

# The mechanism of bone resorption by cyclosporin: Involvement of the NO-cGMP pathway

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## Abstract

Treatment with cyclosporin A (CsA) following solid organ transplantations such as heart or liver generally results in bone loss. However, in vitro studies show that CsA inhibits bone resorption. Our previous in vivo animal studies demonstrated that the effects of nitric oxide (NO) on bone are biphasic; at high doses, NO increases bone resorption. In this study, we have examined in an in vitro setting to determine whether the bone loss caused by CsA administration is dependent on the NO-cyclic guanosine monophosphate (cGMP) pathway. Freshly isolated osteoclast-rich neonatal rat long bone marrow cells were added to 100  $\mu$ M thick dentin sections that had been seeded with neonatal-rat calvarial osteoblasts. These co-cultures were maintained for 48 hrs in a basal medium with CsA (1, 5, and 10  $\mu$ g/ml), both alone and with either L-Arginine (NO substrate; 10-3M), L-NAME (NO synthase enzyme inhibitor; 10-4M), or the combination of the two. The cultures were then fixed in cold 95% ethanol and stained with tartrate resistant acid phosphatase (TRAP) to identify osteoclasts and sites of osteoclastic resorption. Preparations were analyzed using an automated histomorphometry software package. Scanning electron microscopy affirmed that the areas identified by light microscopy as resorption sites contained osteoclastic lacunae. CsA inhibited bone resorption dose-dependently. CsA at 10  $\mu$ g/ml produced a 90% inhibition of bone resorption (control =  $5.5 \pm 2.0\%$ ; CsA =  $0.64 \pm 0.09\%$ ). L-Arginine reversed this inhibition by 90% (Arg + CsA =  $4.23 \pm 1.5\%$ ; CsA =  $0.64 \pm 0.09\%$ ). The application of NOS inhibitor L-NAME inhibited bone resorption by 87% (Arg + CsA + L-NAME =  $0.55 \pm 0.14\%$ ; Arg + CsA =  $4.23 \pm 1.5\%$ ). We conclude that NO-cGMP pathway is involved in the CsA induced bone loss.

**Keywords:** Cyclosporin A (CsA), Bone Loss, Nitric Oxide-cGMP

## Introduction

Osteoporosis and its resulting fractures are often encountered in patients receiving cyclosporin A (CsA) for immunosuppression following solid organ transplantations. In vivo rat studies confirm these deleterious effects of CsA on bone<sup>1,2</sup>. On the other hand, in vitro studies show that CsA inhibits bone resorption<sup>3-5</sup>. Our previous in vivo animal studies demonstrated that the effects of nitric oxide (NO) on bone are biphasic; at high doses, NO increases bone resorption<sup>6,7</sup>. This suggested that the variable effects of CsA might be due to evoked changes in the NO-cGMP pathway. In the current study, we have extended these observations to an in vitro setting to determine whether the bone loss caused by CsA administration might be similarly tied to the NO pathway.

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## Materials and Methods

### Osteoblast culture

Osteoblasts were isolated from the calvaria of 1-day old Sprague-Dawley rats by 6 sequential 20 min digestions in  $Ca^{++}$  and  $Mg^{++}$  free collagenase solution. This procedure was repeated six times and the cells from the last three digestions were pooled and cultured in  $\mu$ -MEM containing 10% FBS and antibiotics.

### Isolation and culture of osteoclasts

Newborn Wistar rats were sacrificed by cervical dislocation and their femora and tibiae were removed. The diaphyses were opened with a scalpel, and the tissues were suspended in HEPES-buffered Medium 199 supplemented with 10% FCS (v/v). Osteoclasts were mechanically desegregated by curetting the bones, agitating the suspension, and sedimenting cells on 22 mm, 0 grade glass coverslips. Osteoclasts were preferentially adherent to the

coverslips and the other cells were washed away. These coverslips were placed in multi-well plates with Medium 199 containing 10% FCS (v/v) and incubated for 20 min at 37 °C.

#### Preparation of dentin slices

Porpoise teeth obtained from the Galveston Stranded Mammal Program (Texas A&M University) were sectioned transversely at 100 µm, and were sonicated and sterilized. Four sections were used for each experimental modality.

#### Effect of NO donor on CsA-induced inhibition of osteoclastic dentin resorption

The freshly isolated osteoclasts were added to osteoblast coated dentin slices and cultured for 48 hrs in a basal medium consisting of µ-MEM, 5% FBS, and 1% antibiotics (Penstrep).

CsA was applied to the co-cultures at concentrations of 1, 5, and 10 µg/ml; the NO substrate L-Arginine was applied at 10-3M.

The cultures were then fixed in cold 95% ethanol and stained for TRAP to identify osteoclasts and sites of osteoclastic resorption. A histomorphometric software package (Optimas Corp., Bothell, WA) that projected a grid with points at 125 µm intervals (magnified x 65) quantitated the percentage of resorption.

#### Effect of NOS inhibitor L-NAME on reversal of CsA-induced inhibition of dentin resorption

Identical protocol to the one described above was performed with the exception of the addition of the NOS inhibitor L-NAME (10-4M). Sections were fixed as described above with 95% ethanol.

Group	CsA dose	Mean ± SEM
Control	0	5.50 ± 2.00
Cyclosporin A [CsA]	1 µg/ml	4.97 ± 1.85
	5 µg/ml	1.44 <sup>‡</sup> ± 0.22
	10 µg/ml	0.64 <sup>‡</sup> ± 0.09
Arginine	3 mM	4.60 <sup>†</sup> ± 1.26
Arginine + CsA	1 µg/ml	12.5 <sup>‡</sup> ± 3.00
	5 µg/ml	6.60 ± 1.40
	10 µg/ml	4.23 <sup>‡</sup> ± 1.50
Arginine + CsA + L-NAME <sup>‡</sup>	1 µg/ml	4.40 ± 2.20
	5 µg/ml	1.10 <sup>‡</sup> ± 0.35
	10 µg/ml	0.55 <sup>‡</sup> ± 0.14

<sup>†</sup>Arginine = 3 mM

<sup>‡</sup>L-NAME = 10-4M

<sup>‡</sup>p<0.05 vs PBS Control

**Table 1:** Effect of CsA on percent dentin resorption in osteoblast-osteoclast co-cultures (n=4 per group). PBS=phosphate buffered saline.

#### Statistical analysis

Results are expressed as mean ± SEM. Comparisons between different treatment groups were made using ANOVA with Students' t-test. P values <0.05 were considered statistically significant.

## Results

The effects of CsA and the various treatment protocols on dentin resorption are summarized in Table 1. CsA attenuated bone resorption in a dose-dependent manner. The percentage of resorption at CsA concentration of 1 µg/ml was not different from those seen in the control. At 5 and 10 µg/ml, however, CsA significantly inhibited dentin resorption. Arginine by itself had little if any effect on dentin resorption.

On the other hand, the CsA-induced inhibition of dentin resorption was antagonized by L-Arginine. Increased concentration of CsA dose-dependency overcame the effects of L-Arginine. Furthermore, blocking NO generation with L-NAME (with or without Arginine) increased dentin resorption. Arginine was able to maintain dentin resorption by osteoclasts at low concentration of CsA (1 µg/ml), but it could not overcome the effects of CsA at its higher concentrations (>5 µg/ml).

## Discussion and Conclusions

First, the concentrations of arginine normally present in the culture medium (0.9 mM) may have been able to inhibit low concentration CsA-induced dentin resorption. Second, the NOS-inhibitor L-NAME was able to obviate the effects of arginine. Therefore, it is possible that CsA inhibits osteoclastic resorptive activity at least in part via the NO pathway. The addition of L-NAME negated the effects of L-Arginine, thereby exhibiting the same inhibition of resorption as demonstrated by the application of only CsA. These experiments test the hypothesis that calcemic hormones are required to demonstrate the bone resorptive effects of CsA in vitro<sup>8</sup>. This study demonstrated that in vitro, the bone resorptive effective of CsA might seldom be evident. It has been proposed that the bone resorptive system requires a repletion of endocrine system with reference to calcemic hormones (i.e., PTH and vitamin D)<sup>9</sup>. Our observations suggest that the CsA-mediated dentin (bone) resorption involves the NO-cGMP pathway. CsA-induced bone resorption is facilitated in in vitro culture media and in vivo situations, because of the presence of high concentrations of NO substrate.

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