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Short communication

L-arginine prevents bone loss and bone collagen breakdown in cyclosporin A-treated rats.

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Abstract

Cyclosporin A is implicated in the pathogenesis of post-transplantation bone disease. Because of recent evidence that cyclosporin A may cause renal and cardiovascular toxicity by inhibiting nitric oxide (NO) activity, and that NO slows bone remodeling and bone loss in animal and human studies, we investigated a possible link between NO production and beneficial effects on bone health in cyclosporin A-treated rats. Thirty-six 10-week-old male rats were assigned to six groups of six animals each, and treated for 4 weeks with: vehicle; cyclosporin A; L-arginine; N^G-nitro-L-arginine methylester (L-NAME, a general inhibitor of NO synthase activity); a combination of cyclosporin A + L-arginine; and a combination of cyclosporin A + L-NAME. Whole body and regional (spine and pelvis) bone mineral content of rats were measured under basal conditions and at the end of the treatment period by dual-energy X-ray absorptiometry (DXA) scanning. Femur weights and serum concentrations of pyridinoline, a reliable marker of bone resorption, were measured at the end of the study period. Cyclosporin A-, L-NAME-, and cyclosporin A + L-NAME-treated rats had significantly lower bone mineral content and femur weights, and significantly higher pyridinoline levels than did control animals. The administration of L-arginine appeared to prevent bone loss caused by cyclosporin A, suggesting that this amino acid, which can be converted to produce NO, might prove useful in preventing disturbed bone modeling and inhibition of bone growth associated with cyclosporin A therapy.

Keywords: Cyclosporin A; Osteopenia; Arginine; Pyridinoline; (Rat)

1. Introduction

Cyclosporin A is a potent immunosuppressive agent that is widely used in the therapy of organ rejection in post-transplantation patients, and in the treatment of several autoimmune diseases. Unfortunately, this agent has major adverse effects, one of which is a dose- and duration-dependent high-turnover osteopenic state (Cvetkovic et al., 1994).

Despite the considerable attention that cyclosporin A's effects on bone metabolism have received, the mechanism by which cyclosporin A induces bone loss is not clearly understood.

A recent study by Oriji and Keiser (1998) has shown that the development of arterial hypertension associated to cyclosporin A treatment is characterized in the rat by inhibition of endothelial nitric oxide (NO) activity, an effect which can be overcome by parenteral administration of L-arginine. Moreover, it is well known that NO is a key signaling molecule in bone also, being produced by bone cells in response to various stimuli (Evans and Ralston, 1996).

This study aimed to investigate further the skeletal effects of cyclosporin A in the rat, endeavoring to determine whether L-arginine (a basic amino acid which can be converted to produce NO) could help maintain bone mass in cyclosporin A-treated animals.

2. Material and methods

Thirty-six 10-week-old Sprague–Dawley male rats, each weighing approximately 220 ± 20 g were used in this study. All animals were housed under similar conditions

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(12/12 h light/dark cycle, ambient temperature 22°C) and maintained on a standard diet containing 0.97% Ca^{2+}, 0.85% phosphorus, and 1045 IU/kg of vitamin D_3, and deionized water ad libitum. All procedures conformed with the guidelines of the Animal Care and Use Committee of Catania University. Rats were randomly divided into six groups of six animals each and treated for 28 days to receive daily: (1) cyclosporin-A (Sandoz Pharmaceutical) 15 mg/kg i.p.; (2) L-arginine (Sigma) 10 mg/kg i.p.; (3) N^G-nitro-L-arginine methylester (L-NAME) (Sigma) 50 mg/kg s.c.; (4) cyclosporin A 15 mg/kg i.p. + L-arginine 10 mg/kg i.p.; (5) cyclosporin A 15 mg/kg i.p. + L-NAME 50 mg/kg s.c. Rats in the group (6) received a weekly subcutaneous injection of 100 μl of sesame oil (vehicle), and were used as controls. The cyclosporin A dose was based on previous studies showing that cyclosporin A at 15 mg/kg caused high-turnover osteopenia in the rat (Movsowitz et al., 1988; Bowman et al., 1996). The L-NAME dose of 50 mg/kg/day s.c. is currently used in rat studies to produce general inhibition of nitric oxide synthase (NOS) activity (Wimalawansa et al., 1996).

Bone mineral content was determined in the whole body (mainly cortical bone), at the spine and at the pelvis (mainly trabecular bone) using a Norland XR36 dual energy X-ray absorptiometry (DXA) bone scanner (Norland, USA) at the beginning and after 28 days of treatment. The instrument was adapted for an ultrahigh resolution mode using the XR-Series Small Subject Scan software provided by Norland. The in vivo precision for the total subject (measured at 0.5×0.5 resolution) is 3% for bone mineral content, and accuracy was within 1.0% of the industry standard. The animals were anesthetized beforehand by i.p. injection of ketamine HCl (40 μg/kg body weight). Total body, spine and pelvis bone mineral content were expressed as milligrams, and the mean values were calculated. Percent changes of bone mineral content (mean ± S.E.) vs. control values were also calculated. Body weights were recorded at the beginning and at the end of the 4 weeks of treatment.

At the end of the study, the animals were killed and the mean weights of the dissected femurs (cortical bone) were measured.

Serum samples were obtained at the end of the study, and analyzed for Ca^{2+} (flame photometry), creatinine, alkaline phosphatase (standard methods), and pyridinoline crosslinks, a specific marker of collagen breakdown (Ureña et al., 1995). Serum pyridinoline was measured with a competitive enzyme immunoassay (using reagents provided by Metra Biosystems (Mountain View, CA). Binding to deoxypyridinoline was negligible. Serum pyridinoline expected values in normal rats are in the range of 5.2–7.1 pmol/ml.

2.1. Statistical analysis

All data were obtained with paired controls. Values are expressed as means ± S.E. Comparison between different treatment groups were made using the analysis of variance (ANOVA), followed by multiple comparison of the means using Dunnet's multiple comparison test. A value of $P < 0.05$ was considered significant.

3. Results

There were no significant differences in the baseline bone mineral content, at the three sites measured, among the six groups of rats studied (mean ± S.E.). The changes in body weight of rats in each group is shown in Table 1. As expected, control rats increased the body weight throughout the study. Although food intake was almost identical in all groups over the whole study, there was a tendency to lose some weight in cyclosporin A + L-Name-treated rats. Rats receiving cyclosporin A alone, arginine alone, L-NAME alone, or cyclosporin A + arginine showed an increase in body weight similar to that observed for the controls.

The changes in bone mineral content 4 weeks after each treatment at each site measured are shown in Fig. 1. For between-group comparison the changes in bone mineral content were calculated against those of the controls. Control rats gained bone at each site, whereas the cyclosporin A-treated group showed a lower increase in bone mineral content. Treatment with L-NAME led to a smaller increase

Table 1
Body weight, weight gain (or loss), femur weight, and serum biochemical data (mean ± S.E.) after 4-week treatment of rats with: CsA = cyclosporin A; LN = N^G-nitro-L-arginine methylester (L-NAME); A = arginine; CsA + LN = combination of cyclosporin A + L-NAME; CsA + A = combination of cyclosporin A + arginine

	Controls	CsA	LN	CsA + LN	CsA + A	A
Body weight (g)	340 ± 26	355 ± 28	348 ± 31	222 ± 36[a]	320 ± 22	370 ± 39
Weight gain (or loss) (g/4 weeks)	115 ± 12	135 ± 14	118 ± 10	−8 ± 4[a]	96 ± 18	145 ± 12
Serum Ca^{2+} (mmol/l)	2.56 ± 0.06	2.51 ± 0.05	2.50 ± 0.05	2.58 ± 0.06	2.54 ± 0.06	2.56 ± 0.05
Serum creatinine (μmol/l)	68.7 ± 3.6	68.1 ± 3	67.2 ± 3.8	71.4 ± 3.9	69.2 ± 4.1	68.8 ± 3.2
Alkaline phosphatase (U/l)	134 ± 11.4	128 ± 8.2	132 ± 6.6	138 ± 10.2	120 ± 11.6	110 ± 8.6
Serum pyridinoline (pmol/ml)	5.4 ± 0.3	7.8 ± 0.7[a]	5.2 ± 0.3	8.9 ± 0.7[a]	6.1 ± 0.9	5.8 ± 0.5
Femur weight (mg)	525 ± 22	272 ± 14[a]	284 ± 16[a]	208 ± 12[a]	532 ± 18	523 ± 24

[a] $P < 0.05$ vs. controls.

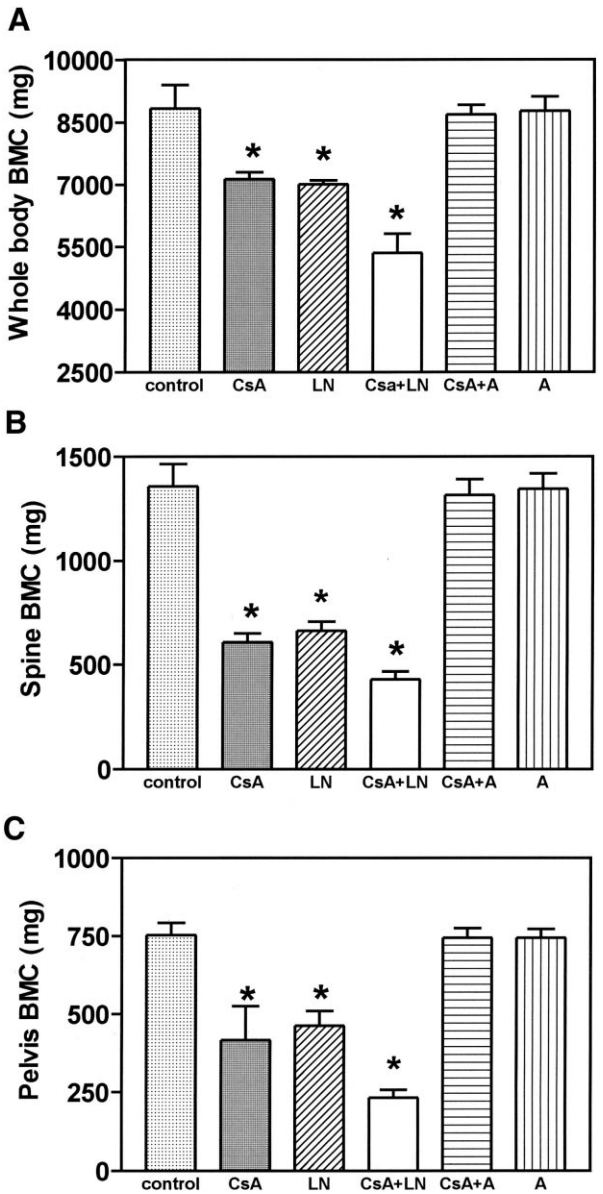

Fig. 1. Bone mineral content (BMC) over the 4 week treatment period in the various treatment groups: control; CsA = cyclosporin A; LN = N^G-nitro-L-arginine methylester (L-NAME); A = arginine; CsA + LN = combination of cyclosporin A and L-NAME; CsA + A = combination of cyclosporin A and arginine; in (A) whole body, (B) spine, and (C) pelvis. Values are means ± S.E. = $^*P < 0.05$ for change in BMC at that site compared with the change in control animals.

in bone mineral content than in the controls ($P < 0.05$). Supplementation with arginine prevented the cyclosporin A-induced bone loss. The addition of L-NAME to cyclosporin A further lowered bone accumulation in the whole body, spine, and pelvis ($P < 0.05$ vs. controls).

Arginine alone had no significant effect on the bone mineral content, compared to that of the controls.

Femoral weights in cyclosporin A- and L-NAME-treated rats were significantly lower compared to those in the controls ($P < 0.02$). The cyclosporin A-induced lowering of femoral weight was prevented by arginine. The serum values for Ca^{2+}, creatinine and alkaline phosphatase were not significantly altered by the treatments. Only rats treated with cyclosporin A and with a combination of cyclosporin A + L-NAME showed a significant increase in pyridinoline serum levels (Table 1).

4. Discussion

This study confirmed previous findings of an osteopenic state, involving both cortical and trabecular bone, induced by cyclosporin A administration to mature rats at a 15 mg/kg daily dose for 4 weeks. This is not due to impaired bone growth or loss of body weight. This experiment, like previous studies (Erben et al., 1998), did not show alterations in serum Ca^{2+}, creatinine and alkaline phosphatase. Although pyridinoline is released more generally by remodeling tissues, the significant increase of pyridinoline serum levels in cyclosporin A-treated animals is consistent with the histomorphometric observation that the deleterious skeletal effects of cyclosporin A are mediated by an increase in bone resorption (Movsowitz et al., 1988). Our data support the hypothesis that cyclosporin A-induced bone loss is a consequence of alteration in L-arginine metabolism, possibly through the NOS enzymes. Drugs that increase nitric oxide levels alleviate ovariectomy-induced bone loss (Wimalawansa et al., 1996), prevent corticosteroid-induced osteopenia in the rat (Wimalawansa et al., 1997), protect post-menopausal women against low bone mineral density by slowing bone resorption (Jamal et al., 1998), and enhance fracture healing (Diwan et al., 2000). Cyclosporin A inhibits endothelial NO activity, with a resulting increase in mean arterial pressure and tension (Oriji and Keiser, 1998), and causes a decrease in glomerular filtration rate (Assis et al., 1997). Both these untoward actions of cyclosporin A can be overcome in the rat by parenteral (Oriji and Keiser, 1998) and oral (Assis et al., 1997) administration of L-arginine. Cyclosporin A itself appears to interfere specifically with the intracellular pathways involved in the action of inducible NO synthase (iNOS) (Trajkovic et al., 1999). In view of this evidence, it is tempting to speculate that cyclosporin A may mediate its osteopenic effect by interfering with NO activity in the bone microenvironment. Our data appear to support this hypothesis, since the observed cyclosporin A-induced disturbance of modeling and remodeling (or focal imbalance of bone formation and resorption during growth) is prevented by the administration of L-arginine. It is also possible that cyclosporin A alters L-arginine uptake or metabolism, which would mimic the effect of L-NAME because it reduces the amount of L-arginine for conversion. The fact that L-NAME and cyclosporin A effects were additive would support this possibility. As evidence from in vitro studies has shown that the effects of NO on bone resorption are biphasic, with increased bone resorption at lower concentrations and inhibition at higher concentra-

tions (Evans and Ralston, 1996), it is likely that L-arginine (substrate of NO) is delivered at the optimal concentration to the skeletal sites.

The present study did not address the likely mechanism(s) by which cyclosporin A induces osteopenia in vivo, nor which step(s) of the putative mechanism would be sensitive to NO action. It is possible that NO delivered to bone cells may represent an important regulator of bone balance, especially under pathological conditions (Wimalawansa et al., 1997). Our findings may however have potential clinical significance for the prevention of cyclosporin A-induced bone loss.

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