Effects of Propranolol on Bone Metabolism in Spontaneously Hypertensive Rats

Takuma Sato, Michitsugu Arai, Shigemi Goto, and Akifumi Togari

Departments of Pharmacology (T.S., M.A., A.T.) and Orthodontics (T.S., S.G.), School of Dentistry, Aichi-Gakuin University, Nagoya, Japan

Received March 1, 2010; accepted April 15, 2010

ABSTRACT

The effects of propranolol (PRO), a nonselective β-adrenergic receptor (β-AR) antagonist with membrane-stabilizing action on bone metabolism, were examined in spontaneously hypertensive rats (SHR) showing osteoporosis with hyperactivity of the sympathetic nervous system. Treatment of SHR with PRO at 1 and 5 mg/kg p.o. for 12 weeks increased bone mass of the lumbar vertebra and proximal tibia without affecting blood pressure, but PRO at 50 and 100 mg/kg with hypotensive action did not increase bone mass. Next, the effects of PRO at 0.1, 1, and 10 mg/kg on bone status were examined in more detail. Compared with the SHR control, not only bone mass but also biomechanical parameters of strength and toughness of the lumbar vertebrae were increased in SHR treated with PRO at 0.1 and 1 mg/kg, suggesting antosteoporotic action. PRO at 1 mg/kg statistically increased histomorphometry indices of bone formation, whereas PRO at doses of 0.1, 1, and 10 mg/kg decreased those of bone resorption. Antosteoporotic effect of PRO is attenuated at 10 mg/kg compared with 0.1 and 1 mg/kg. In addition, treatment with timolol, a nonselective β-AR antagonist without membrane-stabilizing action, or butoxamine, a selective β2-AR antagonist, at 1 mg/kg increased bone mass in SHR. These results suggested that treatment of SHR with β-blockers at low dose improved bone loss and bone fragility. This antosteoporotic effect of β-blockers seems to be caused by the blocking action of β2-AR, regardless of the membrane-stabilizing action.

The possibility of sympathetic nervous regulation of bone metabolism has been documented (Chenu and Marenzana, 2005; Togari et al., 2005; Togari and Arai, 2008). Immunohistochemical studies showed that mammalian bones were widely innervated by sympathetic and sensory nerves, which were particularly abundant in regions of high osteogenic activity, such as the growth plate (Bjurholm, 1991; Hill and Elde, 1991). It has also been established that osteoblasts and osteoclasts express the β2-adrenergic receptor (β2-AR) (Moore et al., 1993; Togari et al., 1997; Togari 2002; Arai et al., 2003). In pharmacological and physiological studies, it was shown that bone-resorbing activity in mice was increased by activation of their sympathetic nervous system by the intracerebroventricular injection of leptin or lipopolysaccharide or by restraint stress (Takeda et al., 2002; Kondo and Togari, 2003). Conversely, bone-resorbing activity was impaired in rats chemically sympathectomized by guanethidine (Cherruau et al., 1989). Accordingly, mice lacking β2-AR develop high bone mass caused by an increase in bone formation and a decrease in bone resorption parameters (Elefantriou et al., 2005). In in vitro studies, adrenaline (α,β-AR agonist) and isoprenaline (β-AR agonist) have been reported to stimulate bone resorption in intact mouse calvaria (Moore et al., 1993), increase the formation of osteoclast-like cells from mouse bone marrow cells (Takeuchi et al., 2001; Ishizuka et al., 2005), and stimulate bone-resorbing activity in human osteoclast-like cells (Arai et al., 2003). These studies suggested that hyperactivity in the sympathetic nervous system might be involved in the development of osteoporosis.

β-Blockers have a well recognized antihypertensive action that is mediated through a reduction in cardiac output, the release of renin from the kidneys, and inhibition of the action of endogenous catecholamines on β-ARs (Graham et al., 2008). β-Blockers are now classified as one of the first-line medicines for the treatment of hypertension and have been widely used in cardiovascular disease. Epidemiological studies have demonstrated that high blood pressure was associated with increased bone loss at the femoral neck and low

ABSTRACT

The effects of propranolol (PRO), a nonselective β-adrenergic receptor (β-AR) antagonist with membrane-stabilizing action on bone metabolism, were examined in spontaneously hypertensive rats (SHR) showing osteoporosis with hyperactivity of the sympathetic nervous system. Treatment of SHR with PRO at 1 and 5 mg/kg p.o. for 12 weeks increased bone mass of the lumbar vertebra and proximal tibia without affecting blood pressure, but PRO at 50 and 100 mg/kg with hypotensive action did not increase bone mass. Next, the effects of PRO at 0.1, 1, and 10 mg/kg on bone status were examined in more detail. Compared with the SHR control, not only bone mass but also biomechanical parameters of strength and toughness of the lumbar vertebrae were increased in SHR treated with PRO at 0.1 and 1 mg/kg, suggesting antosteoporotic action. PRO at 1 mg/kg statistically increased histomorphometry indices of bone formation, whereas PRO at doses of 0.1, 1, and 10 mg/kg decreased those of bone resorption. Antosteoporotic effect of PRO is attenuated at 10 mg/kg compared with 0.1 and 1 mg/kg. In addition, treatment with timolol, a nonselective β-AR antagonist without membrane-stabilizing action, or butoxamine, a selective β2-AR antagonist, at 1 mg/kg increased bone mass in SHR. These results suggested that treatment of SHR with β-blockers at low dose improved bone loss and bone fragility. This antosteoporotic effect of β-blockers seems to be caused by the blocking action of β2-AR, regardless of the membrane-stabilizing action.

The possibility of sympathetic nervous regulation of bone metabolism has been documented (Chenu and Marenzana, 2005; Togari et al., 2005; Togari and Arai, 2008). Immunohistochemical studies showed that mammalian bones were widely innervated by sympathetic and sensory nerves, which were particularly abundant in regions of high osteogenic activity, such as the growth plate (Bjurholm, 1991; Hill and Elde, 1991). It has also been established that osteoblasts and osteoclasts express the β2-adrenergic receptor (β2-AR) (Moore et al., 1993; Togari et al., 1997; Togari 2002; Arai et al., 2003). In pharmacological and physiological studies, it was shown that bone-resorbing activity in mice was increased by activation of their sympathetic nervous system by the intracerebroventricular injection of leptin or lipopolysaccharide or by restraint stress (Takeda et al., 2002; Kondo and Togari, 2003). Conversely, bone-resorbing activity was impaired in rats chemically sympathectomized by guanethidine (Cherruau et al., 1989). Accordingly, mice lacking β2-AR develop high bone mass caused by an increase in bone formation and a decrease in bone resorption parameters (Elefantriou et al., 2005). In in vitro studies, adrenaline (α,β-AR agonist) and isoprenaline (β-AR agonist) have been reported to stimulate bone resorption in intact mouse calvaria (Moore et al., 1993), increase the formation of osteoclast-like cells from mouse bone marrow cells (Takeuchi et al., 2001; Ishizuka et al., 2005), and stimulate bone-resorbing activity in human osteoclast-like cells (Arai et al., 2003). These studies suggested that hyperactivity in the sympathetic nervous system might be involved in the development of osteoporosis.

β-Blockers have a well recognized antihypertensive action that is mediated through a reduction in cardiac output, the release of renin from the kidneys, and inhibition of the action of endogenous catecholamines on β-ARs (Graham et al., 2008). β-Blockers are now classified as one of the first-line medicines for the treatment of hypertension and have been widely used in cardiovascular disease. Epidemiological studies have demonstrated that high blood pressure was associated with increased bone loss at the femoral neck and low
bone mineral density, and β-blockers are potential candidates of therapeutic drugs for osteoporosis and fracture healing (Cappuccio et al., 1999; Pasco et al., 2004; Schlienger et al., 2004; Graham et al., 2008). In the animal study, a lower dose of propranolol (PRO), a nonselective β-blocker with membrane-stabilizing action, has been shown to increase bone mass in ovarioctomized (OVX) rats (Bonnet et al., 2006, 2008); however, conflicting evidence about the antiosteoporotic effects of β-blockers has been also reported. Rejmark et al. (2004) showed in a small group study no differences in bone mineral density between β-blocker users and nonusers, and that the fracture risk was increased in subjects treated with β-blockers compared with the nonuser group.

Spontaneously hypertensive rats (SHR) have been studied as a genetic model of essential hypertension for many years. SHR are characterized by elevated blood pressure, increased heart rate, raised plasma catecholamine level and dopamine-β-hydroxylase activity, and increased adrenal tyrosine hydroxylase and dopamine β-hydroxylase activities compared with Wistar-Kyoto rats (WKY), a normotensive genetic control of SHR (Nagatsu et al., 1971, 1974; Zhang and Thörén, 1998; Klemola et al., 1999), suggesting that the sympathetic nervous system of SHR is overactivated. SHR also showed reductions in cortical and cancellous bone mass and increases in bone turnover, suggesting that osteoporosis is developed in adult SHR (Izawa et al., 1985; Barbagallo et al., 1991; Wang et al., 1993). This evidence suggests that SHR may be a suitable animal model of osteoporosis with hyperactivity of the peripheral sympathetic nervous system.

In this study, the dose effects of PRO on bone metabolism were examined in SHR by analysis of microcomputed tomography (μCT), bone histomorphometry, biomechanical testing, and plasma biochemistry to evaluate the usefulness of β-blockers in preventing osteoporosis with the activated sympathetic nervous system. Effects of timolol, a nonspecific β-blocker without membrane-stabilizing action, and butoxamine, a specific β2-blocker, on bone metabolism were also examined.

### Materials and Methods

**Animals and Reagents.** Seven-week-old male SHR/Izm and WKY/Izm were purchased from Japan SLC Inc. (Hamamatsu, Japan). Rats were housed in the cages (four rats/cage) under automatically controlled conditions of temperature (23 ± 1°C), humidity (50 ± 10%), and a 12:12-h light/dark cycle, and they were given tap water and standard laboratory chow ad libitum. All procedures complied with the Guidelines for Animal Experiments at the School of Dentistry, Aichi-Gakuin University. All rats were acclimatized for 1 week to the housing conditions. Propranolol hydrochloride ([(-)-1-isopropylamino-3-(1-naphthoxy)-2-propanol hydrochloride], timolol maleate salt [S(+)-1-(t-butylamino)-3-[4-morpholino-1,2,5-thiadiazol-3-yl]oxy]-2-propanol maleate salt], butoxamine hydrochloride [α-(1-[t-butylimino]ethyl)-2,5-dimethoxybenzyl alcohol], calcein, and tetracycline hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO).

Groups of SHR (eight rats/group) were administered PRO at doses of 1, 5, 50, or 100 mg/kg p.o. with a gastric tube once every 12 weeks in the first experiment (experiment 1), PRO at doses of 0.1, 1, or 10 mg/kg p.o. in the second experiment (experiment 2), and PRO, timolol, or butoxamine at doses of 1 mg/kg p.o. in the third experiment (experiment 3). SHR control and WKY were administered saline (1 ml/kg) in the same way as PRO administration. Systolic blood pressure of the tail artery was measured at the appropriate times. Each rat received a calcein injection (15 mg/kg i.p.) and a tetracycline injection (25 mg/kg i.p.) at 10 and 3 days before sacrifice, respectively. All rats were sacrificed by drawing whole blood from the abdominal aorta using a heparinized syringe under ether anesthesia, and the tibiae and lumbar vertebrae (L1, L2, L6) were harvested.

**Blood Pressure.** Each rat was warmed for 10 min at 40°C to dilate the tail arteries and restrained in a rat holder. Systolic blood pressure and heart rate were measured with a tail cuff in conscious rats. Three readings were averaged for each rat at each measurement.

**Trabecular Microarchitecture.** The second lumbar vertebra (L2) and the proximal region of the right tibia were subjected to three-dimensional μCT analysis using a SMX 225CT-SV3 μCT scanner (Shimadzu, Kyoto, Japan). A total of 100 consecutive 50-μm-thick sections were analyzed in each bone sample. Cortical bone was excluded from the region of interest with semiautomatically drawn contours. Relative bone volume (BV) per tissue volume (TV) and trabecular number (Tb.N) were calculated.

**Biomechanical Properties.** After sacrifice, the sixth lumbar vertebra (L6) was hermetically stored at −20°C until use. The stored bones were thawed at 4°C, immersed in saline, and used immediately for the biomechanical test. The trunk of the lumbar was separated and made into a column contour for the compression test. The biomechanical test was performed in a computerized materials testing system (EZ Test; Shimadzu). The lumbar vertebrae were placed on the smooth surface of a steel disk and axially compressed by the smooth surface of a steel rod attached to a load cell at a constant speed of 1 mm/min. The force-deflection curve was displayed on a monitoring recorder linked to the tester in each specimen. Four parameters of bone mechanical properties (strength, toughness, ductility, and modulus of elasticity) were assessed from the force-deflection curve. Strength was indicated by maximal force on the force-deflection curve, toughness was determined from the area under the maximal force–maximal deflection curve, ductility was indicated via the maximal deflection at maximal load, and modulus of elasticity was calculated from the initial slope of the force-deflection curve.

**Histomorphometry Analysis.** The lumbar vertebrae (L1 and L2) were used for histomorphometry analyses. L1 vertebrae was stored in 4% paraformaldehyde for 1 week, then stored in 70% ethanol and embedded in resin, a mixture of methyl methacrylate, monomer, benzoyl peroxide, nonylphenyl-polyethylenglycol acetate, and N,N-dimethyl-p-toluidine. Serial undecalcified 7-μm-thick sagittal sections were made. The mineral apposition rate (MAR; μm/ day) was calculated as the mean distance between the first label of calcine and the second label of tetracycline divided by the labeling interval (7 days). The bone formation rate (BFR) per bone surface (BS) (μm²/μm² per day) was calculated by the formula MAR × (single-labeled surface /BS) + double-labeled surface/BS. L2 vertebrae were fixed in 4% paraformaldehyde for 48 h and decalcified in 20% EDTA, pH 7.4, for 3 weeks. The specimens were dehydrated in ethanol, embedded in paraffin, sectioned longitudinally at 4 μm thick with a Leica RM 2255 microtome (Leica, Wetzlar, Germany), and stained for tartrate-resistant acid phosphatase (TRAP). TRAP staining-positive multinucleated cells attached to bone were scored as osteoclasts. Measurements were made within an area of 0.8 mm² with a Leica RM 2255 microtome (Leica, Wetzlar, Germany), and stained for tartrate-resistant acid phosphatase (TRAP). TRAP staining-positive multinucleated cells attached to bone were scored as osteoclasts. Measurements were made within an area of 0.8 mm²

**Blood Pressure.** Each rat was warmed for 10 min at 40°C to dilate the tail arteries and restrained in a rat holder. Systolic blood pressure and heart rate were measured with a tail cuff in conscious rats. Three readings were averaged for each rat at each measurement.

**Trabecular Microarchitecture.** The second lumbar vertebra (L2) and the proximal region of the right tibia were subjected to three-dimensional μCT analysis using a SMX 225CT-SV3 μCT scanner (Shimadzu, Kyoto, Japan). A total of 100 consecutive 50-μm-thick sections were analyzed in each bone sample. Cortical bone was excluded from the region of interest with semiautomatically drawn contours. Relative bone volume (BV) per tissue volume (TV) and trabecular number (Tb.N) were calculated.

**Biomechanical Properties.** After sacrifice, the sixth lumbar vertebra (L6) was hermetically stored at −20°C until use. The stored bones were thawed at 4°C, immersed in saline, and used immediately for the biomechanical test. The trunk of the lumbar was separated and made into a column contour for the compression test. The biomechanical test was performed in a computerized materials testing system (EZ Test; Shimadzu). The lumbar vertebrae were placed on the smooth surface of a steel disk and axially compressed by the smooth surface of a steel rod attached to a load cell at a constant speed of 1 mm/min. The force-deflection curve was displayed on a monitoring recorder linked to the tester in each specimen. Four parameters of bone mechanical properties (strength, toughness, ductility, and modulus of elasticity) were assessed from the force-deflection curve. Strength was indicated by maximal force on the force-deflection curve, toughness was determined from the area under the maximal force–maximal deflection curve, ductility was indicated via the maximal deflection at maximal load, and modulus of elasticity was calculated from the initial slope of the force-deflection curve.

**Histomorphometry Analysis.** The lumbar vertebrae (L1 and L2) were used for histomorphometry analyses. L1 vertebrae was stored in 4% paraformaldehyde for 1 week, then stored in 70% ethanol and embedded in resin, a mixture of methyl methacrylate, monomer, benzoyl peroxide, nonylphenyl-polyethylenglycol acetate, and N,N-dimethyl-p-toluidine. Serial undecalcified 7-μm-thick sagittal sections were made. The mineral apposition rate (MAR; μm/ day) was calculated as the mean distance between the first label of calcine and the second label of tetracycline divided by the labeling interval (7 days). The bone formation rate (BFR) per bone surface (BS) (μm²/μm² per day) was calculated by the formula MAR × (single-labeled surface /BS) + double-labeled surface/BS). L2 vertebrae were fixed in 4% paraformaldehyde for 48 h and decalcified in 20% EDTA, pH 7.4, for 3 weeks. The specimens were dehydrated in ethanol, embedded in paraffin, sectioned longitudinally at 4 μm thick with a Leica RM 2255 microtome (Leica, Wetzlar, Germany), and stained for tartrate-resistant acid phosphatase (TRAP). TRAP staining-positive multinucleated cells attached to bone were scored as osteoclasts. Measurements were made within an area of 0.8 mm² (1.0 ± 0.8 mm²) selected randomly. Histomorphometry was conducted to quantify the osteoclast number (Oc.N) per bone surface (Oc.N/BS) and osteoclast surface (Oc.S) per bone surface (Oc.S/BS), as defined by Parfitt et al. (1987).

**Plasma Biochemical Parameters.** Blood samples obtained from abdominal aorta under ether anesthesia were centrifuged, and the plasma was taken and stored at −80°C. The concentration of osteocalcin and activity of TRAP form 5b (TRAP 5b), the enzymatically active form of TRAP secreted from osteoclasts in the plasma, was tested with the rat osteocalcin EIA kit (Biomedical Technologies, Cambridge, MA) and rat TRAP assay kit (SBA Sciences, Immuno-diagnostic Systems Ltd, Boldon, UK), respectively.
Statistical Analysis. All data are presented as means ± S.E.M., and statistical analysis was carried out by one-way analysis of variance (Tukey’s multiple comparison test). All statistical analyses were performed with Prism version 4 (GraphPad Software Inc., San Diego, CA). p < 0.05 is considered to be significant.

Results
Effect of PRO on the Blood Pressure in SHR. In experiment 1, groups of male SHR at 8 weeks old were administered PRO at doses of 1, 5, 50, or 100 mg/kg p.o. once daily for 12 weeks. Changes in systolic blood pressure are shown in Fig. 1. During the experiment, systolic blood pressure in SHR was statistically higher than in WKY. Four weeks later, decreases in systolic blood pressure were observed in SHR treated with PRO at 50 and 100 mg/kg (p < 0.01 versus SHR control), but not in SHR treated with PRO at 1 and 5 mg/kg. The final body weight (mean ± S.E.M.) of the WKY, SHR control, and SHR treated with PRO at doses of 1, 5, 50, and 100 mg/kg was 393 ± 4.3, 353 ± 5.4, 370 ± 3.9, 372 ± 5.6, 359 ± 9.5, and 360 ± 3.8 g, respectively. Statistical difference in body weight was observed between SHR control and WKY (p < 0.01), but not among SHR groups treated with or without PRO. In experiment 2, blood pressure measured in the last week of the experiment in WKY, SHR control, and SHR treated with PRO at 0.1, 1, and 10 mg/kg was 140.1 ± 3.2, 199.5 ± 13.2, 203.7 ± 11.1, 201.1 ± 5.7, and 198.3 ± 11.0 mm Hg, respectively. All SHR groups treated with or without PRO showed higher blood pressure than WKY (p < 0.001), and no statistical differences were observed among SHR groups.

Effect of PRO on the Trabecular Microarchitecture of Lumbar Vertebra and the Proximal Tibia in SHR. As shown in Fig. 2A, trabecular bone loss was observed in three-dimensional μCT images of the lumbar vertebra (L2) in SHR control compared with WKY. Bone mass measured by BV/TV of the L2 vertebra was significantly decreased in SHR control (−18% p < 0.01, versus WKY), and increased in SHR treated with PRO at doses of 1 and 5 mg/kg (+28% p < 0.001 and +23% p < 0.01 versus SHR control) but not at 50 and 100 mg/kg (Fig. 2B). Microarchitecture analysis (Fig. 2C) showed that Tb.N increased in SHR treated with PRO at doses of 1 and 5 mg/kg (+31% p < 0.05 and +30% p < 0.05 versus SHR control). Likewise, trabecular bone loss was observed in both two- and three-dimensional μCT images of the proximal tibia of SHR control compared with WKY (Fig. 3A). Increases in bone mass and Tb.N were observed in the proximal tibia of SHR treated with PRO at 1 mg/kg (Fig. 3B and C); therefore, the effect of PRO at low doses on bone metabolism was examined in more detail in experiment 2. As shown in Fig. 4, the decreased bone mass and microarchitecture of L2 vertebra in SHR were improved by treatment with PRO at 0.1, 1, and 10 mg/kg (BV/TV: +17% p < 0.001, +18% p < 0.001, and +12% p < 0.05; Tb.N: +25% p < 0.01, +26% p < 0.001, and +20% p < 0.05, versus SHR control). These effects on bone

Fig. 1. Effect of PRO on blood pressure changes in SHR. Eight-week-old SHR were treated with PRO at doses of 1, 5, 50, or 100 mg/kg p.o. daily for 12 weeks, whereas SHR control and WKY were treated with saline in the same way. Systolic blood pressure was measured at 0, 2, 4, and 10 weeks of the experiment. Values are the means ± S.E.M. (n = 8). *p < 0.05, **p < 0.01, and ***p < 0.001 statistically different from SHR control, according to Tukey’s multiple comparison test.

Fig. 2. Effect of PRO on trabecular microarchitecture of lumbar vertebra in SHR. Eight-week-old SHR were treated with PRO at doses of 1, 5, 50, or 100 mg/kg p.o. daily for 12 weeks, whereas SHR control and WKY were treated with saline in the same way. A, representative three-dimensional μCT images of the lumbar vertebra (L2) in WKY (a), SHR control (b), and SHR treated with PRO at doses of 1 mg/kg (c), 5 mg/kg (d), 50 mg/kg (e), or 100 mg/kg (f). B, BV/TV. C, Tb.N. Values are the means ± S.E.M. (n = 8). *p < 0.05, **p < 0.01, and ***p < 0.001, statistically different from WKY; +, p < 0.05, ++, p < 0.01, and ++++, p < 0.001, statistically different from SHR control, according to Tukey’s multiple comparison test.

Fig. 3. Effect of PRO on trabecular microarchitecture of proximal tibia in SHR. Eight-week-old SHR were treated with PRO at a dose of 1, 5, 50, or 100 mg/kg p.o. daily for 12 weeks, whereas SHR control and WKY were treated with saline in the same way. A, representative two-dimensional (top) and three-dimensional (bottom) μCT images of the proximal tibia in WKY (a), SHR control (b), and SHR treated with PRO at doses of 1 mg/kg (c), 5 mg/kg (d), 50 mg/kg (e), or 100 mg/kg (f). B, BV/TV. C, Tb.N. Values are the means ± S.E.M. (n = 8). *p < 0.05, **p < 0.01, and ++++, p < 0.001, statistically different from SHR control, according to Tukey’s multiple comparison test.
were attenuated in SHR treated with PRO at 10 mg/kg, the highest dose used in experiment 2.

**Effect of PRO on the Biomechanical Property of the Lumbar Vertebra in SHR.** The compression test of the lumbar vertebra (L6) showed that the biomechanical property parameters of strength and toughness were lower in the SHR control (strength: −26% \( p < 0.001 \); toughness: −35% \( p < 0.05 \), versus WKY) compared with WKY (Fig. 5). These parameters in L6 vertebra were statistically increased by PRO treatment at doses of 0.1 and 1 mg/kg but not 10 mg/kg. No statistical differences were observed in the lumbar mechanical property parameters of the modulus of elasticity and ductility among all groups.

**Effect of PRO on the Histomorphometry Parameters of the Lumbar Vertebra in SHR.** As shown in Fig. 6A, the distance between the two labeling lines of calcine and tetracycline on the fluorescent micrographs of the lumbar vertebrae was obviously narrow in SHR control in comparison with that in WKY and expanded in SHR treated with PRO at a dose of 1 mg/kg. The values of MAR and BFR/BS, indices of bone formation in SHR control, were statistically lower than that in WKY (Fig. 6, B and C). Both MAR and BFR/BS were increased by PRO treatment at a dose of 1 mg/kg (MAR: +169% \( p < 0.01 \); BFR/BS: +356% \( p < 0.05 \), versus SHR control). In the micrographs of TRAP-stained histological sections of the lumbar vertebrae (Fig. 7A), red-stained osteoclasts were observed on the surface of trabecular bone. Oc.N/BS and Oc.S/BS were higher in SHR control than WKY (Fig. 7, B and C). Both Oc.N/BS and Oc.S/BS were decreased in SHR treated with PRO at doses of 0.1 and 1 mg/kg (Oc.N/BS: −63% \( p < 0.001 \) and −82% \( p < 0.001 \); Oc.S/BS: −70% \( p < 0.001 \) and −89% \( p < 0.001 \) versus SHR control), dose-dependently; however, the degree of decreases in Oc.N/BS and Oc.S/BS was reduced in...
SHR treated with PRO at 10 mg/kg (Oc.N/BS: \(-55\% < 0.001\); Oc.S/BS: \(-68\% < 0.001\) versus SHR control).

**Effect of PRO on Plasma Biochemical Parameters in SHR.** As shown in Fig. 8, the concentration of osteocalcin in plasma was decreased in the SHR control (\(-30\% < 0.001\) versus WKY) and increased by PRO treatment at doses of 0.1, 1, and 10 mg/kg (\(+38\% < 0.001\), \(+35\% < 0.001\), and \(+31\% < 0.001\) versus SHR control). In contrast, the TRAP 5b level was increased in the SHR control (\(+29\% < 0.05\) versus WKY) and decreased by PRO treatment at doses of 0.1 and 1 mg/kg (\(-31\% < 0.001\) and \(-30\% < 0.01\) versus SHR control) but not at 10 mg/kg.

**Effects of Various \(\beta\)-Blockers on Bone Metabolism in SHR.** In experiment 3, the effects of various \(\beta\)-blockers, such as PRO, timolol, and butoxamine, on bone metabolism were examined in SHR. As shown in Fig. 9, treatment with PRO, timolol, or butoxamine at a dose of 1 mg/kg improved bone loss in SHR (BV/TV: \(+18\% < 0.05\), \(+18\% < 0.05\), and \(+25\% < 0.01\); Tb.N: \(+17\% < 0.05\), \(+15\% < 0.05\), and \(+20\% < 0.01\) versus SHR control) with an increased osteocalcin concentration and decreased TRAP 5b level in plasma.

**Discussion**

In the present study, we demonstrated that long-term treatment with low doses of PRO (0.1 and 1 mg/kg p.o.) prevented bone loss and biomechanical fragility developed in SHR without affecting blood pressure.

Evidence in experimental studies has suggested that bone metabolism is under \(\beta\)-adrenergic control and the sympathetic nervous system is involved in the development of bone loss via \(\beta_2\)-AR in osteoblastic and osteoclastic cells (Togari et al., 2005; Togari and Arai, 2008). In clinical studies, the potential of \(\beta\)-blockers as therapeutic drugs for osteoporosis, fracture healing, and hypertension has been demonstrated (Pasco et al., 2004; Schlienger et al., 2004; Graham et al., 2008).
In the literature, the antiosteoporotic effect of PRO was demonstrated recently in OVX rats treated with PRO at low doses (0.1–5 mg/kg/day) subcutaneously (Bonnet et al., 2006, 2008), whereas the hypotensive effect of PRO was demonstrated previously in SHR treated orally with high-dose PRO (30–100 mg/kg/day) (Takeda and Bugia, 1980; Antonacchio et al., 1986), suggesting that relatively low doses of PRO were adequate for antiosteoporotic action and high doses were adequate for antihypertensive action in rats. Therefore, we examined in experiment 1 the effects of PRO at a wide range of doses (1, 5, 50, and 100 mg/kg) on blood pressure and bone mass in SHR. Compared with normotensive WKY, SHR control was significantly lower in trabecular bone volume of the L2 vertebra and the proximal tibia (Figs. 2 and 3), which is consistent with previous observations that osteoporosis is developed in SHR (Izawa et al., 1985; Barbagallo et al., 1991; Wang et al., 1993). Treatment of SHR with PRO at low doses (1 and 5 mg/kg) increased BV/TV of the L2 vertebra and proximal tibia, but did not improve hypertension (Figs. 1–3). In contrast, PRO at high doses (50 and 100 mg/kg) reduced blood pressure, but did not increase bone mass. These results suggested that the presence of hypertension is not a prerequisite for the development of osteoporosis induced in SHR, and conversely, the improvement of hypertension is not a prerequisite for the antiosteoporotic effect of PRO.

In experiment 2, biomechanical parameters of strength and toughness of the lumbar vertebrae and bone mass were decreased in SHR compared with WKY (Figs. 4 and 5), suggesting an increase in the fragility of the cancellous bone of SHR. Treatment of SHR with PRO at 0.1, 1, and 10 mg/kg increased BV/TV and Tb.N of the L2 vertebra compared with the SHR control; however, these effects were attenuated in PRO at 10 mg/kg. Biomechanical parameters of strength and toughness were increased in the lumbar vertebra from SHR treated with PRO at 0.1 and 1 mg/kg, but not 10 mg/kg. Histomorphometry analysis showed that SHR decreased the values of MAR and BFR/BS and increased the values of Oc.N/BS and Oc.S/BS, suggesting that bone loss was induced in SHR by decreasing bone formation and increasing bone resorption (Figs. 6 and 7). The results also demonstrated that PRO treatment increased bone formation (1 mg/kg) and decreased bone resorption (0.1, 1, and 10 mg/kg) significantly in SHR. Furthermore, analysis of plasma biochemistry showed that the osteocalcin level (bone formation marker) was significantly increased by PRO at 0.1, 1, and 10 mg/kg compared with the SHR control, whereas the TRAP 5b level (bone resorption marker) was decreased by PRO at 0.1 and 1 mg/kg (Fig. 8). The bulk of the results in experiment 2 suggested that SHR exhibited bone loss and increased fragility in cancellous bone associated with decreased bone formation and increased bone resorption, and that treatment of SHR with PRO at low doses (0.1 and 1 mg/kg) improved bone status by increasing bone formation and decreasing bone resorption. The antiosteoporotic effect of PRO was attenuated at a dose of 10 mg/kg.

It has been also mentioned that the membrane-stabilizing action of PRO might be involved in controlling bone metabolism. In organ culture, PRO and membrane-stabilizing local anesthetics have been shown to inhibit parathyroid hormone-induced bone resorption (Dietrich et al., 1979). In vivo, Minkowitz et al. (1991) demonstrated in rat that treatment with low doses of PRO (50 and 100 μg/350 g i.p. per rat) might stimulate bone formation in femur through β-adrenergic or membrane-stabilizing mechanisms. In the present study, microarchitecture parameters of the lumbar vertebra were increased in SHR treated with PRO, timolol (nonselective β-AR blocker without membrane-stabilizing action), and butoxamine (selective β2-AR blocker) at 1 mg/kg with increased osteocalcin concentration and decreased TRAP 5b level in plasma (Fig. 9). Considering that only β2-AR was expressed in osteoblasts and osteoclasts (Moore et al., 1993; Togari et al., 1997; Togari, 2002; Arai et al., 2003), these results suggested that the antiosteoporotic effect of β-blockers seems to be caused by the blocking action of β2-AR, regardless of membrane stabilization.

In the present study, the antiosteoporotic effects of PRO were marked by the administration of low doses of 0.1 and 1 mg/kg and moderate by doses of 5 and 10 mg/kg, but they were not shown by doses of 50 and 100 mg/kg. These results were consistent with the report of Bonnet et al. (2006) demonstrating that OVX-induced bone loss was prevented by treatment with PRO at 0.1 and 5 mg/kg s.c. for 10 weeks, but not at 20 mg/kg. OVX rats are a well known high-turnover model for postmenopausal osteoporosis (Wronska et al., 1988). OVX rats showed increases in both bone formation and resorption with unbalanced activities (bone formation < bone resorption). In contrast, SHR showed increased bone resorption and decreased bone formation, suggesting the coupling gap in bone turnover. However, these observations in SHR were not contradicted by previous in vivo evidence demonstrating that activation of the sympathetic nervous system increased bone resorption and decreased bone formation via β2-AR (Takeda et al., 2002; Kondo and Togari, 2003). In the present study and that by Bonnet et al. (2006) similar dose effects of PRO on bone metabolism were observed in SHR and OVX rats, suggesting that β2-AR-mediated pathways might be involved in the development of osteoporosis in OVX rats. Although the reason low, but not high, doses of PRO showed an antiosteoporotic effect on both SHR and OVX rats is not known; similar dose-dependent effects of β-blockers were found on sensory nerve activity in SHR (Okajima et al., 2004). That study demonstrated in SHR that the release of calcitonin gene-related peptide from the capsaicin-sensitive sensory nerve was increased by intravenous injection of β-AR blockers, such as carvedilol (nonselective β-AR blocker) and ICI 118551 [3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol] (selective β2-AR blocker) at a low dose (0.25–0.3 mg/kg) but not at a high dose (1 mg/kg). Although we can not simply compare the acute effect of β-blockers and the chronic effect of β-blockers, it is interesting that the effects of low-dose β-blockers, including a selective β2-AR blocker, were not shown at a high dose in SHR. Additional studies are needed to elucidate the differences in the effects of low-dose and high-dose β-blockers on bone metabolism and differences in the antiosteoporotic and antihypertensive actions of β-blockers.

In conclusion, low doses of PRO (0.1 and 1 mg/kg p.o.) prevented bone loss and biomechanical fragility developed in SHR, suggesting that PRO can be a candidate therapeutic drug for osteoporosis with hyperactivity of the sympathetic nervous system.
References


Address correspondence to: Akifumi Togari, Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan. E-mail: togari@dc.aichi-gakuin.ac.jp