

## Hypergravity Induces Overexpression of Inducible Nitric Oxide Synthase in Mouse Livers

<sup>1</sup>Hyun-Soo Kim and <sup>2</sup>Youn Wha Kim

<sup>1</sup>Laboratory of Molecular Biology, Aerospace Medical Center, Republic of Korea Air Force, P.O. Box 335-21, 635 Danjae-Ro, Namil-Myeon, Cheongwon-Gun, Chungcheongbuk-Do, Postal Code 363-849, Republic of Korea

<sup>2</sup>Department of Pathology, School of Medicine, Kyung Hee University, 26 Kyunghee-Daero, Dongdaemun-Gu, Seoul, Postal Code 130-701, Republic of Korea

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**Abstract:** The molecular responses of tissues and organs to gravity change are one of the most important physiological problems in aviation medicine. Previous studies have shown that changes in the gravity environment produce major changes in blood flow and tissue perfusion and affect several aspects of cellular function. In particular, exposure to hypergravity severely reduces hepatic blood flow, resulting in hypoxic-ischemic insult to the liver. Inducible Nitric Oxide Synthase (iNOS) has been implicated in many pathophysiological states leading to hepatic dysfunction. The aim was to investigate the relationship between hypergravity exposure and hepatic iNOS expression. Using an animal centrifuge, 18 ICR mice were exposed to +3Gz for 1 h and euthanized after 0, 1, 3, 6, 12 and 24 h, respectively. The expression levels of hepatic iNOS were evaluated using quantitative real-time RT-PCR analysis. The quantitative analysis of iNOS mRNA expression showed a significant increase 3 h after hypergravity exposure which persisted for 12 h post-centrifugation. About 0 and 1 h intervals did not result in a significant increase in the amount of iNOS mRNA. By contrast, 3, 6 and 12 h intervals resulted in statistically significant increases in the expression level of iNOS mRNA (relative expression value, 0.587, 2.548 and 3.361, respectively). This study is the first to describe hypergravity exposure-induced alteration in the expression of hepatic iNOS. Researchers observed significantly increased iNOS mRNA expression in the livers of mice exposed to hypergravity. This result suggests that hypergravity exposure has a significant effect on transcription of the *iNOS* gene in the mouse liver.

**Key words:** Hypergravity, inducible nitric oxide synthase, mouse, liver, *iNOS* gene, hypergravity exposure, quantitative real-time reverse transcriptase-polymerase chain reaction analysis

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### INTRODUCTION

Nitric Oxide (NO) is synthesized from L-arginine and oxygen by NO Synthase (NOS), a family of isoenzymes with characteristic functional and regulatory properties (Andrew and Mayer, 1999). Unlike constitutively expressed NOS isoforms (neuronal and endothelial NOS), inducible NOS (iNOS) is regulated primarily at the transcriptional level. At baseline there is little if any, detectable iNOS expression in any cell type. However in response to inflammatory cytokines or endotoxins, there is a robust up-regulation of iNOS mRNA and protein in virtually every nucleated cell type. The activation of iNOS is associated with 100-1,000-fold larger NO output than eNOS (Forstermann *et al.*, 1994; Nadaud and Soubrier, 1996). NO produced by iNOS has been implicated in many pathophysiological states leading to dysfunction of the various tissues and organs.

A high gravitational acceleration force acting along the body axis from the head to the feet (+Gz) causes considerable strain on several organ systems including the brain, heart, kidneys and liver. Exposure to high +Gz has been shown to severely decrease blood flow to the visceral organs including the kidneys, spleen, pancreas and liver in an apparent effort to maintain blood flow to the brain and heart. Previous studies have shown that changes in the gravity environment affect several aspects of cellular function and produce major changes in blood flow and tissue perfusion. In particular, exposure to hypergravity severely reduces hepatic blood flow, resulting in hypoxic-ischemic insult to the liver.

The liver is well recognized as a target during Ischemia Reperfusion (IR) and inflammatory states (Nakamura *et al.*, 1991) and IR-related injury in the liver remains an important clinical problem during shock, liver surgery and liver transplantation. Mediators, such as

oxygen free radicals, NO and their reaction product peroxynitrite have been found to be involved in IR-mediated liver injury (Bowes and Thiernemann, 1998). Controversy exists regarding the effects of NO on the liver. It appears that NO plays a paradoxical role in liver physiology. Small amounts of NO may have a cytoprotective effect while on the other hand, there is an increasing body of evidence indicating that overproduction of NO may damage liver function. Thus, NO may have both cytoprotective and cytotoxic properties, depending on the amount and isoform of NOS that causes NO production.

The effects of hypergravity exposure on the liver have been the subject of many investigations reported in aviation medical literature. However, no information is available on the alteration of iNOS expression in the liver exposed to hypergravity which is a biophysical condition that can adversely affect the liver. The aim of this study was to investigate the relationship between hypergravity exposure and hepatic iNOS expression in mice using an animal centrifuge.

## MATERIALS AND METHODS

The 21 ICR mice at 7 weeks of age were purchased from Samtako Bio Korea Co., Ltd. (Osan-si, Gyeonggi-do, Republic of Korea). Throughout the experimental period, animals were fed standard laboratory mouse chow, provided with free access to water and maintained on a 12:12 h light-dark cycle in pathogen-free conditions with the temperature and moisture level controlled at 20-25°C and 40-45%, respectively. The 18 of the 21 mice were exposed to short-term hypergravity at +3Gz for 1 h using the animal centrifuge at the Animal Testing Laboratory, Aerospace Medical Center, Republic of Korea Air Force (Cheongwon-Gun, Chungcheongbuk-Do, Republic of Korea). The other three mice were used as untreated control. Mice were placed inside a cylindrical plastic restraint device which when mounted in a centrifuge, allowed +Gz to be delivered along their rostral-caudal axes. After the mice were secured, the restraint device was placed onto the animal centrifuge. A cage-mounting module was attached at the end of the arm that allowed one-degree freedom, thereby ensuring that the net G field was perpendicular to the floor of the restraint device. The behavior of the mice was monitored with a CCD camera throughout the centrifugation experiments.

To investigate the time course of iNOS expression, the centrifuged mice were randomly divided into six groups. Each group consisted of three mice. At 0, 1, 3, 6, 12 and 24 h after the cessation of centrifugation, the

mice were euthanized by cervical dislocation and laparotomized via a midline incision. The liver was immediately removed, snap-frozen in liquid nitrogen and stored at -70°C until quantitative real-time Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) analysis was performed.

Expression of iNOS mRNA in the centrifuged mice was detected compared to that in the control mice. Total liver RNA, isolated using the NucleoSpin RNA II extraction kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions was used for cDNA synthesis. The reverse-transcribed cDNA was used for the real-time RT-PCR reaction using SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR was produced using the Bio-Rad CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Inc.). The primer sequence used for iNOS was as follows: Forward 5'-GGAGCGAGTTGTGGATTG-3'; reverse 5'-CCAGGAAGTAGGTGAGGG-3'. The primer sequence used for the housekeeping gene *GAPDH* was as follows: Forward 5'-CAAGAAGGTGGTGAAGCA-3'; reverse 5'-GGTGAAGAGTGGGAGTT-3'. 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 sec, 58°C for 10 sec and 72°C for 20 sec. Each measurement was repeated three times and the values were used to calculate the ratio of iNOS/*GAPDH* with a value of 1.0 used as the control (Calibrator).

All values are provided as mean with standard error. The differences in iNOS/*GAPDH* mRNA ratios between groups were assessed using student's t-test (Statistical Package for the Social Sciences (SPSS) version 15.0 Software, SPSS Inc., Chicago, IL) and  $p < 0.05$  were considered significant.

## RESULTS

The quantitative analysis of iNOS mRNA expression showed a significant increase 3 h after +3Gz exposure which persisted for 12 h post-centrifugation (Fig. 1). The control group and 0 h (relative expression value, 0.049) and 1 h interval (relative expression value, 0.058) groups did not show a significant increase in the amount of iNOS mRNA. By contrast, the 3 h (relative expression value, 0.587), 6 h (relative expression value, 2.548) and 12 h intervals (relative expression value, 3.361) resulted in statistically significant increases in the expression level of iNOS mRNA ( $p < 0.05$ ,  $p < 0.05$  and  $p < 0.05$ , respectively). No iNOS mRNA was detected at 24 h after centrifugation. None of the animals displayed remarkable changes in behavior during or after centrifugation.

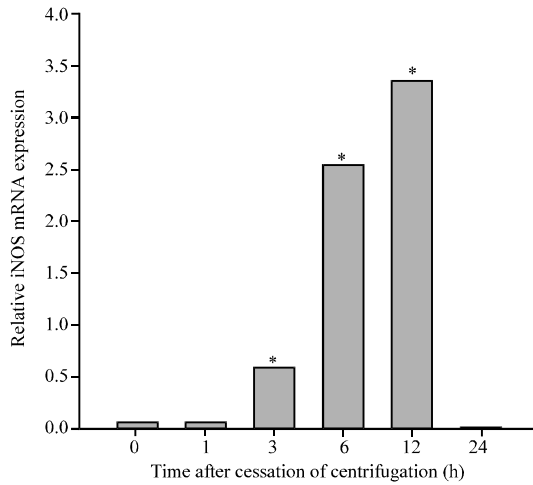


Fig. 1: Effect of hypergravity exposure on iNOS mRNA expression in the livers of centrifuged mice; Quantitative real-time RT-PCR analysis of iNOS mRNA; Experimental groups: 0, 1, 3, 6, 12 and 24 h post-centrifugation; The results are mean values of three independent experiments and three animals in each group; The mean value of iNOS mRNA expression significantly rose 3 h after hypergravity exposure. This increase persisted for 12 h post-centrifugation; \* $p < 0.05$  vs. corresponding value of 0 h interval group

## DISCUSSION

In recent years, both *in vivo* and *in vitro* models of iNOS expression have provided a more in-depth understanding of the molecular mechanisms involved in the regulated expression of iNOS in response to various stimuli. Numerous exogenous conditions and stimuli that are relevant to the hepatic pathophysiology have been shown to alter iNOS expression through the modulation of iNOS at both the transcriptional and post-transcriptional levels. There are however, no investigations on the relationship between iNOS expression and hypergravity, a biophysical condition that can adversely affect the liver.

The study provides the first demonstration of the quantitation of iNOS mRNA expression in mouse liver exposed to high +Gz. When measured using the real-time RT-PCR analysis, the expression level of iNOS mRNA was significantly increased 3 h after hypergravity exposure. In addition, this rise of iNOS mRNA level persisted for 12 h post-centrifugation. These results suggest that hypergravity exposure has a significant effect on transcription of *iNOS* gene in mouse liver. The study is the first to describe hypergravity exposure-induced

alteration of iNOS expression, while the precise mechanism by which hypergravity alters the expression of iNOS in the liver remains unknown. On the basis of a previous study, demonstrating a marked reduction in blood flow to the liver during hypergravity exposure (Laughlin *et al.*, 1982), researchers speculated that hepatocellular hypoxia due to hypergravity-induced inadequate hepatic blood flow might be attributable to the up-regulation of iNOS.

The possibility also, cannot be excluded that the increased expression of hepatic iNOS might be caused by repeated +Gz exposure-induced hepatocellular reperfusion injury. Researchers hypothesized that IR injury might occur when the blood supply returned to the liver after a period of hypoxia-ischemia due to +Gz exposure. This hypothesis is supported by previous data showing that the expression of iNOS was increased after hepatic IR injury. During hepatic IR injury, Kupffer cells are activated and release pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$  and interleukin-6 (Colletti *et al.*, 1990; Wanner *et al.*, 1996). These cytokines up-regulate the expression of iNOS. Further studies are necessary to clarify the relationship between the expression of iNOS and pro-inflammatory cytokines in livers exposed to hypergravity.

## CONCLUSION

This study is the first to report the altered expression of hepatic iNOS associated with high +Gz exposure. Researchers demonstrated significantly increased iNOS mRNA level in the livers of mice exposed to +3Gz, suggesting that hypergravity exposure has a significant effect on transcription of the *iNOS* gene in the mouse liver.

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