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Hepatic inducible nitric oxide synthase expression increases upon exposure to hypergravity

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Abstract

Stimulation by a number of conditions, including infection, cytokines, mechanical injury, and hypoxia, can upregulate inducible nitric oxide synthase (iNOS) in hepatocytes. We observed that exposure to hypergravity significantly upregulated the transcription of the hepatic *iNOS* gene. The aim of this study was to confirm our preliminary data, and to further investigate the distribution of the iNOS protein in the livers of mice exposed to hypergravity. ICR mice were exposed to +3 Gz for 1 h. We investigated the time course of change in the iNOS expression. Hepatic iNOS mRNA expression progressively increased in centrifuged mice from 0 to 12 h, and then decreased rapidly by 18 h. iNOS mRNA levels in the livers of centrifuged mice was significantly higher at 3, 6, and 12 h than in uncentrifuged control mice. The pattern of iNOS protein expression paralleled that of the mRNA expression. At 0 and 1 h, weak cytoplasmic iNOS immunoreactivity was found in some hepatocytes surrounding terminal hepatic venules. It was noted that at 6 h there was an increase in the number of perivenular hepatocytes with moderate to strong cytoplasmic immunoreactivity. The number of iNOS-positive hepatocytes was maximally increased at 12 h. The majority of positively stained cells showed a strong intensity of iNOS expression. The expression levels of iNOS mRNA and protein were significantly increased in the livers of mice exposed to hypergravity. These results suggest that exposure to hypergravity significantly upregulates iNOS at both transcriptional and translational levels.

Key words: Inducible nitric oxide synthase; Mouse; Liver; Hypergravity

Introduction

The generation of nitric oxide (NO) from L-arginine and molecular oxygen has been proposed to mediate or modulate cellular damage in several organs, including the brain, kidneys, and liver (1-5). NO is a gaseous free radical produced mainly by the NO synthase (NOS) family of enzymes. The isoforms of NOS are subdivided into three basic categories: endothelial NOS, neuronal NOS and inducible NOS (iNOS), all of which are encoded by separate genes and, therefore, differently regulated. Unlike endothelial NOS and neuronal NOS, iNOS is not expressed constitutively, but is expressed in most cell types given the appropriate stimulatory conditions, which include infection, cytokines, mechanical injury, and hypoxia (6). In healthy livers, iNOS is not thought to be expressed constitutively. However, it is readily upregulated in the liver under a number of disease conditions, including ischemia-reperfusion injury, hepatic fibrosis, cirrhosis and regeneration (7-12). iNOS is also upregulated *in vitro* in hepatocytes and Kupffer cells in response to endotoxins and cytokines alone or in combination (13-17). The availability of specific antibodies directed against iNOS has prompted attempts to understand their cellular distribution in the liver, and how that may affect the pathogenesis of liver dysfunction (13,18,19).

It is generally accepted that a high gravitational acceleration force acting along the body axis from the head to the feet (+Gz) causes considerable strain on various organs, including the brain, heart, kidneys, and liver. Exposure to hypergravity has been shown to severely reduce blood flow to the visceral organs, including the kidneys, spleen, pancreas, and liver. In a recent preliminary study following exposure to hypergravity (20), we observed a significant elevation of iNOS mRNA expression levels in the livers of mice, suggesting that exposure to hypergravity is a biophysical condition that can adversely affect the liver.

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Based on this finding, we hypothesized that hypergravity exposure may affect the expression of hepatic iNOS protein. In addition, it has been found that high levels of interleukin (IL)-1 or a combination of proinflammatory cytokines, including IL-1, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, can induce iNOS production in hepatocytes under a variety of experimental conditions (14-17). It is also possible that TNF-α could reach high local concentrations in the liver following exposure to hypergravity (21). We, therefore, hypothesized that hypergravity-induced increases in the production of proinflammatory cytokines may be involved in the up-regulation of iNOS. The aim of this study was to confirm our preliminary results and to further investigate whether exposure to hypergravity resulted in a significant change in the expression of proinflammatory cytokines and/or iNOS in the liver.

Material and Methods

Experimental animals

ICR mice at 7 weeks of age were purchased from Samtako Bio Korea (South Korea). Mice were fed standard laboratory mouse chow throughout the experimental period, provided with free access to water, and maintained on a 12-h light-dark cycle under pathogen-free conditions. Temperature and humidity levels were maintained at 20-25°C and 40-45%, respectively. The Institutional Animal Care and Use Committee (IACUC) of the Republic of Korea Air Force Aerospace Medical Center approved all experimental procedures involving the animals (IACUC-2012-ASMC-002).

Centrifugation experiment

The mice were exposed to short-term hypergravity at +3 Gz for 1 h using the small animal centrifuge at the Aerospace Medicine Research Center. The mice were placed inside a cylindrical plastic restraint device that, when mounted in the centrifuge, allowed +Gz to be delivered along the rostrocaudal axis. Once the mice were secured, the restraint device was placed onto the centrifuge. A cage-mounting module was attached at the end of the arm that allowed for one degree of freedom, thereby ensuring that the net gravity field was perpendicular to the floor of the restraint device. The behavior of the mice was monitored with a charge-coupled device camera throughout the centrifugation experiments.

The centrifuged mice were randomly divided into 7 groups to investigate the time course of change in iNOS expression. At least 3 animals were included in each group. For tissue collection, the mice were sacrificed by cervical dislocation and laparotomized via a midline incision at 0 (immediately after cessation of centrifugation), 1, 3, 6, 12, 18, and 24 h after exposure to hypergravity. The control group stayed in the same environment as those of the centrifuged groups, with the exception of the +3 Gz exposure. A portion of each animal's liver was fixed in 10% neutral buffered formalin for immunohistochemical staining. The remaining tissue was sectioned and immediately stored frozen in liquid nitrogen at –80°C until reverse-transcription polymerase chain reaction (RT-PCR) analysis and enzyme-linked immunosorbent assay (ELISA) were performed.

Quantitative RT-PCR analysis (qRT-PCR)

iNOS mRNA expression was detected in the centrifuged mice and compared with that of the control mice. According to the manufacturer's instructions, the total liver RNA was isolated using the NucleoSpin RNA II extraction kit (Macherey-Nagel, Germany). cDNA synthesis was performed with the ReverTra Ace-α-reverse transcriptase kit (Toyobo, Japan). The amount of standard cDNA was determined photometrically. The reverse-transcribed cDNA was used for the RT-PCR using SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, USA). PCR was performed on the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) with C1000 Thermal Cycler (Bio-Rad Laboratories). The primer sequences used for iNOS were forward, 5′-GGAGCGAGTTGTGGATTG-3′ and reverse, 5′-CCAGGAAGTAGGTGAGGG-3′. The primer sequences used for GAPDH were forward, 5′-CAAGAAG GTGGTGAAGCA-3′ and reverse, 5′-GGTGGAAGAGTGG GAGTT-3′. The PCR reactions for iNOS and GAPDH were initiated with a denaturing step at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s, 58°C for 10 s, and 72°C for 20 s. A melting curve, ramping from 65° to 95°C, was performed following each RT-PCR to test for the presence of primer dimers. When primer dimer formation was detected, the PCR was rerun using a separate aliquot of cDNA. Each measurement was repeated 3 times, and the values were used to calculate the iNOS/GAPDH ratio, with a value of 1.0 used as the control (calibrator). The normalized expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method (22).

ELISA

Frozen samples of liver tissue were homogenized in 100 mg tissue/mL lysis buffer (50 mM Tris, pH 7.5, 1% NP-40, 2 mM EDTA, 10 mM NaCl, 20 μg/mL aprotinin, 20 μg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride), and placed on ice for 20 min. After centrifugation at 4500 *g* for 20 min, the supernatant was collected, and the protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories). Samples containing 1-10 mg total protein were quantitatively assayed for IL-1β, IL-6, IFN-γ, and TNF-α by commercial ELISA kits from R&D Systems (USA).

Immunohistochemistry

Formalin-fixed liver tissue was dehydrated in a graded series of ethanol and then embedded in paraffin. Paraffin blocks were sectioned at 4 μm on a standard rotary microtome, and the slices were transferred from a water bath onto cleaned slides. iNOS protein expression was

assessed by immunohistochemical staining using the Bond Polymer Intense Detection System (Vision Bio-Systems, Australia) following the manufacturer's instructions. To summarize, 4-μm sections of formalin-fixed, paraffin-embedded tissue were deparaffinized in Bond Dewax Solution (Vision BioSystems), and an antigen retrieval procedure was performed using Bond ER Solution (Vision BioSystems) for 30 min at 100°C. Endogenous peroxidases were quenched by incubation with hydrogen peroxide for 5 min. The sections were then incubated with a rabbit polyclonal anti-iNOS antibody (1:100, Abcam, USA) for 15 min at ambient temperature. A biotin-free polymeric horseradish peroxidase linker antibody conjugate system was used in the Bond-maX automatic slide stainer (Vision BioSystems), and visualization was performed using a 3.3-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer, pH 7.6, and 0.006% H_2O_2). The sections were then counterstained with hematoxylin. Slides were subsequently dehydrated following a standard procedure, and then sealed with coverslips. Positive and negative control samples were included in each run in order to minimize interassay variation. A sample of normal liver tissue was used as a positive control. The negative control was prepared by substituting nonimmune serum for the primary antibody. No detectable staining was evident.

Statistical analysis

Data are reported as means ± SE. The differences in the normalized mRNA and protein expression ratio between the groups were assessed using the Student *t*-test (SPSS version 18.0 software; IBM SPSS, USA). Statistical significance was set at $P<0.05$.

Results

Expression of hepatic iNOS mRNA in hypergravity-exposed mice

None of the animals displayed remarkable changes in behavior during or after centrifugation. As shown in Figure 1, hepatic iNOS mRNA was almost undetectable in the control group. However, exposure to hypergravity upregulated the transcription of hepatic iNOS gene in the centrifuged groups. In the livers of the 0 and 1 h groups, iNOS mRNA was present, albeit at low levels, and it did not reach statistical significance. The 3 h group showed a 4.8-fold increase in the amount of hepatic iNOS mRNA, which was significantly different from that of the 0 h group ($P=0.002$). Exposure to hypergravity for 6 h induced a further increase in iNOS mRNA. Compared to the 0 h group, the 6 h group showed a 20.8-fold increase in the amount of iNOS mRNA ($P<0.001$). Maximal expression of hepatic iNOS mRNA was observed at 12 h. The livers of the 12 h group demonstrated a 23.6-fold increase in iNOS mRNA level compared with that of the 0 h group ($P<0.001$). In contrast, at 18 and 24 h, hepatic iNOS mRNA expression was undetectable.

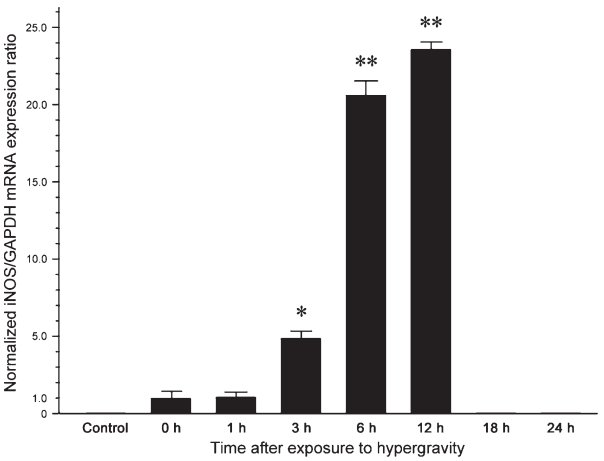

Figure 1. Effect of hypergravity exposure on inducible nitric oxide synthase (iNOS) mRNA expression in the livers of mice. Quantitative real-time RT-PCR analysis of iNOS mRNA was performed. Data are reported as the means ± SE of 3 independent experiments in each group. The 3, 6, and 12 h groups displayed significantly higher iNOS mRNA levels in the liver than the control group (*$P<0.01$ and **$P<0.001$, Student *t*-test). In contrast, in the 18 and 24 h groups, hepatic iNOS mRNA was undetectable.

Expression of hepatic iNOS protein in hypergravity-exposed mice

Immunohistochemical staining for iNOS was performed to determine the qualitative distribution of iNOS protein expression in the liver tissue of the mice exposed to hypergravity. The pattern of iNOS protein expression paralleled that of iNOS mRNA expression. Even though there was a sharp demarcation of the hepatic sinusoidal endothelial cells by iNOS (Figure 2A), the control group showed no cytoplasmic iNOS expression in the hepatocytes. The mice that had been exposed to +3 Gz showed iNOS immunoreactivity in the hepatocyte cytoplasm, although the intensity and proportion of the reactions were uneven. Weak cytoplasmic iNOS immunoreactivity was observed in some hepatocytes surrounding terminal hepatic venules in the 0 (Figure 2B) and 1 h (Figure 2C) groups. In the 3 h group, the number of iNOS-positive perivenular hepatocytes was greater and the intensity of expression was stronger than in the 0 h group (Figure 2D). The 6 h group revealed a higher proportion and stronger intensity of iNOS expression than the 3 h group (Figure 2E). In the 12 h group, the number of iNOS-positive hepatocytes increased, and the majority of those cells exhibited a strong intensity of expression (Figure 2F). In a few foci, strong cytoplasmic iNOS immunoreactivity was observed in the midzonal hepatocytes. In contrast, at 18 (Figure 2G) and 24 h, no iNOS protein expression was observed in hepatocytes. No disorder was observed in the trabecular arrangement of hepatocytes or sinusoidal structure of the livers of either the control or the centrifuged group.

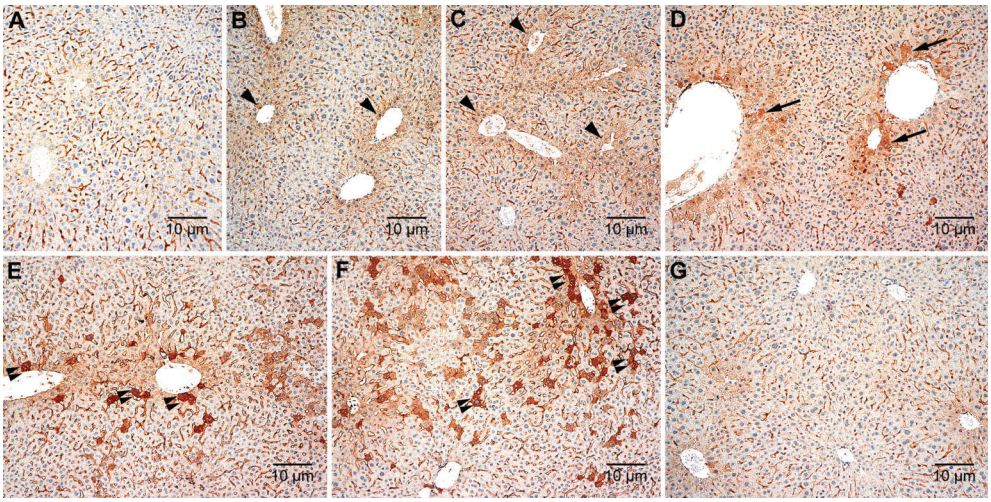

Figure 2. Effect of hypergravity exposure on inducible nitric oxide synthase (iNOS) protein expression in the livers of mice. *A*, The control group showed no cytoplasmic iNOS immunoreactivity in the hepatocytes, except for a demarcation of sinusoidal endothelial cells by iNOS. *B*, The 0 h group showed weak iNOS immunoreactivity in the cytoplasm of some hepatocytes (arrowheads) surrounding the terminal hepatic venules. *C*, In the 1 h group, the intensity and proportion of iNOS expression in the hepatocytes (arrowheads) were nearly identical to those of the 0 h group. *D*, The 3 h group revealed a significantly increased staining intensity of iNOS expression in the perivenular hepatocytes (arrows), compared with that of the 0 or 1 h groups. *E*, The 6 h group displayed an increased number of iNOS-positive hepatocytes, with a moderate to strong intensity of expression (double arrowheads). *F*, In the 12 h group, maximal expression of iNOS protein was observed and the number of iNOS-positive hepatocytes was increased. The majority of those cells exhibited a strong intensity (double arrowheads). *G*, The 18 h group showed no iNOS immunoreactivity in the hepatocytes. Polymer method. Original magnification, 150×.

Hepatic production of proinflammatory cytokines

IL-1β, IL-6, IFN-γ, and TNF-α proteins in the livers of mice exposed to hypergravity were determined by ELISA. Figure 3 reports the concentrations of the cytokines, normalized to controls. There were no significant effects of hypergravity exposure on the hepatic expression levels of IL-1β (Figure 3A), IL-6 (Figure 3B), IFN-γ (Figure 3C), or TNF-α (Figure 3D). Increases in IL-1β and IL-6 levels observed at 0-1 and 12-18 h after centrifugation did not reach statistical significance. Although the TNF-α level rose immediately after centrifugation and peaked at 3 h (a 1.3-fold increase compared to control), there was no significant difference between the time points.

Discussion

Pilots of jet fighter planes experience hypergravity. An understanding of the biological responses to hypergravity using animal models is important to prevent unwanted responses that occur during exposure to hypergravity. We evaluated the levels of iNOS mRNA and protein expression in the livers of mice exposed to hypergravity. Corresponding increases in iNOS immunoreactivity in the cytoplasm of hepatocytes and significant increases in hepatic iNOS mRNA levels were observed from 3 to 12 h after exposure to hypergravity. Hepatic iNOS mRNA was expressed immediately after centrifugation, albeit at low levels. We then observed further increases in iNOS mRNA expression, which rose significantly at 3 h, reached a maximum at 12 h, and then disappeared shortly afterward. These results are consistent with those of our preliminary study (20). iNOS protein expression showed a trend similar to that observed for the levels of iNOS mRNA. No iNOS immunoreactivity was detected in the hepatocytes of the control group. After exposure to hypergravity, iNOS protein was expressed mainly in hepatocytes surrounding terminal hepatic venules, with a substantial increase in the staining intensity and proportion at 6 and 12 h. To the best of our knowledge, alterations in hepatic iNOS protein expression associated with hypergravity exposure have not yet been studied. This is the first study to demonstrate a significant increase in hepatic iNOS protein expression in mice exposed to hypergravity.

Cells of the hepatic lobule are physiologically exposed to different oxygen tensions. Blood flow creates an oxygen gradient that differentiates the periportal area, where oxygen tension is twice as high as in the perivenular area. We showed a distinctive localization of hepatic iNOS expression. Following exposure to hypergravity, hepatocytes in the centrilobular region appeared to be the major source of increased iNOS protein expression. In particular, iNOS protein was mainly contained within the hepatocytes surrounding terminal hepatic venules. The pattern of staining was heterogeneous, with some hepatocytes demonstrating no iNOS staining and others staining intensely. However, it is evident that iNOS was most concentrated in the

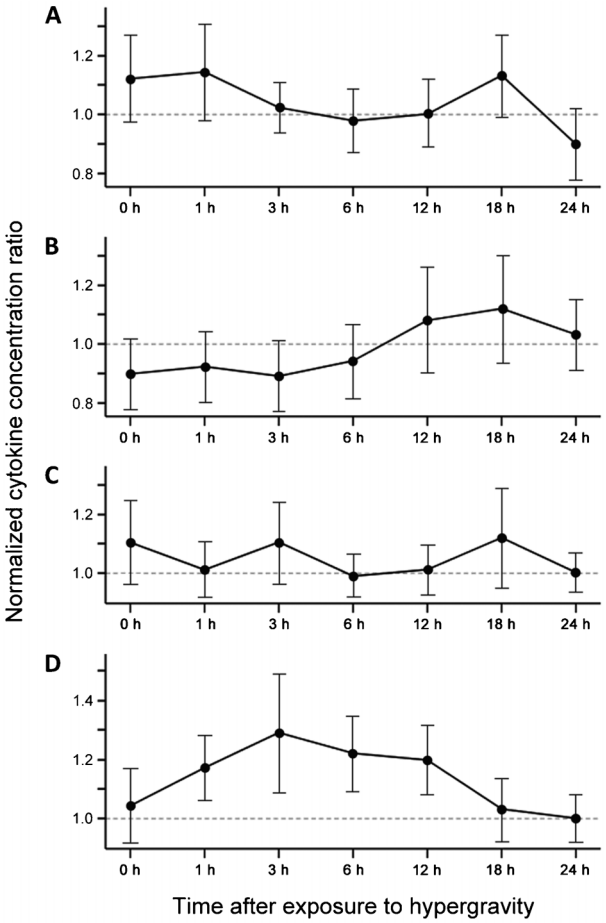

Figure 3. Effect of hypergravity exposure on pro-inflammatory cytokine production in the livers of mice. ELISA was performed to measure the hepatic levels of cytokines. Data are reported as the means ± SE of 3 independent experiments in each group. *A*, IL-1β. *B*, IL-6. *C*, IFN-γ. *D*, TNF-α. IL: interleukin; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α. The centrifuged mice exhibited no significant changes in the concentrations of the 4 cytokines compared to those of the control mice (Student *t*-test).

centrilobular regions. This unique distribution of iNOS expression in the perivenular hepatocytes may represent an indicator for increased stress induced by hypergravity exposure. We speculate that exposure to hypergravity, which causes a considerable decrease in hepatic blood flow, is involved in hypoxia-induced up-regulation of iNOS owing to the fact that the perivenular area would be the most hypoxic region of the hepatic lobule. Our speculation is supported by previous data showing a significant increase in iNOS mRNA expression in the livers of rats subjected to hypoxia or hemorrhagic shock. Vargiu et al. (23) revealed that iNOS expression was increased by low oxygen tension in the hepatocytes. Melillo et al. (24) also showed that hypoxia activated the iNOS promoter and induced iNOS gene transcription, providing evidence that iNOS is a hypoxia-inducible gene. Moreover, Collins et al. (25) observed that the hepatic iNOS protein was localized in the hepatocytes of the shocked rats and increased with time in their analysis, and that the most intense staining was observed in the hepatocytes surrounding terminal hepatic venules. Hypoxia regulates the transcription of genes that possess the hypoxia response elements in their promoters. There are more than 60 genes that are regulated by low oxygen tension, such as endothelin-1, vascular endothelial growth factor and iNOS (26). iNOS is an enzyme directly involved in the production of NO, and NO regulates vascular tone and cell survival. This adaptive response to hypergravity-induced hypoxia is important in stimulating iNOS production, because it is vital for the production of NO. Thus, it could partly regulate the sinusoidal resistance and improve hepatic perfusion to cope with the oxygen deprivation condition.

We observed the absence of iNOS immunoreactivity in hepatocytes of the control group. This finding is not consistent with that of a previous study, which showed constitutive iNOS expression in normal hepatocytes. McNaughton et al. (27) showed that iNOS was localized in the cytoplasm of periportal hepatocytes, but not in those associated with terminal hepatic venules. It was stated that constitutive induction of hepatic iNOS in the periportal area may be caused by constant stimulation of hepatocytes by bacterial or chemical products that are absorbed from the intestine into the portal circulation and then distributed in the liver. When compared with the perivenular hepatocytes, it is possible that the periportal hepatocytes may encounter the greatest concentration of endotoxin. Conversely, we observed the presence of immunoreactive iNOS in the sinusoidal endothelial cells of the control mice despite the absence of iNOS mRNA expression. This finding is not consistent with that of previous studies that found no iNOS immunoreactivity in the sinusoidal endothelial cells of normal livers (28,29). We speculate that the presence of immunostaining might simply represent recognition of a protein homologous to iNOS. Alternatively, it may represent iNOS that is immunoreactive but not physiologically active. According to our findings, Kupffer cells were not a relevant source of iNOS expression in hypergravity-exposed livers. However, we cannot exclude the possibility that hepatic nonparenchymal cells could be potential sources of increased iNOS expression, since Kupffer cells have been identified to generate iNOS following stimulation with proinflammatory cytokines (30). Indirect effects of activated Kupffer cells with increased iNOS gene expression on releasing toxic oxygen radicals and cytokines also need to be taken into account. These discrepancies in iNOS localization may be attributed to differences between animal species (human, rat and mouse), differences in antibodies and/or antigen retrieval, and staining procedures with varying degrees of sensitivity. In light of these conflicting data, further investigations are necessary to confirm or disprove our findings using different antibodies and standardized evaluation methods.

Limitations of the data presented here should be acknowledged. First, this study does not provide direct evidence for the mechanism of iNOS regulation. We did not observe significant alterations in the levels of the proinflammatory cytokines in the livers of centrifuged mice compared to those in the control mice. Our observations suggest that short-term exposure to +3 Gz did not significantly affect hepatic production of proinflammatory cytokines. Proinflammatory cytokines seem less likely to be involved in the upregulation of iNOS in hypergravity-exposed livers. Although there have been two previous studies demonstrating a significant influence of hypergravity on the immune system (21,31), the centrifugation protocol described in those studies was quite different from ours: mice were subjected to ''chronic'' hypergravity for 21 days. Pecaut et al. (21) demonstrated that splenic production of cytokines was primarily affected during 7 days of +3 Gz exposure; IFN-γ decreased, whereas TNF-α increased. After 21 days of exposure, there were no significant differences in cytokine levels between the control and centrifuged groups. In contrast, Gueguinou et al. (31) reported that serum IFN-γ concentration of mice exposed to +3 Gz for 21 days was significantly lower than that of the control mice. Differences in mouse species and centrifuge equipment, pattern of hypergravity exposure including duration and frequency, and/or differences in reagents, and experimental protocols with varying degrees of sensitivity may underlie such discrepancies. Second, we cannot completely exclude the possibility of an involvement of emotional stress and/or neuropsychological responses in the upregulation of iNOS due to the use of a restraint device. A previous study by Madrigal et al. (32) showed that acute restraint stress significantly increased iNOS expression in cerebral cortex of rats. In this study, however, with the exception of hypergravity exposure, the control mice were kept in the same environmental conditions as the centrifuged mice, including light, sound, temperature, humidity, and the application of restraint devices. The absence of iNOS mRNA and protein expression in the hepatocytes of control mice indicates that even though the cylindrical plastic restrainer may have caused stress in the mice, it did not significantly affect iNOS expression in the liver. Further investigations are necessary to clarify the combined effects of hypergravity exposure and restraint use during centrifugation on the expression of iNOS in the brain. Third, we did not explore the alteration of functional and biochemical properties in the liver exposed to hypergravity. We suggest that future studies of changes in levels of serum biochemical parameters, NO metabolites, and/or iNOS enzymatic activity following exposure to hypergravity be conducted in a larger number of animals.

In conclusion, iNOS was upregulated both at transcriptional and translational levels during the course of exposure to hypergravity. Our data can serve as a baseline for further research to evaluate the effect of hypoxic injury on iNOS expression in mice exposed to hypergravity.

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