

Short Communication

Stimulation of Adrenal Chromaffin Cell Proliferation by Hypercalcemia Induced by Intravenous Infusion of Calcium Gluconate in Rats

Kaori Isobe^{1*}, Tsuneo Ito¹, Shun-ichiro Komatsu¹, Kentaro Asanuma¹, Etsuko Fujii¹, Chie Kato¹, Kenji Adachi¹, Atsuhiko Kato¹, Tetsuro Sugimoto¹, and Masami Suzuki¹

¹ Fuji-Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan

Abstract: Increased incidence of adrenal pheochromocytoma is frequently encountered in rat carcinogenicity studies. In some of the studies, the finding is judged to be due to a rat-specific mechanism of carcinogenesis caused by a disturbance of calcium homeostasis. However, direct evidence that the proliferation of chromaffin cells in the adrenal medulla is induced solely by hypercalcemia is not available. In this study, calcium gluconate was intravenously infused for 7 days to rat chromaffin cells by a tail cuff method, and cumulative labeling with bromodeoxyuridine (BrdU) was carried out to evaluate the proliferative activity. The serum calcium concentration was dose-dependently increased, and a high calcium concentration was stably sustained from day 2 to 7. In the adrenal medulla, BrdU-positive chromaffin cells increased in the calcium gluconate-treated animals, and the BrdU-labeling index increased in a dose-dependent manner. In addition, an increased BrdU-labeling index of chromaffin cells was shown to correlate with the serum calcium concentration. Our results demonstrate that hypercalcemia directly enhances the proliferative activity of chromaffin cells and that the proliferative activity is correlated with the serum calcium concentration. (DOI: 10.1293/tox.25.281; J Toxicol Pathol 2012; 25: 281–285)

Key words: cell proliferation activity, adrenal glands, chromaffin cell, hypercalcemia, rat, bromodeoxyuridine

Rodent carcinogenicity studies are widely conducted as bioassays for human safety assessment. Treatment-related increases in the incidence of tumors are often observed in such studies. A large number of compounds induce pheochromocytoma originating from chromaffin cells of the adrenal medulla in rodent carcinogenicity studies. Such compounds include a wide range of chemicals such as metal compounds, hydrocarbons, amines, dyestuffs, pesticides and pharmaceuticals¹. Mechanisms that cause pheochromocytoma in experimental animals include uncoupling of mitochondrial respiration, hypoxia, disturbance of the hypothalamic endocrine axis and disturbance of calcium homeostasis¹.

When assessing the risk of carcinogenicity, it must be taken into consideration that positive results in a rodent carcinogenicity study cannot always be extrapolated to humans. Indeed, several tumors observed in rodent carcinogenicity studies, such as carcinoid tumors of the stomach induced by prolonged suppression of acid secretion, thyroid follicular cell tumors associated with enzyme induction and

kidney tumors related to $\alpha_2\mu$ -globulin nephropathy, show no carcinogenic risk in humans². Among them, adrenal pheochromocytoma is commonly enhanced in rodent carcinogenicity studies when triggered by disturbance of calcium homeostasis, and its tumorigenesis has been concluded to have no carcinogenic risk in human^{1, 3–5}. For instance, high doses of slowly or poorly absorbed sugars or sugar alcohols cause disturbance of calcium homeostasis by increasing calcium absorption from the small intestine in rats and thereby induces adrenal pheochromocytoma³. Vitamin D and Vitamin D-derivative compounds also cause adrenal pheochromocytomas in rats due to disturbance of calcium homeostasis^{4, 5}. The positive results in these carcinogenicity studies are judged to be caused by rat-specific mechanisms of carcinogenesis activated by a non-genotoxic mechanism. Although disturbance of calcium homeostasis is strongly indicated to be a promotive cause of adrenal pheochromocytoma, compounds that cause the increase of this tumor have various biological functions other than their influence on calcium homeostasis, and direct evidence that proliferation of chromaffin cells in the adrenal medulla is induced solely by hypercalcemia is not available as far as we know.

We therefore induced a disturbance in calcium homeostasis by hypercalcemia in rats without using chemical compounds that are known to induce pheochromocytoma in rodent carcinogenicity studies, and examined the proliferative activity of chromaffin cells in the adrenal medulla. A sustained high serum calcium concentration was induced in

Received: 10 April 2012, Accepted: 5 July 2012

*Corresponding author: K Isobe (e-mail: isobe.kaori@chugai-pharm.co.jp)

©2012 The Japanese Society of Toxicologic Pathology

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/3.0/>>.

rats by intravenous infusion of calcium gluconate (GACa) for 7 days, and 5-bromo-2'-deoxyuridine (BrdU) cumulative labeling was carried out to evaluate the proliferative activity of chromaffin cells in the adrenal medulla. All animal experiments were approved by the Ethical Committee for Treatment of Laboratory Animals at Chugai Pharmaceutical Co., Ltd.

In this study, 21 eight-week-old male Sprague-Dawley rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in cages in an animal room maintained at a temperature of $24 \pm 2^\circ\text{C}$ and a humidity of $55 \pm 10\%$, with 14 to 16 air changes per hour and a 14-hour light and 10-hour dark cycle, and were fed pelleted chow (CE-2; Clea Japan, Inc., Tokyo, Japan) and tap water *ad libitum*.

At 9 weeks of age, a polyurethane catheter (0.6-mm ID \times 0.9-mm OD, Access Technologies, Skokie, IL, USA) was inserted from the femoral vein to the posterior vena cava under anesthesia by a tail cuff method. The swivel was connected via polyethylene PE-50 tubing (Becton, Dickinson and Company, Sparks, MD, USA) with a 23-gauge needle to a 0.22 μm filter (Millipore Japan Co., Ltd., Tokyo, Japan), which was connected to a 50-ml syringe. The detailed procedures for preparing a rat model for intravenous infusion were reported by Asanuma *et al.*⁶ After surgery, the rats were infused with saline at 1.0 ml/head/hour until the start of this study at 11 weeks of age.

In order to set the dose levels of GACa, we carried out a preliminary study. In the preliminary study, all animals infused at dose levels of 60 mg/ml (5,760 mg/kg/day) were found dead or were sacrificed in moribund condition from 1 to 3 days after the start of administration of GACa. At dose levels of 10 mg/ml (960 mg/kg/day), a high serum calcium concentration was not sustained. On the other hand, rats infused at dose levels of 20 mg/ml (1,920 mg/kg/day) and 40 mg/ml (3,840 mg/kg/day) showed a stable increase in serum calcium concentration, which was sustained for a period of 7 days. On the basis of these preliminary results, dose levels of 20 mg/ml (1,920 mg/kg/day) and 40 mg/ml (3,840 mg/kg/day) were selected.

In this study, 14 rats were divided into 2 treatment groups ($n=7$) and subjected to administration of GACa (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at dose levels of either 20 mg/ml (1,920 mg/kg/day) or 40 mg/ml (3,840 mg/kg/day). Each dose of GACa was prepared using saline as a vehicle. The remaining 7 rats were used as the control group and administered saline. All rats were infused with GACa or saline at 4 ml/kg/hour for a period of 7 days.

The proliferative activity of chromaffin cells in the adrenal glands was determined by cumulative labeling with BrdU. BrdU labeling was carried out according to the method described by Suzuki *et al.*⁷ At day 0 (first dosing day), mini-osmotic pumps (Alza Co., Palo Alto, CA, USA) filled with BrdU (Sigma Chemical Co., St. Louis, MO, USA) were implanted into the peritoneal cavity in rats under anesthesia, and the rats were then injected with 240 μg of BrdU per hour for 7 days before sacrifice.

All animals were weighed on days 0 (before dosing), 1, 3 and 7. During the infusion period, clinical signs of all animals were observed every day. Blood was taken from the jugular vein to examine the serum calcium concentration on days 0 (before dosing), 1, 2, 3 and 6. Approximately 24 hours of cumulative pooled urine was collected to measure urine volume and urinary calcium excretion. Urinary samples were collected on days 2 and 6. Measurement of the serum calcium concentration and urinary calcium excretion was determined by the OCPC method.

Animals were sacrificed by exsanguination from the abdominal artery under anesthesia. At necropsy, the adrenal glands and kidneys were removed from each animal, and the adrenal glands were weighed. The specimens were fixed in 10% neutral-buffered formalin for 24 hours. After fixation, the tissues were embedded into paraffin according to the standard method. Thin sections from the paraffin blocks were prepared and used for HE and immunohistochemical staining.

BrdU-incorporated nuclei were immunohistochemically identified using anti-BrdU antibody as described previously⁷. Immunohistochemical staining was performed according to the LSAB method (Dako LSAB kit, Dako, Carpinteria, CA, USA) using an anti-BrdU monoclonal antibody (Amersham, Bucks, UK). The immunoreaction was visualized by a peroxidase-diaminobenzidine reaction. The sections were finally counterstained lightly with hematoxylin. To evaluate the proliferative activity of chromaffin cells in the adrenal glands, analysis was conducted on 500 cells for each animal. The BrdU labeling index was determined by the following formula: (BrdU-positive nuclei count) \div 500 (nuclei count) \times 100.

Group mean values and standard deviations were calculated for body weight, serum calcium concentration, urinary calcium excretion, adrenal glands weight and BrdU labeling index. These data were analyzed for statistical significance of any differences between the control group and each drug-treated group using the Student's or Aspin-Welch's *t*-test at concentrations of 1% and 5%. To analyze the correlation between serum calcium concentration and BrdU-labeling index, the average calcium concentration of each animal on days 2 and 6 was calculated, and then the significance of the correlation value between the 2 parameters was tested using the Pearson's correlation coefficient test with the Mini StatMate software (ATMS Co., Ltd., Tokyo, Japan). Difference with a P value less than 0.05 were considered to be significant.

The serum calcium concentration was dose-dependently increased on day 1, and a high and stable calcium concentration was sustained from day 2 to the end of the administration period in the GACa-treated groups (Table 1). Urinary calcium excretion was dose-dependently increased on both days 2 and 6 in the GACa-treated groups (Table 1). It is well known that maintaining hypercalcemia by bolus administration of calcium solutions is difficult because of the rapid excretion of calcium in urine. In this study, however, we intravenously infused GACa to rats by a tail cuff

Table 1. Changes in Serum Calcium Concentration and Urinary Calcium Excretion

	Dose groups (mg/kg/day)	Days of infusion				
		0	1	2	3	6
Serum calcium concentration (mg/ml)	0	10.52 ± 0.26	10.48 ± 0.25	10.65 ± 0.29	10.48 ± 0.26	10.53 ± 0.15
	1,920	10.56 ± 0.17	11.07 ± 0.25**	12.38 ± 0.42**	11.77 ± 0.33**	12.55 ± 0.80**
	3,840	10.54 ± 0.21	12.66 ± 0.37**	15.23 ± 1.17**	14.70 ± 1.00**	15.26 ± 1.51**
Urinary calcium excretion (mg/day)	0	NE	NE	0.79 ± 0.24	NE	1.34 ± 0.62
	1,920	NE	NE	10.37 ± 1.62**	NE	9.73 ± 2.29**
	3,840	NE	NE	14.22 ± 2.94**	NE	12.78 ± 4.37**

Values are presented as the mean ± standard deviation. Significantly different from the control (** P<0.01; Student's or Aspin-Welch's *t*-test). Each group is composed of 7 rats. NE, not examined.

Table 2. Body Weight and Adrenal Glands Weight

Dose groups (mg/kg/day)	Body weight (g)		Adrenal glands weights (mg)
	Initial	Final	
0	362.1 ± 12.5	377.0 ± 15.4	61.9 ± 4.4
1,920	365.5 ± 36.3	379.6 ± 37.5	63.8 ± 3.1
3,840	360.4 ± 17.4	359.1 ± 27.7	83.5 ± 22.2*

Values are presented as the mean ± standard deviation. Significantly different from the control (* P<0.05; Student's or Aspin-Welch's *t*-test). Each groups is composed of 7 rats.

method, successfully induced a dose-dependent increase in the serum concentration and urinary excretion of calcium and were able to sustain a high and stable serum calcium level during the administration period.

No deaths or treatment-related clinical signs were observed in any dose group. There was suppression of body weight gain in the 3,840-mg/kg/day group from day 1 that continued throughout the study (Table 2).

At necropsy, enlargement of the adrenal glands and white foci of the kidneys were seen in 2/7 rats in the 3,840-mg/kg/day group. No macroscopic abnormality was observed in the 1,920-mg/kg/day group. In the kidney, the histopathological changes in the 3,840-mg/kg/day group were characterized by slight nephrocalcinosis, which coincided with the white foci observed at necropsy. No histopathological findings were observed in the 1,920-mg/kg/day group. In the adrenal glands, organ weight was significantly increased in the 3,840-mg/kg/day group in addition to the enlargement observed at necropsy. Histopathologically, slight hypertrophy of cortical cells was observed in the 3,840-mg/kg/day group. In addition, BrdU-positive cortical cells also slightly increased. These findings were considered to coincide with an increased adrenal gland weight (Table 2). There were no histopathological findings in the adrenal medulla in any dose group.

In summary, the influence on the rats of infusion of GACa for 7 days was characterized by sustained hypercalcemia with a slight suppression of body weight, slight mineralization in the kidneys and no marked deterioration of clinical signs. Therefore, we considered that the changes in the adrenal cortex were appropriately evaluated in this study without the interference of secondary effects such as marked deterioration of general condition. In addition, it was indicated that intravenous infusion of calcium prepara-

tions to rats by a tail cuff method is highly useful and valuable for inducing sustained hypercalcemia and evaluating the influence of that state on the organs and tissues in rats.

In the adrenal medulla, BrdU-positive nuclei were found in the chromaffin cells and endothelial cells. BrdU-positive chromaffin cells were sporadically localized in the medulla of both the control and GACa-treated groups, and an increase in BrdU positivity was clearly identified in the GACa-treated groups. On the other hand, BrdU-positivity of endothelial cells was smaller (approximately 5%) compared to that of chromaffin cells in the control group, and the increase in BrdU-positive endothelial cells was histopathologically marginal in the GACa-treated groups. Therefore, morphometric analysis using BrdU labeling was conducted for chromaffin cells in this study. The number of BrdU-positive chromaffin cells increased in the animals of the GACa-treated groups compared with the animals of the control group (Fig. 1A). The BrdU labeling index was significantly and dose-dependently increased in the GACa-treated groups (Fig. 1B). These results indicated proliferation of chromaffin cells in the GACa-treated groups, although there were no histopathological findings in the adrenal medulla in any dose group. We considered that this was due to the short term of this study. Moreover, cumulative labeling with BrdU enables the observation of any cell that passes through the S-phase and consequently might increase the sensitivity of detection of cell proliferation compared with histopathological evaluation.

A significant correlation was noted between the serum calcium concentration and BrdU labeling index of chromaffin cells (Fig. 2). In this study, sustained hypercalcemia induced by intravenous infusion of GACa was demonstrated to increase the proliferative activity of chromaffin cells in the adrenal medulla. Moreover, the proliferative activity

In conclusion, GACa was intravenously infused into rats by a tail cuff method, and a high serum calcium concentration was successfully sustained. The results of this study indicate that hypercalcemia directly stimulates the proliferation of chromaffin cells in the adrenal medulla of rats. Additionally, the proliferative activity of chromaffin cells is correlated with of serum calcium concentration. Our results will be highly useful in assessing adrenal toxicity, especially for interpreting the increased incidence of adrenal pheochromocytoma in rat carcinogenicity studies.

Acknowledgments: We would like to thank Ms. Yayoi Takai at Chugai Research Institute for Medical Science, Inc. for technical assistance.

References

1. Greim H, Hartwig A, Reuter U, Richter-Reichhelm HB, and Thielmann HW. Chemically induced pheochromocytomas in rats: mechanisms and relevance for human risk assessment. *Crit Rev Toxicol.* **39**: 695–718. 2009. [[Medline](#)] [[CrossRef](#)]
2. Alison RH, Capen CC, and Prentice DE. Neoplastic lesions of questionable significance to humans. *Toxicol Pathol.* **22**: 179–186. 1994. [[Medline](#)] [[CrossRef](#)]
3. Tischler AS, Powers JF, Downing JC, Riseberg JC, Shahsavari M, Ziar J, and McClain RM. Vitamin D₃, lactose, and xylitol stimulate chromaffin cell proliferation in the rat adrenal medulla. *Toxicol Appl Pharmacol.* **140**: 115–123. 1996. [[Medline](#)] [[CrossRef](#)]
4. Ikezaki S, Nishikawa A, Furukawa F, Tanakamaru Z, Nakamura H, Mori H, and Hirose M. Influences of long-term administration of 24R, 25-dihydroxyvitamin D₃, a vitamin D₃ derivative, in rats. *J Toxicol Sci.* **24**: 133–139. 1999. [[Medline](#)] [[CrossRef](#)]
5. Tischler AS, Powers JF, Pignatello M, Tsokas P, Downing JC, and McClain RM. Vitamin D₃-induced proliferative lesions in the rat adrenal medulla. *Toxicol Sci.* **51**: 9–18. 1999. [[Medline](#)] [[CrossRef](#)]
6. Asanuma K, Komatsu S, Sakurai T, Takai R, and Chiba S. Total parenteral nutrition using continuous intravenous infusion via the posterior vena cava in rats. *J Toxicol Sci.* **31**: 139–147. 2006. [[Medline](#)] [[CrossRef](#)]
7. Suzuki M, Adachi K, Sugimoto T, Nakayama H, and Doi K. Application of flash and cumulative labeling with bromodeoxyuridine for analysis of cell kinetics of bone cells and chondrocytes in young growing rats. *J Toxicol Pathol.* **11**: 65–68. 1998. [[CrossRef](#)]
8. Pitot HC, and Dragan YP. Chemical carcinogenesis. In: Casarett & Doull's Toxicology, 6th ed. Klaassen CD (ed). McGraw-Hill, New York. 241-319. 2001.
9. Baer A. Sugars and adrenomedullary proliferative lesions: the effects of lactose and various polyalcohols. *J Am Coll Toxicol.* **7**: 71–81. 1988. [[CrossRef](#)]
10. Lynch BS, Tischler AS, Capen C, Munro IC, McGirr LM, and McClain RM. Low digestible carbohydrates (polyols and lactose): significance of adrenal medullary proliferative lesions in the rat. *Regul Toxicol Pharmacol.* **23**: 256–297. 1996. [[Medline](#)] [[CrossRef](#)]