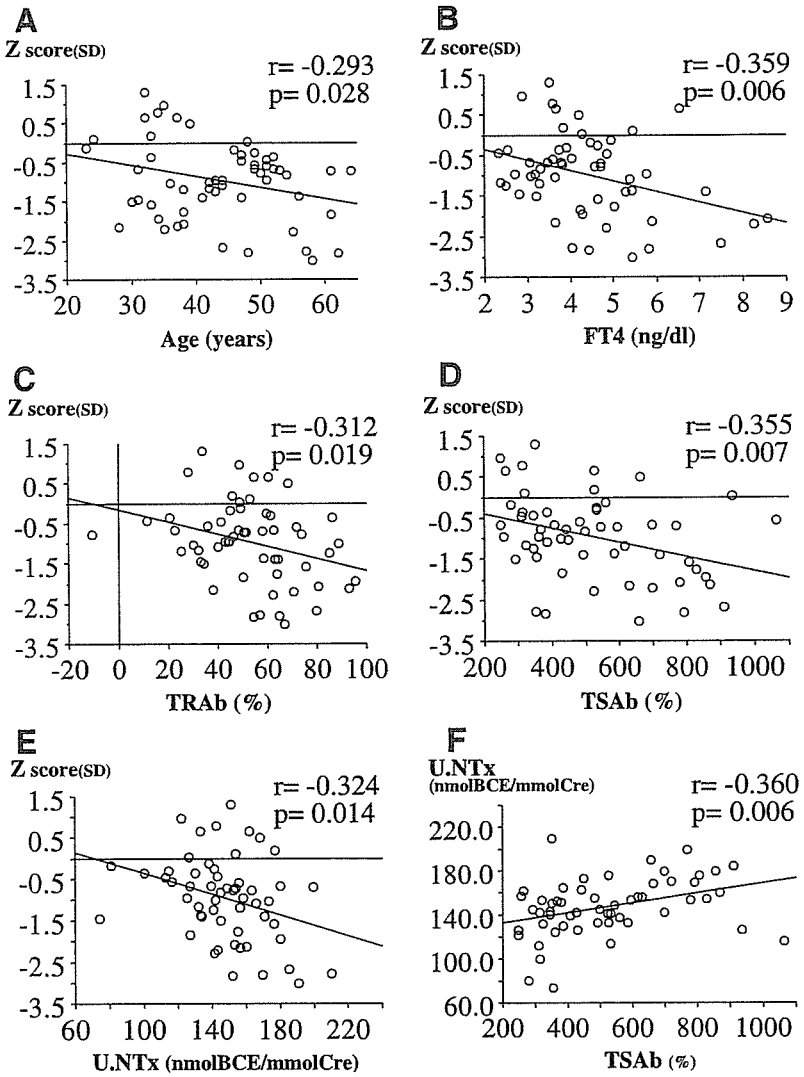

Fig. 3 Correlations between Z-score at the radius and clinical parameters (**a** age, **b** FT4, **c** TSAb, **d** TRAb, **e** U.NTx) and between TSAb and U.NTx (**f**) for patients with Graves' disease. For each figure, Z-score measurements at the radius were plotted against the clinical parameters **a**~**e**, and U.NTx measurements were plotted against TSAb (**f**). The line reflects the regression, and r mean the correlation coefficients and *P*-values for correlations between Z-score at the radius and the clinical parameters (**a**–**e**), and between TSAb and U.NTx (**f**)

In the study presented here, we examined men with untreated GD accompanied by active hyperthyroidism and found that their BMD (Z-scores) at all three sites was significantly lower than that of age-matched controls. Toh et al. [24], using single photon absorptiometry, also observed significant radial bone loss in 24 male patients with active hyperthyroidism. Our results agree with theirs, thus suggesting the importance of measuring the BMD of male patients with thyrotoxic GD. There is ample evidence to indicate that osteoporosis in men is already a public health problem, and most osteoporotic men were found to have secondary causes of osteoporosis, including hyperthyroidism [25]. Therefore, we should not underestimate the significance of reduced BMD in male patients with hyperthyroidism.

We were able to show that the Z-score is lowest at the DR among the three sites (LS, FN, and DR) we studied in male patients with GD. In addition, we found a negative correlation between the Z-score at the DR and FT4, TRAb, and TSAb in our patients. These results suggest that selective loss of cortical rather than cancellous bone may occur in GD. This selective cortical bone loss in hyperthyroidism has often been postulated in the literature [6, 8, 13], although again mostly in female patients, and these findings are supported by a great deal of evidence in rats

Table 4 Correlations of bone metabolic markers with thyroid function and thyroid autoantibodies in patients with Graves' disease (*FT3* free T3, *FT4* free T4, *TRAb* TSH hormone receptor antibodies, *TSAb* thyroid stimulating antibody, *BAP* bone-specific alkaline phosphatase, *U.NTx* N-terminal telopeptide of type I collagen normalized by creatinine)

	FT3	FT4	TRAb	TSAb
BAP	0.256	0.221	0.097	0.239
U.NTx	0.204	0.189	0.198	0.360[a]

Values are correlation coefficients

P-values for correlations of bone metabolic markers with thyroid function and thyroid autoantibodies in patients with Graves' disease: [a]$P<0.01$

[26]. Bone morphometric studies have also demonstrated that enhanced osteoclastic bone resorption is more prominent in cortical than in cancellous bone [13], which may lead to an increased risk of forearm fracture in patients with hyperthyroidism [27, 28]. These observations are compatible with our results and suggest the clinical importance of evaluating BMD at the radius.

Likewise, an increased risk of hip fracture [27–31] and reduced BMD at the FN [1, 3, 6, 7, 15, 16, 21], which contains much cortical bone, has been reported in mostly female patients with hyperthyroidism. In our study, however, BMD at the FN showed no significant difference between male patients with GD and matched controls. A possible reason might be the higher impact of gravity or exercise on bone mass, or the difference in the cortical-cancellous bone ratio between FN and DR (FN<DR). This finding agrees the results of some previous studies that showed unaltered BMD at the FN [2, 14, 20, 22]. It is different, however, from the findings of other studies [1, 3, 6, 7, 15, 16, 21] that show a reduction in BMD of the FN. Gender and racial differences of the subjects or the severity and duration of hyperthyroidism could explain these discrepancies between our study and other investigations [20].

It is clinically important to predict which patients with GD are at higher risk of losing bone, although that remains unclear at present [20]. We found a significant correlation between BMD at the DR and age, FT4, TRAb, TSAb, and U.NTx. After stepwise multiple regression analyses, higher FT4 and higher age remained as independent risk factors for reduced BMD at the DR in our study. Mudde et al. [17] also reported the significant inverse relationship between FT4 and Z-score at the DR in 23 women with subclinical hyperthyroidism. On the other hand, Fraser et al. [18] also reported that the loss of metacarpal bone mass worsened with age. Furthermore, Vestergaard et al. [29] concluded in their review of the literature that the increased risk of fractures in patients with hyperthyroidism [27, 28, 30, 31] became larger with advancing age and that the close relationship between observed and estimated fracture risk from BMD could indicate that most of the changes in fracture risk were related to changes in BMD. These results indicate the importance of examining BMD at the DR as well as at the LS or the FN, especially in elderly patients with active hyperthyroidism, in the context of fracture prevention.

Both higher TRAb and TSAb significantly correlated with a reduction in BMD at the DR in our study. In addition, higher TSAb significantly correlated with higher U.NTx. These results suggest some deleterious effects of TRAb and TSAb on bone metabolism, probably via TSH receptors in osteoblasts or osteoclasts [32, 33]. Previous studies have suggested that the past history of GD itself, not the current state of thyroid function, is responsible for bone loss in women receiving long-term levothyroxine therapy [34], indicating some autoimmune effects of TRAb on bone metabolism. A decreased fracture risk in patients with hyperthyroidism treated with surgery and an increased fracture risk in patients treated with radioiodine [28] could also support this hypothesis of TRAb being involved in bone metabolism, considering that surgical treatment of GD is associated with a less pronounced elevation of TRAb, whereas radioiodine treatment is associated with its higher elevation [35]. In addition, Wakasugi et al. [15] and Jodar et al. [16] previously showed significant negative correlation between TRAb and lumbar BMD in hyperthyroid patients, and Kumeda et al. [19] reported that TRAb did not correlate with FT3 or FT4, but correlated closely with bone metabolic markers in GD. They suggested direct adverse effect of higher TRAb on bone metabolism regardless of thyroid function [15, 16, 19].

Furthermore, it has been recently demonstrated that TSH directly affects bone metabolism via the TSH receptor found on osteoblast and osteoclast precursors in mice [32]. These studies support our findings of an inverse correlation between both TRAb and TSAb and BMD at the DR, and a positive correlation between TSAb and U.NTx. One of the questions would concern why, if these antibodies play a direct role in reducing BMD by accelerating bone turnover in similar mechanisms to TSH, the present results seem to be opposite those reported in the study by Abe et al. [32], where reduced TSH receptor expression led to profound osteoporosis, suggesting an inhibitory effect of TSH on bone turnover. However, more recently, Morimura et al. [33] showed that TSH positively regulates intracellular T3 production by controlling type 2 iodothyronine deiodinase in human osteoblasts. It is, therefore, tempting to speculate that both higher TSH receptor antibodies and lower TSH might be directly associated with lower bone mass, but not in the same way. Namely, higher TSH receptor antibodies might overproduce intracellular T3 to decrease bone mass by accelerating bone turnover while lower TSH could hardly produce intracellular T3, essential for bone growth, to decrease bone mass as well. Alternatively, higher TSH receptor antibodies might reduce bone by their effect of lowering TSH [36].

Further studies are needed to clarify these issues. Although we cannot completely exclude the possibility that these correlations in our study may have only reflected the correlation with the severity of hyperthyroidism, as Kumeda et al. [19] stated, we believe our results are clinically important in indicating that efforts should be directed not only to early restoration of euthyroidism but also to correction of TRAb and TSAb to prevent bone loss in GD.

It also remains unclear whether the gender difference in the effects of thyroid hormone on BMD exists. Compared with previous reports, our patients' Z-scores seem to be rather high, which may indicate less susceptibility to hyperthyroid-induced bone loss in male patients. Greenspan et al. [8] also referred to the possibility that the influence of excessive thyroid hormones on bone is not as striking in men as in women. And more recently, Schneider et al. [37] showed in their review of the literature that BMD in men did not decline as a result of TSH suppression in several studies [8–10], whereas in women it has often been reported to diminish [8]. In addition, while several animal studies have

shown that T3 reduced BMD in ovariectomized female rats [38], Fitts et al. [38] found that T3 alone had no effect on BMD in male rats. Larger studies are needed to identify gender differences, if any, in the effects of hyperthyroidism on bone metabolism.

In summary, we found a significant reduction in the BMD of male patients with hyperthyroidism associated with GD. We also found that the Z-score at the DR was significantly lower than that at the LS and FN in male patients with GD. The Z-score at the DR correlated negatively with age, FT4, TRAb, TSAb, and U.NTx, and TSAb correlated positively with U.NTx in male patients with GD. These results indicate a high prevalence of cortical bone loss in male patients with GD, especially elderly patients. We conclude that BMD measurement is crucial in all Graves' patients regardless of gender and that the radial BMD as well as that in the LS and the FN should be monitored to effectively prevent bone loss and subsequent fracture.

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Direct Effects of Aldosterone on Cardiomyocytes in the Presence of Normal and Elevated Extracellular Sodium

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It is now recognized that aldosterone is potentially cardiotoxic, although its local effects in the heart are not well understood. We examined the effects of aldosterone on cultured neonatal rat cardiomyocytes in the presence of normal and elevated extracellular Na^+ ($[Na^+]_o$). We evaluated the intracellular volume of cardiomyocytes in the presence of normal (141 mEq/liter) and elevated (146 mEq/liter) $[Na^+]_o$ by measuring cell size. Intracellular Na^+ was measured using sodium-binding-benzofuran-isophthalate as a fluorescent sodium indicator, and cardiac hypertrophy was assessed using B-type natriuretic peptide transcription and ^{3}H-leucine incorporation. Cardiomyocytes shrank in the presence of 146 mEq/liter Na^+ due to the increased extracellular osmolarity at early phase. Aldosterone (10^{-7} mol/liter) mitigated the shrinkage by stimulating Na^+ uptake by the cells. This effect of aldosterone was blocked by SM 20220, a Na^+/H^+ exchanger 1 (NHE1) inhibitor, but not by eplerenone, a mineralocorticoid receptor blocker. Seventy-two hours of exposure to aldosterone in the presence of 146 mEq/liter Na^+ led to increases in cardiomyocyte size, ^{3}H-leucine incorporation, and B-type natriuretic peptide and NHE1 transcription that were significantly greater than were seen in the presence of 141 mEq/liter Na^+. All but the last were blocked by either eplerenone or SM 20220; the increase in NHE1 transcription was blocked only by eplerenone. Aldosterone exerts a beneficial effect via NHE1 to block cardiomyocyte shrinkage in the presence of elevated $[Na^+]_o$ at early phase, but long-time exposure to aldosterone in the presence of elevated $[Na^+]_o$ leads to cardiomyocyte hypertrophy via genomic effects mediated by the mineralocorticoid receptor. (*Endocrinology* 147: 1314–1321, 2006)

THE BODY RESPONDS to a salty meal by decreasing the secretion of aldosterone from the adrenal cortex, increasing the secretion of antidiuretic hormone from the posterior pituitary, and activating drinking behavior, thereby stabilizing levels of circulating Na^+ (1, 2). However, if one's diet is continuously high in Na^+ or if a large amount of Na^+ is acutely taken in, the circulating Na^+ level will tend to rise. When that happens, somatic cells are subject to fluid loss due to the increase in the extracellular osmolarity, which causes them to shrink. Oxidative stress is also increased, and a life-threatening crisis can occur in severe cases (3, 4). But despite its vital importance, the regulatory system that strictly governs the levels of circulating Na^+ is not well understood.

Aldosterone has traditionally been seen as a key regulator of fluid and electrolyte balance, acting via the mineralocorticoid receptor (MR) in the epithelium of the kidney (distal nephron), colon, and salivary glands (5). However, we recently showed that aldosterone is also synthesized in the hearts of patients with heart failure or hypertension (6–8), and that in neonatal rat cardiomyocytes it induces expression of angiotensin-converting enzyme, creating a vicious circular cascade involving the renin-angiotensin-aldosterone-system (9). In addition, others have shown that aldosterone induces vascular inflammation and apoptosis within the cardiovascular system (10, 11). The molecular mechanisms by which aldosterone exerts its local effects are not fully characterized, however.

The Na^+/H^+ exchanger 1 (NHE1) is a ubiquitously expressed housekeeping transporter that catalyzes the electroneutral countertransport of extracellular Na^+ and intracellular protons (12, 13). In addition to mediating the transcellular absorption of Na^+, NHE1 plays a major role in the regulation of intracellular pH, cell volume and, possibly, cell proliferation (13). Aldosterone regulates Na^+ homeostasis and, consequently, extracellular volume in large part by controlling NHE1 activity in the kidney (12–14). There are also reports that aldosterone up-regulates NHE1 activity by both genomic or nongenomic means (15, 16); that NHE1 serves as a critical downstream regulator contributing to cardiac remodeling in response to various hypertrophic factors (16, 17); and that inhibition of NHE1 suppresses progression of cardiac hypertrophy (18).

With that as background, we hypothesized that, in the face of an acute increase in the extracellular Na^+ concentration ($[Na^+]_o$), aldosterone would act nongenomically on NHE1 to promote cellular Na^+ uptake and fluid retention to attenuate cell shrinkage. If those conditions persisted, however, aldo-

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Abbreviations: BNP, B-type natriuretic peptide; ENaC, epithelial Na^+ channel; MR, mineralocorticoid receptor; Na^+/K^+ ATPase, Na^+/K^+ adenosine triphosphatase; NHE1, Na^+/H^+ exchanger 1; SBFI, sodium-binding-benzofuran-isophthalate; SBFI-AM, SBFI-acetoxymethyl ester.

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sterone would act genomically, causing cardiomyocyte hypertrophy by way of NHE1. To test this hypothesis, we investigated the direct actions of aldosterone—*i.e.* those independent of hemodynamic overload—in cultured neonatal rat cardiomyocytes.

Materials and Methods

In this *in vitro* study, $[Na^+]_o$ was either 141 mEq/liter or 146 mEq/liter, which is within the physiological range. Cellular fluid changes were evaluated by measuring cell size (19) and changes in the intracellular Na^+ concentration ($[Na^+]_i$) were measured using sodium-binding benzofuran isophthalate (SBFI), a fluorescent Na^+ indicator (20). To examine NHE1 activity, intracellular H^+ concentration ($[H^+]_i$) was measured using LysoSensor Green DND-153 (21). Cardiac hypertrophy was assessed using ^{3}H-leucine incorporation (22) and B-type natriuretic peptide (BNP) gene expression (9, 19, 23), two sensitive makers of cardiac hypertrophy, as indices. In addition, we also tested the effects of eplerenone, a specific MR blocker (24), and SM 20220, a NHE1 inhibitor (25), on the genomic and nongenomic actions of aldosterone.

Agents used

Aldosterone was purchased from Steraloid Co. (Wilton, NH). SBFI-acetoxymethyl ester (SBFI-AM) was purchased from Sigma Chemical Co. (St. Louis, MO) (20). LysoSensor Green DND-153 as a pH indicator was purchased from Molecular Probes (Eugene, OR) (21). Eplerenone was provided by Pfizer Co., Ltd. (New York, NY) (24). SM 20220, which is a specific inhibitor of NHE1 in cultured neurons and glial cells with an IC_{50} of 5 and 20 nM, respectively, was provided by Sumitomo Pharmaceuticals Co., Ltd., Research Division (Osaka, Japan) (25, 26).

Preparation of cardiomyocytes

All animal procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Committee at Kumamoto University. Cardiomyocytes were obtained from 1- to 2-d-old Wistar rats. Ventricular cells were dispersed in a balanced Na^+ solution containing 0.04% collagenase II (Sigma) and 0.06% pancreatin (Sigma) (9, 19, 22, 23). The cardiomyocytes were isolated on a discontinuous Percoll gradient using 40.5% and 58.5% Percoll (Sigma) prepared in balanced Na^+ solution. Ventricular cells were initially suspended in the 58.5% Percoll layer (19, 22, 23). After centrifugation at 3000 rpm for 30 min at 15 C, the cardiomyocytes had migrated to the interface between the layers.

Cell culture

Purified cardiomyocytes were plated at a density of 3.0×10^4 cells/cm^2 in six-well plates (2.9×10^5 cells/well) in DMEM (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (Moregate Bio Tech, Bulimba, Australia) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin; GIBCO). The cells were allowed to attach for 30 h, after which the medium was replaced with serum-free DMEM, and the cells were incubated for an additional 12 h. After this preconditioning period, the cells were incubated in serum-free DMEM containing 1 mg/ml BSA (Sigma) with the indicated test substances (9, 19, 22, 23). Medium containing 146 mEq/liter Na^+ was made by simply adding NaCl (Wako, Osaka, Japan) to the normal 141 mEq/liter Na^+ medium (27). pH was 7.3 ± 0.2 in both media.

Measurement of cell size

Surface areas of cardiomyocytes were measured using Lumina Vision (Mitani Co., Fukui, Japan). Cardiomyocytes in the culture wells were chosen at random for measuring cell sizes from five to eight preparations and two different people blindly measured cell sizes.

^{3}H-leucine incorporation

Cardiomyocytes were plated at 3.0×10^4 cells/cm^2 in 96-well dishes and treated as described above. ^{3}H-leucine (3 μCi/ml, 2.15×10^{-8} mol/liter; PerkinElmer, Yokohama, Japan) was then added to each well just after the last treatment, as previously described (22). After incubating 72 h, the cells were harvested using an Omnifilter-96 Harvester (PerkinElmer) (28), and ^{3}H-leucine incorporation was measured using a MicroBeta TriLux (PerkinElmer) (28).

Quantitative real-time RT-PCR

For real-time RT-PCR, total RNA was extracted from cardiomyocytes cultured in six-well plates using an RNeasy Mini Kit (QIAGEN, Bulimba, Germany) (9, 23) and treated with deoxyribonuclease I (QIAGEN) to eliminate any contaminating genomic DNA (9, 23). The oligonucleotide primers and TaqMan probes used to analyze expression of rat BNP mRNA were designed from GenBank databases (M25297) using Primer Express version 1.0 (Applied Biosystems, Foster City, CA) as previously described (9, 23). The forward primer was 5′-181-CAG AAG CTG CTG GAG CTG ATA AG-203-3′; the reverse primer was 5′-258-TGT AGG GCC TTG GTC CTT TG-239-3′; and the TaqMan probe was 5′-207-AAA GTC AGA GGA AAT GGC TCA GAG ACA GCT C-237-3′. Primers and the TaqMan probe set for rat NHE1 (No. 185248084) were purchased from Assays-on-Demand Gene Expression Products (Applied Biosystems), and those for rat glyceraldehyde-3-phosphate dehydrogenase were from PerkinElmer Applied Biosystems. Two-step real time RT-PCR was carried out using TaqMan Reverse Transcription Reagents (Applied Biosystems) and a TaqMan Universal Master Mix kit (Applied Biosystems) (29) with an ABI Prism 7900 sequence detection system (Applied Biosystems) (29).

Measurement of BNP and NHE1 protein levels

We measured BNP levels in these culture mediums after 72 h using a BNP ELISA Kit (Peninsula Laboratories Inc., San Carlos, CA). NHE1 protein levels were measured by Western blotting using a polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) after 72 h culture as described (16).

Measurement of intracellular Na^+

After incubating cardiomyocytes for 2 h in medium containing 141 or 146 mEq/liter Na^+, $[Na^+]_i$ was determined as previously described (20). Briefly, cardiomyocytes grown on glass-bottomed dishes were incubated with 10 μmol/liter SBFI-AM for 90 min at room temperature in presence of the nonionic surfactant Pluronic F-127 (0.05% wt/vol; Sigma). After washing out the external dye, we allowed the intracellular SBFI-AM to be deesterified to active SBFI for 20 min before proceeding with $[Na^+]_i$ measurements. The cells were incubated in DMEM containing 141 and 146 mEq/liter Na^+, and $[Na^+]_i$ levels at a single cell were measured as a function of SBFI fluorescence using an Ion Optix dual-wavelength ratiometric photon counting system (11). The cells were pretreated with SM 20220 or eplerenone just before this measuring. After measuring for 60 sec, 10^{-7} mol/liter aldosterone was added to the medium.

Measurement of intracellular H^+

After incubating cardiomyocytes for 2 h in a medium containing 141 or 146 mEq/liter Na^+, intracellular H^+ levels ($[H^+]_i$) were examined (21). Cardiomyocytes grown on glass-bottomed dishes were incubated with 1 μmol/liter LysoSensor Green DND-153 for 30 min. After washing out the external dye, the cells were incubated in DMEM containing 141 and 146 mEq/liter Na^+, and $[H^+]_i$ levels in a single cell with 10^{-7} mol/liter aldosterone were measured using LysoSensor Green DND-153 fluorescence with Lumina Vision (Mitani Co., Fukui, Japan). The cells were pretreated with SM 20220 or eplerenone just before this measurement.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by multiple comparisons using Fisher's protected least-significant difference and unpaired Student's *t* tests, as appropriate. Values of $P < 0.05$ were considered significant.

Results

In the present study, we evaluate the actions of aldosterone in time course divided into early phase (0–6 h) and late phase (72 h).

Effect of aldosterone on cardiomyocyte size in the presence of normal and elevated $[Na^+]_o$ at early phase

Figure 1A shows the morphology of neonatal rat cardiomyocytes after 2 h in the presence of normal (141 mEq/liter) or elevated (146 mEq/liter) $[Na^+]_o$ with and without 10^{-7} mol/liter aldosterone. There was no change in the size of the cells in the presence of 141 mEq/liter Na^+ with or without aldosterone. In the presence of 146 mEq/liter Na^+ without aldosterone, however, the cells became substantially smaller, and this shrinkage was blocked by the addition of aldosterone.

Figure 1B shows the time-dependent changes in the size of cardiomyocytes over a period of 24 h. In the absence of aldosterone, the cells shrank significantly in the presence of 146 mEq/liter Na^+ over the course of 6 h ($P < 0.0001$; *vs.* 141 mEq/liter Na^+ without aldosterone at 1, 2, 3, and 6 h); again, this shrinkage was blocked by addition of 10^{-7} mol/liter aldosterone. The ability of aldosterone to block cell shrinkage in the presence of elevated $[Na^+]_o$ apparently reflects its ability to stimulate Na^+ uptake by the cells and thus increase $[Na^+]_i$. Figure 1C showed the effects of eplerenone and SM 20220 on the cell sizes of cardiomyocytes with 10^{-7} mol/liter aldosterone in the presence of 146 mEq/liter Na^+ at 2 h. SM 20220, but not eplerenone, blocked cell recovery induced by aldosterone in the presence of 146 mEq/liter Na^+.

Effect of aldosterone on the level of intracellular Na^+ and intracellular H^+ in the presence of normal and elevated $[Na^+]_o$ at early phase

In Fig. 2, 10^{-7} mol/liter aldosterone did not elevate intracellular sodium concentrations in SBFI-loaded cardiomyocytes with 141 mEq/liter Na^+ (Fig. 2A). There was an in-

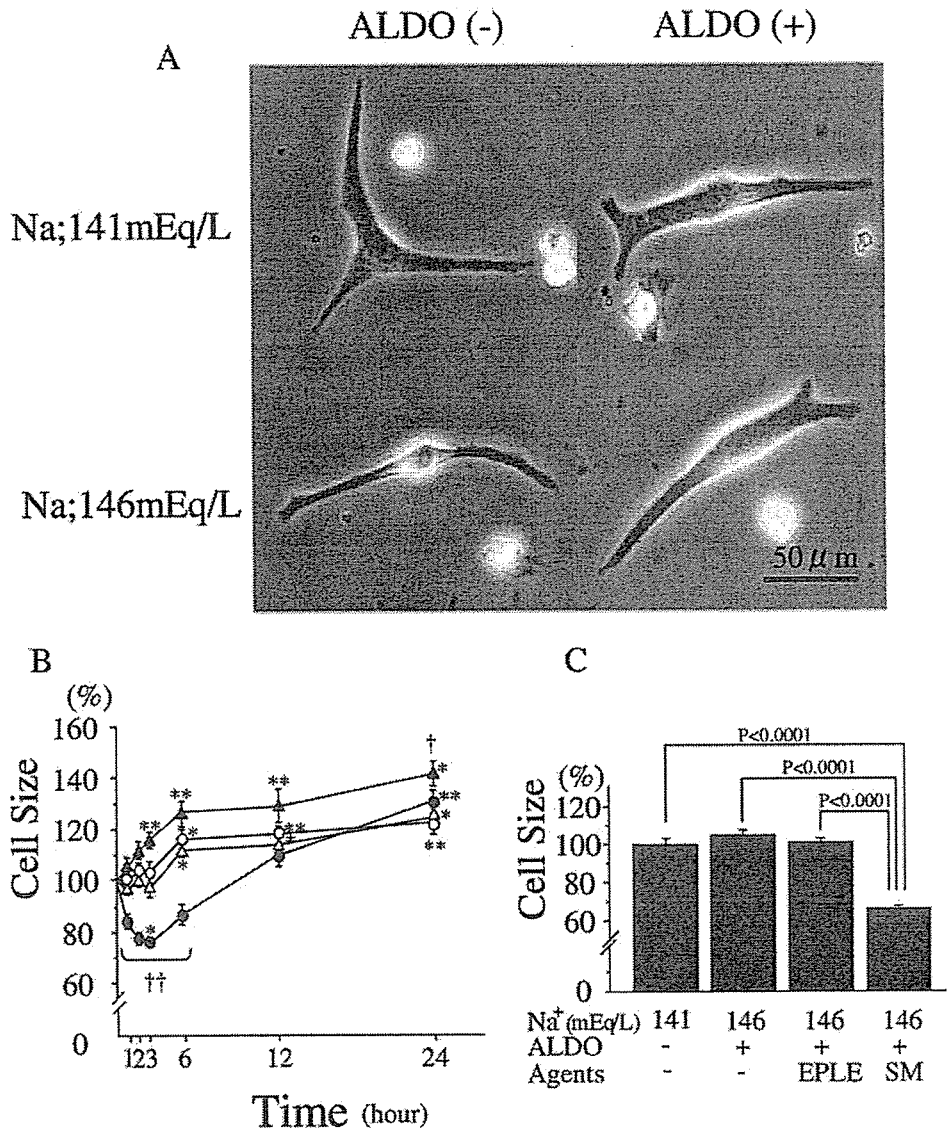

FIG. 1. A, Photomicrographs showing cardiomyocytes after 2 h in the presence of 141 mEq/liter or 146 mEq/liter Na^+ with (+) or without (−) 10^{-7} mol/liter aldosterone (ALDO). B, Time-dependent changes in the sizes of cardiomyocytes during incubation in the presence of 141 mEq/liter or 146 mEq/liter Na^+ with or without 10^{-7} mol/liter aldosterone. *Open circle*, Without 10^{-7} mol/liter aldosterone in the presence of 141 mEq/liter Na^+; *closed circle*, without 10^{-7} mol/liter aldosterone in the presence of 146 mEq/liter Na^+; *open triangle*, with 10^{-7} mol/liter aldosterone in the presence of 141 mEq/liter Na^+; *closed triangle*, with 10^{-7} mol/liter aldosterone in the presence of 146 mEq/liter Na^+. *, $P < 0.01$; **, $P < 0.0001$ *vs.* 0 h; †, $P < 0.01$; ††, $P < 0.0001$ *vs.* 141 mEq/liter Na^+ without aldosterone; n = 20. C, Sizes of cardiomyocytes incubated for 2 h in the presence of 146 mEq/liter Na^+ with 10^{-7} mol/liter aldosterone (ALDO) alone or in combination with 10^{-5} mol/liter eplerenone (EPLE) or 10^{-7} mol/liter SM 20220 (SM); n = 20.

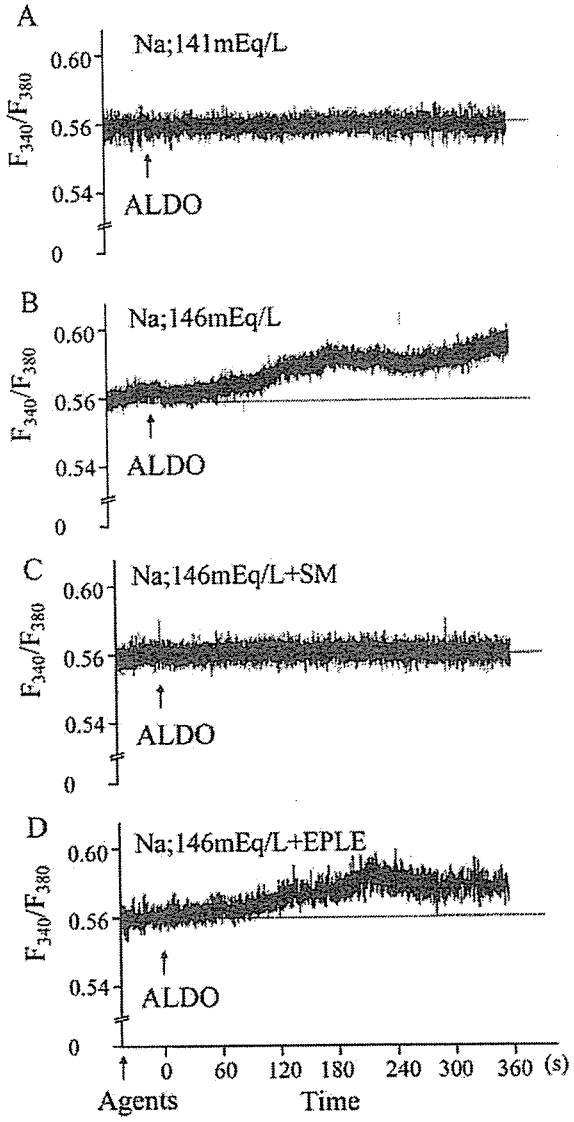

FIG. 2. Fluorescence ratios indicating the time-dependent changes in $[Na^+]_i$ at $[Na^+]_o$ = 141 or 146 mEq/liter. The *arrow* indicates addition of 10^{-7} mol/liter aldosterone (ALDO). A, In the presence of 141 mEq/liter Na^+; B, in the presence of 146 mEq/liter Na^+; C, with 10^{-7} mol/liter SM 20220 (SM) in the presence of 146 mEq/liter Na^+; D, with 10^{-5} mol/liter eplerenone (EPLE) in the presence of 146 mEq/liter Na^+.

crease in $[Na^+]_i$ after adding 10^{-7} mol/liter aldosterone to cardiomyocytes in the presence of 141 and 146 mEq/liter Na^+ (Fig. 2B). It appears that the aldosterone-induced Na^+ uptake was mediated by the NHE1, as the effect was significantly inhibited by the NHE1 antagonist SM 20220 (10^{-7} mol/liter) (Fig. 2C). In contrast, the MR antagonist eplerenone (10^{-5} mol/liter) had no effect (Fig. 2D).

In Fig. 3, we measured the level of intracellular H^+ ($[H^+]_i$) in the cardiomyocytes using LysoSensor Green DND-153 with 10^{-7} mol/liter aldosterone for examination of the activity of NHE1. Levels of $[H^+]_i$ in the cardiomyocytes with 10^{-7} mol/liter aldosterone in the presence of 146 mEq/liter Na^+ were significantly lower than those in the presence of 141 mEq/liter Na^+. SM 20220 blocked aldosterone-induced

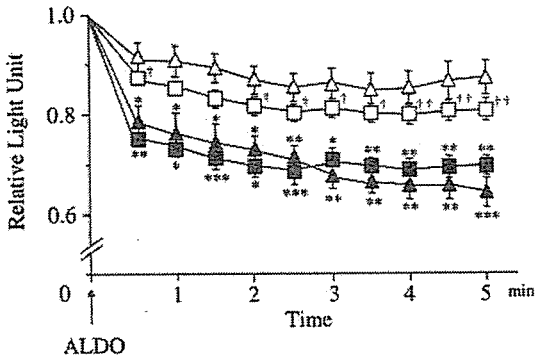

FIG. 3. Fluorescence ratios with 10^{-7} mol/liter aldosterone (ALDO) indicating the time-dependent changes in $[H^+]_i$ at $[Na^+]_o$ = 141 or 146 mEq/liter. *Open triangle*, In the presence of 141 mEq/liter Na^+, *closed triangle*, in the presence of 146 mEq/liter Na^+, *open square*, with 10^{-7} mol/liter SM 20220 in the presence of 146 mEq/liter Na^+; *closed square*, with 10^{-5} mol/liter eplerenone in the presence of 146 mEq/liter Na^+. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ *vs.* fluorescence ratios with 10^{-7} mol/liter aldosterone in the presence of 141 mEq/liter Na^+ at each time; †, $P < 0.05$; ††, $P < 0.005$ *vs.* fluorescence ratios with 10^{-7} mol/liter aldosterone in the presence of 146 mEq/liter Na^+ at each time; n = 8.

H^+ discharge, but eplerenone did not block aldosterone-induced H^+ discharge in the presence of 146 mEq/liter Na^+.

Effect of aldosterone on cardiomyocytes size in the presence of normal and elevated $[Na^+]_o$ at late phase

Seventy-two hours of exposure to 10^{-7} mol/liter aldosterone significantly increased cardiomyocyte size in the presence of both 141 mEq/liter and 146 mEq/liter Na^+ ($P < 0.005$, $P < 0.0005$, respectively), although the increase was significantly greater the presence of the latter ($P < 0.0005$ *vs.* normal Na^+) (Figs. 4A and 5A). The effect of aldosterone on cell size was significantly attenuated by either eplerenone or SM 20220 in the presence of 146 mEq/liter $[Na^+]_o$ (both $P < 0.0005$) (Figs. 4B and 5A). In the absence of aldosterone, neither eplerenone nor SM 20220 affected the cell size in the presence of 141 mEq/liter Na^+ at 72 h. In Figs. 1A and 4, they were different cells before and after aldosterone stimulation.

Effect of aldosterone on ^{3}H-leucine incorporation by cardiomyocytes at late phase

Indicative of induction of cell hypertrophy, 10^{-7} mol/liter aldosterone significantly increased ^{3}H-leucine incorporation by cardiomyocytes in the presence of either 141 mEq/liter or 146 mEq/liter Na^+ at late phase ($P < 0.05$, $P < 0.05$, respectively) and, as with cell size, the effect was more pronounced in the presence of the latter (Fig. 5B). This effect in the presence of 146 mEq/liter Na^+ was significantly attenuated by inhibiting NHE1 using SM 20220 ($P < 0.0005$). In the absence of aldosterone, neither eplerenone nor SM 20220 affected ^{3}H-leucine incorporation in the presence of 141 mEq/liter Na^+ at 72 h.

Effect of aldosterone on BNP gene expression and BNP expression by cardiomyocytes at late phase

We found that long-time exposure to 10^{-7} mol/liter aldosterone in the presence of 146 mEq/liter Na^+ induced a significant increase in BNP gene expression by cardiomyo-

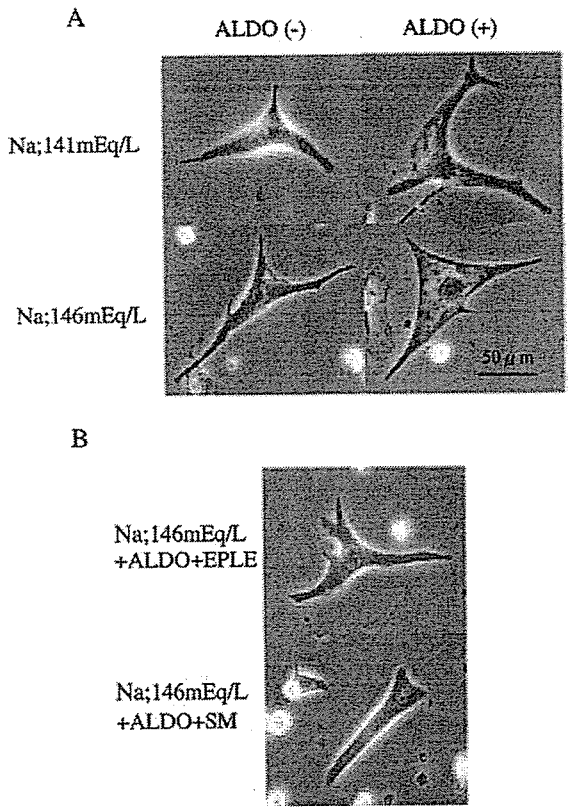

FIG. 4. A, Photomicrographs showing cardiomyocytes after 72 h in the presence of 141 mEq/liter or 146 mEq/liter Na^+ with (+) or without (−) 10^{-7} mol/liter aldosterone (ALDO). B, Photomicrographs showing cardiomyocytes after 72 h in the presence of 146 mEq/liter Na^+ with 10^{-7} mol/liter aldosterone (ALDO) alone or in combination with 10^{-5} mol/liter eplerenone (EPLE) or 10^{-7} mol/liter SM 20220 (SM).

cytes ($P < 0.0005$) that was accompanied by a substantial increase in cell size (Fig. 5C). Moreover, the effect of aldosterone on BNP gene expression was significantly greater in the presence of 146 mEq/liter Na^+ than in the presence of 141 mEq/liter Na^+ ($P < 0.05$). Both the BNP gene expression and the hypertrophy were attenuated by either eplerenone or SM 20220 ($P < 0.005$, $P < 0.0005$, respectively, for BNP gene expression). Eplerenone and SM 20220, without aldosterone, did not affect BNP gene expression levels in the presence of 141 mEq/liter Na^+ at 72 h. SM 20220 or eplerenone with 10^{-7} mol/liter aldosterone did not inhibit the effect of aldosterone by these parameters in the presence of 141 mEq/liter Na^+ at 72 h.

In the supplemental figure published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org, 10^{-7} mol/liter aldosterone significantly increased BNP levels in cardiomyocytes in the presence of either 141 mEq/liter or 146 mEq/liter Na^+ at the late phase ($P < 0.05$, $P < 0.05$, respectively) and, as with BNP gene expression, the effect was more pronounced in the presence of the latter. This effect in the presence of 146 mEq/liter Na^+ was significantly attenuated by inhibiting NHE1 using SM 20220 ($P < 0.05$). In the absence of aldosterone, eplerenone and SM 20220 did not affect BNP levels in the presence of 141 mEq/liter Na^+ at 72 h.

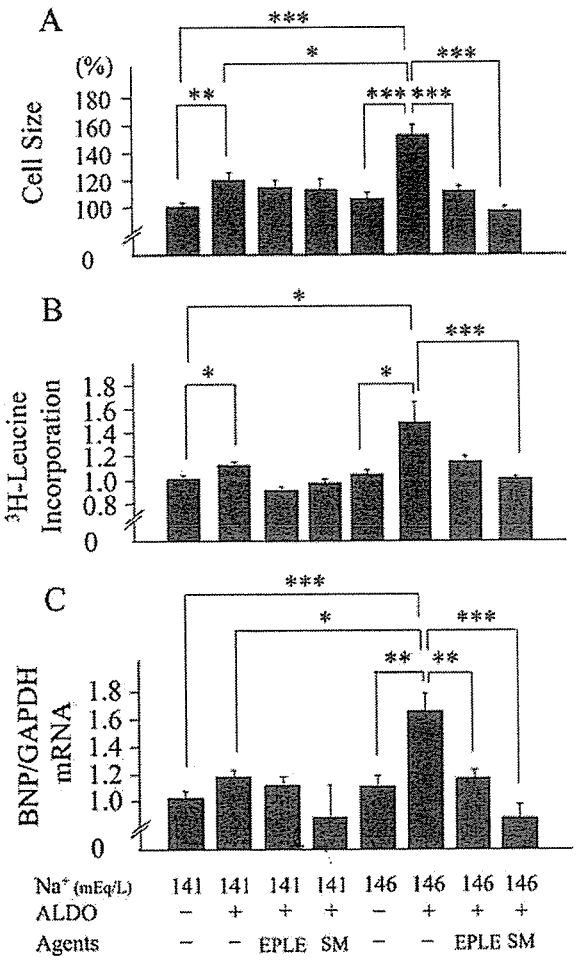

FIG. 5. Effects of aldosterone and $[Na^+]_o$ on cell size (A), ^{3}H-leucine incorporation (B), and BNP gene expression (C) at late phase. Cardiomyocytes were incubated for 72 h in the presence 141 mEq/liter or 146 mEq/liter Na^+ with or without 10^{-7} mol/liter aldosterone (ALDO) alone or in combination with 10^{-5} mol/liter eplerenone (EPLE) or 10^{-7} mol/liter SM 20220 (SM). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; n = 10–20. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Effects of aldosterone on NHE1 gene and protein expression by cardiomyocytes at late phase

NHE1 gene expression was also significantly increased by 10^{-7} mol/liter aldosterone ($P < 0.05$) in the presence of Na^+141 and 146 mEq/liter (Fig. 6A). Aldosterone also increased NHE1 protein levels in the presence of Na^+ 141 and 146 mEq/liter (Fig. 6B). The effect of aldosterone on NHE1 gene expression was significantly attenuated by eplerenone ($P < 0.05$); in contrast, SM 20220 had no effect (Fig. 6A).

Discussion

We found that acute exposure to elevated $[Na^+]_o$ caused cardiomyocytes to rapidly shrink as a result of fluid loss to the outside driven by the increase in extracellular osmolarity. Aldosterone strongly suppressed this loss of fluid by inducing Na^+ uptake, as indicated by the observed increase in SBFI fluorescence, which diminished the osmotic pressure gradient across the cell membrane. We believe these findings show that aldosterone exerts a protective effect against cardiomy-

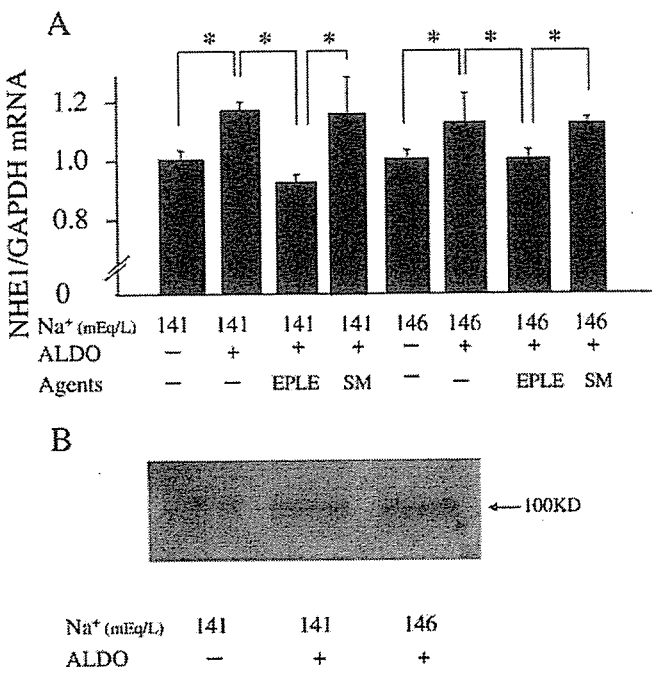

FIG. 6. A, Effect of aldosterone on NHE1 gene expression in cardiomyocytes incubated for 72 h in the presence of 141 or 146 mEq/liter Na^+ with or without 10^{-7} mol/liter aldosterone (ALDO) alone or in combination with 10^{-5} mol/liter eplerenone (EPLE) or 10^{-7} mol/liter SM 20220 (SM). *, $P < 0.05$, n = 10. B, Western blots of NHE1 with or without 10^{-7} mol/liter aldosterone (ALDO) in the presence of 141 or 146 mEq/liter Na^+. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

ocyte dehydration in the presence of elevated $[Na^+]_o$ at early phase. It appears that this effect is mediated by the NHE1 because it was blocked by the NHE1 antagonist SM 20220. At early phase, we found no evidence that aldosterone acts acutely via the MR, as indicated by eplerenone's lack of effect, or that it acutely induces cellular hypertrophy, as indicated by the absence of up-regulated BNP transcription or ^{3}H-leucine incorporation (data not shown). These findings also suggest that aldosterone may contribute to the rapid regulation and maintenance of circulating Na^+ levels by regulating the movement of Na^+ into cardiomyocytes like principal cells of the kidney distal tubules (30). It has been reported that the nongenomic effects of aldosterone on NHE were blocked by eplerenone in mesenteric resistance vessels (31). We cannot positively deny the effects of aldosterone on NHE1 via the classical MR in cardiomyocytes (31, 32).

In one recent study, aldosterone was shown to induce Na^+ influx into human umbilical vein endothelial cells, leading to cell swelling, even when the Na^+ concentration in the culture medium was unchanged (33). It was suggested that this effect was mediated via the epithelial Na^+ channel (ENaC). At one time, ENaCs were thought to exist only in the kidney; that was until the ENaC δ-subunit was identified in human heart (34). The function and significance of the ENaC in heart is not yet known, but it is possible that, along with NHE1, it contributes to mediating the acute effects of aldosterone in response to a rise in $[Na^+]_o$.

Na^+/K^+ adenosine triphosphatase (Na^+/K^+ ATPase), which mediates the active transport of Na^+ out and K^+ into the cells, is present in cardiomyocytes (35). It is notable that Na^+/K^+ ATPase has been reported to be reversely suppressed by aldosterone in the myocardium (36). Therefore, the inhibitory action of aldosterone on Na^+/K^+ ATPase in the myocardium would not conflict with the possible effect of aldosterone on the intracellular influx of sodium by NHE1 because both actions take sodium into cells in the myocardium.

Secretion of aldosterone from the adrenal gland is diminished by a high Na^+ diet, reducing circulating levels of the hormone (2). On the other hand, it was recently reported that, in rats, the concentration of aldosterone in cardiac and vascular tissues are increased by high Na^+ intake (37). In view of our present findings, we suggest that cell shrinkage caused by the increase in extracellular osmolarity associated with high Na^+ intake would stimulate local synthesis of aldosterone, which in turn stimulates uptake of extracellular Na^+ and water into the cells, thereby stabilizing the electrolyte and fluid balance across the cell membrane. Naturally, we understand that there are many reports disputing the theory about cardiac aldosterone synthesis (38, 39). It is still unclear, however, which of sodium, potassium, chloride, angiotensin II, ACTH, or others is actually the key regulator for cardiac aldosterone synthesis *in vivo* (40, 41). There are many issues that we should study in the future.

In sharp contrast to the effects of aldosterone at early phase, we found that long-time aldosterone exposure induces cardiomyocyte hypertrophy, as indicated by increased cell size, increased incorporation of ^{3}H-leucine, and increased BNP transcription. That these effects could be suppressed by either eplerenone or SM 20220 means that the effects of aldosterone are mediated via both the MR and NHE1 at late phase. In addition, we also found that aldosterone induces NHE1 gene expression via the MR (eplerenone sensitive) at late phase, which is consistent with earlier reports (15, 16).

The effects of aldosterone at late phase observed in the present study are in agreement with those of Karmazyn *et al.* (16) and complement them by adding the observation that aldosterone-induced myocardial hypertrophy is dependent on $[Na^+]_o$ as well as on the NHE1 activity. Elevation of $[Na^+]_i$ via NHE1 likely leads to Ca^{2+} overload via the Na^+/Ca^{2+} exchanger, which would in turn stimulate hypertrophic signaling (42). Also, it is possible that the increase in cell pH directly resulting from NHE1 activation might be the signal for the induction of the hypertrophic response. Furthermore, inflammatory cytokines may be involved in this system of aldosterone-induced myocardial hypertrophy depending on the $[Na^+]_o$ level. In any event, eplerenone would be useful for treating and/or preventing myocardial hypertrophy. The results of both the RALES trial and the EPHESUS trial support this idea for the MR antagonist (43, 44). Also, as suggested by Young and Funder (45), the NHE1 antagonist might also be good for reducing cardiac fibrosis, although this agent has not been used in a clinical stage yet.

We used aldosterone at a concentration of 10^{-7} mol/liter, which is a close approximation to the circulating levels seen *in vivo*, particularly under hyperaldosteronemic conditions. This actually may be somewhat conservative, however, because aldosterone concentrations are reportedly an order of

magnitude higher in cardiac tissues than in the peripheral circulation (40).

The effect of aldosterone in the heart should be discussed because cardiomyocyte MR is normally occupied by endogenous glucocorticoid in physiological status because 11β-hydroxysteroid dehydrogenase type 2 is not normally expressed in the cardiomyocytes and there are high levels of circulating cortisol (46); however, in pathophysiological states, such as hypertension, heart failure, or neonatal stage, it has been hypothesized that mineralocorticoids can access cardiac MR and thereby produce cardiac damage (46).

We previously reported that aldosterone synthesis is activated in both the adrenal gland and hearts of patients with heart failure or hypertension (6–8). Moreover, as mentioned above, aldosterone levels are higher in the myocardium than in the circulation (40). Taken together, these findings suggest that a continuous intake of excess salt stimulates cardiac hypertrophy together with local production of aldosterone in the heart, irrespective of circulating aldosterone levels. Consistent with that idea, we observed that long-time elevated $[Na^+]_o$ induced a small increase in cardiac hypertrophy even in the absence of added aldosterone. This may be explained by the endogenous production of aldosterone by the cells. We have to regardless the fact that there are many reports disputing the theory about cardiac aldosterone synthesis (38, 39) and also that the detrimental effects of aldosterone on the heart may be due to adrenal derived aldosterone (47).

From a clinical viewpoint, the results of the present study highlight the benefit of reducing salt in the diet and are consistent with earlier reports emphasizing the importance maintaining a low-salt diet to prevent cardiac hypertrophy and subsequent heart failure (27). In that regard, our findings indicate that by maintaining a low-salt diet and thus reducing local cardiovascular levels of aldosterone, one mitigates the chronic effects of this potent mediator of cardiac hypertrophy. Observational studies of the Yanomamo Indians, a no-salt culture, reinforce this view (48, 49).

In conclusion, aldosterone acutely induces Na^+ uptake via NHE1 in the presence of elevated $[Na^+]_o$. This rapid, nongenomic protective effect against cellular fluid loss is a positive and physiological attribute. In the face of elevated $[Na^+]_o$, however, long-time exposure to aldosterone induces pathological genomic effects via MR that lead to cardiomyocyte hypertrophy. The MR antagonist eplerenone could thus be useful for suppressing cardiac hypertrophy, without affecting the beneficial effects of aldosterone at early phase.

Acknowledgments

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ORIGINAL ARTICLE

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Clinical significance of risedronate for osteoporosis in the initial treatment of male patients with Graves' disease

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Abstract It has been well established that hyperthyroidism leads to diminished bone mineral density (BMD), and that a previous history of hyperthyroidism remains a risk factor for fractures. However, little is known about how to manage the reduction in BMD caused by hyperthyroidism. The purpose of this study was to evaluate the efficacy of risedronate for the treatment of osteoporosis/osteopenia in patients with Graves' disease (GD). Of 34 Japanese male patients with newly diagnosed GD, 27 with osteoporosis/osteopenia were included in this study. They were randomly divided into two groups by therapeutic regimen. Group A consisted of 14 patients treated with an antithyroid drug and risedronate. Group B consisted of 13 patients treated with the same antithyroid drug only. We used dual-energy X-ray absorptiometry to measure BMD at the lumber spine, femoral neck, and distal radius at baseline, and at 6 and 12 months after the trial. Bone-specific alkaline phosphatase and urinary N-terminal telopeptide of type I collagen normalized by creatinine were significantly more reduced in group A than in group B after both 6 and 12 months. The percentage increases in BMD at the lumbar spine and distal radius were significantly greater in group A than in group B. These beneficial effects of risedronate for patients with osteoporosis/osteopenia caused by GD may lead to a reduced risk of future fractures. We thus conclude that risedronate should be considered for the treatment of decreased bone mass associated with GD.

Key words bisphosphonate · Graves' disease · thyroid hormones · bone mineral density · N-terminal telopeptide of type I collagen

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Introduction

It has been well established that hyperthyroidism leads to diminished bone mineral density (BMD) [1–3] accompanied by an increase in bone turnover in favor of bone resorption [4,5]. After the successful treatment of hyperthyroidism, some improvement of the reduction in BMD has generally been observed [4,6–8]. However, it is not clear whether the reduced bone mass is completely normalized after the attainment of euthyroidism [2]. Some clinical studies have suggested that the reduction in bone mass caused by hyperthyroidism will be completely corrected after several years of continuous euthyroidism [6–8]. However, other studies have shown that the recovery from the reduction in bone mass remains incomplete despite effective treatment for hyperthyroidism [9–14]. Moreover, the risk of fracture for patients with hyperthyroidism has been reported to remain high, even many years after the attainment of euthyroidism [15–17]. This is probably due to the poor bone quality of patients with a past history of hyperthyroidism even if their BMD has apparently recovered as a result of treatment for hyperthyroidism. While the accelerated bone turnover accompanying hyperthyroidism is known to improve rapidly after the beginning of antithyroid treatment [4,5], some investigators have reported slightly but continuously elevated bone turnover 1 year after the attainment of euthyroidism [4,18]. These observations suggest the need for some form of therapeutic intervention, with the increased bone turnover in hyperthyroidism leading to the reduction in bone mass. However, little is known

about how to manage the abnormal bone metabolism in hyperthyroidism.

Likewise, little is known about bone mineral metabolism in male patients with active hyperthyroidism, as Greenspan and Greenspan [1] stated in their review of the published data. In addition, an interpretation of the effects of hyperthyroidism and its treatment on bone metabolism is hampered by the background of involutional osteoporosis in female patients [19]. Our assessment of the potential effects of hyperthyroidism and its treatment on bone metabolism was therefore limited to male patients in an effort to largely eliminate the influence of involutional osteoporosis.

Antiresorptive agents are widely used to treat osteoporosis, Risedronate (RIS), a potent pyridinyl bisphosphonate, has been shown to reduce the risk of both vertebral and nonvertebral fractures by reducing bone turnover, increasing bone mass, and preserving trabecular bone architecture [20–23]. However, there have been no clinical studies to evaluate the utility of RIS for the treatment of osteoporosis/osteopenia in patients with Graves' disease (GD).

The aim of our study was thus to examine and assess the effects of RIS on BMD and bone turnover in male patients with GD, both at diagnosis and prospectively after 6 and 12 months of antithyroid treatment.

Subjects and methods

Subjects

Thirty-four male Japanese patients (mean age 43.7 ± 11.2 years) with newly diagnosed GD, who attended the clinic of Rakuwakai Otowa Hospital between April 2003 and June 2004, were selected for this study. The control group comprised 34 healthy male Japanese volunteers without a past or present history of thyroid disease. All were employees of the Rakuwakai Otowa Hospital, and were similar to the patients in their exercise history and calcium intake. The diagnosis of GD was established on the basis of clinical signs and symptoms, as well as of laboratory finding, including positive thyroid stimulating antibody (TSAb) and/or thyroid-stimulating hormone receptor antibodies (TRAb) and an elevated technetium-99m thyroid scan (>5%). GD in all patients was treated with methimazole (MMI) alone. The initial dose of MMI was 30 mg daily given in three 10-mg doses. During the course of this study, the dose of MMI was adjusted according to the biochemical thyroid status of each patient. Of the 34 patients with GD, seven showed no signs of osteoporosis/osteopenia at the lumbar spine (LS), femoral neck (FN), or distal radius (DR). The other 27 patients had reduced BMD (*T* score below −1 SD) in at least one site (14 in the LS, 8 in the FN, and 22 in the DR). This study involved a 1-year (at baseline, and at 6 months and 12 months after the diagnosis) longitudinal examination of these 27 patients, who were randomly divided into two groups according to their therapeutic regimen. Group A consisted of 14 patients treated with MMI and bisphosphonate (RIS, 2.5 mg/day orally). Group B, the control group, consisted of 13 patients treated with MMI only. Their clinical data at baseline are shown in Table 1.

All subjects completed a questionnaire administered by a doctor or nurse prior to entry into the study, and the results showed that the two subgroups of patients were similar with regard to physical activity and calcium intake. All subjects underwent laboratory blood and urinary tests. We excluded subjects who had a history of fractures and/or of other diseases (liver disease, renal dysfunction, malignancy, diabetes mellitus, hyperparathyroidism, hypercorticoidism, or hypogonadism) and those taking medication (active vitamin D3, bisphosphonates, calcitonin, testosterones, steroids, thyroid hormones, diuretics, heparin, or anticonvulsants) that could influence bone metabolism. Plain X-rays (anteroposterior and lateral views) of the lumbar spine were administered to all patients and controls at baseline, and to all patients at 6 months and 12 months after the diagnosis, and patients with scoliosis, compression fractures, or ectopic calcifications that could interfere with the bone mineral results were excluded. None of the subjects were smokers or substance abusers.

This study was performed in accordance with the recommendations of the Declaration of Helsinki and approved by the Ethical Committee of Rakuwakai Otowa Hospital, and all participants provided informed written consent.

BMD measurements

BMD was measured at the LS (L2–L4), FN, and DR by means of dual-energy X-ray absorptiometry (DXA) (Hologic QDR 4500c; Hologic, Waltham, MA, USA) at baseline, and at 6 months and 12 months after the diagnosis in patients, and at baseline only in controls. To eliminate technical discrepancies, the same operator measured all the subjects. The reproducibility was calculated as the coefficients of variation obtained by daily measurements of a standard phantom over a period of 2 years. The CV of our instrument is 0.43% with the standard phantom. Values of BMD at the LS were expressed as the mean of those at the L2–L4. *T* scores and *Z* scores were calculated on the basis of the normal reference values of the age- and sex-matched Japanese group provided by the DXA system manufacturer.

Biochemical measurements

Serum samples were obtained before 0800 hours after overnight fasting, and were immediately processed and kept frozen at −20°C until the assays were carried out. Serum-free T3 (FT3), free T4 (FT4), and thyroid-stimulating hormone (TSH) were measured with the aid of an electrochemiluminescence immunoassay (ECLUSISU; Roche Diagnostics, Tokyo, Japan; normal values: FT3, 2.30–4.30 pg/ml; FT4, 0.90–1.70 ng/dl; TSH, 0.500–5.000 μIU/ml). The minimum detection limit of the TSH assay was 0.005 μIU/ml. TRAb was measured with a

Table 1. Baseline characteristics of examined subjects

	Patients with Graves' disease		Controls ($n = 34$)	Overall P-value
	Group A ($n = 14$)	Group B ($n = 13$)		
Age (years)	43.64 ± 11.0	45.46 ± 13.1	43.8 ± 10.5	0.8850
Height (cm)	169.79 ± 6.0	169.67 ± 5.4	170.1 ± 6.3	0.9740
Weight (kg)	65.79 ± 5.1	64.20 ± 8.5	66.1 ± 5.2	0.6089
BMI (kg/m^2)	22.86 ± 1.9	22.34 ± 1.8	22.9 ± 2.0	0.6550
FT3 (pg/ml)	11.99 ± 4.3**	12.51 ± 4.5**	3.30 ± 0.48	<0.0001
FT4 (ng/dl)	4.71 ± 1.7**	4.90 ± 1.5**	1.34 ± 0.22	<0.0001
TSH (μIU/ml)	<0.005	<0.005	2.17 ± 0.51	–
TRAb (%)	56.68 ± 20.9	47.39 ± 22.5	N.D.	–
TSAb (%)	611.93 ± 299.2	550.00 ± 169.1	N.D.	–
Calcium (mg/dl)	10.01 ± 0.4*	9.99 ± 0.4	9.77 ± 0.31	0.0488
ALP (IU/l)	364.07 ± 49.4**	353.46 ± 57.3**	199.1 ± 37.0	<0.0001
BAP (U/l)	52.82 ± 10.1**	55.73 ± 10.4**	23.8 ± 3.6	<0.0001
UrinaryNTx/Cre (nmolBCE/mmolCr)	157.69 ± 26.7**	151.59 ± 32.2**	34.6 ± 11.2	<0.0001
Lumbar spine				
BMD (g/cm^2)	0.939 ± 0.102*	0.949 ± 0.124*	1.016 ± 0.078	0.0157
T score (SD)	−0.811 ± 1.019*	−0.714 ± 1.236*	−0.035 ± 0.715	0.0157
Z score (SD)	−0.471 ± 0.608**	−0.334 ± 0.830*	0.097 ± 0.501	0.0075
Femoral neck				
BMD (g/cm^2)	0.830 ± 0.137	0.774 ± 0.104	0.841 ± 0.070	0.1028
T score (SD)	−0.263 ± 1.075	−0.703 ± 0.821	−0.170 ± 0.549	0.1028
Z score (SD)	0.055 ± 0.985	−0.291 ± 0.781	0.192 ± 0.492	0.1113
Distal radius				
BMD (g/cm^2)	0.633 ± 0.072**	0.643 ± 0.062**	0.751 ± 0.052	<0.0001
T score (SD)	−1.745 ± 1.136**	−1.582 ± 0.987**	0.129 ± 0.823	<0.0001
Z score (SD)	−1.583 ± 0.964**	−1.418 ± 0.907**	0.045 ± 0.724	<0.0001

Data represent mean ± SD
BMI, body mass index; FT3, free T_3; FT4, free T_4; TRAb, thyroid-stimulating hormone receptor antibodies; TSAb, Thyroid stimulating antibody; ALP, alkaline phosphatase; BAP, bone type alkaline phosphatase; U.NTx, N-terminal telopeptide of type I collagen normalized by creatinine; N.D., not done
* $P < 0.05$ vs. controls, ** $P < 0.01$ vs. controls

radioreceptor assay (Cosmic III, Cosmic, Tokyo, Japan; normal range: 0.0%–15.0%), and TSAb with a bioassay radioimmunoassay (TSAB-Kit-Yamasa; Yamasa, Chiba, Japan; normal range: <180.0%). Serum calcium, phosphate, creatinine, and alkaline phosphatase (ALP) were measured by standard laboratory methods. Bone-specific alkaline phosphatase (BAP) was measured with an enzyme immunoassay kit (Osteolinks-BAP; Sumitomo Pharmaceuticals, Tokyo, Japan; normal range: 13.0–33.9 U/l) as a marker of bone formation. Urinary N-terminal telopeptide of type I collagen normalized by creatinine (U.NTx) was measured in the morning in the second voided urine sample by means of an enzyme-linked immunosorbent assay (Osteomark; Mochida Pharmaceutical, Tokyo, Japan; normal range: 13.0–66.2 nmolBCE/mmolCre) as a marker of bone resorption.

Statistical analysis

The differences between two groups were analyzed with the unpaired t-test, and longitudinal differences in the same group with the paired t-test. Differences among three groups were analyzed with the one-way factorial ANOVA and Fisher's protected least significant difference (PLSD) method. Statistics were calculated using Statview version 5.0 (Abacus Concepts, Berkeley, CA, USA) A P value < 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the baseline values between GD patients and controls. There was no significant differences among the two subgroups of patients (group A and group B) and the control subjects in age, height, weight, and BMI. However, the thyroid hormones, serum calcium, ALP, BAP, and U.NTx levels of the GD patients were significantly higher than those of controls. BMD at the LS and the DR in the GD patients were significantly lower than those of controls. However, BMD at all three sites at the baseline were not different between group A and group B (Fig. 1A).

Table 2 shows the longitudinal characteristics of the patients in group A and group B at the time of diagnosis and at 6 and 12 months after the baseline. FT3, FT4, TRAb, and TSAb significantly decreased after antithyroid treatment in both groups. There was no significant difference in age,

height, weight, body mass index (BMI), thyroid hormones, or thyroid autoantibodies between the two groups at any of the three times. However, BAP and U.NTx were significantly lower in group A than in group B at both 6 months (P = 0.0171 and 0.0012, respectively) and 12 months (P = 0.0079 and 0.0080, respectively) after the baseline, while both BAP and U.NTx decreased significantly compared with the initial values in group A and group B (Table 2).

The percentage values of the decreases (mean ± SD) in U.NTx from the initial value in group A were 72.77% ± 9.5% and 75.65% ± 6.6% at 6 and 12 months, respectively, which were significantly higher than the corresponding values in group B (58.34% ± 17.5% and 66.56% ± 11.5%, respectively; P = 0.0125 and 0.0179, respectively) (Fig. 2). The percentage values of the decreases (mean ± SD) in BAP from the initial value in group A were 26.70% ± 8.9% and 52.22% ± 9.7% at 6 and 12 months, respectively, which were also significantly higher than the corresponding values in group B (11.06% ± 13.2% and 42.88% ± 11.5%, respectively; P = 0.0012 and 0.0311, respectively) (Fig. 2). However, neither BAP nor U.NTx in group A dropped below the normal range at either 6 or 12 months after the baseline.

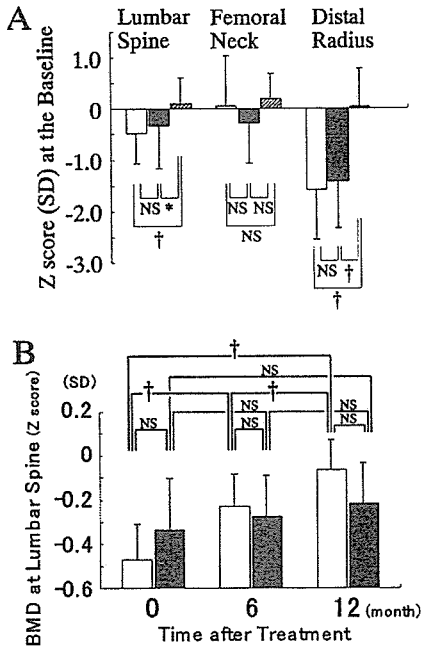

Fig. 1. **A** Comparison of bone mineral density (BMD) at the lumbar spine, femoral neck, and distal radius evaluated at baseline between patients with Graves' disease (group A, *white columns*; group B, *black columns*) and control subjects (*shaded columns*). Each column represents mean ± SD. *NS*, $P \geq 0.05$; *, $P < 0.05$; †, $P < 001$. **B** Z score at the lumbar spine evaluated at baseline and after 6 and 12 months for patients in group A (*white columns*) and group B (*black columns*). Data are shown as mean ± SEM. *NS*, $P \geq 0.05$; †, $P < 0.01$ vs. baseline. All differences between group A and group B were nonsignificant at all three times

Table 3 and Fig. 1B show the changes in BMD, T score, and Z score for both groups during the course of this study. While only BMD and the T score at the FN were significantly higher at 6 months than at the baseline in group B, BMD, T score, and Z score at the LS and FN were significantly higher at 6 months, and those at all three sites were significantly higher at 12 months compared with those at the baseline in group A. On the other hand, BMD, T score, and Z score for the two groups at any site were not significantly different at any of the three times.

However, the percentage change in BMD from the baseline was considerably different between group A and group B at both 6 and 12 months (Fig. 3). In group A, the percentage increases (mean ± SD) in BMD at the LS, FN, and DR were 3.53% ± 3.3%, 2.55% ± 1.7%, and 0.63% ± 1.4%, respectively, at 6 months, and 6.07% ± 4.3%, 4.41% ± 2.3%, and 2.41% ± 2.3%, respectively, at 12 months (Fig. 3). In group B, the percentage increases (mean ± SD) in BMD at the LS, FN, and DR were 1.18% ± 2.3%, 1.62% ± 2.5%, and –0.55% ± 3.0%, respectively, at 6 months, and 1.90% ± 3.2%, 2.61% ± 4.5%, and 0.08% ± 3.5%, respectively, at 12 months (Fig. 3). The percentage increases in BMD at the LS were significantly higher in group A than in group B at both 6 and 12 months (P = 0.0428 and 0.0094, respectively), at the FN it was higher, although not significantly so, in group A than in group B, and at the DR it was significantly higher in group A than in group B at 12 months (P = 0.0499), but not at 6 months.

During the course of this study, one patient with RIS and one patient without RIS experienced epigastric discomfort, and one patient with RIS and one patient without RIS experienced diarrhea. Two patients in group A and 2 patients in group B experienced urticaria or general itchiness. However, their symptoms were not so severe that this study had to be discontinued.

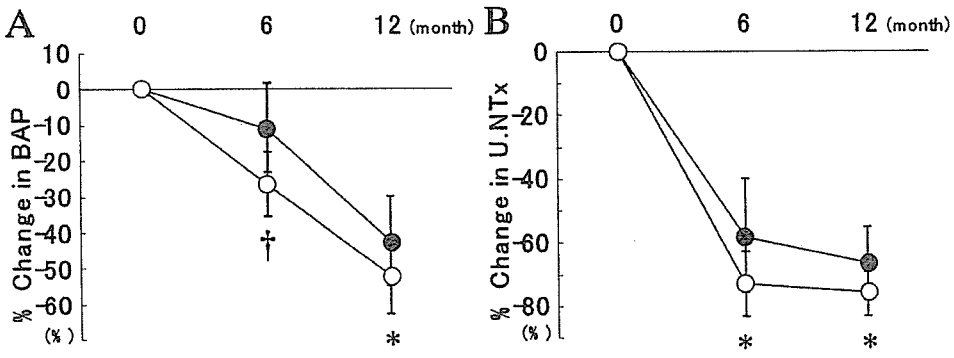

Fig. 2. The percentage change compared with the initial values of BAP (**A**) and U.NTx (**B**) evaluated after 6 and 12 months of treatment. For each figure, *white circles* represent the data of group A, and *black circles* the data of group B. Data are shown as mean ± SD. *, $P < 0.05$ vs. group B; †, $P < 0.01$ vs. group B

Table 2. Longitudinal variables assessed in patients with Graves' disease

	Baseline	6 months	*P*-value[a]	12 months	*P*-value[b]	*P*-value[c]
Group A ($n = 14$)						
Age (years)	43.64 ± 11.0*					
Height (cm)	169.79 ± 6.0*	169.76 ± 6.0*	0.3019	169.74 ± 6.0*	0.2123	0.1648
Weight (kg)	65.79 ± 5.1*	69.56 ± 5.4*	<0.0001	69.26 ± 5.3*	<0.0001	0.3280
BMI (kg/m^2)	22.86 ± 1.9*	24.17 ± 1.8*	<0.0001	24.01 ± 1.9*	<0.0001	0.1840
FT3 (pg/ml)	11.99 ± 4.3*	3.29 ± 0.4*	<0.0001	3.20 ± 0.6*	<0.0001	0.6825
FT4 (ng/dl)	4.71 ± 1.7*	1.21 ± 0.3*	<0.0001	1.11 ± 0.3*	<0.0001	0.3490
TSH (μIU/ml)	<0.005	2.71 ± 1.3*	–	3.27 ± 1.4*	–	0.1247
TRAb (%)	56.68 ± 20.9*	37.81 ± 13.3*	<0.0001	29.91 ± 10.8*	<0.0001	0.0030
TSAb (%)	611.93 ± 299.2	358.57 ± 173.1	0.0003	259.5 ± 92.2*	0.0003	0.0058
Calcium (mg/dl)	10.01 ± 0.4*	9.40 ± 0.3*	<0.0001	9.37 ± 0.2**	<0.0001	0.7271
ALP (IU/l)	364.07 ± 49.4*	274.07 ± 36.4*	<0.0001	226.50 ± 37.0*	<0.0001	0.0002
BAP (U/l)	52.82 ± 10.1*	38.77 ± 8.9**	<0.0001	24.59 ± 3.8†	<0.0001	<0.0001
UrinaryNTx/Cre (nmolBCE/mmolC)	157.69 ± 26.7*	41.66 ± 11.0†	<0.0001	37.53 ± 8.6†	<0.0001	0.0553
Group B ($n = 13$)						
Age (years)	45.46 ± 13.1					
Height (cm)	169.67 ± 5.4	169.60 ± 5.4	0.1205	169.61 ± 5.4	0.8193	0.0876
Weight (kg)	64.20 ± 8.5	67.90 ± 9.6	<0.0001	67.77 ± 8.8	<0.0001	0.6781
BMI (kg/m^2)	22.34 ± 1.8	32.62 ± 2.0	<0.0001	23.60 ± 1.8	<0.0001	0.9199
FT3 (pg/ml)	12.51 ± 4.5	3.14 ± 0.6	<0.0001	3.19 ± 0.5	<0.0001	0.8074
FT4 (ng/dl)	4.90 ± 1.5	1.17 ± 0.3	<0.0001	1.16 ± 0.3	<0.0001	0.8755
TSH (μIU/ml)	<0.005	2.508 ± 1.3	–	2.633 ± 1.4	–	0.7280
TRAb (%)	47.39 ± 22.5	29.9 ± 15.9	0.0006	26.18 ± 13.2	0.0001	0.1257
TSAb (%)	550.00 ± 169.1	359.00 ± 125.7	0.0011	263.23 ± 56.9	<0.0001	0.0010
Calcium (mg/dl)	9.99 ± 0.4	9.54 ± 0.3	0.0010	9.53 ± 0.2	0.0011	0.9254
ALP (IU/l)	353.46 ± 57.3	296.5 ± 42.7	<0.0001	235.9 ± 40.8	<0.0001	0.0001
BAP (U/l)	55.73 ± 10.4	49.64 ± 13.0	0.0179	31.77 ± 8.4	<0.0001	<0.0001
UrinaryNTx/Cre (nmolBCE/mmolC)	151.59 ± 32.2	59.06 ± 13.7	<0.0001	48.32 ± 10.8	<0.0001	0.0047

Data represent mean ± SD
BMI, body mass index; FT3, free T_3; FT4, free T_4, TRAb, thyroid-stimulating hormone receptor antibodies; TSAb, thyroid stimulating antibody; ALP, alkaline phosphatase; BAP, bone type alkaline phosphatase; U.NTx, N-terminal telopeptide of type I collagen normalized by creatinine
P-values[a] for comparisons of the parameters at the baseline and at 6 months, *P*-values[b] for comparisons of the parameters at the baseline and at 12 months, and *P*-values[c] for comparisons of the parameters at 6 and at 12 months
P-values for comparisons of the parameters for group A and group B: * $P > 0.05$; ** $P < 0.05$

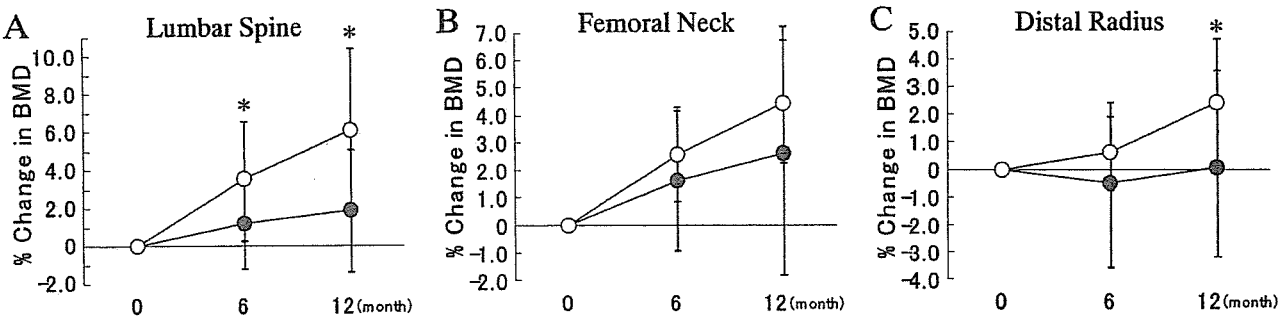

Fig. 3. The percentage change in BMD compared with the initial values at the lumbar spine (**A**), at the femoral neck (**B**), and at the distal radius (**C**) evaluate after 6 and 12 months of treatment. For each figure, *white circles* represent the data of group A, and *black circles* the data of group B. Data are shown as mean ± SD. *, $P < 0.05$ vs. group B

Discussion

It has been suggested that the reduction in BMD in hyperthyroid patients is potentially reversible in association with the cure of hyperthyroidism [2,4,6–8,10]. However, it remains unclear whether the reduction can be completely reversed [2]. Our study showed that 1 year of antithyroid treatment alone, even without any therapeutic intervention for the osteopenia/osteoporosis, increased BMD at the LS and FN by 2.3% and 3.1%, respectively. However, the patients' recovery was deemed unsatisfactory because their *Z*-score was still slightly below zero. On the other hand, BMD at the DR showed no increase during the 1 year of antithyroid treatment. Some longitudinal studies have examined the restoration of bone mass after treatment for hyperthy-

Table 3. BMD, T score, and Z score of patients with Graves' disease at the baseline, at 6 and 12 months

	Baseline	6 months	*P*-value[a]	12 months	*P*-value[b]	*P*-value[c]
Group A ($n = 14$)						
Lumbar spine						
BMD (g/cm^2)	0.939 ± 0.102*	0.970 ± 0.088*	0.0018	0.993 ± 0.085*	0.0002	<0.0001
T score (SD)	−0.811 ± 1.019*	−0.499 ± 0.875*	0.0018	−0.268 ± 0.850*	0.0002	<0.0001
Z score (SD)	−0.471 ± 0.608*	−0.228 ± 0.535*	0.0003	−0.067 ± 0.511*	<0.0001	<0.0001
Femoral neck						
BMD (g/cm^2)	0.830 ± 0.137*	0.850 ± 0.136*	<0.0001	0.866 ± 0.140*	<0.0001	<0.0001
T score (SD)	−0.263 ± 1.075*	−0.101 ± 1.074*	<0.0001	0.021 ± 1.098*	<0.0001	<0.0001
Z score (SD)	0.055 ± 0.985*	0.257 ± 1.023*	0.0002	0.383 ± 1.041*	<0.0001	<0.0001
Distal radius						
BMD (g/cm^2)	0.633 ± 0.072*	0.637 ± 0.073*	0.1134	0.648 ± 0.072*	0.0027	0.0002
T score (SD)	−1.745 ± 1.136*	−1.680 ± 1.155*	0.1133	−1.506 ± 1.140*	0.0027	0.0002
Z score (SD)	−1.583 ± 0.964*	−1.527 ± 0.963*	0.1390	−1.373 ± 0.94*	0.0042	0.0003
Group B ($n = 13$)						
Lumbar spine						
BMD (g/cm^2)	0.949 ± 0.124	0.958 ± 0.107	0.1483	0.965 ± 0.113	0.0624	0.1743
T score (SD)	−0.714 ± 1.236	−0.623 ± 1.074	0.1483	−0.552 ± 1.129	0.0624	0.1743
Z score (SD)	−0.334 ± 0.830	−0.274 ± 0.654	0.3786	−0.217 ± 0.660	0.1835	0.1788
Femoral neck						
BMD (g/cm^2)	0.774 ± 0.104	0.785 ± 0.099	0.0419	0.793 ± 0.100	0.0570	0.1094
T score (SD)	−0.703 ± 0.821	−0.611 ± 0.776	0.0419	−0.552 ± 0.791	0.0570	0.1094
Z score (SD)	−0.291 ± 0.781	−0.228 ± 0.711	0.1818	−0.170 ± 0.688	0.1086	0.1311
Distal radius						
BMD (g/cm^2)	0.643 ± 0.062	0.640 ± 0.067	0.5450	0.644 ± 0.068	0.9243	0.0725
T score (SD)	−1.582 ± 0.987	−1.636 ± 1.061	0.5450	−1.573 ± 1.079	0.9243	0.0725
Z score (SD)	−1.418 ± 0.907	−1.438 ± 0.890	0.8539	−1.379 ± 0.908	0.7635	0.0749

Values are mean ± SD
BMD, bone mineral density
P-values[a] for comparisons of the parameters at the baseline and at 6 months, *P*-values[b] for comparisons of the parameters at the baseline and at 12 months, and *P*-values[c] for comparisons of the parameters at 6 and at 12 months
P-values for comparisons of the parameters for group A and group B: * $P > 0.05$

roidism. Diamond et al. [10] showed that after 1 year of antithyroid therapy, BMD at the LS and FN increased by 6.6% and 1.2%, respectively, while Jodar et al. [11] found that the diminished BMD at the LS and FN increased significantly as early as after 9 months of antithyroid treatment, but remained 5% lower than that of matched controls even at the 18-month follow-up. On the other hand, Toh et al. [9] reported that BMD at the DR in their male patients had decreased 1 year after antithyroid treatment, but returned to pretreatment levels after 2 years, although it remained significantly (16%) lower than that of controls. Their results are in agreement with ours, suggesting that the decrease in BMD is partially, but not completely, reversed in the first year after antithyroid treatment. Jodar et al. [11] maintained that even this small bone mass deficit early after the antithyroid treatment may leave the patients at risk of fracture in later life.

Other studies have suggested that the reduced bone mass can be completely restored after several years of continued euthyroidism [6–8]. However, epidemiological studies have shown that patients with a past history of hyperthyroidism remain at a considerably higher risk of fractures [15–17]. These seemingly inconsistent observations suggest that even complete restoration of BMD with antithyroid treatment cannot always lead to sufficient recovery of bone quality to prevent fractures. Therefore, some therapeutic intervention which can restore not only the bone mass but also the bone quality is needed for osteoporotic/osteopenic patients with GD, and we consider bisphosphonate to be one of the prime candidates.

Bisphosphonates are the most effective antiresorptive agents currently available [21], and RIS has been shown to reduce the rate of bone resorption, increase BMD, and decrease fracture risk in patients with osteoporosis [20–22]. In our study, we found that RIS, in addition to MMI, increased BMD at the LS, FN, and DR by 6.6%, 4.2%, and 2.4%, respectively, during the 1-year treatment, which was significantly more than MMI alone. Two clinical studies have been reported which examined the efficacy of bisphosphonates for bone loss in GD. Lupoli et al. [24] evaluated the effects of alendronate on BMD in female patients with hyperthyroidism. They found that after 12 months of treatment, alendronate significantly increased BMD at the LS in both pre- and postmenopausal female patients compared with the results obtained without alendronate. Fittipaldi et al. [25] investigated the increase in BMD in elderly osteoporotic and hyperthyroid male patients treated with MMI alone vs. alendronate in addition to MMI. The mean changes in BMD at the LS and FN after 12 months of treatment were significantly higher in patients treated with alendronate in addition to MMI (6.2% and 2.1%, respectively) than in those with MMI alone (2% and 1.4%, respectively). Unfortunately, BMD was measured at only a single site in the former study, and the latter did not show the BMD values. Other clinical studies suggest that pamidronate is effective for abnormal bone

metabolism in patients with suppressive doses of thyroid hormone [26]. Studies in rats have also demonstrated that bisphosphonates improve the excessive bone loss caused by hyperthyroidism [27,28]. All these observations agree with ours, and are thus indicative of the potential benefits of bisphosphonates or RIS for osteoporosis/osteopenia in GD.

As far as we know, however, there are no reports in the literature regarding the effect of bisphosphonates on the reduced bone mass at the DR in patients with active hyperthyroidism. As noted above, our study found that RIS caused a significant increase in BMD at the DR as well as at the LS and FN in patients with active hyperthyroidism. Rosen et al. [26] investigated the efficacy of APD on bone metabolism in 55 patients (18 males and 37 females) with thyroid cancer who were being medicated with suppressive doses of levothyroxine. In their study, the administration of pamidronate produced no significant increase in BMD at the DR compared with a placebo, while pamidronate significantly increased BMD at the LS, trochanter, and total hip. However, BMD at the DR treated with pamidronate was significantly increased at 12 and 18 months compared with that at the baseline. In addition, actual increases in radial BMD after bisphosphonate therapy have been observed in postmenopausal osteoporosis [29,30]. These findings are also consistent with ours. Moreover, even if bisphosphonates do not influence BMD at the DR, they are known to reduce the risk of fracture at the DR by reducing cortical porosity [29,31,32], indicating the potential benefit of bisphosphonates for reduced bone mass at the DR in hyperthyroid patients as well.

Numerous reported studies have examined the effects of antithyroid therapy on bone metabolism by measuring bone formation and bone resorption markers in patients with hyperthyroidism [4,5]. Bone turnover rapidly decreases after normalization of the thyroid function, and is accompanied by a more rapid and prominent decrease in bone resorption marker [4,5], as was also seen in our study. Bone resorption markers thus seem to be better markers for monitoring bone loss in hyperthyroidism than bone formation markers. However, there are no previous studies on how the bone resorption marker (U.NTx) in GD patients after antithyroid treatment is longitudinally influenced by bisphosphonate, although Lupoli et al. [24] previously showed a significant reduction in a bone formation marker (osteocalcin) resulting from bisphosphonate in patients with Graves' thyrotoxicosis. Our study found that, as expected, RIS in addition to MMI significantly diminished the accelerated bone turnover in our GD patients compared with MMI alone, but not to a level below the normal range. In view of the fact that the bone metabolic markers are good predictors for the change in BMD in response to antiresorptive treatment [33], we speculate that this significant improvement in bone turnover brought about by RIS may account for the significant increase in BMD produced by RIS in our study. Furthermore, some clinical studies have suggested that increased bone turnover independently contributes to an increase in fracture risk [34]. RIS therefore seems all the more beneficial for these patients because a significant improvement in bone turnover by RIS in our GD patients is expected not only to increase their BMD, but also to reduce the risk of future fractures.

Using nasal calcitonin (CT), Jodar et al. [11] evaluated the effects of another antiresorptive therapy on BMD at the LS, the FN, and the whole body in hyperthyroid patients (8 men and 35 women). BMD increased in all the subgroups treated with different doses of CT (0, 800, and 1400IU/month), but no significant difference was found in the improvement in either BMD or biochemical bone markers among the three subgroups, suggesting that the treatment with nasal CT has no additional effect beyond the attainment of the euthyroid state. Kung and Yeung [35] also found that calcium supplementation prevented bone loss induced by thyroxine suppressive therapy regardless of whether it was with or without the use of nasal CT. Although Akcay et al. [36] recently found that nasal CT siginificantly lowered urinary deoxypyridinoline in 10 patients with hyperthyroidism, they also showed that BMD was similarly increased after treating hyperthyroidism regardless of nasal CT. These results and ours suggest that RIS is superior to CT for improving the reduced BMD in hyperthyroidism.

Although our study demonstrated that RIS significantly increased BMD and improved bone turnover in male patients with thyrotoxic GD, it remains unclear whether RIS can reduce the risk of fractures for these patients. Some clinical studies have reported that changes in BMD may explain only a small portion of fracture risk reduction [37], so it remains a matter of much debate whether changes in BMD are directly linked to changes in fracture risk [38]. However, even if increased BMD does not lead to fracture risk reduction, RIS has been reported to actually decrease the risk of new vertebral fractures by up to 70% within 1 year of treatment for patients with osteoporosis [39], although the specific effect of RIS or bisphosphonates on fracture risk for patients with GD is not known. It also remains unclear whether the added bone mass benefit of RIS is worth the additional cost. As is well known, fractures associated with osteoporosis, especially compressed fracture of the spine or hip fracture, can have a devastating effect on disability and medical costs. However, although the present study suggested some beneficial effects of RIS on the increased risk of fractures in Graves' disease, it was not designed to calculate the cost-effectiveness of RIS. Further studies are therefore needed to clarify these issues.

Fortunately, RIS was well tolerated by our patients, except for some minor febrile reactions that have been reported in the literature, including epigastric discomfort and diarrhea [40]. On the other hand, MMI was also well tolerated whether with or without RIS, except for transient itchiness. Our finding that these adverse events were comparable for patients with or without RIS indicates the adequate safety and tolerability of RIS for patients with GD.

In conclusion, our study has provided new and supportive information to the existing literature. We found that both 6- and 12-month treatment with RIS significantly in-

creased BMD at the LS, FN, and DR, and improved the accelerated bone metabolic markers in male patients with thyrotoxic GD. These potentially beneficial effects of RIS upon the abnormal bone metabolism in GD suggest that osteoporosis/osteopenia in patients with GD should be treated with RS in order to improve their bone metabolism, and consequently to reduce the risk of future fractures.

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