Antioxidant Properties of Rimantadine in Influenza Virus Infected Mice and in Some Model Systems

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Influenza virus infection is associated with development of oxidative stress in lung and blood plasma, viz. increase of primary and secondary lipid peroxidation products. It was established that rimantadine treatment led to a decrease of the products of lipid peroxidation in tissues of mice experimentally infected with influenza virus A/Aichi/2/68 (H3N2). The effect is strongest in blood plasma (a decrease of about 50%) and weaker in the lung (about 20%). To elucidate the mechanism of this action of rimantadine, experiments were carried out with some model systems. The capability of rimantadine to scavenge superoxide radicals (scavenging properties) was studied in a system of xanthine-xanthine oxidase to generate superoxide. The amount of superoxide was measured spectrophotometrically by the NBT-test and chemiluminesce. Rimantadine does not show scavenging properties and its antioxidant effect observed *in vivo*, is not a result of its direct action on the processes of lipid peroxidation and/or interaction with antioxidant enzymes. The antioxidant properties of rimantadine were investigated by measurement of induced lipid peroxidation in a Fe^{2+} and $(Fe^{2+} - EDTA)$ system with an egg liposomal suspension. Our findings with model systems do not prove an antioxidant or prooxidant effect of the drug on the processes of lipid peroxidation. Apparently, the observed antioxidant effect of rimantadine *in vivo* is not connected directly with free radical processes in the organism.

Introduction

The acute influenza virus infections are accompanied by the so-called "oxidative stress", which is manifested in increased generation of reactive oxygen species (ROS). This generation eventually results in activation of free radical processes and particularly of lipid peroxidation (LPO) (Hennet et al., 1992; Peterhans, 1996, 1997) and inhibits drug metabolism (Mileva et al., 2000). The endogenous antioxidant systems are effective only at the initial steps of free radical damages. Later, the generation of ROS and free radicals increases and exceeds many-fold the capacity of endogenous utilization, hence in the course of the disease the quenching capacity of the antioxidant protection of the organism decreases (Inozemtseva and Chetverikova, 1991; Hennet et al., 1992). Recently, clinicians and researchers are interested in the action of the antioxidants, and the possibility of their use for prevention and treatment of influenza virus infection, assigned to so-called "free radical diseases" (Jacoby and Choi, 1994; Akaike et al., 1996; Peterhans, 1997). Seemingly, the net therapeutic effect of the drugs is a combination of their specific and antioxidant effect.

In recent years the chemotherapeutic rimantadine hydrochloride (Rim) is used on a large scale in the extreme prevention and treatment of influenza virus infections. It has a manifested antiflu effect upon the disease caused by influenza virus A(H3N2) and A(H2N2) (Hayden, 1997; Mossad, 1999).

The objective of the present paper is to establish whether Rim has an effect on the processes of lipid peroxidation (LPO), which accompanies the experimental viral infection with virus A/Aichi/2/68 (H3N2). To elucidate this process the ability of Rim was studied to interact with superoxide radicals, to influence their generation, and eventually to change the level of LPO processes in lung and blood plasma of infected mice and in some model systems.

Materials and Methods

Animals and treatment

Albino male mice, line ICR, were used with a body weight of 14–16 g. The animals were distributed into four groups as follows: Group I – control group (healthy animals); Group II – mice treated orally with Rim every day for 4 days; Group III – mice infected with influenza virus A / Aichi/2/68 (H3N2) – 1.5 of LD50 by intranasal inoculation; Group IV – Rim – supplemented mice infected with influenza virus A /Aichi/2/68 (H3N2) – 1.5 of LD50 by intranasal inoculation. Rim was applied in a dose of 20 mg/kg orally 2 h before and 2 h after inoculation and on 24, 48 and 72 h after infection.

The animals (n=8 for each day per group) were sacrificed on the 5th and 7th day, respectively. We collected the blood in test tubes for isolation of blood plasma. Lungs were perfused with ice-cold 1.15% KCl and were homogenized at 4 °C in 0.1 M K,Na-phosphate buffer, pH 7.4, 1:3 (w:v). The supernatant fraction was isolated by centrifugation at 10 000×g for 20 min.

Influenza virus A /Aichi/2/68 (H3N2) is from the collection of D. I. Ivanovsky Institute of Virology, Russian Academy of Medicine, Moscow.

Analysis of LPO products

Conjugated dienes were determined spectrophotometrically after previous total lipid extraction according to Folch *et al.* (1957). The chloroform phase was separated, the solvent removed in a rotation vacuum evaporator and the residue dissolved in ethanol. The optical density at 232 nm was measured by the method of Rechnagel and Glende (1984).

Endogenous LPO products reacting with 2– thiobarbituric acid (TBA – reactive substances, TBARS) were measured spectrophotometrically (λ_{max} = 532 nm) according to Asakawa and Matsushita (1980). TBARS in the blood plasma was assayed after Ledwozyw *et al.* (1986).

Protein concentracion was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Model systems and tissue homogenates for in vitro study

Liposomal suspension. A liposomal suspension obtained from phospholipids of egg yolk extracted according to Folch *et al.* (1957) was used. After evaporation to dryness, the chloroform fraction was dissolved in 50 mmol/l K-Na phosphate buffer pH 7.4 (PBS) to a final concentration of 5 mg lipid/ml.

Lung homogenate. Wistar rats (180–200 g) were used. Lungs were perfused *in situ* with ice-cold 1.15% KCl . The homogenization was carried out in PBS at a ratio tissue: PBS = 1:3 (w/v). The homogenate was dissolved with PBS to contain 1 mg/ ml protein.

Registration of TBARS

The TBARS of LPO was measured in liposomal suspension and in lung homogenate after incubation for 30 min at 37 °C. Each sample included 1 ml homogenate and 0.8 ml PBS (or 1.8 ml liposomal suspension with concentration of 1 mg lipid/ ml). The induction of LPO was initiated by adding of $FeCl_2$ or the complex (Fe^{2+} – EDTA) to the samples. The complex was obtained by equimolar mixing of $FeCl_2$ and EDTA to a final concentration of 1 mmol/l. The TBARS generated in the system was determined according to Asakava and Matsushita (1980).

Superoxide – scavenging properties of the preparation

The generation of ROS in the model system xanthine-xanthine oxidase (XO) and the changes occurring upon the Rim effect were investigated by two methods – by luminol-depending chemiluminescence (CL) and photometrically – by the nitro blue tetrazolium (NBT) test. The volume of the measured CL sample was 1 ml PBS containing as follows: 1 mmol/l xanthine, 0.1 mmol/l luminol, as well as Rim at concentration from 0 (for the controls) to 100 mmol/l. After incubation for 10 min at 37 °C the measurement was started by addition of 20 μl (100 IU/l) xanthine oxidase (XO). The CL scavenger index was calculated by the obtained data. It represents the ratio between the area under the CL curve, obtained for the sample, and the same area for the control. The spectrophotometric regis-

tration of superoxide $O_2 \cdot^-$ was carried out measuring the amount of formazan generated by $O_2 \cdot^-$ induced reduction of NBT. The investigated samples of a volume 1 ml PBS contained: 1 mmol/l xanthine, 2.10^{-3} IU XO, 0.04 mmol/l NBT, as well as Rim at concentrations from 0 to 100 μmol/l. The samples were incubated at 37 °C and the amount of the formed formazan measured by absorption at 560 nm. The time of incubation was selected so that the absorption for the controls was 0.2. The ratio of the absorption at 560 nm for the sample, containing Rim and the same absorption for the control in percents is called spectrophotometric scavenger index (Traykov *et al.*, 1997).

Activity of XO, catalase (CTS) and SOD in lung homogenate

The enzyme activities were measured after incubation of the lung homogenate for 30 min at 37 °C with Rim in the investigated concentration. The results are presented as units of enzyme activity per milligram protein. *The activity of SOD* in lung homogenate was determined by Geller and Winge (1984) method by reduction of NBT to formazan. The volume of each sample was 2 ml and each sample contained: 50 mmol/l Na_2CO_3, 0.1 mmol/l EDTA, 0.25 mmol/l NBT, 0.1 mmol/l xanthine in 50 mmol/l K-Na phosphate buffer pH 7.8. The reaction was initiated by adding 20 μl (100 IU/l) XO.

The activity of the CTS was determined according to the Claireborne's method (1985). Prior to analysis, 2% Triton X 100 in proportion of 1:9 (v/v) was added. The samples contained 2 ml 50 mmol/l K-Na phosphate buffer pH 7.0 and 50 μl homogenate. The reaction was initiated with 100 μl 30 mmol/l H_2O_2. The kinetics of the absorption at 240 nm was registered for 1 min. *The activity of XO* in the lung homogenate was determined in samples, which contained in 2 ml PBS: 1 mmol/l xanthine, homogenate (2 mg protein/ml), 0.04 mmol/l NBT. The amount of formazan obtained for 30 min (when the absorption reaches its maximum) was determined at 560 nm.

Rimantadine hydrochloride were obtained from prof. V. I. Ilyenko, Institute for Influenza, St. Petersburg, Russia.

All reagents of analycal grade were obtained from Aldrich Chem. Co., Henkel Co., Merck, Sigma Chem.Co.

The statistic processing of the results was performed with the program for multifactor analysis ANOVA . The statistic differentiation of the results was determined by Bonferroni's test. The data are presented as value ± SD.

Results

Effect of Rim on lipid peroxidation in lung of influenza virus infected mice

Lipid peroxidation is a complex process, including the generation of different kind of products, e.g. initial products such as conjugated dienes and end products such as TBARS. The virus inoculation was found to cause significant increase of conjugated dienes in lungs – ~3 times and ~1.3 times, on the 5th (Fig. 1A) and 7th day (Fig. 1B), respectively, as compared to control animals. Rim administration leads to a significant, ~1.2 times, decrease of conjugated dienes on the 5th day in comparison of untreated and virus infected mice and decreases to the level of the control group on the 7th day. Influenza virus infection had the same effect on the lung levels of TBARS. Significant increase of TBARS in lungs was recorded as compared to controls, ~45% on the 5th day (Fig. 1C) and ~25% on the 7th day (Fig. 1D) after virus inoculation. Rim administration resulted in a significant decrease of TBARS in lungs in comparison of non-supplemented virus inoculated animals. The TBARS was reduced to the control levels, on both the 5th and 7th day of influenza virus infection.

Effect of Rim on lipid peroxidation in blood of influenza virus infected mice

Our further experiments focused on changes of lipid peroxidation products in blood plasma. The levels of endogenous conjugated dienes in the blood of mice are shown in Fig. 1E and Fig. 1F. Significant increase in these early products of LPO was found on the 5th and 7th day after virus inoculation, being ~ 2.5 and ~ 2 times higher than in control animals. Rim led to a significant decrease of conjugated dienes in blood. The results are similar on the 5th and 7th day of influenza virus infection. The same effect of Rim on the blood levels of TBARS products was found. TBARS increased ~2 times and ~1.5 times, respectively, on the 5th
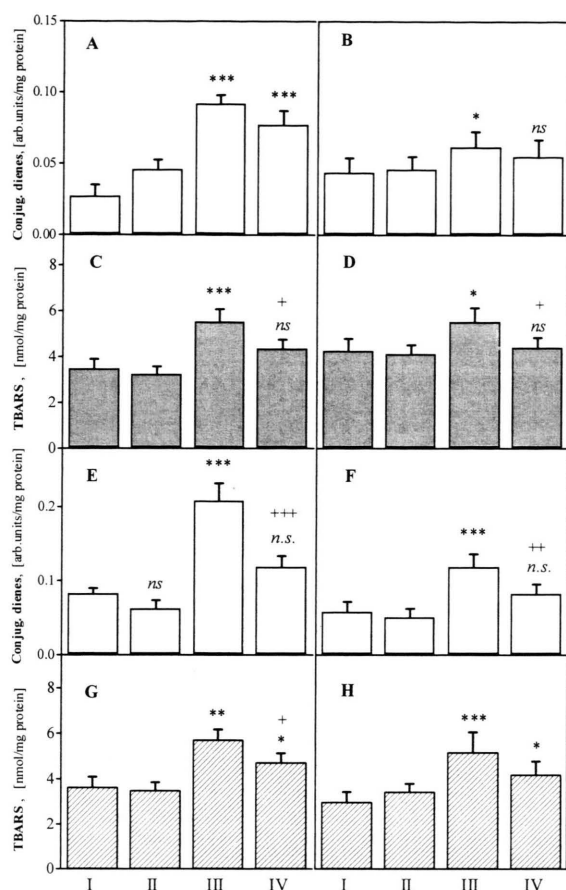
Fig. 1. Effect of influenza virus on the level of lipid peroxidation products in the lung (A, B, C, D) and in the blood (E, F, G, H) of mice on the 5th (A, C, E, G) and 7th day (B, D, F, H). I, control; II, rimantadine (healthy controls); III, influenza virus infection; IV, rimantadine (in scheme as described in Materials and Methods); * P < 0.05 versus group I; ** P < 0.01 versus group I; *** P < 0.001 versus group I; + P < 0.05 versus group III, ++ P < 0.01 versus group III, +++ P < 0.001 versus group III, ns non-significant.

(Fig. 1G) and 7th day (Fig. 1H) after virus inoculation. The blood levels of TBARS increased about 30% and 20%, respectively, on the 5th and 7th day. Rim led to a significant decrease of TBARS levels.

Investigation of superoxide – scavenging properties of the drug

We used a xanthine – xanthine oxidase system to generate superoxide, and two methods for reg-

istration of superoxide: a chemiluminescent method and a photometric one (NBT-test). As shown in Table I Rim does not show any scavenging properties in the systems used.

XO activity of Fig. 2 shows that the availability of Rim results in a slight increase of absorption, depending on concentration which indicates an increased generation of $O_2 \bullet^-$. This is probably due to an increased conversion of xanthine dehydrogenase into xanthine oxidase activity.

SOD activity in lung homogenate with Rim present at a concentration of 10^{-4} M showed a slight decrease of the enzyme activity (Fig. 2). Values close to the control group were measured at lower concentrations.

The activity of CTS decreased upon increase of the drug concentration and reached a 50% decrease at 10^{-4} M Rim (Fig. 2). Results obtained by initiation of LPO by adding of Fe^{2+} or (Fe^{2+} – EDTA) (Table I) indicate that the addition of Rim in this model systems has no effect on the amount of TBARS of lipid peroxidation. Experiments carried out with lung homogenate were repeated with liver homogenate. No significant difference was observed.

Discussion

The results represented by Fig. 1 indicate that the influenza virus infection increases both the level of the primary lipid peroxidation products and the amount of the late products on the 5th and 7-th day after virus inoculation (Group III). Comparing the data obtained for healthy animals from Groups I and II we conclude that administration of Rim *per se* does not lower the level of LPO products in blood plasma and lungs.

The Rim therapy also decreases the contents of the conjugated dienes and TBARS, which suggests an antioxidant effects of the drug. On the one hand, this might be due to properties of Rim to scavenge ROS. As reported, the change of production of ROS in the organism leads to a change of the level of LPO (Darley – Usmar *et al.*, 1995). On the other hand, it could be suggested that Rim probably has a direct effect on the ROS generation in biological tissues and/or has and indirect effect on the processes of lipid peroxidation by potentiating the capacity of the antioxidant protective systems.

Table I. Effect of rimantadine on the content of thiobarbituric acid – reactive substances in liposome suspension and lung homogenate containing Fe^{2+} or (Fe^{2+} – EDTA) and scavenging properties in a xanthine-xanthine oxidase system for superoxide generation. TBA test was aplicated after 30 min incubation of the probes at 37 C.

Rimantadine (μmol/l)	Liposome suspension MDA (nmol/mg lipid)		Lung homogenate MDA (nmol/mg protein)		Xanthine-xanthine oxidase system for O_2^--generation	
	Fe^{2+}	Fe^{2+} – EDTA	Fe^{2+}	Fe^{2+} – EDTA	CL – SI*(%)	SPh – SI** (%)
0.0	6.73±0.33	4.73±0.20	4.43±0.20	0.26±0.01	–	–
1.0	6.02±0.55	5.14±0.49	5.14±0.49	0.24±0.03	92.63±5.44	97.98± 8.04
10.0	5.77±0.35	4.29±0.63	4.28±0.63	0.22±0.03	100.43±3.45	98.76±12.00
100.0	6.25±0.59	4.61±0.37	4.61±0.37	0.24±0.02	99.65±5.71	100.46±10.31

* Chemiluminescence scavenging index (CL – SI) – the ratio (in percentage) between chemiluminescence in the presence and absence of the tested drug. ** Spectrophotometric scavenging index (SPh – SI) – the ratio (in percent) between changes of absorbance at 560 nm with and without the tested drug.
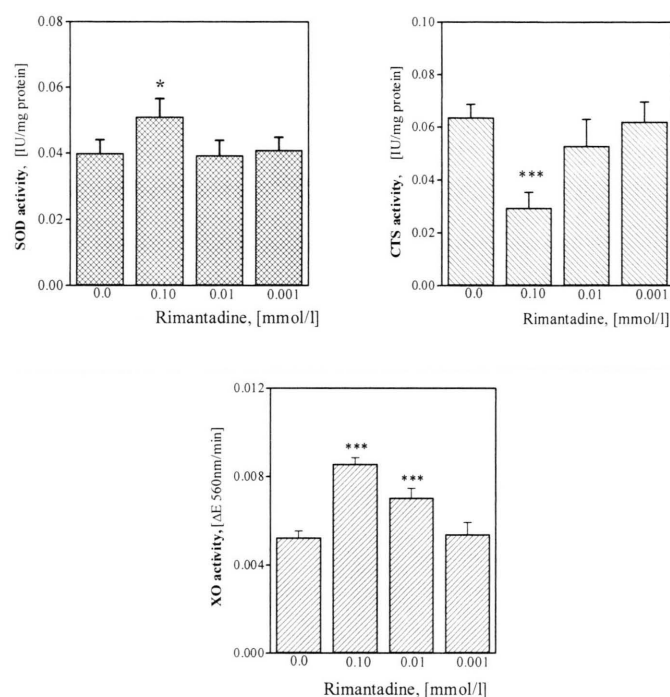

Fig. 2. Effect of rimantadine on the superoxide dismutase, catalase and endogenous xanthine oxidase activity in lung homogenate measured as described in Materials and Methods.

We checked whether the antioxidant-like effect of Rim in experimental influenza infection is connected with the Riminduced decrease of the ROS level. Results of Table I show that Rim does not scavenge $O_2\bullet^-$, indicating that a possible antioxidant effect of Rim should be due to other mechanisms. Such an effect is the influence of Rim on the transformation of the endogenous xanthine dehydrogenase into xanthine oxidase activity (Fig. 2). The weak and concentration-dependent increase of absorption suggests such an enhanced conversion, although this can not explain the anti-oxidant – like effect of Rim in influenza infection. The level of ROS in biological tissues is controlled to a high degree by SOD and catalase. Fig. 2 shows that Rim lowers slightly the SOD activity at 10^{-4} M. The tendency is similar to the activity of CTS in the presence of Rim (Fig. 2). This suggests a slight prooxidant effect of Rim, and contradicts the well-manifested antioxidant effect of Rim observed *in vivo*. Our findings exclude the potential of Rim to influence the lipid peroxidation in sus-

pension of phospholipid liposomes and lung homogenate (Table I). Rim has no direct effect on the amount of TBARS. The experiments carried out in model systems do not provide sufficient arguments to prove the antioxidant or prooxidant effect of the drug on the processes of LPO.

The results obtained indicate that the observed antioxidant effect of Rim *in vivo* is not connected directly with the effect on the free radical processes in the organism. Undoubtedly, the therapeutic effect of Rim is due to its specific effect on the virus replication (Hay *et al.*, 1985; Wharton *et al.*, 1990; Shigeta, 1997). In the course of the disease Rim interacts with M2 protein of the influ-

enza virus and lowers both its ability to form a hydrophobic channel, and its affinity towards the lipid bilayer of the host cell (Kharitonenkov *et al.*, 1988). Rim prevents the conformation changes of influenza virus haemagglutinin as well as the morphological changes of haemagglutinin spikes on the virus surface (Mikheeva *et al.*, 1989). Inclusion of Rim at this early stage of development of the infection is a possible cause for the subsequently observed lowered free radical pathology.

The metabolism of Rim by the liver monooxygenases (Hoffman *et al.*, 1988) which results in products with antioxidant effect is another possible cause for the *in vivo* effects.

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