Physiol. Res. 50: 83-90, 2001

Permanent Depression of Plasma cGMP during Long-Term Space Flight

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Received November 18, 1999
Accepted July 4, 2000

Summary

The purpose of this study was to investigate plasma concentrations of cyclic guanosine monophosphate (cGMP) and atrial natriuretic peptide (ANP) during and after real and simulated space flight. Venous blood was obtained 3 min after the beginning and 2 min after the lower body negative pressure maneuver in two cosmonauts preflight (supine), inflight, and postflight (supine) and in five other subjects before, at the end, and 4 days after a 5-day head-down tilt (–6°) bed rest. In cosmonaut 1 (10 days in space), plasma cGMP fell from preflight 4.3 to 1.4 nM on flight day 6, and was 3.0 nM on the fourth day after landing. In cosmonaut 2 (438 days in space), it fell from preflight 4.9 to 0.5 nM on on flight day 3, and stayed <0.1 nM with 5, 9, and 14 months in space, as well as on the fourth day after landing. Three months after the flight his plasma cGMP was back to normal (6.3 nM). Cosmonaut 2 also displayed relatively low inflight ANP values but returned to preflight level immediately after landing. In a ground-based simulation on five other persons, supine plasma cGMP was reduced by an average of 30 % within 5 days of 6° head-down tilt bed rest. The data consistently demonstrate lowered plasma cGMP with real and simulated weightlessness, and a complete disappearance of cGMP from plasma during, and shortly after long-duration space flight.

Key words

Long-duration space flight • Head-down tilt • Cyclic guanosine monophosphate • Atrial natriuretic peptide • Bed rest

Introduction

As part of the project ”RLF” (Russian long-term flight), our group accompanied the medical-physiological part of a cosmonaut’s continuous 438-day stay (current world record) onboard the space station MIR. Studies on volume regulation and cardiovascular adaptation were performed during and after the space flight. To create an analogue to orthostatic stress, we used lower body negative pressure (LBNP) as a stimulus to induce, *via* a decreased central and peripheral baroreceptor load (Ebert *et al.* 1982), endocrine responses (Tidgren *et al.* 1990, Smith and Ebert 1991) before, during, and after sojourn in the MIR space station. These reactions are indicative of the adaptive state of cardiovascular-endocrine regulation during and after space flight.

LBNP is used routinely as an indicator of cardiovascular functions. We employed this technique to

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investigate, on quantitative grounds, hormone concentration changes as a function of cardiovascular stress. During space flight, blood is redistributed throughout the vasculature along the body axis, vascular mechanoreceptor loads are altered (central engorgement), "excess" fluid is lost from the organism, and reflexly connected endocrine systems adapt by accompanying changes of hormonal output. After returning to earth, the systems are forced to re-adapt to 1-G conditions. Altered steady-state plasma concentration values of volume sensitive hormones have been observed inflight as well as postflight (Grigoriev *et al.* 1994, Hinghofer-Szalkay *et al.* 1999, Kvetňanský *et al.* 1988, Macho *et al.* 1991, Nixon *et al.* 1979).

Cyclic guanosine monophosphate (cGMP) is produced from GTP by means of various guanylate cyclases (Anand-Srivastava and Trachte 1993, Tremblay *et al.* 1985) and acts as an intracellular second messenger. The activity of guanylate cyclase is regulated by various signal substances, including the atrial natriuretic peptide (ANP). Plasma cGMP correlates well with plasma ANP both during basic conditions and after ANP infusion (Tremblay *et al.* 1985, Vorderwinkler *et al.* 1991) and consequently plasma cGMP is considered to follow closely the changes in ANP (Heim *et al.* 1988, Lijnen *et al.* 1987).

Due to conflicting results about volume regulating hormones, especially ANP during space flight, we determined cGMP as well as ANP during the longest space flight proving a close parallelity, as mentioned above.

We are not aware of any determinations of plasma cGMP under real or simulated weightlessness. Our results clearly depict a complete suppression of plasma cGMP besides unchanged ANP concentrations and indicate that other factors than ANP, such as reduced shear stress, may be involved in this second messenger mechanism.

Methods

Experimental protocol

The protocol was designed for investigations in space and approved by an International Review Board and by the University's Ethics Committee.

Investigations on cosmonauts

Cosmonaut FV (31 yrs, 75 kg preflight) spent 10 days in space, cosmonaut VP (52 yrs, 88 kg preflight) 438 days in space. Investigations were performed 3 months prior to launch, on different days inflight (FV: 6 days, VP: 3, 170, 287, and 430 days) and on different days after the missions (FV: 4 days, VP: 4 and 90 days). After equipment stowage and preparation (LBNP device, blood collection kit "Vene", blood centrifuge, plasma container and freezer), the cosmonaut moved into the onboard LBNP device ("Chibis"-suit), which was identical to the device used for pre- and postflight investigations on the earth where the test subjects lay in a supine position during the whole experiment (60 min pre-LBNP). A venous catheter was inserted into the left antecubital vein by the cosmonaut himself before LBNP was started. Three minutes into LBNP (–15/–30/–35 mm Hg for 15/15/10 min), the first blood sample (sample "A") was withdrawn. A second blood sample (sample "B") was taken 2 min after termination of LBNP. In both cases, two 10-ml syringes (EDTA) were filled. Plasma was prepared as described previously (Kvetňanský *et al.* 1998) and samples were frozen (–25 °C) immediately after spinning. For operational reasons, pre- and inflight studies were done between 15:00 and 18:00 h, whereas the postflight experiments were performed before noon.

Head-down tilt bed rest (HDBR) experiments

Five male volunteers (24-30 yr, 62-83 kg) familiar with the test situation, purpose and nature of the study gave their informed consent, underwent an LBNP test as indicated above 4 days before, at the end of, and 4 days after a 5-day head-down period. The 6° HDBR (antiorthostatic position) was chosen since many changes in cardiovascular responses caused by loss of gravity are closely simulated by this ground-based model. Room temperature was maintained at 20 °C and room lighting was on between 07:00 and 23:00 h. The movements of the subjects were restricted to turning in bed, and they were allowed to use one pillow (under their head). All activities, including meal consumption, were carried out in this position. Fluid and food intake were controlled and logged over the whole HDBR. Their daily caloric intake was 7.8-15.9 MJ, daily fluid intake 1.54 ±0.04 liters, and daily urine output was 0.66-2.1 liters.

Hormone determinations

Plasma ANP and cGMP measurements were based on radioimmunoassay (RIA), Cyclic guanosine monophosphate (cGMP) measurements were performed by a commercially available RIA kit (Biomedica, Austria) according to (Steiner *et al.* 1970). The assay

employs competition between succinylated cGMP (sample) and a radioactive tracer for binding to polyclonal antibodies onto the tubes. The sensitivity, defined as the minimum concentration of cGMP significantly different from the standard with probability of 95 %, was 10 pM. For atrial natriuretic peptide 99-126 (ANP) determinations we used a RIA kit which does not require prior extraction (Nichols Institute, Diagnostics BV, the Netherlands). Sensitivity, defined as the apparent concentration at 3 SD from the counts at maximum binding, was 11 pg/ml.

Statistical treatment of data

Data were analyzed using the statistics package Statistica for Windows, StatSoft Inc., Tulsa, OK,. Repeated ANOVA measures were used to compare the means among treatments where applicable. Different experimental periods were compared by paired Wilcoxon or Students t-tests according to the respective variable distribution. Differences are considered as significant if $p<0.05$. The whiskers of the box plots in Figure 1 indicate the 95 % confidence interval for the mean at each measuring point. For each pair of box plots with non-overlapping whiskers the difference of the mean values is in accordance with a t-test difference on a significant level of 0.01.

Validation of cGMP measurements

Heparin has been found to destroy cGMP in plasma samples (Patterson *et al.* 1971, Heim *et al.* 1988). To quantify this effect, we applied the specific calcon reaction on plasma samples returned from space. The reaction results in a change of color from blue to violet in the presence of free calcium. Some samples displayed an alteration in color indicating an insufficient EDTA admixture. To test the validity of our originally determined cGMP values, additionally collected plasma samples with either heparin or EDTA as preservative added were frozen and stored at –20 °C before cGMP determination, in order to simulate conditions on the space station.

Table 1. ANP and cGMP concentrations in two cosmonauts pre-, in- and postflight.

Cosmonaut		ANP (pg/ml)		CGMP (nM)	
	Time	3 min	42 min	3 min	42 min
VP	preflight	171	136	4.9	3.9
	FD3	37	27	0.5	0.4
	FD170	42	89	nd	nd
	FD287	48	7.9	nd	nd
	FD430	4.9	15	nd	nd
	PD4	94	180	nd	nd
	PD90	105	89	6.3	4.5
FV	preflight	42	128	4.3	3.6
	FD6	52	106	1.4	0.4
	PD4	106	166	3.0	0.3

Nd – non-detectable

Results

Table 1 indicates all preflight, inflight and postflight data from the two cosmonauts. During space flight, a 25 % increase of ANP could be observed in cosmonaut FV on FD6, whereas in cosmonaut VP we found a steady decrease in ANP levels during the whole duration of flight. Postflight ANP values increased by 25 % inflight in cosmonaut FV and 2.5 fold postflight, whereas in cosmonaut VP we found a steady decrease in ANP levels during the whole duration of the flight. Reexposure of cosmonaut VP to gravity after a long-lasting stay in microgravity led to higher ANP levels which also decreased 90 days after landing compared to preflight conditions (Table 1). During the long-term flight cGMP decreased continuously in both cosmonauts. But

despite very low cGMP levels, LBNP-induced relative changes were similar throughout (Table 1).

During a 5-day bed rest study, no differences in ANP concentrations could be observed (Fig. 1). In contrast, cGMP was significantly decreased during simulated weightlessness, but returned to control values within four days after head-down bed rest (Fig. 1).

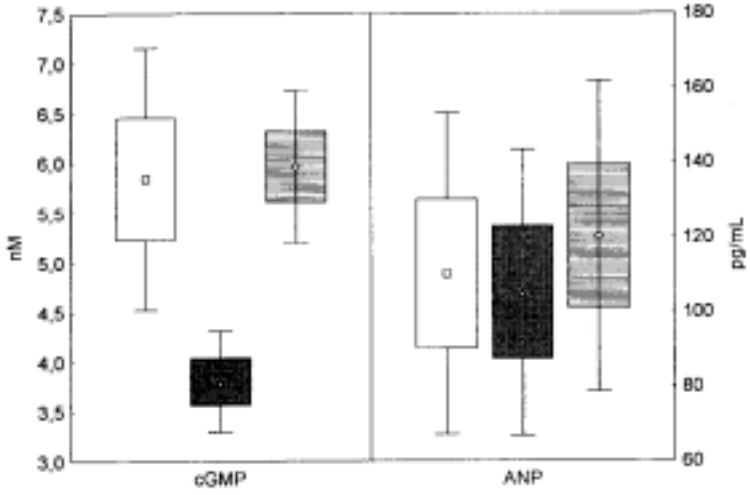

Fig. 1. *Mean cGMP and ANP concentrations before (open boxes), at the end of the bed rest period (gray boxes) and after bed rest (hatched boxes). Boxes represent means ± S.E.M. with whiskers indicating 95 % confidence interval of variables in 5 test subjects. Each value represents the mean of three samples per test subject.*

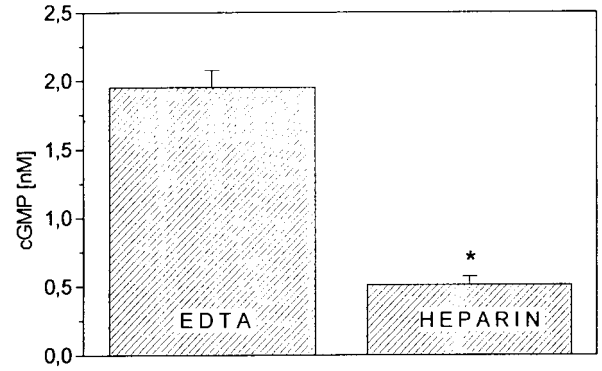

Fig. 2. *Cyclic GMP content in identical plasma samples stabilized by either EDTA or heparin (n = 16). Note the significant fourfold decrease in heparin-treated samples compared to those treated with EDTA.*

Discussion

Cyclic guanosine monophosphate (cGMP) is produced from GTP by means of various guanylate cyclases (Anand-Srivastava and Trachte 1993, Tremblay *et al.* 1985) and acts as an intracellular second messenger. Guanylate cyclase activation is regulated by various signal substances, including atrial natriuretic peptide (ANP) which mediates vasorelaxation. Cyclic GMP, as its second messenger, is therefore a mediator and a marker of ANP activity (Hamet *et al.* 1986). Accordingly, plasma cGMP correlates well with plasma ANP both during basic conditions and after ANP infusion (Tremblay *et al.* 1985, Vorderwinkler *et al.* 1991). In longer time periods, plasma cGMP is considered to follow changes of ANP closely (Heim *et al.* 1988, Lijnen *et al.* 1987).

cGMP is quite stable in the plasma and for that reason cGMP has been proposed, on certain occasions, to be a more reliable marker of ANP turnover than ANP itself (Vorderwinkler *et al.* 1991). Consequently, plasma cGMP analysis would be an acceptable chemical marker as an indirect monitor of possible ANP effects. Previous investigations have shown that plasma cGMP concentrations increases several fold after infusions of brain or atrial natriuretic peptides in humans (Holmes *et al.* 1993, Florowski *et al.* 1994), whereas their short-term stimulus-response time course can differ significantly. After extracellular volume expansion, cGMP maximum occurs 10 min after a preceding ANP peak (Weil *et al.* 1985).

However, in our experiments, cGMP changes were different from those in ANP. While ANP inflight data are partly controversial, showing also decreased

Validation of our cGMP determinations with the calcon reaction resulted in a significant reduction of cGMP content in heparin-treated samples (Fig. 2). Nevertheless, we detected a 4.2 fold reduction in the cGMP concentration, whereas the cGMP content in the samples from the space station was close to the detection limit.

levels during long-term flight, no significant changes occurred during short-term flight or HDT-bed rest (Table 1). Not only that cGMP was decreased (–65 %) inflight in both subjects as well as during bed rest, LBNP consistently depressed cGMP at min 2 post-LBNP as compared to min 3 during LBNP**.** Additionally, other factors than ANP, such as bradykinin or adrenergic receptor stimulation could contribute to the changes in cGMP concentrations (Lijnen *et al.* 1987). Thus, the dynamics of responses after acute cardiovascular stress might cause changes in these hormonal systems with different time courses.

There is a multiple connection between vasoactive substances and cGMP, for example nitric oxide (NO) and the recently discovered peptide adrenomedullin act at least in part *via* cGMP (Griendling and Alexander 1996, Schell *et al.* 1996). Therefore, two important characteristics, namely vascular relaxation and regulation of vascular permeability, are induced by various nitrate vasodilators or by endogenously released vasodilators such as NO as well as inhibition of platelet adhesion and aggregation (Murad 1986, Radomski *et al.* 1987). Obviously, cGMP is of paramount importance, in relation to vascular tone, blood pressure, and perfusion parameters. These properties may complicate the interpretation of altered cGMP levels as a response to different stimuli. A functional disturbance of vascular functions and endothelium due to weightlessness may therefore be responsible for depressed cGMP values. Accordingly, we postulate that changes in plasma cGMP may indicate fundamental cardiovascular adaptational rearrangements as they occur during and after space flight. Significant changes in the fluid-regulating systems of the body have been observed since the earliest space flights. Obviously the cardiovascular and fluid-electrolyte systems need continuous gravitational (hydrostatic) challenging in order to function properly in a 1-G environment. To study the effects of the absence of these forces, hormone values, which are obtained before or at the beginning of a certain cardiovascular stressor like LBNP, may be considered indicative for adaptation to space flight or bed rest, whereas actual stress-induced changes (with and after LBNP) conceivably reflect the sensitivity of corresponding endocrine system. Previous experimental findings in our laboratory have shown that no significant changes in the concentration of any of the metabolites studied occurred within the first 3 min of LBNP. Therefore, ANP and cGMP concentrations in the first samples withdrawn from the cosmonauts can be considered as control values (Hinghofer-Szalkay *et al.* 1996).

We found only one study measuring cGMP during bed rest (Sigaudo *et al.* 1996). These authors found no decrease in urinary cGMP, but noticed diminished cGMP concentrations in the saliva of cosmonaut 2 during space flight (Gharib, personal communication). Since we cannot yet decide which factors brought about the observed decrease of plasma cGMP during the stay in orbit, the possible influence of various factors, such as radiation, needs to be investigated. Nevertheless, the hypothesis that radiation produces (or destroys) substances like oxygen-derived radicals which may interfere with cGMP production and metabolism and can contribute to a reduction in NO (Rubanyi 1988) seems unlikely, as during HDBR on the earth the concentration of plasma cGMP also decreased.

It is also possible that the depressed cGMP level is due to the absence of gravitational pull causing a shift of the intravascular balance between cGMP production and degradation. Although cyclic nucleotides are intracellular products, they can leak by an unknown mechanism (probably *via* chloride channels) from cells as an overflow reaction, reflecting alterations of the intracellular pool and expressing the vasoreactive status. Since cyclic nucleotides in the plasma seem to be in a dynamic steady-state relationship with intracellular pools (Broadus *et al.* 1970), a decreased intracellular level of cGMP might also be reflected by a reduced plasma cGMP level. As the vasodilator effect of ANP or NO requires the integrity of the vascular endothelium, it can be speculated that a reduction of intra- or extracellular cGMP levels is considered to be an important factor in the development of increased vascular resistance. The present study tested this hypothesis by subjecting volunteers on the ground to simulated weightlessness, i.e. head-down tilt bed rest.

The release and action of NO is the main physiological regulatory system controlling vascular tone. Many hormones and neurotransmitters stimulate NO, activate the soluble guanylate cyclase and thereby raise intracellular cGMP. Prostaglandins, NO, adrenomedullin, histamine, substance P and bradykinin induce the production and liberation of NO, which in turn activates soluble guanylate cyclase in vascular smooth muscle that increases cGMP production. Enhanced fluid shear stress is effective in altering endothelial NO synthesis (Griendling and Alexander 1996) and chronic increase in blood flow can modulate the release of NO and hence the concentration of cGMP. Following 6 weeks of elevated

blood flow, both basal and stimulated NO release from the artery are augmented (Miller and Vanhoutte 1988). As there is an adaptation to increased blood flow, there might also be an adaptation process in the opposite direction, a hypothesis requiring testing in cellular cultures. It is even tempting to speculate that the proposed endothelial dysfunction or endothelial injuries resulting from prolonged stay in weightlessness might be responsible for inactivation of the NO system (Rowe 1998). Taken together, it is probable that a diminished release of NO from the vascular endothelium might be caused by decreased activity of the endothelial constitutive nitric oxide synthase (ecNOS) or the down-regulation of gene expression of ecNOS which results in lower cGMP concentrations.

Cytoplasmatic signal transduction can be activated by microgravity (De Groot *et al.* 1991). Membrane receptor activity is influenced by a multitude of factors that thereby modulate cell sensitivity to hormones. Possible changes of receptor expression or ANP signal transduction during weightlessness are poorly investigated, weightlessness causes an increase of ANP binding site number in the rat choroid plexus (Herbute *et al.* 1994). However, endothelial clearance receptors (R_2 or C), which bind more than 85% of ANP were not examined. The number and affinity of smooth muscle ANP receptors depend on the blood volume as well as the blood pressure (Hirata *et al.* 1986, Resink *et al.* 1989). A greater number of clearance receptors could directly be involved in a low cGMP content. Short (30 min) head-out water immersion fails to alter B-receptor activity, but volume expansion, mineralocorticoid administration or 4 days increased sodium intake down-regulate vascular ANP receptors (Schiffrin and St-Louis 1985, Schiffrin and St-Louis 1987, Dikshit *et al.* 1994).

The density of vascular ANP binding sites and plasma ANP concentration seem to be inversely related (Schiffrin and St-Louis 1987). Altered ANP concentrations during weightlessness may sensitize clearance receptors. R_2-receptors might constitute the function of capacity receptors that buffer free ANP; their increased expression may reduce receptor affinity. C-receptors are down regulated by the cGMP-analogue 8-bromo-cyclic GMP (Kato *et al.* 1991) or by β_2-adrenergic stimulation (Kishimoto *et al.* 1994). Furthermore, changed densities of adrenergic receptors after 10 days of head-down tilt bed rest have been reported (Maass *et al.* 1992). The measurement of messenger RNA from endothelium or platelet receptors, which is not coupled to particulate guanylate cyclase should help to answer the question whether the altered receptor-mediated clearance is indeed cause of ANP and ultimately cGMP concentration changes during space flight or simulated weightlessness.

Finally, diminished mechanical stress in the lower body, particularly the sole of the foot, during space flight may reduce wear and tear of blood cells and thereby decrease cGMP "spillover", resulting in low plasma cGMP.

In conclusion, the most pronounced change as observed in this study was a continuous decrease in ANP during the whole period of 430 days, which returned to normal values within 90 days after landing. As a result of this decrease complete suppression of plasma cGMP during the long-term stay in space occurred, but returned to control values within three months after landing. This effect was not dependent on a low EDTA content of the samples. Furthermore, an attenuated decrease of plasma cGMP concentrations was found under conditions simulating weightlessness, such as 5-day head-down tilt bed rest. As many factors may contribute to decreased plasma cGMP, reasons for changes in these second messenger mechanisms cannot as yet be explained but the absence of major hydrostatic pressure, decreased shear stress and/or altered receptor properties might contribute to this phenomena.

Acknowledgements

We thank Ulrike Wöllik, MTA and Andreas Rothaler CTA for their skilled assistance. This work was supported by the Austrian Ministry of Sciences and Research, and the Austrian Society for Aerospace Medicine, Vienna.

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