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ORGAN TOXICITY AND MECHANISMS

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Abstract The present study provides a direct experimental evidence that the combination of influenza A/Aichi/2/68 (H3N2) infection with different models of ''oxidative stress'', such as immobilization, cold and cold-restraint, is associated with graduated oxidative disturbances in the liver of mice, despite the absence of virus and inflammation in this tissue. It was found that experimental influenza virus infection is accompanied with a significant increase of lipid peroxidation products, a decrease of natural antioxidants (vitamin E, glutathione) and cytochrome P-450, an inhibition of cytochrome *c* reductase and liver monooxygenases (analgin-*N*-demethylase and amidopyrine-*N*-demethylase). Immobilization and cold stress, applied separately or in combination (cold-restraint), did not influence significantly any of the analysed parameters compared to those of the control group of non-infected mice. Preliminary exposure of mice to immobilization or cold stress and subsequent inoculation of influenza virus resulted in a significant increase of lipid peroxidation products and a significant decrease of vitamin E and reduced glutathione, compared with levels in control (non-infected) animals. Compared to influenza virus-infected and non-stressed animals, the changes in all these parameters were negligible. Immobilization or cold stress, applied in combination with influenza virus infection, partially prevented the suppressive effect of influenza virus on cytochrome P-450 and liver monooxygenases. A tendency towards normalization of these parameters to the control levels was observed. However, after application of cold-restraint plus influenza virus infection, the level of cytochrome P-450 and activity of cytochrome *c* reductase stayed markedly lower than in infected and non-stressed animals. The activities of liver monooxygenases were slightly increased compared with those of infected and non-stressed animals, but stayed relatively low compared to control (non-infected) mice. Combination of cold-restraint and influenza virus infection resulted in a greater synergistic increase of lipid peroxidation products and a greater synergistic decrease of vitamin E and reduced glutathione compared to controls, as well as to influenza virus-infected and non-stressed animals.

Keywords Influenza virus infection · Immobilization · Cold-restraint stress · Lipid peroxidation · Antioxidants

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Introduction

The pathogenesis of influenza virus infection involves various factors and, despite considerable research over many decades, the exact mechanism(s) underlying influenza virus toxicity still remains to be elucidated. The acute form of influenza was shown to result in a release of several biogenic amines (Han et al. 2000), a production of interferon (Samuel 1991; Cox and Hughes 1999), an activation of nitric oxide synthase and xanthine oxidase (Oda et al. 1989; Akaike et al. 1996; Peterhans et al. 1997a; Murphy et al. 1998), a stimulation of respiratory burst in phagocytic cells (Peterhans 1987, 1997a) and an accumulation of lipid peroxidation products in blood and target organs (Oda et al. 1989; Schwarz 1996; Peterhans 1997a, 1997b; Murphy et al. 1998; Mileva et al. 2000). The liver is one of the most widely investigated targets for influenza. Although it is not a site of virus replication, this organ has been shown to be influenced to a certain extent in influenza virus infection (Gorbunov et al. 1992; Hennet et al. 1992; Kang et al. 1992).

The data obtained support the important role of ''oxidative stress'' in the development of liver toxicosis

under acute influenza virus infection. The oxidative stress is defined as a disturbance of the pro-oxidant/antioxidant balance in favour of pro-oxidants (Kagan et al. 1988; Schwarz 1996). It was observed that the oxidative stress that occurs with influenza virus infection is followed by a compensatory increase of activities of antioxidative enzymes and a decrease of lipid- and water-soluble antioxidants in the liver (Jacoby and Choi 1994; Choi et al. 1996; Peterhans 1997a, 1997b). For chemotherapeutic management of influenza virus infection it is important to know what's the relative role of antioxidative capacity and oxidative damage of a liver among the other mechanisms underlying liver toxicosis in influenza. Strategies are being devised to incorporate antioxidants and agents interfering with the harmful effects of cytokines, catecholamines and other mediators into the therapeutic armamentarium for control of influenza virus infection.

In our previous work we demonstrated that influenza virus infection decreases the activities of cytochrome P-450-dependent monooxygenases and thus could enhance the toxic effects of some hydrophobic drugs with low therapeutic indices, if they are administered at the same time (Mileva et al. 2000). On the other hand, treatment of animals with antioxidant supplements led to elimination of the effects of influenza virus on liver monooxygenase activities as well as to a suppression of lipid peroxidation in the liver (Asakawa and Matsushita 1980; Akaike et al. 1990; Hayek et al. 1997; Peterhans 1997b; Mileva et al. 2000). The suppression of liver antioxidant defence system before influenza virus inoculation would, probably, result in an acceleration of oxidative stress and graduated liver damages.

Immobilization and cold-restraint stress are widely used experimental models that are accompanied by considerable decrease of antioxidative capacity of the animal organism (Simmons et al. 1991; Das and Banerjee 1993; Kovacheva-Ivanova et al. 1994; Oishi et al. 1999). Thus, these models may be used for indirect modulation of antioxidant deficiency in the liver. To our knowledge there are few reports demonstrating the hepatotoxic effects of influenza virus infection in animals that have undergone to oxidative stress (Chetverikova et al. 1987; Misheneva et al. 1988).

Based on the data mentioned above, the present study was designed to investigate the effect of immobilization, cold and cold-restraint stress on the levels of lipid peroxidation products, endogenous antioxidants and the activities of drug-metabolizing enzymes in liver of mice infected with influenza virus A/Aichi/2/68 (H3N2).

Materials and methods

Animals and treatments

The experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Bulgarian Academy of Science. Propofol anesthesia was used, which does not influence liver monooxygenase system.

Albino male mice, line ICR (14–16 g) were used. The animals were maintained on a standard diet and were fasted for 24 h before the study. The mice were assigned to eight groups, as follows:

- Group I ($n = 10$), control group (healthy animals)
- Group II ($n = 10$), mice subjected to immobilization stress in stereotactic frames (cages) for 4 h
- Group III ($n = 10$), mice subjected to cold stress in a refrigerated cabinet for 4 h at 4°C
- Group IV ($n = 10$), mice subjected to cold-restraint stress (immobilized in stereotactic frames and subjected to cold stress in refrigerated cabinet for 4 h at 4°C)
- Group V ($n = 10$), mice infected with influenza virus A/Aichi/2/68 (H3N2) (0.5 of LD_{50}) by intranasal inoculation
- Group VI ($n = 16$), mice subjected to immobilization stress for 4 h and subsequently infected with influenza virus A (0.5 of LD_{50})
- Group VII (n = 16), mice subjected to cold stress for 4 h at 4°C and subsequently infected with influenza virus A (0.5 of LD_{50})
- Group VIII (n = 16), mice subjected to cold-restraint stress and subsequently infected with influenza virus A (0.5 of LD_{50})

The influenza virus A/Aichi/2/68 (H3N2) was from the collection of the Institute of Virology, Russian Academy of Science, Moscow.

The animals were decapitated on the day 5. Livers and 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). Virus titre in lung was determined by a haemagglutination test (Akaike et al. 1989). Lung lesions were analysed macroscopically and were expressed as percentage of control.

Liver microsomal fraction was isolated by differential centrifugation (10,000 *g* for 20 min, 100,000 *g* for 60 min).

Measurements

Endogenous lipid peroxidation (LPO) products reacting with 2-thiobarbituric acid (thiobarbituric acid-reactive substances, TBARS) were measured spectrophotometrically ($\lambda_{max} = 532$ nm) by the method of Asakawa and Matsushita (1980).

Total lipids were extracted from liver homogenates by a chloroform:methanol mixture (2:1, v/v), according to the method of Folch et al. (1957). Fluorescent lipofuscine-like products were measured at $\lambda_{ex} = 360$ nm and $\lambda_{em} = 420$ nm (Dillard and Tappel 1984).

Vitamin E content was analysed by high performance liquid chromatography (HPLC) using the method of Lang et al. (1986). Glutathione content was analysed spectrophotometrically by the GSSG-reductase assay (Adams et al. 1983).

Cytochrome P-450 was assayed according to the method of Matsubara et al. (1976) and NADPH-cytochrome *c* reductase (CCR) activity according to the procedure of Roering et al. (1972). Analgin-*N*-demethylase (ANND) and amidopyrine-*N*-demethylase (APND) activities were determined by the method of Nash (1953).

Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard. Lipid concentration was determined by the method of Richard et al. (1974).

All reagents were of analytical or HPLC grade and were obtained from Aldrich Chemical Co. (Munich, Germany), Henkel Co. (Dusseldorf, Germany), Merck (Darmstadt, Germany) or Sigma Chemical Co. (Deisenhofen, Germany).

Statistical analysis

One-way analysis of variance (ANOVA) was used, followed by Bonferroni's test for significant differences. Statistical significance was defined at $P < 0.05$. The statistical procedures were performed with GraphPad InStat software (San Diego, Calif., USA). Data were expressed as means ± SEM. The data, illustrating the effects

Table 1. Titre of influenza virus and incidence of lesions in lungs of experimental animals. Titre values are expressed a mean haemagglutination (*HA*) units ± SEM

Group	Experimental procedure	Virus titre, (HA units)	Lung lesions, (%)
I	Control	0	0
II	Immobilization	0	0
III	Cold stress	0	0
IV	Cold-restraint stress	0	24**
V	Influenza virus infection	4.32 ± 1.40	31***
VI	Influenza virus infection + immobilization	7.88 ± 2.84	52***
VII	Influenza virus infection + cold stress	7.92 ± 2.90	64***$^{+}$
VIII	Influenza virus infection + cold restraint stress	16.50 ± 3.73^{+}	89***$^{++}$

$^{+}P < 0.05$
$^{++}P < 0.01$ versus group V
$^{**}P < 0.01$
$^{***}P < 0.001$ versus group I

of combined application of influenza virus infection, immobilization and/or cold-restraint stress, were analysed statistically by comparison with (i) control, non-infected animals, and (ii) non-stressed influenza virus-infected animals.

Results

Influenza virus yields in lungs and livers

Virus yields in the lungs of experimental animals are shown in Table 1. It was established that the virus titre in the lungs of influenza-infected mice was about 4 haemagglutination units (HA units) and increased in lungs of mice, subjected to combined application of influenza virus infection and stress model as follows:

- Immobilization plus influenza virus infection, 8 HA units (non significant)
- Cold stress plus influenza virus infection, 8 HA units (non-significant)
- Cold-restraint stress plus influenza virus infection, 16 HA units ($P < 0.05$)

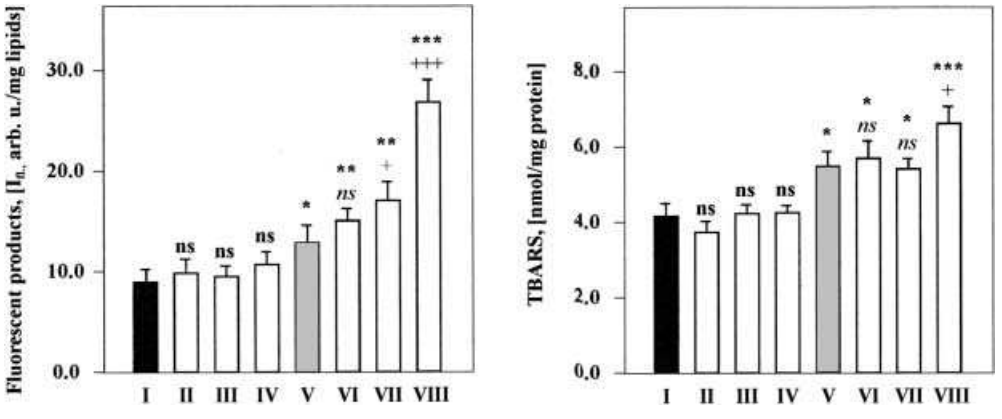
The same trend was also observed in macroscopically described lung lesions (Table 1).

Virus titres were not determined in the livers of all experimental animals.

Levels of LPO products

Figure 1 illustrates the endogenous levels of LPO products in liver homogenates isolated from mice subjected to different models of oxidative stress. It was found that immobilization, cold and cold-restraint stress did not influence the endogenous levels of TBARS or fluorescent lipofuscine-like products in the liver of mice that had not been infected with influenza virus. Inoculation of mice with influenza virus A/Aichi/2/68 (H3N2) resulted in a significant increase of both TBARS and fluorescent lipofuscine-like products of 30 and 40%, respectively, compared with levels in non-infected animals. Combined application of immobilization stress and influenza virus infection led to a significant increase

Fig. 1. Effect of immobilization, cold and cold-restraint stress on the level of lipid peroxidation products in the liver of mice before and after influenza virus inoculation. Fluorescent lipofuscine-like products and thiobarbituric acid-reactive substances (*TBARS*) were determined in the following groups: *I* control, *II* immobilization, *III* cold stress, *IV* cold-restraint stress, *V* influenza virus infection, *VI* immobilization + influenza virus infection, *VII* cold stress + influenza virus infection, *VIII* cold-restraint stress + influenza virus infection. Values are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate significances versus group *I*; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ indicate significances versus group *V*; ns non-significant versus group *I*; *ns* non-significant versus group *V*



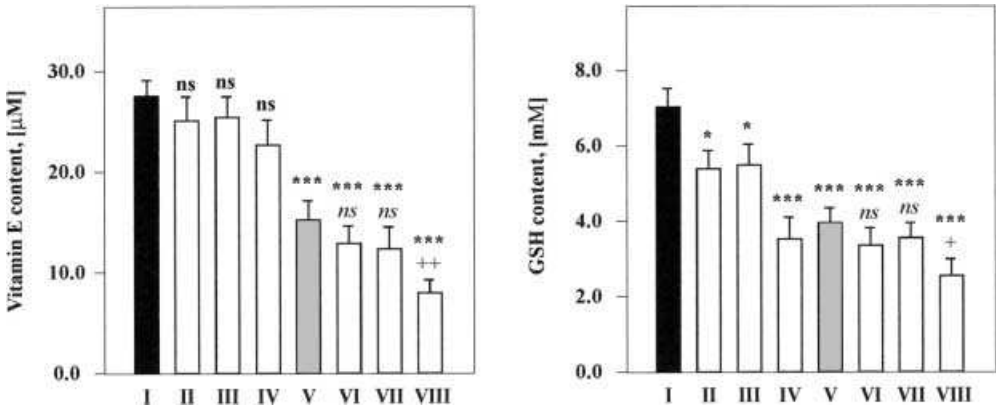


Fig. 2. Effect of immobilization, cold and cold-restraint stress on the levels of vitamin E and glutathione (GSH) in the liver of mice before and after influenza virus inoculation. Values are expressed as means ±SEM for the following groups: *I* control, *II* immobilization, *III* cold stress, *IV* cold-restraint stress, *V* influenza virus infection, *VI* immobilization + influenza virus infection, *VII* cold stress + influenza virus infection, *VIII* cold-restraint stress + influenza virus infection. $*P < 0.05$, *** $P < 0.001$ indicate significances versus group *I*; $^{+}P < 0.05$, $^{++}P < 0.01$ indicate significances versus group *V*; ns non-significant versus group *I*.; *ns* non-significant versus group *V*

in LPO products relative to those of control non-infected animals and did not change significantly from the LPO products in infected non-immobilized animals. Similar results were observed after combined application of cold stress and influenza virus infection. In this case, LPO products increased significantly compared with those of control non-infected animals. In mice subjected to cold stress and influenza virus infection the level of fluorescent products was significantly higher than that in infected and non-stressed animals, too. The level of TBARS in mice subjected to cold-restraint stress plus influenza virus infection was the same as in infected and non-stressed animals. Combination of influenza virus infection and cold-restraint stress led to a strong increase of LPO products. In this case, fluorescent lipofuscine-like products increased about three-fold compared to those in control animals, and about two-fold compared to those in influenza virus-inoculated and non-stressed animals; TBARS increased about 1.5-fold compared to control animals and by about 20% compared to virus-infected and non-stressed animals.

Levels of natural antioxidants

As can be seen from the data presented in Fig. 2, vitamin E levels were not changed significantly in the liver of mice subjected to immobilization, cold and cold-restraint stress. On the other hand, glutathione decreased by 30% after immobilization or cold stress, and by about one-half after cold-restraint, compared with values for control animals. Influenza virus infection caused obvious decreases of vitamin E and glutathione, too: about 80% and 70%, respectively, compared with levels in control animals. Combination of immobilization stress and influenza virus infection led to significant decreases of vitamin E and glutathione in the liver to about one-half of the levels in control non-infected animals, but there were no significant changes compared to infected and non-stressed animals. Similar results were observed after combined application of influenza virus infection and cold stress. In this case, vitamin E and glutathione decreased, too, by about one-half compared with levels in control non-infected animals, and did not change relative to levels in infected and non-stressed animals. A greater decrease of both natural antioxidants was characteristic of mice subjected to combined application of cold-restraint stress and influenza virus infection. The contents of vitamin E and glutathione significantly decreased compared with those in control non-infected animals as well those of infected and non-stressed animals.

Liver monooxygenase activities

Figure 3 represents the effect of different models of oxidative stress on cytochrome P-450 and CCR activity. The content of cytochrome P-450 and the activity of CCR were not changed after immobilization, cold or cold-restraint stress compared to those of control animals. However, influenza virus infection led to strong decreases in the content of cytochrome P-450 and CCR activity by about two-fold in both cases, compared to control animals. Combination of influenza virus infection with immobilization or cold stress resulted in significant decreases of cytochrome P-450 and CCR activity compared with levels in control animals, and to a significant increase of both parameters compared to influenza virus-inoculated and non-stressed animals. Combination of influenza virus infection and cold-restraint stress led to significant decreases of cytochrome P-450 and CCR activity relative to control animals, by about three- and five-fold, respectively. There were no significant differences in either parameter between influenza virus-infected non-stressed mice and mice subjected to combination of cold-restraint and influenza virus infection.

Similar results were observed with liver monooxygenases (Fig. 4). Immobilization, cold or cold-restraint

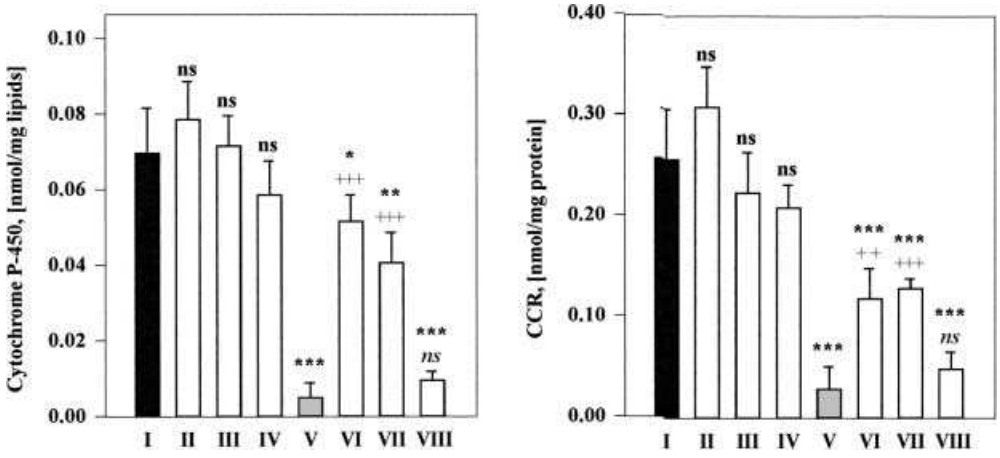


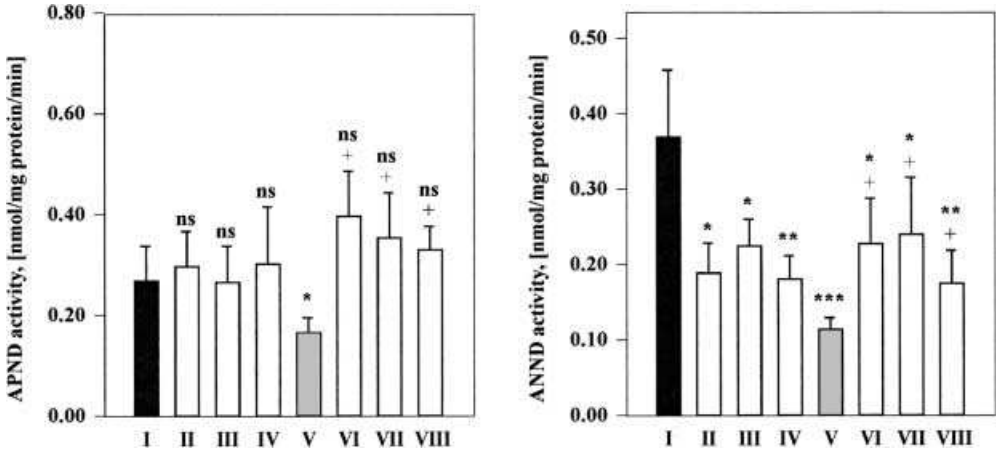
Fig. 3. Effect of immobilization, cold and cold-restraint stress on the concentration of cytochrome P-450 and the activity of NADPH-cytochrome *c* reductase (*CCR*) in the liver of mice before and after influenza virus inoculation. Values are expressed as means ± SEM for the following groups: *I* control, *II* immobilization, *III* cold stress, *IV* cold-restraint stress, *V* influenza virus infection, *VI* immobilization + influenza virus infection, *VII* cold stress + influenza virus infection, *VIII* cold-restraint stress + influenza virus infection. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicate significances versus group *I*; $^{++}P<0.01$, $^{+++}P<0.001$ indicate significances versus group *V*; ns non-significant versus group *I*, *ns* non-significant versus group *V*

stress did not affect the activity of APND, but decreased significantly the ANND activity to about one-half that of control non-infected animals. The effect of cold-restraint stress on ANND activity was the same as the effect of the separately applied models of stress. Influenza virus infection led to significant decreases of both enzymes APND and ANND, by about 1.7-fold and 3-fold, respectively, compared with activities in control animals. Both enzyme activities increased significantly after combined application of influenza virus infection and cold-restraint relative to non-stressed and influenza-infected animals – the activity of APND increased to control level, and the activity of ANND increased too, but remained lower than the control group level.

Fig. 4. Effect of immobilization, cold and cold-restraint stress on the activities of liver monooxygenases in mice before and after influenza virus inoculation. Amidopyrine-*N*-demethylase (*APND*) and analgin-*N*-demethylase (*ANND*) activities were determined in the following groups: *I* control, *II* immobilization, *III* cold stress, *IV* cold-restraint stress, *V* influenza virus infection, *VI* immobilization + influenza virus infection, *VII* cold stress + influenza virus infection, *VIII* cold-restraint stress + influenza virus infection. Values are expressed as means ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicate significances versus group *I*; $^{+}P<0.05$ indicates significance versus group *V*; ns non-significant versus group *I*; *ns* non-significant versus group *V*

Discussion

In the present study we demonstrated that the influenza virus infection resulted in an activation of free radical processes accompanied by an accumulation in the liver of LPO products and a decrease of natural antioxidants (vitamin E and reduced glutathione), despite of the absence of a virus in that organ. These results confirm previous data, proposing the role of LPO products in the hepatotoxicity of influenza virus (Oda et al. 1989; Hennet et al. 1992; Akaike et al. 1996; Schwarz 1996; Peterhans 1997a, 1997b; Murphy et al. 1998; Mileva et al. 2000). However, the biochemical nature of production of free radicals and LPO products in the liver under influenza remains to be established. We observed that influenza virus infection was accompanied by a



decrease of the content of cytochrome P-450, CCR activity and activities of liver monooxygenases. Therefore, the induction of LPO in the liver is not mediated directly by the cytochrome P-450 system. Several studies have suggested that an overreaction of the host's immune system to influenza virus involves free radical generation outside the liver (Oda et al. 1989; Peterhans 1987, 1997a, 1997b). Some mechanisms responsible for acceleration of LPO in target organs during the course of infection have been discussed: activation of neutrophils and macrophages to produce superoxide free radicals and hydrogen peroxide, which normally are involved in the killing of microbes (Oda et al. 1989; Peterhans 1997a, 1997b; Schwarz 1996), activation of proteases and subsequent acidosis (Goto and Kawaoka 1998), activation of superoxide-generating enzyme xanthine oxidase (Oda et al. 1989; Akaike et al. 1990, 1996; Peterhans 1997a, 1997b), virus-induced increase in free iron with subsequent iron-induced LPO (Schwarz 1996), activation of lipoxygenases (Antal et al. 1986), and accumulation of nitric oxide and nitrosyl complexes (Peterhans 1997a; Murphy et al. 1998). It is most likely that the reactive metabolites generated outside the liver can cross the hepatocyte barrier and initiate LPO in the liver as well as modifying the activities of liver cytochromes and monooxygenases (Kagan et al. 1988; Gorbunov et al. 1992; Mileva et al. 2000). Whereas the most reactive oxygen species do not diffuse more than a few femtometres, the lipid peroxides resulting from the free radical-induced peroxidation of membrane phospholipids can traverse the circulation and cell membranes, and result in dysfunction in other organs of the host, e.g. liver (Schwarz 1996).

Some authors supposed that the effects of influenza virus infection seen in the liver could be due to a soluble mediator(s) released early during infection (Hennet et al. 1992). Certain immune mediators at high concentrations exert toxic effects on the liver. For example, interferons, cytokines and tumor necrosis factor have been recognized as acting on the liver metabolism and to produce toxic effects on the liver (Grunfeld et al. 1988; Kishimoto 1989; Hennet et al. 1992).

The activation of free radical processes in the liver in the course of infection was followed by a compensatory enhancement in the activities of antioxidant enzymes and a decrease in the content of non-enzymic antioxidants (Hennet et al. 1992; Jacoby and Choi 1994; Choi et al. 1996; Peterhans 1997a, 1997b). In the present paper we demonstrated that influenza virus infection decreased significantly the endogenous vitamin E and reduced glutathione in liver homogenates. Hennet et al. (1992) reported a significant decrease of vitamin E, reduced glutathione and uric acid in lung and liver, too. It was established also that application of superoxide dismutase or vitamin C protects mice infected with a lethal dose of influenza virus (Oda et al. 1989; Gorton and Jarvis 1999). We have reported previously, also, a protective role of vitamin E supplements against lipid peroxidation in the liver of influenza virus infected mice (Mileva et al. 2000). Vitamin E supplements suppressed the effects of influenza virus infection on liver cytochromes and monooxygenases, too. Therefore, the liver pro-oxidant/antioxidant balance is extremely important for the protection against influenza virus infection. It follows that the exposure of animals to oxidative stress and antioxidant deficiency in the liver before influenza virus inoculation is likely to result in an increase of the ratio pro-oxidants/antioxidants and a graduated hepatotoxicity of influenza virus infection. To verify this hypothesis we studied the effects of combined application of influenza virus infection with two models of oxidative stress, which are accompanied by strong decrease in natural antioxidants in the early stage of stress – immobilization, cold and cold-restraint.

We found that immobilization, cold and cold-restraint stress did not influence endogenous levels of LPO products in the liver of mice non-infected with influenza virus. Immobilization and/or cold stress decreased significantly ANND activity, but did not influence the cytochrome P-450 content or activities of CCR and APND in mice non-infected with influenza virus. There is a discrepancy between our results and the previously published data, which indicates a significant increase of LPO products in the target organs of animals subjected to immobilization or cold stress (Simmons et al. 1991; Das and Banerjee 1993; Kovacheva-Ivanova et al. 1994; Oishi et al. 1999). The discrepancies in the results cited above may be explained by the different experimental protocols. In the present study we measured the level of LPO products and activities of liver monooxygenases 5 days after termination of immobilization and/or cold stress, whereas in the other studies the measurements were made immediately after immobilization or cold stress.

The preliminary exposure of mice to immobilization or cold stress and subsequent inoculation with influenza virus resulted in both a significant increase of LPO products and significant decreases of vitamin E and reduced glutathione, compared to control non-infected animals. The changes in all these parameters were insignificant compared with levels in infected and non-stressed animals. On the other hand, the preliminary exposure of mice to cold-restraint stress and subsequent inoculation with influenza virus resulted in a greater increase of LPO products and greater decreases of vitamin E and reduced glutathione than seen in controls, as well as in infected and non-stressed animals. In this case, the effect of combined application of influenza virus infection and cold-restraint on the levels of LPO products and natural antioxidants was synergistic.

Unusual results were observed with cytochrome P-450 and activities of CCR and liver monooxygenases after combined application of influenza virus infection, immobilization and/or cold stress. Immobilization or cold stress, applied separately but in combination with influenza virus infection, partially prevented the suppressive effects of influenza virus on cytochrome P-450 and the activities of CCR, APND and ANND. A ten-

dency for normalization of all these parameters to the control levels was observed. It was found that restraint stress elevates plasma corticoid and tissue catecholamine levels and reduces cellular accumulation and inflammation in target organs associated with influenza virus infection (Scheridan et al. 1991). This fact partially explains the stabilizing role of combined application of influenza virus infection and immobilization on liver cytochromes and monooxygenases. However, in our study, after combined application of influenza virus infection and cold-restraint stress, the level of cytochrome P-450 and CCR activity remained markedly lower than that in infected and non-stressed animals. The activities of APND and ANND were slightly increased compared with those of infected and non-stressed animals, but remained relatively low compared with the activities in control non-infected animals.

The observed additive and synergistic effects of combined application of influenza virus infection, immobilization, cold and cold-restraint stress on liver cytochromes and monooxygenases are very complicated and difficult to explain. There is scant information about the mechanisms of modulation of liver monooxygenases and cytochromes under immobilization and/or cold stress (Rabovsky et al. 1986; Gorbunov et al. 1992; Nagyova and Ginter 1993), and there are no data about their combination with influenza virus infection. There are a few works reporting that immobilization influences the activity of macrophages, as well as the release of interferon and viral polypeptides in early stages of influenza virus infection (Chetverikova et al. 1987). These substances probably modulate directly or indirectly the activities of liver monooxygenases and cytochromes. The more likely mechanism of upregulation of liver cytochromes and monooxygenases under combined application of influenza virus infection, immobilization, cold and cold-restraint stress is that activated oxygen species, generated through the activation of xanthine oxidase and nitric oxide synthase, and/or glucocorticoids play a role in activation of proteases and phospholipases, subsequent acidosis and pH-dependent long-lasting changes in cytochromes and liver monooxygenases. However, both hypothetical mechanisms need of verification.

In conclusion, the present study describes two major observations:

(i) The influenza virus infection A/Aichi/2/68 (H3N2) is associated with graduated oxidative disturbances in the liver and changes of cytochromes and liver monooxygenase activities, despite the apparent absence of virus and inflammation in this organ. At least two hypothetical mechanisms, explaining the hepatotoxicity of influenza virus infection, may exist: production of hepatotoxic mediators outside the liver (in virus replication areas) and subsequent diffusion or transportation of these compounds across the hepatocyte barrier may provoke liver disorders; and, induction of liver pro-oxidant systems and abnormal production of free radicals and/or activated oxygen species in the liver. In both cases, antioxidant capacity of the liver is extremely important for protection against influenza virus infection.

(ii) Cold-restraint, but not immobilization and cold stress, acts synergistically with influenza virus infection on the increase of lipid peroxidation products and the decrease of endogenous antioxidants in the liver. Immobilization and cold stress prevent the loss of monooxygenase activities due to influenza virus infection. This phenomenon may be explained at least partially by the increase of interferon (Chetverikova et al. 1987; Samuel 1991) and/or influence on different isoforms of cytochrome P-450 (Rabovsky et al. 1986) induced by the stress models.

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