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Calbindins Decreased After Space Flight

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Exposure of the body to microgravity during space flight causes a series of well-documented changes in Ca^{2+} metabolism, yet the cellular and molecular mechanisms leading to these changes are poorly understood. Calbindins, vitamin D-dependent Ca^{2+} binding proteins, are believed to have a significant role in maintaining cellular Ca^{2+} homeostasis. In this study, we used biochemical and immunocytochemical approaches to analyze the expression of calbindin-D_{28k} and calbindin-D_{9k} in kidneys, small intestine, and pancreas of rats flown for 9 d aboard the space shuttle. The effects of microgravity on calbindins in rats from space were compared with synchronous Animal Enclosure Module controls, modeled weightlessness animals (tail suspension), and their controls. Exposure to microgravity resulted in a significant and sustained decrease in calbindin-D_{28k} content in the kidney and calbindin-D_{9k} in the small intestine of flight animals, as measured by enzyme-linked immunosorbent assay (ELISA). Modeled weightlessness animals exhibited a similar decrease in calbindins by ELISA. Immunocytochemistry (ICC) in combination with quantitative computer image analysis was used to measure *in situ* the expression of calbindins in the kidney and the small intestine, and the expression of insulin in pancreas. There was a large decrease of immunoreactivity in renal distal tubular cell-associated calbindin-D_{28k} and in intestinal absorptive cell-associated calbindin-D_{9k} of space flight and modeled weightlessness animals compared with matched controls. No consistent difference in pancreatic insulin immunoreactivity between space flight, modeled weightlessness, and controls was observed. Regression analysis of results obtained by quantitative ICC and ELISA for space flight, modeled weightlessness animals, and their controls demonstrated a significant correlation. These findings after a short-term exposure to microgravity or modeled weightlessness suggest that a decreased expression of calbindins may contribute to the disorders of Ca^{2+} metabolism induced by space flight.

Key Words: Calbindin-D_{28k}; calbindin-D_{9k}; space flight; microgravity.

Introduction

Calbindins, vitamin D-dependent Ca^{2+}-binding proteins, are believed to be essential, as intracellular Ca^{2+} sequestrants/buffers, to the process of intestinal Ca^{2+} absorption (Nemere and Norman, 1991; Norman et al., 1992) and renal Ca^{2+} reabsorption (Johnson and Kumar, 1994; Hemmingsen et al.,1995). Calbindin-D_{28k}, functioning as an intracellular Ca^{2+} buffer, is crucial for preventing accumulation of excessive levels of cytosolic free Ca^{2+} (Iacopino et al., 1992; Rhoten and Sergeev, 1994) and, thus, determining cell fate (Dowd, 1995). Vitamin D is the precursor of the steroid hormone, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Norman et al., 1982; Henry and Norman, 1984; Sergeev, 1989). $1,25(OH)_2D_3$ produces a wide spectrum of biological effects via both receptor-mediated regulation of nuclear events (Minghetti and Norman, 1988; Lowe et al., 1992; Sergeev and Norman, 1992) and rapid actions independent of the genomic pathway (Farach-Carson et al., 1991; Norman et al., 1992; Sergeev and Rhoten, 1995). The vitamin D receptor regulates genes associated with Ca^{2+} homeostasis, such as calbindins, with the proliferation pathway, the differentiation pathway, and the developmental cascade (Lowe et al., 1992; Norman et al., 1993).

There is evidence that the integrated operation of the vitamin D endocrine system is affected by factors of space flight, including evidence from human and rat models of microgravity (Sergeev et al., 1982a,b, 1983, 1984, 1985; Morey-Holton et al., 1988; Spirichev and Sergeev, 1988; Arnaud et al., 1991). Serum $1,25(OH)_2D_3$ concentration decreased in healthy volunteers after 1 yr of bed rest with the head lower than the feet (Sergeev and Morukov, unpublished observations). The $1,25(OH)_2D_3$ production in the kidney and accumulation of the hormone in the bone and intestine markedly decreased in rats after long-term

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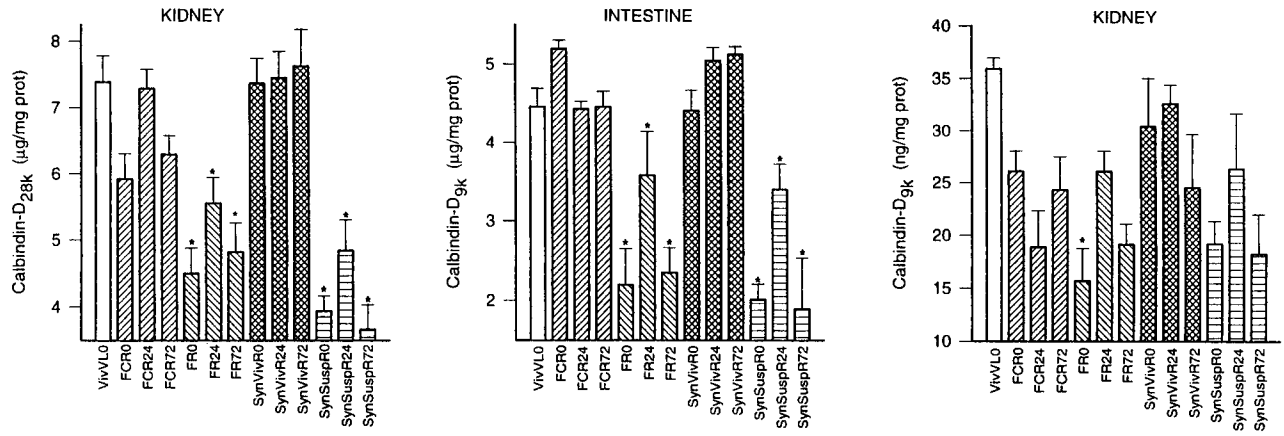

Fig. 1. Calbindin contents in the small intestine and the kidney of space flight, tail suspension, and control animals. Calbindin-D_{28k} and calbindin-D_{9k} were measured by ELISA, as described in Materials and Methods. Group abbreviations are: VivL0, basal control group dissected at launch; FCR0, FCR24, and FCR72, flight control animals that were housed in flight hardware (Animal Enclosure Module); FR0, FR24, and FR72, flight animals housed in Animal Enclosure Modules; SynVivR0, SynVivR24, and SynVivR72, synchronous vivarium control animals; SynSuspR0, SynSuspR24, and SynSuspR72, synchronous tail suspension animals. FC, F, SynViv, and SynSusp groups were divided into three subgroups (R0, R24, and R72) for dissection at 0, 24, and 72 h after landing. The data, analyzed by ANOVA, represent mean values ± SEM. Only significance of differences between F vs FC and SynSusp vs SynViv groups are presented. (*), $p < 0.05$.

hypokinesia (Sergeev et al., 1983, 1984). These were accompanied by a decrease in the intestinal Ca^{2+} absorption (Sergeev and Spirichev, 1986) and osteopenia (Kabitskaya et al., 1984; Sergeev et al., 1987). Prophylactic treatment with vitamin D_3 active metabolites prevented bone loss to a significant extent in rats during long-term hypokinesia (Sergeev et al., 1982a,b, 1985, 1987; Ushakov et al., 1982, 1983a,b, 1984), indicating a crucial role for the vitamin D endocrine system in regulation of bone and Ca^{2+} metabolism in modeled weightlessness. It seems probable that the vitamin D hormone-mediated regulation, particularly that of calbindins, may be a critical factor in adaptational and readaptational changes of Ca^{2+} metabolism that are associated with the effects of weightlessness and return to earth gravity.

We participated in the organ-sharing program for tissues from rats flown aboard the Spacelab 3 mission, and compared the effects of microgravity on calbindins in rats in space vs ground, control animals (synchronous Animal Enclosure Module, tail suspension, and vivarium controls). We hypothesized that exposure to microgravity might affect expression of vitamin D-dependent Ca^{2+}-binding proteins, calbindin-D_{28k}, and calbindin-D_{9k}. To test this hypothesis, we evaluated content and immunocytochemical expression of calbindin-D_{28k} and calbindin-D_{9k} in kidney and small intestine.

Results

Measurement of Calbindin-D_{28k} and Calbindin-D_{9k} in Kidney and Calbindin-D_{9k} in Small Intestine by ELISA

ELISA was used to measure calbindins in the kidney and the intestinal mucosa of space flight, tail suspension, and control animals. The calbindin-D_{28k} content in the kidney and the calbindin-D_{9k} content in the small intestine of space flight and suspension animals was reduced when compared with their respective controls (Fig. 1, left and central panels). In terms of the flight animals, the decrease was 24.0, 23.9, and 23.5% for calbindin-D_{28k} in the kidney at 0, 24, and 72 h postflight, respectively (*see also* Fig. 1, left panel), and for calbindin-D_{9k} in the intestine the decrease was 57.8, 19.2, and 47.3% at 0, 24, and 72 h postflight (*see also* Fig. 1, central panel). Quantitatively similar decreases were found when synchronous tail suspension and vivarium groups were compared at 0, 24, and 72 h postflight (*see* Fig. 1, left and central panels).

The content of calbindin-D_{9k} in the kidney was very low as compared with the renal calbindin-D_{28k} content. Levels were in the ng/mg protein range for calbindin-D_{9k} vs the µg/mg protein range for calbindin-D_{28k} (Fig. 1, right panel). The trend toward a decreased calbindin-D_{9k} content in the kidney of flight and suspension animals at 0 and 72 h, but not 24 h postflight was observed. However, this change was statistically significant only for flight rats at 0 h postflight (*see* Fig. 1, right panel).

In Situ *Quantification of Calbindin-D_{28k} in Kidneys, Calbindin-D_{9k} in Intestine, and Insulin in Pancreas Using Immunocytochemistry*

Immunocytochemistry (ICC) in combination with quantitative computer image analysis was used to measure *in situ* the expression of calbindins in kidneys and intestine, and insulin in pancreas.

The predominate immunolocalization and gene expression of renal calbindin-D_{28k} was similar to that first reported by us (Rhoten and Christakos, 1981, 1990), i.e., cells of the

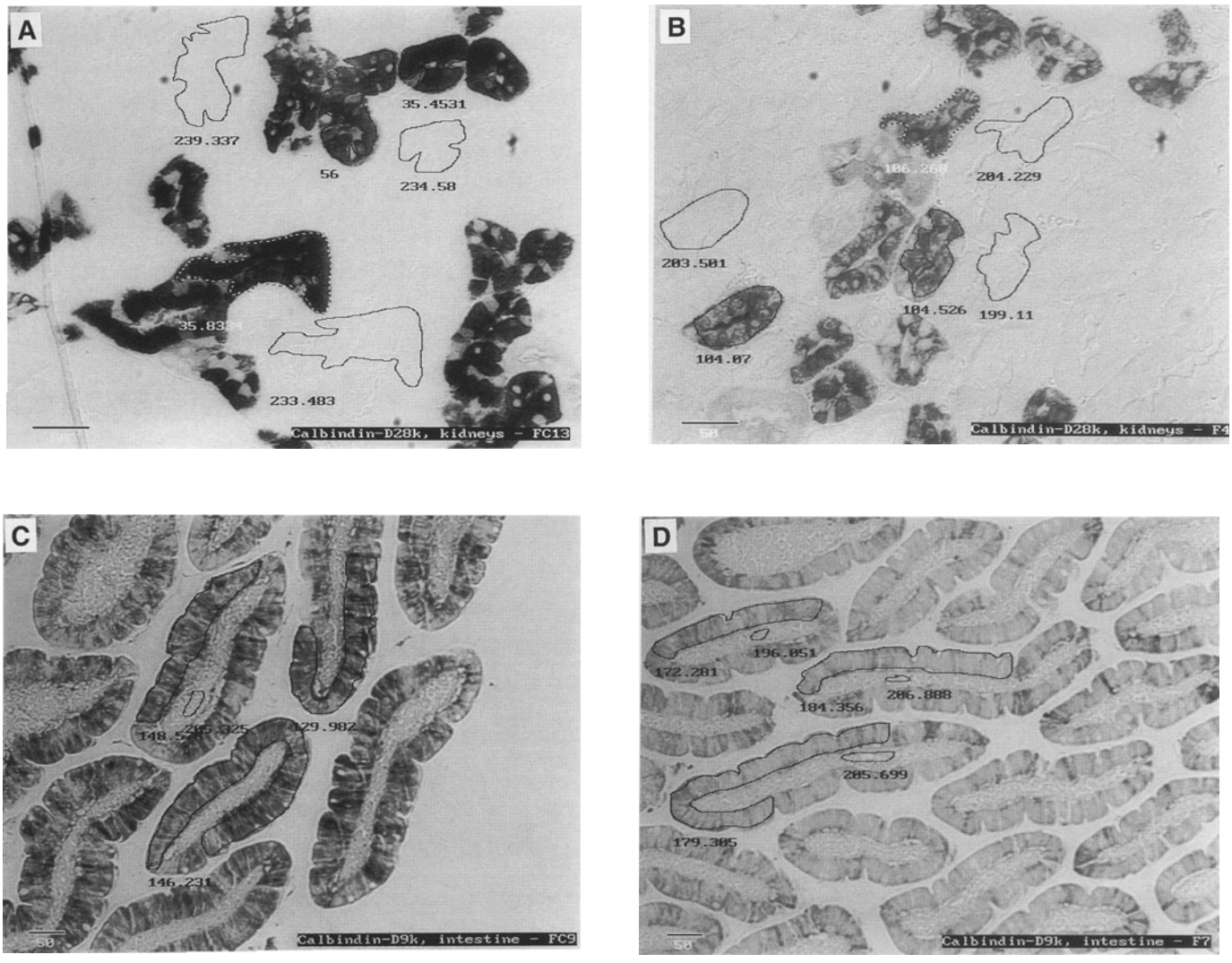

Fig. 2. Immunocytochemical localization and quantification of calbindins. Tissue sections were immunoreacted and calbindin-D_{28k} in the kidney (upper panels) and calbindin-D_{9k} in the intestine (lower panels) were measured, as described in Materials and Methods. Scale bars, 50 μm.

distal convoluted tubules, the connecting tubules, and the cortical collecting tubules. Some flight and ground-based animals had kidneys exhibiting a highly variable amount of immunoreactivity for calbindin-D_{28k} in the medullary collecting ducts and papillary ducts (not shown). The absence of consistent immunolocalization in medullary collecting ducts and papillary ducts argued against quantifying the calbindin-D_{28k} found in these sites. Intestinal localization of calbindin-D_{9k} appeared to be confined to the absorptive cells. As seen in Fig. 2, there was a large decrease in the distal tubular cell-associated calbindin-D_{28k} immunoreactivity and in the absorptive cell-associated calbindin-D_{9k} immunoreactivity in the space flight (F) kidney and small intestine, as compared with matched ground control (FC) animals. Insulin was localized to relatively large numbers of cells making up the core of pancreatic islets. This result is consistent with the localization of insulin in β-cells of the rat. There was no consistent difference in pancreatic insulin immunoreactivity of space flight and control animals (not shown). No specific immunoreactivity for calbindin-D_{28k} was observed in the pancreatic islet cells of the flight or ground-based animals.

The results of quantitative image analysis of tissues from space flight, suspension, and control animals are summarized in Fig. 3. Comparison of groups was done as described above for ELISA. Reductions in the calbindin-D_{28k} level in the kidney and the calbindin-D_{9k} level in the small intestine were similar to those found with ELISA (*see* Fig. 3, left and central panels). Linear regression analysis of data obtained using ELISA and quantitative ICC showed a statistically significant correlation between two methods (Fig. 4). The insulin level in pancreas varied widely among animals within a group, and no apparent trend toward a decreased insulin immunoreactivity was revealed in space flight and suspension animals (*see* Fig. 3, right panel).

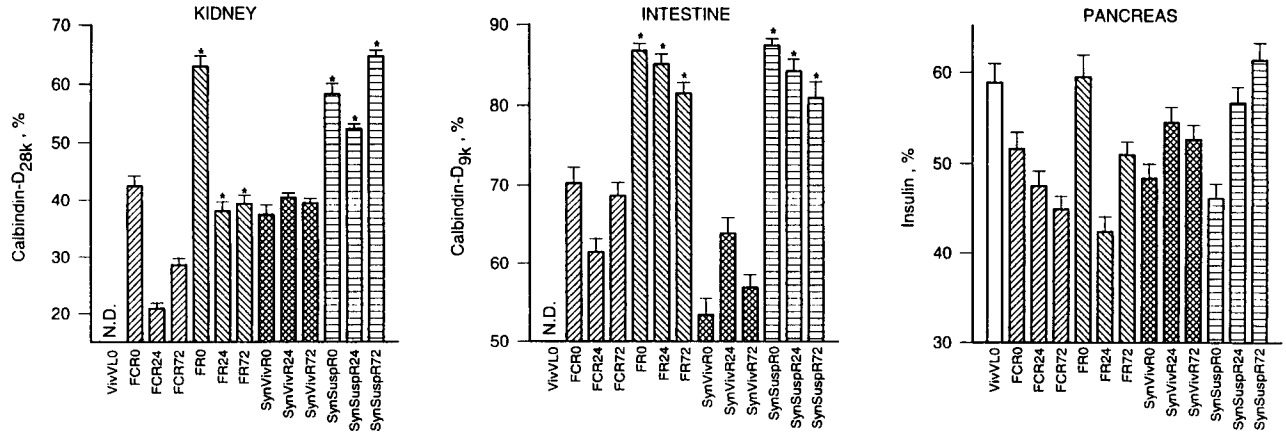

Fig. 3. Relative labeling intensity of calbindins in the kidney and small intestine, and of insulin in pancreas of space flight, tail suspension, and control animals. Calbindin-D_{28k} and calbindin-D_{9k} were quantified by ICC, as described in Materials and Methods. Note that higher percentages correspond to lower levels of calbindins and insulin (*see also* Materials and Methods). Group abbreviations are explained in the legend to Fig. 1. The data, analyzed by ANOVA, represent mean values ± SEM. Only significance of differences between F vs FC and SynSusp vs SynViv groups are presented. (*), $p < 0.05$; N.D., not determined.

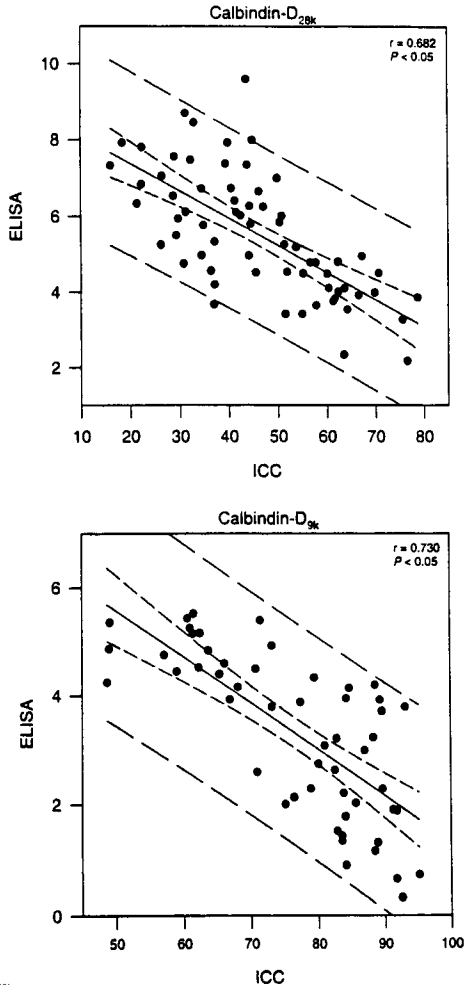

Fig. 4. Correlation between ELISA and immunocytochemical quantification of renal calbindin-D_{28k} and intestinal calbindin-D_{9k}. Linear regression analysis revealed for two methods a correlation coefficient of ≥0.7 and $p < 0.05$. A 95% confidence interval is shown by short dashed lines; long dashed lines fit values predicted by the regression model.

Discussion

Calcium metabolism and its regulation change promptly (within days) with exposure of the body to microgravity or simulated weightlessness (hypokinesia, suspension) (Spirichev and Sergeev, 1988; Arnaud et al., 1992). The vitamin D hormone, $1,25(OH)_2D_3$, is a critical component of the Ca^{2+}-regulating endocrine system (Norman et al., 1982, 1992). Vitamin D-dependent Ca^{2+}-binding proteins, calbindin-D_{28k} and calbindin-D_{9k}, play essential roles in the regulation of Ca^{2+} metabolism and maintenance of cellular Ca^{2+} homeostasis. Calbindins are involved in the intestinal Ca^{2+} absorption, renal Ca^{2+} reabsorption, and intracellular Ca^{2+} buffering (Christakos et al., 1989; Rhoten and Sergeev, 1994; Johnson and Kumar, 1994).

In this study, we have demonstrated, using two independent approaches, a significant decrease in the renal calbindin-D_{28k} and intestinal calbindin-D_{9k} content and immunocytochemical expression in rats exposed for 9 d to microgravity on board the space shuttle (Spacelab 3 mission). Importantly, tail suspension animals demonstrated virtually identical changes in calbindins. These findings indicate that factors of modeled weightlessness (suspension) can mimic the effects of microgravity on calbindins.

Because calbindins are vitamin D-regulated proteins and because vitamin D receptors do not seem to be not affected by the factors of space flight (Chaudhry, Sergeev, and Rhoten, unpublished observations), the decreased circulating concentration of the hormonal form of vitamin D, $1,25(OH)_2D_3$, might be primarily responsible for the reduction in calbindin contents and levels of expression in kidneys and intestine of flight and suspension animals. Decreased production of $1,25(OH)_2D_3$ in kidneys may determine, to a large extent, such a reduction in the serum $1,25(OH)_2D_3$ concentration. As we have shown earlier, this

is the case for hypokinetic model of weightlessness in rats (Sergeev et al., 1984; Spirichev and Sergeev, 1988).

The depression of calbindin-D_{28k} in the kidney and calbindin-D_{9k} in the small intestine appears to be relatively selective, because the insulin level in pancreas, evaluated by ICC, was not changed in space flight and suspension animals. It is also noteworthy that the decrease in calbindins was independent of the time of harvesting the tissues after re-entry (0, 24, or 72 h postflight). This suggests that the reduction in amounts of calbindins and gene expression for these Ca^{2+}-binding proteins is a long-lasting effect of microgravity and suspension *per se*, rather than a rapid, transient stress response.

Functional consequences of decreased calbindin expression at the organismal level might be the decreased absorption of Ca^{2+} in the small intestine and the increased excretion of Ca^{2+} in the urine. At the cellular level, the decreased expression of calbindins may lead to a sustained increase in the concentration of cytosolic free Ca^{2+}. Such an increase in free Ca^{2+} could interfere with Ca^{2+} signaling and biological responses, including disorders of Ca^{2+} metabolism induced by space flight.

Thus, our study implies that the reduced calbindin expression in kidneys and small intestine from rats exposed to microgravity and modeled weightlessness (tail suspension) may relate directly to changes in Ca^{2+} metabolism under these conditions. Changes in calbindins may be attributed to an interference of microgravity and suspension with functioning of the vitamin D-endocrine system. Future space and ground-based experiments are necessary to test this hypothesis and to determine the specific effects of calbindins at the cellular level.

Materials and Methods

Sample Handling

Male Sprague-Dawley rats (weighing ca. 150 g and aged 6 wk at launch) were flown for 9 d aboard the Spacelab 3 mission. On return to earth, the animals were dissected at 0, 24, and 72 h postflight (groups FR0, FR24, and FR72). Age- and sex-matched ground control animals were maintained in flight hardware where factors of the space flight, except microgravity, were synchronously reproduced (groups FCR0, FCR24, and FCR72). Tail suspension rats were used as a model that mimics some effects of microgravity (groups SynSuspR0, SynSuspR24, and SynSuspR72); corresponding controls for these animals were rats kept in a vivarium (SynVivR0, SynVivR24, and SynVivR72). Moreover, a preflight, basal control group (VivL0) was dissected at launch.

The organs (right kidney and washed upper portion, ca. 10 cm, of the small intestine) were snap-frozen in liquid nitrogen, stored at −70°C, and shipped on dry ice. Left kidney, duodenum, and pancreas were fixed in neutral buffered formalin.

Enzyme-Linked Immunosorbent Assay

Calbindin-D_{28k} and calbindin-D_{9k} in kidneys and calbindin-D_{9k} in intestine were measured by means of an ELISA, as described previously (Miller and Norman, 1983; Rhoten and Sergeev, 1994). Diluted cytosol aliquots (50 µL; 0.5 mg/mL total protein of kidney cytosol for calbindin-D_{28k}, 0.1 mg/mL total protein of kidney cytosol for calbindin-D_{9k}, and 0.1 mg/mL total protein of intestinal cytosol for calbindin-D_{9k}) were assayed in calbindin-D_{28k}-coated (10 ng/well) or calbindin-D_{9k}-coated (2.5 ng/well) multiwell flat-bottomed immunoassay plates (Falcon Pro-Bind; Fisher, Pittsburgh, PA). Chicken intestinal calbindin-D_{28k} was a gift from A. W. Norman (University of California-Riverside), and bovine intestinal calbindin-D_{9k} was purchased from Sigma (St. Louis, MO). Calbindin-coated plates were preblocked with 1 % bovine serum albumin, 0.5% Tween-20 in phosphate-buffered saline (PBS), and then incubated for 2 h at room temperature with unknown, standards (0–400 ng calbindin-D_{28k}/well or 0–12.5 ng calbindin-D_{9k}/well), and primary antibody (150 µL; mouse monoclonal anticalbindin-D_{28k}, clone CL-300, Sigma, 1: 140,000 dilution, or rabbit antiserum against calbindin-D_{9k}, 1:5000 dilution, a gift from M. E. Bruns, University of Virginia, Charlottesville). The washed plates were then incubated for 2 h with a secondary antibody (alkaline phosphatase-labeled goat antimouse or antirabbit IgG, 1:1500 dilution, Sigma). Substrate, *p*-nitrophenylphosphate (1 mg/mL in diethanolamine buffer, pH 9.8), was used to produce a chromogen, which was quantitated at 405 nm in the microplate reader (Molecular Devices Corp., Sunnyvale, CA). Protein concentration in cytosols was measured with a Bio-Rad detergent-compatible protein microassay (Bio-Rad Laboratories, Hercules, CA) using a microplate format.

Immunocytochemistry

Cellular localization and quantification of calbindin-D_{28k} in kidneys, calbindin-D_{9k} in duodenum, and insulin in pancreas were carried out on the formalin-fixed tissues, as described previously (Rhoten et al., 1985; Rhoten, 1987; Rhoten and Christakos, 1990). Fixed tissues were embedded in Paraplast (Monoject Scientific, St. Louis, MO). Microtome sections were affixed to Superfrost-Plus microscope slides (Fisher Scientific) and stored at room temperature until use. Paraffin was removed, and sections rehydrated. Slides were treated with 3% H_2O_2 in PBS for 10 min, rinsed in PBS, and then incubated overnight in a humid chamber at 4°C with primary antibody. The antibodies used were mouse anticalbindin-D_{28k}, clone CL-300, 1:200 dilution in Tris-buffered saline, pH 7.6, containing 2% normal goat serum, and 1% albumin (SA-TBS); rabbit antirat intestinal calbindin-D_{9k}, 1:400 dilution in SA-TBS; and guinea pig antiinsulin (Incstar, Stillwater, MN), 1:200 dilution in SA-TBS. Slides were then washed with SA-TBS, and primary antibodies detected using goat peroxidase-labeled

antimouse IgG, 1:100 dilution (Sigma); goat peroxidase-labeled antirabbit IgG, 1:100 dilution (Incstar); and rat peroxidase-labeled antiguinea pig IgG, 1:200 dilution (Sigma). Slides were incubated with secondary antibodies for 45 min at room temperature. Chromogen used was 3,3'-diaminobenzidine.

In situ levels of the calbindins and insulin were quantified on the basis of the intensity of the oxidized diaminobenzidine reaction product present in individual cells, using an Image-1 image acquisition, processing, and analysis system (Universal Imaging, West Chester, PA). Labeling intensity was measured on digitized images in arbitrary optical density units based on a 255 tone gray scale, where the value of 0 is completely black and 255 is completely white (transparent). The intensity value encompassed both the number of labeled cells and their individual brightness values, and was obtained by defining the outline of the cell cluster and determining the average brightness value over the entire area. The same defined area was placed on the image close to the measured positive (more dense) area to determine the level of background nonspecific staining. Data are expressed as relative labeling intensity in percent, i.e. (brightness of the positive area)/(brightness of the background area) × 100, so that lower numbers, i.e., greater density, correspond to higher levels of calbindins. For each stained slide, two to three fields of cells were captured and at least three areas of labeled cells were counted.

Statistics

Statistical analysis of the data was performed using Sigma Stat v. 1.0 software (Jandel Scientific, San Rafael, CA).

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