms. A dramatic example of illness caused by an excess of serotonin is the mental depression and feeling of exhaustion that develop with a rise of the positive ion content in the inhaled air. This physiological state can be induced by dry hot winds that have various names in different countries. In the Near East they are known as Sharaf or Hamsin. Sulman obtained pronounced relief in the state of patients affected by Sharaf conditions through the inhalation of NAI. He showed that such inhalation also decreased the excessive level of serotonin (Sulman, Levy, Levy, Pfeifer, Superstine & Tal, 1974). It was found in rats that negative air ion inhalation abolishes the stress-induced impairment of the mitochondria in the brain and liver, and improves mitochondrial energy processes under adrenalin administration (Kondrashova & Grigorenko, 1985; Saakyan, 1998). Treatment with air ions *in vitro* promotes stability of physiological structure and energy functions of mitochondria (Stavrovskaya, Sirota, Saakyan & Kondrashova, 1998). Many other examples of the beneficial medical/biological effects of negative air ions are described in the reviews cited here and elsewhere (Krueger, 1973; Soyka & Edmonds, 1978; Garman, 1981). Two main mechanisms were proposed for beneficial

biological action of NAI, namely, an exchange of NAI for charged species in tissues and a decrease in serotonin level (Vasilev, 1953; Tchijevsky, 1960; Krueger & Reed, 1976), but the variety of biochemical processes induced by air ions suggested that each of them is hardly a primary act. We suggested that the search of primary mechanism of NAI action should be issued from their chemical nature. The superoxide radical serves as the main negatively charged species in air (Kellog, Yost, Barthakur & Krueger, 1979; Goldshtein, 1988; Goldshtein, Goldshtein & Merzlyak, 1992). Generation of superoxide and of stable product of its dismutation, hydrogen peroxide by "Tchijevsky Lustre" was shown in our laboratory (Kosenko, Kaminsky, Stavrovskaya, Sirota & Kondrashova, 1997; Kondrashova, Grigorenko, Tikhonov, Sirota, Temnov, Stavrovskaja, Kosyakova, Lange & Tikhonov, 2000). An increase of SOD activity in solutions, treated with air ions was also shown (Kosenko *et al.*, 1997), as well as regulatory action of air ion inhalation on the level of products of peroxidative oxidation of lipids in rats (Saakyan, Gogvadze, Sirota, Stavrovskaya & Kondrashova, 1998) and on SOD activity in patient blood (Kondrashova *et al.*, 2000). Recent data show that ROS in physiological concentrations, μM and lower, are essential for living activity (Khan & Wilson, 1995; Gamaley & Klybin, 1999; Kondrashova, 1999; Voeikov, 1999; Skurlatov, 1984). Own and literature data allow to state that the primary physicochemical mechanism of beneficial biological/medical effects of NAI is a mild stimulation of free radical peroxidative oxidation within a physiological range that is lower than in tissues under pathology and that can activate antioxidant defense of the organism, such as SOD (Kondrashova *et al.*, 1999). The presence of many pollutants, particularly HNO_2, in city and room air suggested formation of NO and CO during NAI treatment of air. These signal molecules induce relaxation blood vessels. They seem to be responsible for such effects of NAI as a quick relief from asthma attack reported earlier and relaxation and sleep of rats observed in our experiments (Tchijevsky, 1960; Saakyan *et al.*, 1998). Therefore we attempted to detect NO in ionized air using oxyhemoglobin as a trap. The results are presented in this paper.

MATERIALS AND METHODS

As a source of NAI we used the electroeffluvial ionizer, ELION-131 M (DIOD, Moscow) that generates air ions by electrical discharge from negatively charged spikes (for details see Kondrashova *et al.*, 2000). The construction of ionizer prevents ozone formation. Concentration of air ions was measured by aspiration condenser method with an ion counter SAI-TGU-66m (Tartu, Estonia). The negative, positive, light and heavy air ions can be detected separately. The source of ions is completely unipolar, only negative air ions are generated.

The oxyhemoglobin assay was used for NO detection as described by Feelisch, Kubitzek and Werringloer (1996). This method was developed for the determination of NO in the presence of molecular oxygen. The method is based on the reaction of oxyHb with NO to form metHb and nitrate:

$$HbO_2 + NO \rightarrow metHb + NO_3^{-\cdot}$$

No Hb-NO forms in the presence of O_2. The molar amount of NO generated during fixed time period is identical to the increase in molar concentration of metHb.

As a source of oxy-Hb the hemolysate of human red blood cells prepared as described by Riggs (1981) was chosen. 100 μl of blood in 900 μl of 10% sodium citrate were centrifuged at 1000 × *g* 10 min and erythrocytes were washed twice with 0.9% saline. Then 360 μl of cold bidistilled water was added to the erythrocyte pellet. After 10 min at 4°C the suspension was intensively stirred and centrifuged at 7000 × *g* for 20 min. Aliquots of the supernatant were frozen and kept at –20°C. Before experiment the stock solution of hemolysate was thawed and diluted to about 5 μM of oxyHb with 0.1 M phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The resulting solution was used in the experiments. In some cases SOD and catalase (1000 U/ml and 300 U/ml, respectively) were added to the incubation medium. For the treatment, 3 ml of oxyHb solutions in broad glass flasks were exposed to room air (control) or to NAI. The layer of solution was 1.3 mm deep. For NAI treatment the experimental flasks were left for 3–6 hours under the ionizer. The intensity of ionization was of 8,000,000 negative charges in cm^3 at the point of application. Control samples of the solution were kept in identical flasks in another room without ionization. Absolute absorption spectra of both ionized and control solutions were recorded every hour by a Uvicon-923 spectrophotometer (Kontron Instruments, Italy) using the buffer or the buffer plus enzymes as a reference. In some experiments the samples were incubated with a chemical source of NO, SNP, (2 mM) and the

spectra were recorded after 6 and 30 minutes of incubation. The resulting spectra were compared with ones from NAI treated samples. The amount of metHb increase as the result of NAI treatment was calculated using the millimolar extinction coefficient at 630 nm that is 3.7 mM^{-1} cm^{-1}. All measured values for metHb formation were corrected for the "basal" oxidation of oxyHb (at room air exposure) to compensate absorbance changes unrelated to the formation of NO:

$$\Delta C_{NO} = \Delta C_{metHb} = \Delta(\Delta D)/\varepsilon$$

where: ΔC is the increase in metHb concentration during fixed time period;
$\Delta(\Delta D) - \Delta D_{exp} - \Delta D_{control}$ is the difference in absorbance increase at 630 nm during the same time period for experimental and control samples respectively and ε is millimolar extinction coefficient at 630 nm, 3.7 mM^{-1} cm^{-1}.

SOD and catalase were obtained from Sigma. Other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Fig. 1 shows the changes in the absorption spectrum of oxy-Hb solution after exposure with NO donor nitroprusside. Initial spectrum had maxima at 415, 542 and 577 nm and no absorbance at 630 nm. This suggests that our hemolysate contained predominantly oxyHb and the amount of metHb is negligible. Spectral characteristics of the Hb-containing solution changed from those of oxyHb at the beginning to those of metHb at the end of the incubation. Three main points of changes were seen: the shift of γ-band from 415 to 407 nm, the decrease of absorbance in β- and α- bands and appearance "D" and "E" bands at 630 and at 500 nm, respectively. This process was time dependent. The upper spectrum corresponds to oxy-Hb, the lower to met-Hb and the middle one represents the combination of these two forms. Because SNP is a well known source of NO the observed changes are the results of reaction of NO with oxyHb. After exposure of oxy-Hb solution to NAI all three changes could be seen (Fig. 2) and simultaneously there was an increase in the concentration of both forms of Hb because of long exposure of solution in broad open flasks. After exposure of oxy-Hb solution to room air only an increase in concentration of oxy-Hb was detected (Fig. 3). The absorbance was increased at all wavelengths without shifts and decreased at 542 and 577 nm without shift of the Soret band. No increase in absorbance at 630 nm was detected during 3-hour incubation. Thus the metHb production from oxy-Hb was the result of the influence of NAI on oxy-Hb solution but not of the autoxidation of oxy-Hb. The presence of superoxide in NAI was shown earlier (Kondrashova *at al.*, 2000). NO can rapidly react with superoxide to form peroxynitrite ($ONOO^-$). It can decrease the trapping of NO by oxyHb. We used SOD and catalase to increase the trapping efficiency for NO by oxyHb and to prevent the possible oxidation of

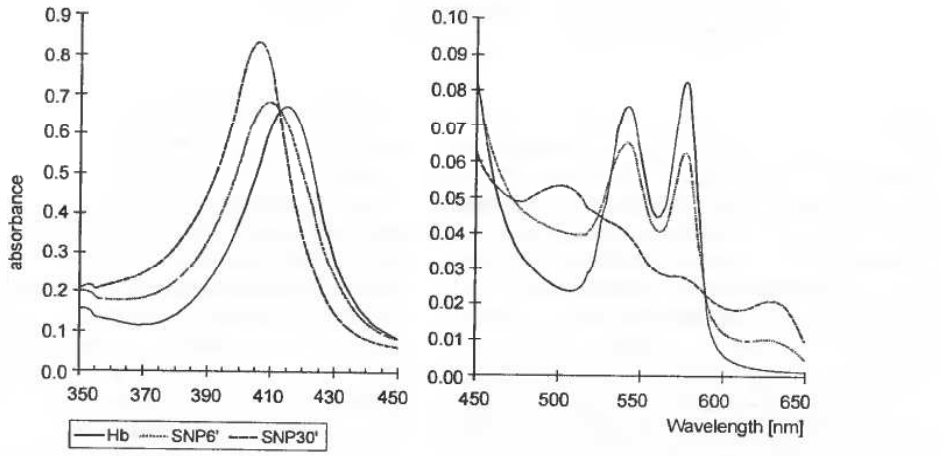

Fig. 1. Absorption spectra of oxyhemoglobin after exposure to sodium nitroprusside. Incubation of oxy Hb was performed as described in Materials and Methods. The spectra were recorded in 1 cm quartz cuvette at room temperature. Medium: 5 μM oxyHb; 0.1 M phosphate buffer; pH 7.0; EDTA 0.5 mM; SNP 2 mM. Line (——) – initial solution; (·····) – 6 minutes of incubation with SNP; (----) – 30 minutes of incubation with SNP

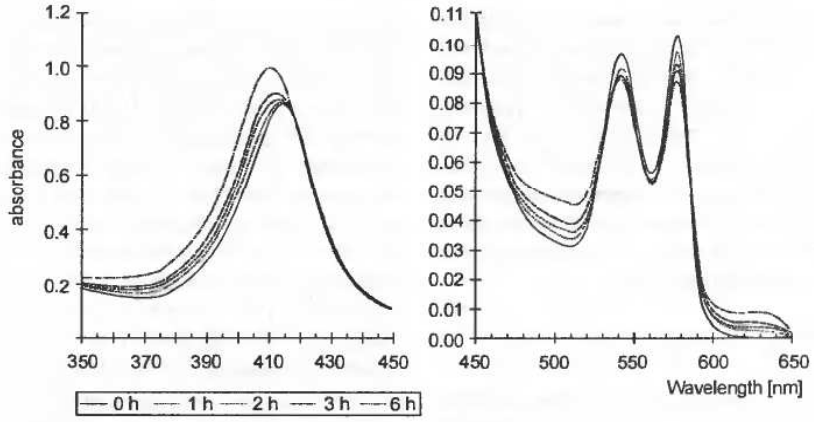

Fig. 2. Absorption spectra of oxyhemoglobin after exposure to air ions. The oxyHb solution was exposed to NAI as described in Materails and Methods and the spectra were recorded every hour in 1 cm quartz cuvette at room temperature. Medium: 6.5 μM oxyHb; 0.1 M phosphate buffer, pH 7.0; EDTA 0.5 mM. Line (——) – initial solution of oxyHb; other lines correspond respectively to 1, 2, 3 and 6 hour exposure to NAI

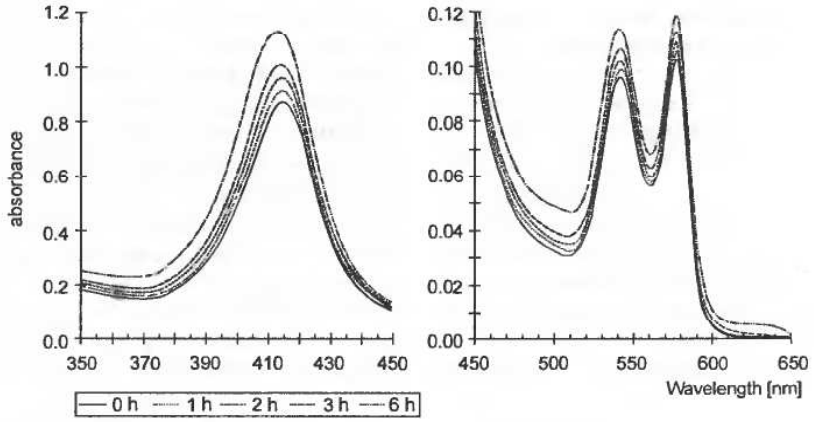

Fig. 3. Absorption spectra of oxyhemoglobin after exposure to room air. Medium and indications as in Fig. 2 but the samples were exposed to room air instead of NAI

oxyHb to metHb by superoxide. Fig. 4 and 5 show the same spectra as in Fig. 2 and 3, respectively, but SOD (1000 U/ml) and catalase (300 U/ml) were added to Hb solution. It can be seen that SOD and catalase had no effect on oxy-Hb oxidation. This suggested that oxidation of oxy-Hb to met-Hb in NAI is not due to interaction with superoxide or hydrogen peroxide. Because it is known that the rate of reaction between NO and oxy-Hb is rapid (Feelisch *et al.*, 1996) the latter serves as a trap for NO not only in solution (Ghafourifar & Richter, 1997) and also in air (Friebe, Malkewitz, Schultz & Koesling, 1996; Friebe A., Schultz & Koesling, 1998). Therefore we suggest that the active species generated by ionization and oxidizing Hb is NO.

The oxyhemoglobin technique is one of the most frequently applied assays for detection of nitric oxide. Using the oxy-Hb as a trap for NO we found the SOD- and catalase-insensitive formation of metHb in solutions treated with NAI and interpreted it as the accumulation of NO. The calculated rate of NO generation by ionizer was very small (0.15–0.38 μM/h) comparable with generation of superoxide, detected with an EPR trap, Tiron (0.5–1 μM/min) (Kondrashova *et al.*, 2000). We propose such pollutants of air as HNO_2, NO_2, and NO_3 to be the sources of NO. It is known that SOD can inhibit the reaction of NO derived from

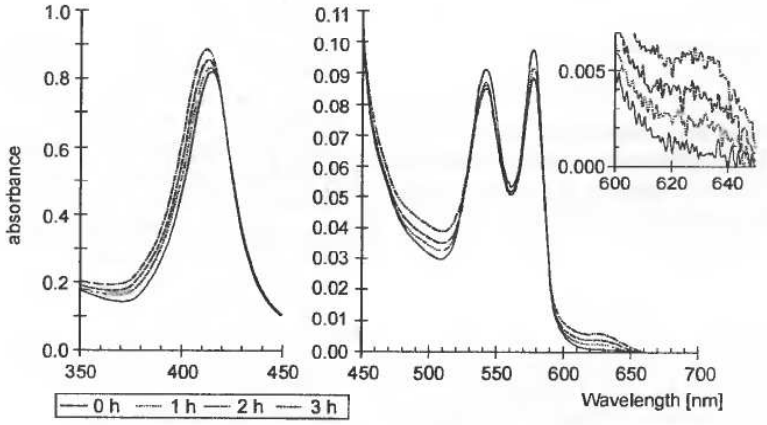

Fig. 4. Absorption spectra of oxyhemoglobin after exposure to air ions in the presence of SOD and catalase. Medium as in Fig. 2 but SOD (1000 U/ml) and catalase (300 U/ml) were added. Line (——) – initial spectra of oxyHb in the presence of enzymes; other lines correspond respectively to 1-, 2- and 3-hour exposure to NAI

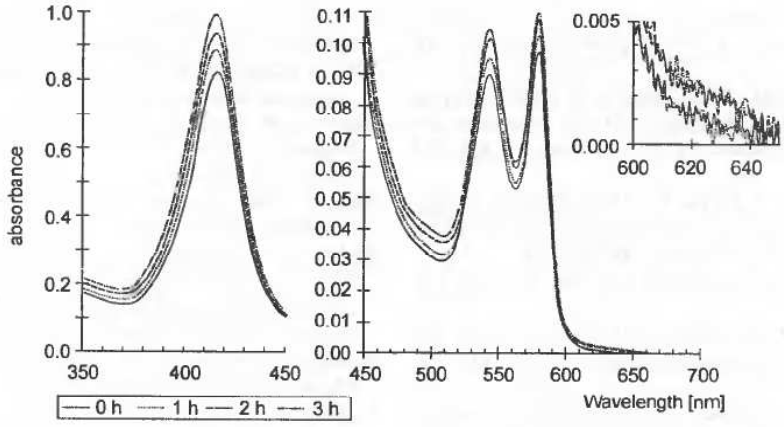

Fig. 5. Absorption spectra of oxyhemoglobin after exposure to room air in the presence of SOD and catalase. Medium and indications as in Fig. 4 but the samples were exposed to room air instead of NAI

the atmosphere with heme (Friebe *et al.*, 1998) and usually enhances the sensitivity of the hemoglobin assay for NO. In our experiments the oxy-Hb oxidation was insensitive to SOD. It seems possible to propose a mechanism which includes $O_2^{\pm}$:

$$HO_2^{\cdot} + NO_2 \rightarrow O_2 + HONO$$
$$2HONO \leftrightarrow NO^{\cdot} + NO_2 + H_2O$$

$HO_2^{\cdot}$ can be regenerated in the Haber-Weiss cycle (Fridovich, 1976):

$$O_2^{\pm} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + O_2$$
$$OH^{\cdot} + H_2O_2 \rightarrow H_2O + HO_2^{\cdot}$$

If superoxide really participates then SOD has a double effect on the yield of NO. It is well known that the mechanism of NO effect in the organism is the increase of soluble guanylate cyclase activity. This enzyme can be activated even by very slight concentrations of NO in atmosphere (Friebe *et al.*, 1998). Activation of the enzyme leads to relaxation of smooth muscles. Thus our finding that NO can be generated by aeroionizer may explain such facts as a quick relief from asthma attack and relaxation and sleep of rats during of NAI exposure. Krueger and Smith (1960) saw the relaxation of trachea constricted by positive air ions after influence of negative air ions on rabbits. They postulated that this effect is realized through decrease of serotonine concentration. Our experiments suggest also involvement of an NO-dependent mechanism.

Acknowledgments

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Abbreviations: NAI, negative air ions; EDTA, ethylenediamine tetraacetate; SOD, superoxide dismutase; Hb, hemoglobin; NO, nitric oxide; SNP, sodium nitroprusside.

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