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Clinorotation Upregulates Inducible Nitric Oxide Synthase by Inhibiting AP-1 Activation in Human Umbilical Vein Endothelial Cells

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ABSTRACT

Alterations of nitric oxide contribute to post-flight orthostatic intolerance. The aim of this study was to investigate the changes of inducible nitric oxide synthase (iNOS) and the mechanisms underlying regulation of iNOS by simulated microgravity in human umbilical vein endothelial cells (HUVECs). Clinorotation, a simulated-model of microgravity, increased iNOS expression and promoter activity in HUVECs. The transactivations of NF-κB and AP-1 were suppressed by 24 h clinorotation. A key role for AP-1, but not NF-κB in the regulation of iNOS was shown. (1) PDTC, a NF-κB inhibitor, had no effect on clinorotation upregulation of iNOS. (2) SP600125, a JNK-specific inhibitor, which resulted in inhibition of AP-1 activity, enhanced the iNOS expression and promoter activity in clinorotation. (3) Overexpression of AP-1 remarkably attenuated the upregulation effect of clinorotation. These findings indicate that clinorotation upregulates iNOS in HUVECs by a mechanism dependent on suppression of AP-1, but not NF-κB. These results support a key role for AP-1 in the signaling of postflight orthostatic intolerance. J. Cell. Biochem. 107: 357–363, 2009.

KEY WORDS: CLINOROTATION; INDUCIBLE NITRIC OXIDE SYNTHASE; NF-κB; AP-1; ENDOTHELIAL CELLS

Individuals exposed to extended periods of microgravity during spaceflight or prolonged 6° head-down-tilt often suffer from symptoms of cardiovascular deconditioning when returning to Earth or upright posture, respectively. The cardiovascular dysfunctions include reduced exercise capacity, resting tachycardia, increased incidence of orthostatic intolerance, and frank syncope. Many astronauts are unable to maintain adequate perfusion of the brain after assuming an upright posture immediately following spaceflight, this alteration in orthostatic stability named postflight orthostatic intolerance (POI) can adversely affect the performance of physical work. Recent works shows that this is mostly due to reduce plasma volume, attenuated baroreflex sensitivity, inadequate peripheral resistance response, increased venous compliance, and impaired vasoconstrictive response in such individuals, but the exact etiology remains elusive [Convertino et al., 1990; Hargens and Watenpaugh, 1996]. Evidence from human and animal studies indicates that vascular contractile hyporesponsiveness is a valuable contributor in the occurrence of POI [Purdy et al., 1998; Shoemaker et al., 1998]. Yet, the cellular and molecular mechanism underlying vascular contractile hyporesponsiveness remain to be fully elucidated.

The endothelium is located in a strategic anatomical position within the blood vessel wall and thereby plays a crucial role in maintaining the integrity of the vasculature. The endothelial cells (ECs) are in close contact and form a smooth layer that prevents blood cell interaction with the vessel wall. Due to its unique position in the vessel wall, ECs may respond to changes in local conditions such as blood pressure, oxygen tension and blood flow by secreting substances, which have powerful effects on the tone of vascular smooth muscle, causing either contraction or relaxation. The studies in the Hind-limb unloading (HLU) rodents model, which has been extensively used as a model to simulate cardiovascular consequences of microgravity in humans, have clearly shown that nitric oxide (NO) synthesis was strongly enhanced and NO released from the endothelium is an important factor inducing vasodilation [Zhang, 2001].

NO, an endogenous modulator which is produced by various cell types in different tissues, has been shown to regulate numerous

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biological processes. This potent endogenous vasodilator plays an important role in the regulation of cardiovascular function [Moncada et al., 1991]. NO is synthesized by the enzyme nitric oxide synthase (NOS) from an L-arginine substrate. Three isoforms of NOS enzymes have been characterized, two of which are constitutively expressed and a third which is inducible. In contrast to the two constitutive enzymes, iNOS produces large, sustained amounts of NO. It has been reported that both activity and expression of iNOS are increased in many vasculatures such as the femoral artery from HLU rodents [Vaziri et al., 2000].

It has recently become evident that ECs are highly sensitive to microgravity, including the alterations in morphology and gene expression [Carlsson et al., 2003; Morbidelli et al., 2005; Infanger et al., 2007]. However, to our knowledge, the alterations of the iNOS and the possible mechanisms underlying the regulation of iNOS in ECs cultured in real or simulated microgravity remain to be determined. In this study, we found that the expression and promoter activity of iNOS in HUVECs could be enhanced markedly as a result of simulated microgravity. And suppression of AP-1 signaling by simulated microgravity appeared to be essential for the enhancement of iNOS, but alteration of NF-κB had no effect on iNOS expression and promoter activity.

MATERIALS AND METHODS

CELL CULTURE

The HUVEC cell lines were purchased from ATCC (ATCC Number CRL-1730^{TM}), and cultured in gelatin-coated 75-cm^2 flasks in RPMI-1640 (HyClone Laboratories, Logan, UT) containing 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 5 units/ml heparin under normal cell culture conditions (37°C and 5% CO_2).

CLINOROTATION TO SIMULATE MICROGRAVITY

The clinostat is an effective, ground-based tool to simulate microgravity [Kacena et al., 2002; Barjaktarovic et al., 2007]. The clinostat consisted of two groups of turntables, one vertical and the other horizontal, to which chambers were affixed during the incubation period. The vertical chambers were rotated around the horizontal axis, which designates clinorotation. Clinorotation mimics certain aspects of the microgravity environment by nullifying the integrated gravitational vector through continuous averaging. The horizontal chambers were rotated around the vertical axis, which designates rotational control. HUVECs were exposed to clinorotation for 24 h at 30 rpm. In the present study, cells were seeded at a density of 1×10^5 cells on 2.5 cm × 3.0 cm coverslips which were placed in 6-well plates at 37°C in 5% CO_2. After the cells grew for 24 h and adhered to the coverslips, the coverslips were inserted into the fixture of the chambers which were subsequently filled completely with RPMI-1640 with 10% FBS and aspirated to eliminate the presence of air bubbles. The chambers were divided into three groups: stationary control without rotation, horizontal rotation control and clinorotation. The clinostat is located in an incubator and thermostated at 37°C.

RNA EXTRACTION AND RT-PCR ANALYSES

Reverse transcription-polymerase chain reaction (RT-PCR) was used to measure the steady-state levels of mRNA using primers for human iNOS and GAPDH induced by the predetermined periods of clinostat. RT-PCR was performed with total RNA which was extracted from cultured HUVECs by Trizol reagent according to the manufacturer's instructions. The first-strand cDNA was synthesized from 500 ng of total RNA using a reverse transcription kit (Takara, Japan). PCR for iNOS was preformed in an automated Thermal Cycler ThermoHybrid with an initial activation step for 3 min at 94°C followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 50 s at 56°C (iNOS) or 52°C (GAPDH), extension for 7 min at 72°C. The primers used in this passage were designed by Primer 5.0. The sense and antisense primers for iNOS (GenBank accession no. NM000625) were 5′-CAAGTTCCATCTTTCACCCAC-3′ and 5′-CCCAGCCTCAAGTCTTA-TTTC-3′, respectively. The sense and antisense primers for GAPDH (GenBank accession no. NM002046) were 5′-AAAGGTGGAG-GAGTGGGT-3′ and 5′-GGGAAACTGTGGCGTGAT-3′. The amplified PCR products were separated by a 2% PAGE and visualized with ethidium bromide under UV light illumination. Quantitative analysis of the electrophoretically isolated PCR product for iNOS showed the cycle number-dependent exponential amplification, and the middle portion of the semilogarithmic plot was chosen for examination of the gene expression. The housekeeping gene GAPDH was used for normalization. The ratios of the emissions incorporated into the PCR products of iNOS mRNA to the GAPDH products were calculated to evaluate relative changes in the mRNA expression levels of iNOS.

WESTERN BLOT ANALYSIS

After being stimulated for the indicated times, HUVECs were washed three times with PBS. HUVECs were scraped off the coverslips and lysed with the cell lysis buffer (Cell Signal Technology, MA). Fifty mg protein/slot was resolved on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA) at 400 mA for 2 h at 4°C. The transblotted membranes were washed twice with TBS containing 0.1% Tween 20 (TBST) and incubated with blocking solution (5% skim milk) for 2 h. The membranes were incubated overnight at 4°C with the antibodies to iNOS (1:1,000) and GAPDH (1:500), respectively. Blots were washed three times with TBST for 20 min and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz, CA) for 1 h at room temperature. Blots were again washed three times in TBST and then developed for visualization using ECL Plus detection kit (Amersham, UK). The intensity of each band was quantitatively determined using UN-SCAN-IT^{TM} software (Silk Scientific, UT) and the density ratio represents the relative intensity of each band against those of controls in each experiment.

TRANSFECTIONS AND REPORTER GENE ASSAYS

Cells were plated at 60–70% confluence in 6-well plates before transfection. The following plasmids were transfected: (i) 3 μg of plasmid phiNOS(7.2)Luc [Michael et al., 1996], (ii) 3 μg of plasmid pNF-κB-luc, (iii) 3 μg of plasmid pAP-1-luc. Three microgram of plasmid phiNOS(7.2)Luc and 3 μg pRSV-c-Jun and 3 μg pRSV-c-

Fos were cotransfected. The cells were transfected by Lipofectamine Plus (Invitrogen, CA) following the manufacturer's protocol. To control for transfection efficiency between groups, 150 ng of pRL-TK (a plasmid encoding Renilla luciferase) was added to each well. Twenty-four hours after cotransfection, cells were exposed to clinorotation for 24 h and then lysed with reporter lysis buffer (Promega, WI). Firefly and Renilla luciferase activities were obtained by analyzing the total cell extract according to standard instructions provided in the Dual Luciferase Kit (Promega, WI) in a TD-20/20 Luminometer (Turner Designs, USA). Relative luciferase activity of cell extracts was typically represented as the ratio of firefly luciferase value/Renilla luciferase value.

CHEMICALS AND REAGENTS

Chemicals and reagents included dimethyl sulfoxide (DMSO), PBS, EDTA, Tris–HCl. Pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma–Aldrich (St. Louis, MO). SP600125 was from Alexis Biochemicals (Switzerland). Taq-polymerase for long PCR, restriction enzymes, and the DNA ligation kit were from Takara (Kyoto, Japan). The plasmid piNOS(7.2)Luc was kindly provided by David A. Geller (University of Pittsburgh, Pittsburgh, USA). The pGL3-basic plasmid, the Dual Luciferase Kit and Passive Lysis Buffer were from Promega (Madison, WI), the plasmids pNF-κB-Luc and pAP-1-Luc were from Stratagene (La Jolla, CA). pRSV-c-Jun and pRSV-c-Fos were kindly provided by Ying-Zhong Niu (Department of Conservative Dentistry, FMMU, Xi'an, China). pRSV-Lipofectamine 2000 Plus and Trizol reagent were from Invitrogen Corp. Polyclonal anti-mouse iNOS antibody and polyclonal anti-rabbit GAPDH antibody were from BD Biosciences (San Jose, CA) and ABcom (Cambridge, UK), respectively.

STATISTICAL ANALYSIS

The data were shown as Mean ± SEM. All the experiments were performed three times with similar results for each time. Differences between three or more groups were analyzed by repeated-measures one-way ANOVA and Fisher's PSLD. Statistical significance was accepted at $P < 0.05$.

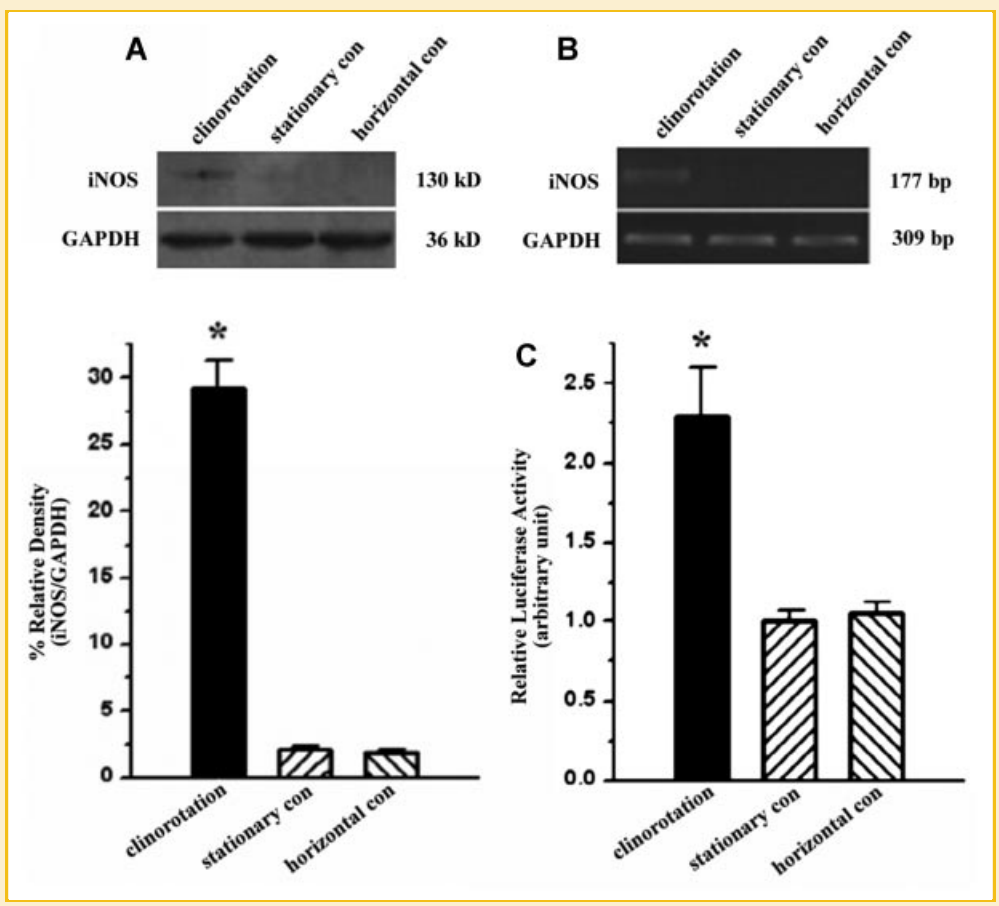

Fig. 1. Clinorotation upregulates expression and promoter activity of iNOS. A: The expression of iNOS protein was upregulated by 24 h clinorotation. The expression of iNOS protein was measured by Western blot. The lower panel shows the relative level of iNOS protein (iNOS/GAPDH) which was determined after densitometric scanning of iNOS and GAPDH bands. B: RT-PCR shows iNOS mRNA induction in response to 24 h clinorotation. RT-PCR was performed using standard method with total RNA which was extracted from cultured HUVECs. C: Relative promoter activity was evaluated using phiNOS(7.2)Luc. HUVECs transfected with the phiNOS(7.2)Luc constructs, which contained sequentially 5′-flanking regions of the 7.2 kb hiNOS promoter gene and a luciferase reporter gene, were treated with 24 h clinorotation. Relative ratio in stationary control was expressed as 1 arbitrary unit. $^{*}P < 0.05$, compared with stationary control, n = 3.

RESULTS

UPREGULATION OF iNOS EXPRESSION AND PROMOTER ACTIVITY BY CLINOROTATION

To understand the influence of simulated microgravity on the expression of iNOS, Western blot and RT-PCR were performed. HUVECs were cultured in clinorotation condition for 24 h. The level of iNOS protein was significantly increased in clinorotation group. And the level of iNOS protein maintained very low level in stationary and rotational controls (Fig. 1A). Subsequently, RT-PCR for iNOS mRNA also showed markedly higher expression of iNOS mRNA in clinorotation group than that in controls (Fig. 1B). Next, to characterize alteration of iNOS in transcriptional level, iNOS promoter-driven luciferase activity was studied. Reporter gene assays showed 2.29-fold induction of luciferase activity in clinorotation HUVECs transfected with phiNOS(7.2)Luc compared with that in controls (Fig. 1C).

DOWNREGULATION OF NF-κB AND AP-1 BY CLINOROTATION

The upregulation of iNOS expression and promoter activity involves a significant transcriptional component. The transcription factors nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) are known to play an important role in cytokine induction of the human iNOS [Kleinert et al., 2004]. However, their roles in the regulation of iNOS expression in real or simulated microgravity still remain unclear. Therefore, we next investigated whether NF-κB and AP-1 were altered by clinorotation. To address the influence of clinorotation on NF-κB transactivation, the HUVECs were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNF-κB-Luc, Stratagene). As shown in Figure 2A, the relative luciferase activity in the clinorotation cells cotransfected with pNF-κB-Luc and pRL-TK was markedly decreased about 48% in comparison to that of stationary or horizontal rotation controls. Furthermore, to detect the alteration of AP-1 transactivation in clinorotation, a reporter gene analysis was carried out by using the luciferase reporter plasmids pAP-1-Luc. Figure 2B shows that the relative luciferase activity in the clinorotation cells cotransfected with pAP-1-Luc and pRL-TK was markedly decreased approximately 38% in comparison to that of stationary or horizontal rotation controls.

A DECREASE IN AP-1 ACTIVITY, BUT NOT NF-κB, CONTRIBUTES TO INCREASED EXPRESSION OF iNOS BY CLINOROTATION

In order to further clarify whether the alteration of NF-κB protein activity was involved in the upregulation of iNOS, we examined the changes of iNOS by addition of PDTC (50 μM), a specific NF-κB inhibitor, for 4 h followed with 24 h clinorotation. The expression of iNOS protein (Fig. 3A) and mRNA (Fig. 3B) were not altered by PDTC. And there was no significant change in iNOS promoter-driven luciferase activity with treatment of PDTC (Fig. 3C). The data shown here indicated that NF-κB was not involved in the regulation of iNOS by clinorotation.

To determine whether AP-1 was involved in clinorotation-induced iNOS expression, HUVECs were pretreated for 4 h with 20 μM SP600125, a specific Jun N-terminal kinase (JNK) inhibitor, which resulted in inhibition of AP-1. Then HUVECs were cultured in clinorotation condition for 24 h. The expression of iNOS protein (Fig. 3A) and mRNA (Fig. 3B) were markedly enhanced with the

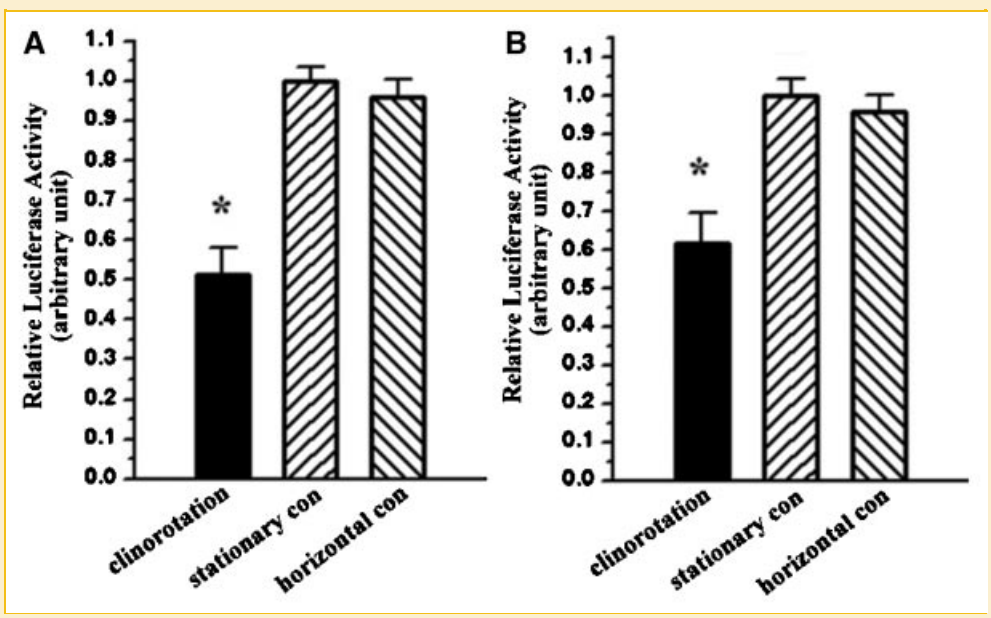

Fig. 2. Downregulation of NF-κB and AP-1 dependent luciferase activities by clinorotation in HUVECs. Cells plated at 60–70% confluence in 6-well plates were cotransfected with 150 ng of pRL-TK and 3 μg of pNF-κB-Luc or pAP-1-Luc using Lipofectamine 2000. After transfection for 24 h, cells were exposed to clinorotation for 24 h. Relative luciferase activity of cell extracts was typically represented as the ratio of firefly luciferase value/Renilla luciferase value. A,B: Effects of clinorotation on relative luciferase activity of NF-κB and AP-1 over stationary control, respectively. Data are expressed as mean ± SEM of the relative luciferase activity over stationary control. Relative ratio in stationary control was expressed as 1 arbitrary unit. $^{*}P < 0.05$, compared with stationary control, n = 3.

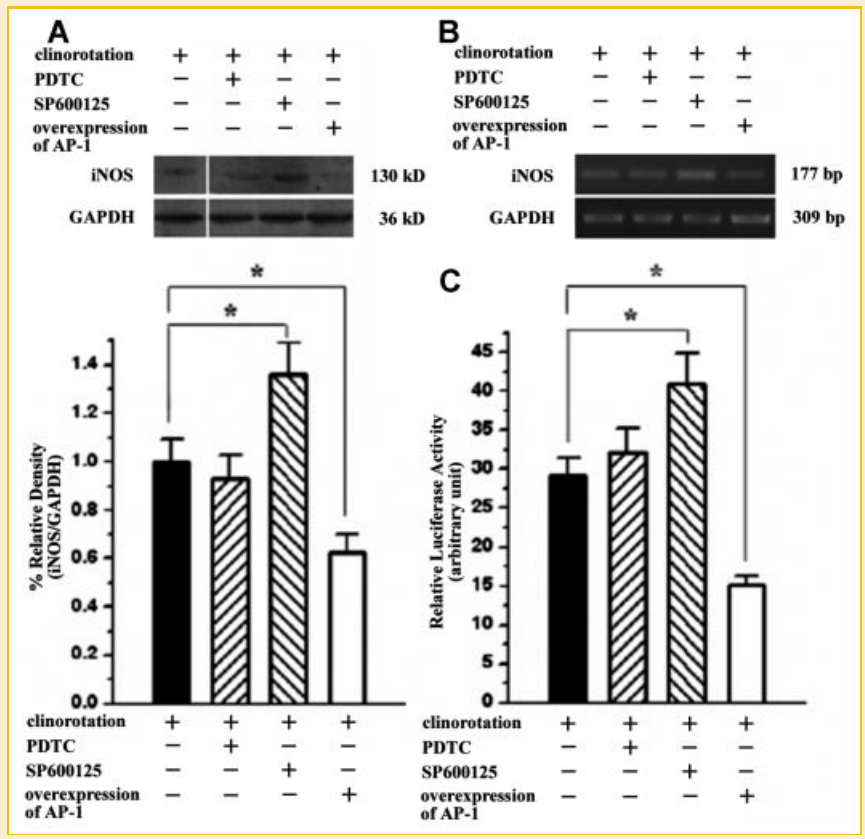

Fig. 3. AP-1 signaling is involved in the upregulation of expression and promoter activity of iNOS in HUVECs exposed to 24 h clinorotation. A: Effects of 24 h clinorotation following 4 h pretreatment with PDTC (50 μM), SP600125 (20 μM) and 24 h incubation with cotransfection of c-Jun/c-Fos respectively on expression of iNOS protein. Western blot showed the expression of iNOS protein (upper). The relative level of iNOS protein was determined after densitometric scanning of iNOS and GAPDH bands (lower). B: Effects of 24 h clinorotation following 4 h pretreatment with PDTC (50 μM), SP600125 (20 μM) and 24 h incubation with cotransfection of c-Jun/c-Fos respectively on iNOS mRNA induction in HUVECs. RT-PCR was performed using standard method with total RNA which was extracted from cultured HUVECs. C: Effects of 24 h clinorotation following 4 h pretreatment with PDTC (50 μM), SP600125 (20 μM) and 24 h incubation with cotransfection of c-Jun/c-Fos respectively on relative luciferase activity of hiNOS promoter constructs in HUVECs. Promoter activity was evaluated using phiNOS(7.2)Luc. Data are expressed as mean ± SEM of the relative luciferase activity over clinorotation group without pretreatment. Relative ratio in clinorotation group without pretreatment was expressed as 1 arbitrary unit. $^{*}P < 0.05$, compared with clinorotation group without pretreatment, n = 3.

inhibition of AP-1. Consistent with the result of iNOS expression, relative luciferase activity of iNOS was significantly increased in clinorotation with treatment of SP600125 compared with that in no treatment cells (Fig. 3C). Next, to explore the effects of excessive AP-1 by overexpression of c-Fos and c-Jun on the regulation of iNOS in clinorotation, HUVECs were cotransfected with pRSV-c-Jun and pRSV-c-Fos. After 24 h incubation, cells were cultured in clinorotation for 24 h. Then, the cells were lysed and light units determined. The expression of iNOS protein (Fig. 3A) and mRNA (Fig. 3B) were evidently attenuated by cotransfection of c-Jun/c-Fos. Overexpression of AP-1 significantly reduced the luciferase activity in phiNOS(7.2)Luc transfected cells (Fig. 3C).

DISCUSSION

In this study, we demonstrated that simulated microgravity could upregulate the expression and promoter activity of iNOS and attenuate the activities of NF-κB and AP-1 in HUVECs. The alteration of AP-1 signaling was involved in the upregulation of iNOS by simulated microgravity in HUVECs.

Orbital spaceflight have clearly demonstrated that the absence or the reduction of gravity significantly affects on astronauts. Health hazards in astronauts are represented by cardiovascular problems and impaired bone healing. Cardiovascular deconditioning such as reduced exercise capacity and orthostatic hypotension has been described in astronauts after spaceflight or prolonged head-down-tilt bed rest [Levine et al., 1996]. The impairment of cardiovascular system induced by microgravity has been the main limitation of long-term spaceflight and received general concern by researchers. Although a lot of works have been done, the basic mechanisms underlying cardiovascular dysfunction are still not fully understood.

Recent works showed that the vasoactive substance such as NO from vascular system may account, in part, for the orthostatic hypotension and the HU-induced hyporesponsiveness of vessels to norepinephrine (NE) [Ma et al., 2003]. NO, released from the endothelium following activation of NOS, acts in a paracrine fashion to stimulate smooth muscle cells via cGMP messenger to induce

vascular relaxation of the blood vessels [Moncada et al., 1988]. Since simulated microgravity stimulates NO synthesis, we hypothesized that activities of NOS would be enhanced under clinorotation conditions. Here we confirmed our hypothesis by observing an increase of iNOS promoter activity, an upregulation of iNOS at both protein and mRNA levels after 24 h clinorotation in respect to controls. Our data obtained are in agreement with the results obtained in HU rats. For example, functional and morphological evidences that upregulation of iNOS expression associated with NO-induced hypotension have been found in many cardiovascular and noncardiovascular tissues from rats subjected to HU [Vaziri et al., 2000; Ma et al., 2003]. It has been demonstrated, by using both a vessel ring bioassay and expression data, that iNOS is increased in the aorta of HU rats [Sangha et al., 2000].

Transcription factors play an important role in the expression of genetic information in the final stage of intracellular signaling. The upregulation of iNOS expression and promoter activity indicated that at least a significant transcriptional component was involved. As we all known, the pathways regulating iNOS expression are very complex and seem to vary in different cells or different species. In general, activation of the transcription factors NF-κB and thereby activation of the iNOS promoter seems to be an essential step in regulating iNOS expression in most cells induced by cytokines [Marks-Konczalik et al., 1998; Taylor and Geller, 2000]. To clarify the involvement of NF-κB in the regulation of iNOS in HUVECs subjected to 24 h clinorotation, the alteration of NF-κB transactivation was determined. Our result showed that the NF-κB dependent transactivation of a luciferase reporter gene was markedly suppressed by clinorotation, but not in controls. Our results on the suppression of NF-κB were in accordance with other reports [Kobayashi et al., 2000; Hirasakaa et al., 2005], though they were observed in different cells by different devices, indicating that simulated microgravity could suppress the NF-κB signaling in various kinds of cells. To further determine the role of NF-κB in iNOS expression, we observed the effects of PDTC, an inhibitor of NF-κB, on iNOS induction in HUVECs subjects to 24 h clinorotation. Data reported here showed that there were no alterations of iNOS expression and promoter activity by addition of PDTC, while the activity of NF-κB protein was significantly inhibited. According to the results above, unlike the role of regulation to iNOS by cytokines, NF-κB seemed not to have an impact on the iNOS expression induced by simulated microgravity in HUVECs.

Furthermore, apart from NF-κB, we examined the alteration of another transcriptional factor AP-1. AP-1, a heterodimer formed by the products of the Fos and Jun, is implicated in cell growth, differentiation and transformation [Angel and Karin, 1991]. Therefore, we determined the luciferase activity of AP-1 dependent transactivation reporter gene to explore the influence of simulated microgravity on AP-1. The negative effect of clinorotation on the transactivation of AP-1 was detected in this part of our study. Recently, several evidences indicate that blockade of AP-1 in mammalian cells leads to the upregulation of iNOS expression by cytokines [Xu et al., 2003; Hartmut et al., 2004]. In this experiment, we performed tests to determine whether AP-1 was involved in the upregulation of iNOS by clinorotation. Our findings showed that the iNOS expression and promoter activity were markedly enhanced by addition of SP600125 compared with that in no SP600125 treatment cells. SP600125 (anthrax[1,9-cd]pyrazol-6(2*H*)-one) is a specific JNK inhibitor, which results in inhibition of AP-1 activity. Recent studies have shown that SP600125 completely blocked IL-induced accumulation of phospho-Jun and induction of c-Jun transcription [Han et al., 2001]. Then, we cotransfected c-Jun and c-Fos expression plasmids into the HUVECs to investigate the influence of overexpression of AP-1 on the regulation of iNOS in clinorotation. The results demonstrated that cotransfection of c-Jun and c-Fos significantly attenuated the expression and promoter activity of iNOS in clinorotation. Collectively, these studies suggested that the upregulation of iNOS in clinorotation was, at least in part, via the suppression of AP-1 signaling.

In conclusion, suppression of AP-1, rather than NF-κB, was involved in the pathway of upregulating iNOS activation and expression by clinorotation. To our knowledge, this is the first report demonstrating the relationship of suppression of AP-1 signaling and activation of iNOS expression under conditions of simulated microgravity in HUVECs in vitro. This finding is consistent with the role of AP-1 in the regulation of iNOS expression in mammalian cells. The mechanisms accounting for the suppressive effect of simulated microgravity on AP-1 signaling and a more detailed analysis of specific AP-1 family members involved in this response are under investigation.

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