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High Calcium in the Diet and Vitamin D Inhibit a Bone Matrix Degrading Enzyme β-Glucuronidase Activity

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Abstract

A lysosomal and bone matrix degrading enzyme β-glucuronidase activity was measured in BALB/c mice fed high, medium and low Ca in combination with the i.p. administration of calcium-regulating hormones including parathyroid hormone(PTH), calcitonin(CT) and cholecalciferol(Vit D). After feeding experimental diets for five weeks, mice were sacrificed by cervical dislocation and the enzyme was fluometrically measured at 440nm. β-Glucuronidase activity was inhibited by high calcium in the diet. In addition, vitamin D also inhibited the enzyme activity in the serum regardless of the level of dietary calcium. In contrast, PTH has shown to stimulate the enzyme at all the levels of dietary calcium. Calcitonin, an inhibitor of PTH action for bone resorption, revealed to curb PTH effect on this enzyme, whereas CT stimulated the action of vitamin D in the serum. The above results led us to conclude that osteoclastic bone resorption and senile osteoporosis may be reduced by adequate dietary calcium and vitamin D.

Key words: calcium, β-glucuronidase, parathyroid hormone, vitamin D

INTRODUCTION

β-Glucuronidase(GUS) is a heat-stable and matrix degrading enzyme such as collagenase, gelatinase and elastase. β-Glucuronidase enzyme activity corresponds to the action of osteoclast which acts as a receptor to PTH hormone. PTH is known to stimulate bone resorption(1-3) through the action of osteoclast. This hormone is also reported to stimulate the process of proliferation from osteoprogenitor cell to osteoclast(4,5).

Osteoporosis, a degenerative bone disease, is constantly increasing as human life expectancy is extended. In the elderly, PTH hormone is enhanced for the mobilization of minerals from bone matrix, consequently leading to osteoporosis. At the same time, GUS activity is also increased as PTH hormone level is up. GUS serves as a marker for osteoclastic activity and is responsible for resortive action in the bone. While most of the studies had focused on the catabolic effect of PTH, several reports(6-10) indicate possible anabolic effects of PTH in bone metabolism.

As an essential nutrient, acting like a hormone, vitamin D involves in the maintenance of plasma calcium balance and bone mineralization. And the synthesis of vitamin D and its function is facilitated by PTH hormone. The role of PTH hormone in GUS related to the dietary Ca level and other hormonal status has not been elucidated clearly.

This study has examined the *in vivo* effects of PTH, vitamin D and calcitonin hormones and dietary Ca levels on GUS bone enzyme related to bone resorption and senile osteoporosis.

MATERIALS AND METHODS

Experimental animals and diet compositions

Three months-old BALB/C mice were maintained in a 12 : 12 dark and light cycled room. Before the start of the experiment animals were fed chow diet and then divided into 3 dietary groups of high and medium Ca and Ca-free. Diet groups and the experimental diet composition is shown in Table 1.

Experimental design and hormonal treatment

Animals were divided into 18 groups according to

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Table 1. Compositions of experimental diet

	High Ca group[1]	Medium Ca group	Ca-free group
Casein[2]	14%	14%	14%
Fat(corn oil)	5%	5%	5%
Corn starch	70.32%	70.82%	70.82%
DL-Methionine	0.18%	0.18%	0.18%
Fiber[3]	5%	5%	5%
Vitamin[4]	1%	1%	1%
Mineral[5]			
Mineral mixture	4%	4%	-
Calcium-free	-	-	4%
$CaCO_3$	0.5%	-	-
Total	100%	100%	100%

[1]Total Ca contents of high, medium, and Ca-free diet were 1.09, 0.59 and 0% respectively
[2]Casein: Purified high nitrogen casein(ICN)
[3]Fiber: Aalphacel(non nutritive bulk, ICN)
[4]Vitamin: AIN vitamin mixture 76(ICN)
[5]Mineral: AIN mineral mixture 76(ICN) for high and medium Ca group and calcium-free salt mixture for Ca-free group

diet and hormones. Hormones including PTH(1-34, bovine, Sigma), calcitonin(CT, from salmon, Sigma), and cholecalciferol(Vit D, Sigma) were injected intraperitoneally in physiological levels and the initial solution was prepared at 0.83μg/ml, 41.67μg/ml, 83mg/ml, respectively. 100μl of PTH and 25μl of vitamin D was injected in the first week and to avoid ethanol overdose half-dose of vitamin D was injected in the first and second week. 100μl of CT was injected at the third week of experimental diet feeding. The injection of these hormones were performed a week after the feeding of experimental diet and animals were sacrificed in the week 5(Table 2).

Table 2. Total design of the experiment[1]

	I.P. injections[2]	Diet
Control H	Saline	High calcium
Control M	Saline	Medium calcium
Control L	Saline	Calcium-free
PTH H	PTH	High calcium
PTH M	PTH	Medium calcium
PTH L	PTH	Calcium-free
VD_3 H	Cholecalciferol	High calcium
VD_3 M	Cholecalciferol	Medium calcium
VD_3 L	Cholecalciferol	Calcium-free
PTH+CT H	PTH & Calcitonin	High calcium
PTH+CT M	PTH & Calcitonin	Medium calcium
PTH+CT L	PTH & Calcitonin	Calcium-free
VD_3+CT H	Cholecalciferol & CT	High calcium
VD_3+CT M	Cholecalciferol & CT	Medium calcium
VD_3+CT L	Cholecalciferol & CT	Calcium-free
ALL H	PTH & Cholecalciferol & CT	High calcium
ALL M	PTH & Cholecalciferol & CT	Medium calcium
ALL L	PTH & Cholecalciferol & CT	Calcium-free

[1]An independent group of calcitonin(CT) was not established in this experimental design as it is a known inhibitor of bone resorption acting coupled with PTH or Vit D rather than acting independently
[2]Hormones were I.P. injected during the experimental period as indicated above

Tissue and sample preparation

Animals were fasted for 12 hours and weighed before sacrificed by ether. Blood was taken by cardiac puncture, organs including liver and kidney were taken, weighed and rinsed by physiological saline.

Serum was obtained by centrifugation of blood at 2000RPM(Microspin 24S, Sorvall instruments) for 20 minutes and 1g of liver was homogenized with sucrose buffer and centrifuged at 2000RPM for 15 minutes, pellets were discarded and supernatants were obtained and kept frozen at -70°C until assay.

Measurement of β-glucuronidase enzyme activity

1.0mM of 4-methylumbelliferone-β-D-glucuronide was dissolved in β-glucuronidase assay buffer containing 0.05% BSA and 0.08M sodium acetate(pH 3.75). 10μl of sample and 50μl of assay buffer were mixed in a test tube sealed with parafilm and reacted for an hour at 37°C water bath then 2ml of termination buffer, 0.02M 2-amino-2-methyl-1-propanol was added and stopped the reaction. Emission was measured at 440nm(scanning in the range of 400~480nm) using fluorometer(KONTRON, SFM 25) with excitation at 365nm. Serum and liver specimen were diluted by 9 and 50 times before assay, respectively. 2mM 4-methylumbelliferone(sodium salt, Sigma) served as a standard(11) for the assay.

Protein measurement

Protein was determined by the method of Bradford(12) using Coomassie Brillant Blue G-250 and bovine serum albumin as a standard. Supernatants were diluted with deionized distilled water. 3ml of Coomassie solution was added to each 100μl of sample. Absorption was measured at 595nm(Kontron Instruments, Uvikon Spec.).

Statistical analysis

Data from individual experiment were expressed as

Table 3. β-Glucuronidase activity in mouse serum (unit: mmole/mg pro.)

Hormone	High Ca	Medium Ca	Ca-free	Average
Control	$7.57\pm0.245^{1)bcA}$	8.40 ± 0.171	8.86 ± 0.435^{bB}	8.28^{c}
PTH	9.76 ± 1.194^{ab}	9.73 ± 1.397	11.16 ± 0.883^{a}	10.18^{a}
VD_3	6.93 ± 0.323^{cA}	8.29 ± 0.555	8.55 ± 0.543^{bB}	7.81^{c}
PTH+CT	10.59 ± 1.166^{a}	7.95 ± 0.667	8.57 ± 0.523^{b}	9.04^{abc}
VD_3+CT	7.51 ± 0.999^{bcA}	9.41 ± 0.337	11.76 ± 0.923^{aB}	9.73^{ab}
PTH+VD_3+CT	7.85 ± 0.528^{bc}	8.90 ± 0.382	8.71 ± 0.553^{b}	8.45^{bc}
Average	8.37*	8.80	9.69**	

1)Mean±S.E.M.(Standard error of mean)

a,b,cValues within the same column with different superscripts are significantly different($p<0.05$) among groups by Duncan's multiple range test

A,BValues within the same row with different superscripts are significantly different($p<0.05$) among groups by Duncan's multiple range test

Values with no statistical significance among groups were not indicated

the means±standard error of means(SEM). Statistical analysis was performed by SAS(statistical analysis system) and Duncan's multivariate analysis.

RESULTS

Effects of dietary calcium

Calcium in the diet showed a statistically significant effect on the activity of GUS both in the serum and liver. GUS activity was stimulated by low calcium rather than high calcium. As shown in Table 3, the overall increase of serum GUS activity at medium and low Ca group was 5.2% and 16.0%, respectively compared to high calcium group. Likewise, the overall increase of liver GUS activity at medium and low Ca group was 12.2% and 17.6%, respectively compared to high Ca group.

Effects of PTH hormone

PTH has shown to stimulate the enzyme activity at all the levels of dietary calcium-high, medium and calcium-free. The average increase of the enzyme activity in the serum of PTH group over the control was 23%(Table 3). Particularly when dietary calcium supply was sufficient PTH together with CT stimulated GUS activity even more in the serum. On the other hand, when dietary Ca supply was not sufficient enough the combination of PTH and CT did not stimulate the enzyme activity in the serum. Liver GUS activity was enhanced only when high dietary Ca was supplied.

The overall increase of serum GUS at high and medium Ca and calcium-free group showed a constant trend with more than 15% of increment(Fig. 1), whereas liver GUS was constantly inhibited by PTH when

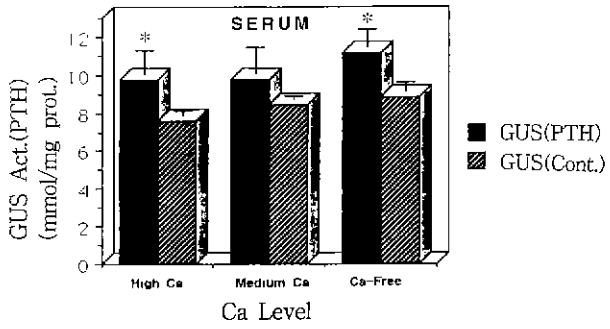

Fig. 1. Stimulation of GUS activity in mice serum by PTH at all levels of dietary Ca.
*Indicates that values are significantly different from the control at $p<0.05$. Data are means±SEM.

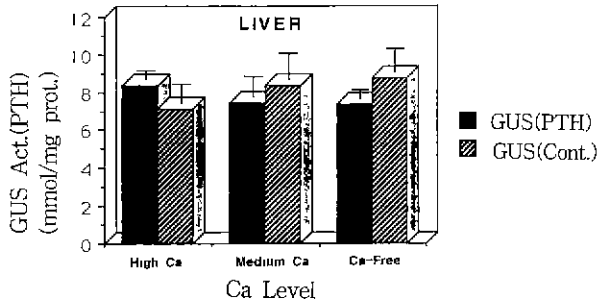

Fig. 2. GUS activity in mice liver was enhanced by PTH only in the presence of high dietary Ca.
Data are means±SEM.
Values are not significantly different from the control at $p<0.05$.

Ca-free or medium Ca was supplied(Fig. 2). With high dietary Ca, the enzyme activity both in the serum and liver was enhanced by PTH.

Effects of vitamin D

Vitamin D inhibited GUS activity at all the dietary

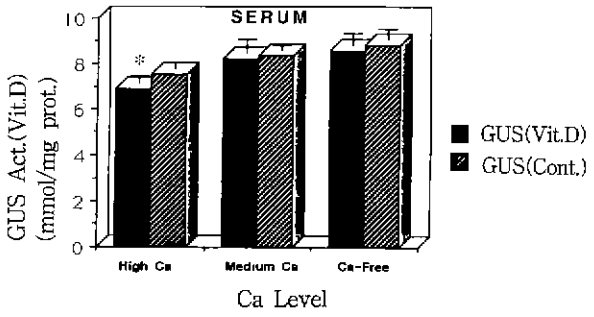

Fig. 3. Inhibition of GUS activity in mice serum by vitamin D at all levels of dietary Ca.
*Indicates that values are significantly different from the control at $p<0.05$. Data are means±SEM.

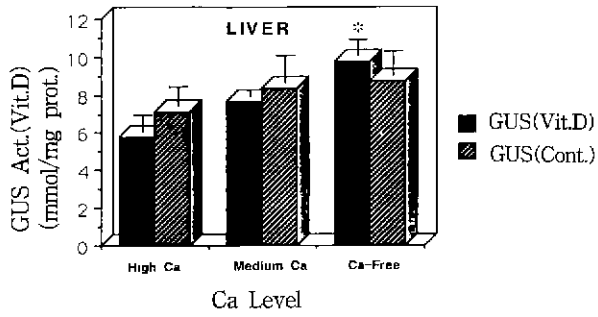

Fig. 4. GUS activity in mice liver was inhibited by Vit D only in the presence of calcium-free diet.
*Indicates that values are significantly different from the control at $p<0.05$. Data are means±SEM.

levels of Ca, particularly more in the presence of high dietary Ca. As shown in Fig. 3, T/C ratio of serum GUS activity in high, medium Ca and Ca-free group was 0.92, 0.99, and 0.97, respectively.

Also the liver GUS T/C ratio(Fig. 4) in high, medium Ca and Ca-free group was 0.81, 0.91 and 1.11, respectively.

Effects of calcitonin

As shown in Table 3 and 4, CT revealed to curb PTH effect on the enzyme in the serum when dietary Ca was not adequate. CT along with PTH stimulated this enzyme activity by 40% in the serum in the presence of high Ca. On the contrary, CT along with vitamin D significantly stimulated serum GUS at low dietary Ca, whereas the enzyme was inhibited in the liver. The combination of PTH, Vit D and CT did neither stimulate nor inhibit this enzyme regardless of the level of Ca supply.

Protein synthesis

Although a slight increase of protein synthesis was observed in PTH and Vit D treated liver tissue there was no statistical differences between dietary and hormonal groups(Table 5).

DISCUSSION

An *in vitro* study has revealed an inverse correlation between osteoclastic GUS enzyme activity and osteoblastic collagen synthesis(13,14). *In vivo* studies have also found elevated GUS enzyme activity in gingiva and skin of diabetic Sprague Dawley rats(15) and in the liver and heart of bone marrow transplanted patients (16), suggesting that active bone resorption is occuring through the action of the osteoclastic enzyme.

In this study, statistically significant inhibition of GUS in the presence of high Ca was observed both in the serum and liver. The inhibition of lysosomal GUS activity in the presence of high Ca rather than low Ca indicates the existence of matrix mineral protection mechanism when dietary Ca supply was sufficient.

The average increase of the enzyme activity in the serum of PTH group over the control was 23%. Particularly when dietary calcium supply was sufficient PTH

Table 4. β-Glucuronidase activity in mouse liver (unit: mmole/mg pro.)

Hormone	High Ca	Medium Ca	Ca-free	Average
Control	7.13±0.986[1)]	8.38±1.371	8.77±1.204^{ab}	8.09^{ab}
PTH	8.33±0.523	7.44±1.041	7.33±0.503^{ab}	7.68^{ab}
VD_3	5.80±0.880^{A}	7.65±0.315	9.72±0.860^{aB}	7.73^{ab}
PTH+CT	6.83±0.909^{A}	9.64±0.445^{B}	10.29±1.311^{aB}	8.98^{a}
VD_3+CT	7.39±0.619	6.53±0.729	6.06±0.636^{b}	6.66^{b}
PTH+VD_3+CT	7.27±0.982	7.76±0.917	7.97±1.019^{ab}	7.70^{ab}
Average	7.12*	7.99	8.37**	

[1)]Mean±S.E.M.(Standard error of mean)
^{a,b,c}Values within the same column with different superscripts are significantly different among groups at $p<0.05$
^{A,B}Values within the same row with different superscripts are significantly different among groups at $p<0.05$
Values with no statistical significance among groups were not indicated

Table 5. Protein contents in mouse serum and liver

	Hormone	High Ca	Medium Ca	Ca-free	Average
					(mg/100μl)
Serum	Control	3.97±0.133[1)]	3.86±0.159	4.27±0.589	4.03[NS]
	PTH	3.97±0.064	3.59±0.325	3.76±0.093	3.89
	VD_3	3.84±0.190	4.20±0.471	3.93±0.125	3.97
	PTH+CT	3.71±0.153	4.23±0.379	3.49±0.122	3.81
	VD_3+CT	4.13±0.215	4.88±0.609	4.02±0.173	3.95
	PTH+VD_3+CT	3.55±0.183	4.28±0.376	3.60±0.123	3.85
	Average	3.88[NS]	4.16	3.83	
					(mg/g liver)
Liver	Control	77.76±4.175[1)]	85.92±9.588	88.40±12.318	84.04[NS]
	PTH	85.52±5.145	80.32±8.650	84.96±8.192	83.60
	VD_3	86.40±10.378	84.53±6.468	89.36±4.640	87.12
	PTH+CT	86.32±6.228	86.72±8.805	91.28±10.944	88.12
	VD_3+CT	92.00±7.416	83.10±2.763	95.84±8.370	90.84
	PTH+VD_3+CT	95.00±9.241	83.00±7.846	93.20±8.297	90.60
	Average	86.88[NS]	83.96	90.52	

[1)]Mean±S.E.M.(Standard error of mean)
[NS]Not significant among groups at $p<0.05$ level by Duncan's multiple range test
Values with no statistical significance among groups were not indicated

together with CT stimulated GUS activity even more in the serum. In the previous study(17), PTH has also enhanced alkaline phosphatase(AP) activity by average 26% in the serum. The stimulation of both lysosomal GUS and bone forming AP enzymes by PTH hormone (18-20) suggests that PTH hormone is not only involved in bone resorption but also contributes to bone forming process.

Calcitonin, an inhibitor of PTH action for bone resorption, revealed to curb PTH effect on this enzyme whereas it stimulated the action of vitamin D in the serum. As shown in the results when CT was administered with PTH, GUS enzyme activity was stimulated by 40% in the serum in the presence of high dietary Ca. On the contrary, CT along with vitamin D significantly(33%) stimulated serum GUS at low dietary Ca. This results indicate that when dietary calcium is not sufficient enough Vit D may increase GUS activity in the blood to facilitate osteoclastic action and eventually increasing blood Ca levels.

The fact that the combination of PTH, vitamin D and CT did neither stimulate nor inhibit this enzyme regardless of dietary Ca level indicates the existence of *in vivo* cooperation in the control of the enzyme. Further, the balance of the enzyme can protect the bone from excessive resorptive action for the mobilization of matrix minerals possibly leading to type I or II ostoeporosis(21-25). The *in vivo* control mechanism among PTH, vitamin D and CT in accordance with dietary mineral regimen may be an essential biological function for the maintenance of bone mineral density(26).

In conclusion, the observation that inhibition of serum β-glucuronidase activity by high dietary calcium and vitamin D regardless of the level of dietary calcium and the stimulation of the enzyme by PTH at all the levels of dietry calcium led us to conclude that osteoclastic bone resorption and senile osteoporosis may be reduced by adequate dietary calcium and vitamin D.

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