Effect of endothelin receptor antagonist on parathyroid gland growth, PTH values and cell proliferation in azotemic rats

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Abstract

Background. A variety of stimuli are involved in the pathogenesis of parathyroid gland hyperplasia in renal failure. Recently, it was shown that blocking the signal from the endothelin-1 (ET-1) receptor (ETAR/ETBR) by a non-selective receptor antagonist, bosentan, reduced parathyroid cell proliferation, parathyroid gland hyperplasia and parathyroid hormone (PTH) levels in normal rats on a calcium deficient diet. Our goal was to determine whether in 5/6 nephrectomized (NPX) rats with developing or established hyperparathyroidism, the endothelin receptor blocker, bosentan, reduced the increase in parathyroid cell proliferation, parathyroid gland hyperplasia and PTH values.

Methods. High (HPD, 1.2%) or normal phosphorus diets (PD) (NPD, 0.6%) were given to 5/6 NPX rats for 15 days (NPX15). In each dietary group, one-half the rats were given bosentan (B) i.p. 100 mg/kg/day. The four groups of rats were: (1) NPX15-1.2% P; (2) NPX15-1.2% P+B; (3) NPX15-0.6% P; and (4) NPX15-0.6% P+B. In a second study in which hyperparathyroidism was already established in 5/6 NPX rats fed a HPD for 15 days, rats were divided into two groups in which one group was maintained on a HPD and the other group was changed to very low PD (VLPD, < 0.05%) for an additional 15 days. In each dietary group, one-half the rats were given bosentan i.p. 100 mg/kg/day. The four groups of rats were: (1) NPX30-1.2% P; (2) NPX30-1.2% P+B; (3) NPX30-0.05% P and (4) NPX30-0.05% P+B. Parathyroid cell proliferation was measured by proliferating cell nuclear antigen (PCNA) staining and ET-1 expression by immunohistochemical techniques.

Results. In the study of developing hyperparathyroidism, bosentan reduced ET-1 expression in the parathyroid glands of rats on the NPD and HPD (P<0.05). But only in rats on the NPD did bosentan result in a reduced increase in parathyroid gland weight (P<0.05). In the study of established hyperparathyroidism, in which 5/6 NPX rats were given a HPD for 15 days, bosentan started on day 15 reduced (P<0.05) ET-1 expression in rats maintained for 15 additional days on the HPD or the VLPD. On the VLPD, parathyroid gland weight was less (P<0.05) than that in rats on the HPD sacrificed at 15 or 30 days. Bosentan did not reduce parathyroid cell proliferation or parathyroid gland weight in rats maintained on the HPD or further reduce these parameters beyond that obtained with dietary phosphorus restriction. PTH values were lowest in the VLPD group, intermediate in the NPD group, and highest in the HPD group, but in none of the three groups did bosentan decrease PTH values.

Conclusions. In azotemic rats with developing hyperparathyroidism, bosentan resulted in a reduced increase in parathyroid gland weight when dietary phosphorus content was normal. Despite a reduction in ET-1 expression in rats on a HPD with developing or established hyperparathyroidism, bosentan did not reduce the increase in parathyroid cell proliferation, parathyroid gland growth or PTH values. Thus, ET-1 blockade with bosentan did not prevent parathyroid gland growth in the azotemic rat.

Keywords: bosentan; dietary phosphorus; endothelin; parathyroid hyperplasia
Introduction

Endothelin-1 (ET-1) was first isolated in 1988 from cultured porcine aortic endothelial cells and its major action was observed to be a potent vasoconstrictor and pressor peptide [1]. However, ET-1 was subsequently reported to exert biological effects on hormonal cells, including inhibition and stimulation of hormones [2]. Furthermore, ET-1 has been shown to be a mitogenic factor functioning in an autocrine/paracrine manner in a number of different cell lines [3,4]. ET-1 interacts with two homologous G-protein coupled receptors, endothelin A receptor (ET_{A}R) and endothelin B receptor (ET_{B}R). Both endothelin receptors are expressed in the bovine and human parathyroid gland [5]. Fujii et al. showed that ET-1 increased parathyroid hormone (PTH) release in a dose-dependent manner in bovine parathyroid cells [6]. Furthermore, they also reported that the rat parathyroid cell lines expressed only ET_{A}R. As a result of these in vitro studies, Kanesaka et al. [7] studied the effect of endothelin blockade in the normal rat maintained on a calcium deficient diet and showed that the non-selective endothelin receptor antagonist, bosentan, prevented or reduced the increase in parathyroid gland weight, parathyroid cell proliferation and PTH values.

Marked secondary hyperparathyroidism is a common complication in chronic renal failure and the disorder is characterized by parathyroid cell hyperplasia. Parathyroid hyperplasia appears early in renal failure [8] and results from increased parathyroid cell proliferation. Although the mechanisms involved in the pathogenesis of parathyroid hyperplasia in uremia are still incompletely understood, recent studies have suggested that phosphorus retention plays an important role in its development in azotemic patients and animals [9,10].

High dietary phosphorus in azotemic rats is known to produce severe hyperparathyroidism by decreasing the calcemic action of PTH, which in turn, increases the demand for PTH to maintain calcium homeostasis [11]. Moreover, phosphorus has been reported to directly affect PTH secretion [12] and to control the stability of PTH mRNA possibly acting via the specific nuclear protein AUF1 [13]. A high phosphorus diet (HPD) and hyperphosphataemia induce hyperparathyroid hyperplasia and conversely, a low phosphorus diet (LPD) inhibits parathyroid gland growth. Associated with the phosphorus-induced increased parathyroid cell proliferation in early renal failure has been an increased expression of transforming growth factor-alpha (TGF-α) and its receptor (EGFR) [14]. Thus, phosphorus loading in azotemic animals may act not only by decreasing the efficiency of PTH, but also as a result of a direct effect on the parathyroid cell.

The goal of the present study was to determine whether the endothelin receptor blocker, bosentan, reduced the increase in parathyroid cell proliferation, parathyroid gland hyperplasia and PTH values in 5/6 nephrectomized (NPX) rats. To evaluate endothelin receptor blockade, the effect of bosentan was studied during the development of secondary hyperparathyroidism and after secondary hyperparathyroidism was established.

Methods

Male Sprague–Dawley rats weighing 180–200 g were studied. Weights were similar among the different groups studied. Renal failure was induced by arterial ligation of two of the three hilar branches of the left main artery and 1 week later, a right nephrectomy was performed. During surgical procedures, rats were anaesthetized with intraperitoneally administered ketamine 7.5 mg/100 g (Ketaset; Fort Dodge Laboratories, Fort Dodge, IA, USA) and xylazine 0.5 mg/100 g (AnaSed; Lloyd Laboratories, Shenandoah, IA, USA). Rats were housed in individual cages, given 14 g food daily and allowed free access to water. Rats were removed from the study if they did not consume at least 90% of their diet.

Experiment 1–developing hyperparathyroidism. After the right nephrectomy, rats received either a HPD (1.2%, H) or normal phosphorus diet (NPD) (0.6%, N). Dietary calcium was 0.6%, vitamin D 100 IU/100 g and dietary protein 20% for each phosphorus diet (ICN, Cleveland, OH). In half of each group, bosentan 100 mg/kg/day was intraperitoneally administered (kindly supplied by Dr Martine Clozel, Actelion Co. Ltd., Allschwil, Switzerland). Fifteen days after the right nephrectomy, rats were sacrificed after an overnight fast. After anaesthesia, the abdomen was opened and blood was obtained from the aorta for chemistry measurements. The two parathyroid glands were then selectively removed and weighed on a microbalance (Sartorius 1712, Sartorius, Edgewood, NY) to determine the wet weight.

Experiment 2–established hyperparathyroidism. After the right nephrectomy, rats were maintained on a HPD (1.2%) for 15 days. Previous studies have shown that this diet in azotemic rats has increased PTH levels by at least ten times and produced marked hyperparathyroid hyperplasia [15–17]. At 15 days, rats were divided into two dietary phosphorus groups. One group was maintained on a HPD (1.2%) and the other group was changed to a VLPD (<0.05%). In half of each group, bosentan 100 mg/kg/day was administered intraperitoneally, and 15 days later, rats were sacrificed and the parathyroid glands removed. Sham-operated rats maintained on a NPD (0.6%) were used for comparison. In half of this group, bosentan 100 mg/kg/day was administered intraperitoneally. 15 days later, rats were sacrificed and the parathyroid glands were removed and weighed on a microbalance.

Biochemical measurements

Serum calcium and phosphorus were measured with specific kits (Sigma, St Louis, MO, USA). Serum creatinine was measured with a creatinine analyzer (Beckman, Fullerton, CA, USA). PTH was measured with an immunoradiometric assay specific for intact PTH in the rat (Nichols, San Juan Capistrano, CA, USA). The intra-assay coefficient of variation was 4%.
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**Immunohistochemistry**

For proliferating cell nuclear antigen (PCNA) and ET-1 staining, the parathyroid glands were fixed in 4% buffered formalin for 12–24 h, embedded in paraffin, cut into 5 μm sections, adhered to polylysine-coated glass and fixed overnight at 56 °C. After deparaffinizing through xylene, alcohol and distilled water, endogenous peroxidase was blocked by 3% H2O2 for 20 min. The sections were then treated in a microwave oven in a solution of 0.1 mM citrate buffer pH 6.0 for 20 min and transferred to distilled water. After rinsing in Tris-HCl-phosphate-buffered saline (TPS), the sections were incubated with 1:10 normal rabbit serum in TPS/1% BSA. The sections were then incubated overnight at 4°C with unlabelled polyclonal and monoclonal specific primary antibodies. For detection of PCNA, the sections were incubated overnight at 4°C with mouse monoclonal anti-PCNA antibody (diluted 1:400, DAKO Corp., Glostrup, Denmark). Quantification was done twice and the results were similar. The staining score is expressed as nuclei/mm².

For detection of ET-1, the sections were incubated overnight at 4°C with mouse monoclonal anti-PCNA antibody (diluted 1:400, DAKO Corp., Glostrup, Denmark). For detection of ET-1, the sections were incubated overnight at 4°C with mouse monoclonal anti-PCNA antibody (diluted 1:400, DAKO Corp., Glostrup, Denmark). After three rinses in TPS, they were incubated with streptavidin–peroxidase (Dako) 1:500 for 30 min. Colour was developed with DAB and then counterstained with haematoxylin, dehydrated and mounted with Canadian balsam (Polysciences, Inc.).

**Statistics**

Statistical analysis was performed using the software program NCSS (Kaysville, UT). The non-parametric Mann–Whitney test was used for the comparison between two groups. A P-value of <0.05 was considered to be significant. Results are shown as the mean value ± SE.

**Results**

In the study of developing hyperparathyroidism, biochemical values in the 5/6 NPX rats fed the NPD or a HPD for 15 days are shown in Table 1. As expected, rats fed the HPD had lower serum calcium and higher serum phosphorus values than rats on the NPD. Serum calcium, phosphorus and creatinine values did not differ between rats on the same diet that received vehicle or bosentan. Serum PTH levels were less in rats on the NPD than in those on the HPD. In both the NPD and HPD groups, bosentan did not reduce PTH values. Weight gain in the four groups was 15.2 ± 5.3 g (NPX-0.6% P), 33.4 ± 3.4 g (NPX-0.6% P+B), 22.4 ± 4.0 g (NPX-1.2% P) and 23.5 ± 5.1 g (NPX-1.2%P+B). The only difference was a greater weight gain in the NPX-0.6% + B group than in the NPX-0.6% P group (P < 0.05).

As shown in Figure 1, ET-1 expression in the parathyroid glands of rats on the NPD and HPD was less in the groups given bosentan. Parathyroid gland weight was less in the NPD but not in the HPD group given bosentan (Figure 2).

In the study of established hyperparathyroidism, 5/6 NPX rats were fed the HPD for the first 15 days and then divided into two groups which received either the HPD (1.2%) or VLPD (<0.05%) for the next 15 days. As expected, serum calcium values were greater and serum phosphorus values were less in rats on the VLPD (<0.05%) compared to rats on the HPD (Table 2). Serum creatinine values were not different among groups except for a lower value in the NPX-0.05% P group than in the NPX-1.2% P group. Serum PTH values were markedly reduced in rats fed the VLPD compared to rats fed the HPD. There was no difference in weight gain among the four groups: 28.5 ± 7.4 g (NPX-1.2% P), 39.0 ± 9.9 g (NPX-1.2% P+B), 44.5 ± 5.7 g (NPX-0.05% P) and 32.0 ± 6.0 g (NPX-0.05% P+B). As shown in Figure 3, there were no differences in ET-1 expression between rats fed HPD and VLPD. Bosentan similarly reduced ET-1 expression in both the HPD and VLPD groups. In 5/6 NPX rats on the HPD,
parathyroid gland weight was greater than that in sham rats fed a NPD (Figure 4). In 5/6 nephrectomized rats changed to the VLPD at 15 days, parathyroid gland weight was less than that in the group maintained on a HPD for 30 days and was not different from that in sham rats. In 5/6 NPX rats maintained on a HPD for the first 15 days, parathyroid gland weight was greater than that of the group given a VLPD from 15 to 30 days. This latter result suggests that the marked dietary phosphorus restriction decreased the parathyroid gland weight. As shown in Figure 5, bosentan did not produce any change in parathyroid gland weight in rats maintained on the HPD nor reduce parathyroid gland weight further in rats changed to the VLPD. Finally, dietary phosphorus restriction markedly decreased parathyroid cell proliferation as measured by PCNA (Figure 6). However, bosentan did not lower PCNA values in the HPD group nor did it further lower PCNA values in the group given the VLPD.

**Discussion**

Our study in azotemic rats was designed to determine whether treatment with the ET-1 receptor (ETAR/ETBR) blocker, bosentan, would reduce the magnitude of the increase in parathyroid gland weight, parathyroid cell proliferation and PTH values. Dietary phosphorus content was varied to control the stimulus for the development of hyperparathyroidism. The effect of bosentan administration was studied both during the development of hyperparathyroidism and in established hyperparathyroidism. The primary results of our study were that the bosentan-induced decrease in ET-1

![Fig. 1. Endothelin-1 (ET-1) expression in parathyroid cells from azotemic rats in -bosentan and +bosentan groups. In 5/6 NPX rats (NPX) on NPD (0.6%) and HPD (1.2%), ET-1 was significantly reduced in the +bosentan groups as compared to the -bosentan groups (P<0.05).](https://academic.oup.com/ndt/article-abstract/21/4/917/1932266/)

![Fig. 2. Parathyroid gland weight in -bosentan and +bosentan groups. Between the 5/6 NPX rats (NPX) on a NPD (0.6% P), parathyroid gland weight was reduced in the +bosentan group as compared to the -bosentan group (**P<0.05**). In the 5/6 NPX rats on a HPD (1.2% P), parathyroid gland weight was not different between the +bosentan and -bosentan groups. *P<0.05 vs NPX rats on a NPD without bosentan. #P<0.05 vs NPX rats on a NPD with bosentan.](https://academic.oup.com/ndt/article-abstract/21/4/917/1932266/)

**Table 2. Serum biochemical values in 5/6 NPX rats continued on HPD or placed on LPD for 15 days**

<table>
<thead>
<tr>
<th></th>
<th>NPX-1.2% P (n=15)</th>
<th>NPX-1.2% P+B (n=12)</th>
<th>NPX-0.05% P (n=12)</th>
<th>NPX-0.05% P+B (n=12)</th>
<th>Sham-0.6% P (n=12)</th>
<th>Sham-0.6% P+B (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium, mg/dl</td>
<td>7.21±0.29</td>
<td>8.05±0.33*</td>
<td>11.5±0.33*</td>
<td>10.9±0.17**</td>
<td>9.27±0.08</td>
<td>9.61±0.06</td>
</tr>
<tr>
<td>PO4, mg/dl</td>
<td>12.4±0.55</td>
<td>11.5±1.08</td>
<td>4.35±0.60*</td>
<td>4.30±0.47**</td>
<td>7.21±0.25</td>
<td>6.89±0.14</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>1.01±0.04</td>
<td>0.86±0.06</td>
<td>0.80±0.06*</td>
<td>0.71±0.04</td>
<td>0.38±0.01</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td>PTH, pg/ml</td>
<td>1048±79.9</td>
<td>1106±121</td>
<td>12.9±3.2*</td>
<td>12.2±1.8**</td>
<td>102±10.1</td>
<td>109±7.1</td>
</tr>
</tbody>
</table>

Mean ± SE

*a Data in sham-operated groups provided for informational purpose and statistical analysis is not applied.

*P<0.05 vs NPX-1.2% P

**P<0.05 vs NPX-1.2% P+B
expression did not prevent: (1) the increase in PTH values on both the NPD and HPD; and (2) the increase in parathyroid gland growth on the HPD, but did modestly reduce the increase in parathyroid gland weight on the NPD.

We decided to study bosentan in the azotemic rat because Kanesaka et al. [7] reported that bosentan prevented or reduced the increase in parathyroid cell proliferation, parathyroid gland weight and PTH values in normal rats maintained on a calcium deficient diet for 8 weeks. We were able to show that bosentan did reduce ET-1 staining in the parathyroid glands of azotemic rats on NPDs and HPDs. In the study in which high dietary phosphorus had already established hyperparathyroidism in azotemic rats, bosentan reduced ET-1 staining in rats maintained on the HPD and in rats changed to the phosphorus restricted diet. Thus, it was shown that bosentan did reduce ET-1 expression in the rat parathyroid gland.

Our study also showed that bosentan reduced the increase in the growth of the parathyroid gland in rats given a NPD for the first 15 days after the right nephrectomy. However, when azotemic rats were given a HPD for 15 days, the increase in parathyroid gland weight was not reduced by bosentan. After a more than doubling of parathyroid gland weight during the first 15 days, no further increase in parathyroid gland weight was seen between 15 and 30 days despite continuation of the HPD. A similar result has been reported in two previous studies [10,17]. Moreover, in an unpublished study, it was observed in azotemic rats that parathyroid gland volume increased almost linearly as PTH values increased from normal values to 500 pg/ml. However, as PTH values increased further from 500 to 2000 pg/ml, the increase in parathyroid gland volume was minimal (personal communication, John Fox and Edward Nemeth). Thus, our result that parathyroid gland weight, after an initial doubling during the first 15 days, did not increase between 15 and 30 days despite continuation of the HPD is consistent with previous observations.

In the groups sacrificed at 15 days and in those sacrificed at 30 days, the only difference in weight gain was in the two groups on a 0.6% P diet for 15 days in which the group on bosentan gained more weight. Parathyroid gland weight was factored for body weight to correct for any differences in weight gain. But it is still possible that the difference in parathyroid gland weight between the two groups on the 0.6% P diet could be because growth in body weight and growth in parathyroid gland weight was not proportional. However, this latter possibility seems remote because even when not factored for body weight, parathyroid gland weight...
Parathyroid gland weight was still less \((P<0.05)\) in the 0.6% P group on bosentan.

Fifteen days after the right nephrectomy, a group of rats, which had been on the HPD for 15 days, was placed on the phosphorus restricted diet either with or without bosentan. The presumed parathyroid gland weight at the change to the phosphorus restricted diet would be similar to that in the group on the high phosphorus sacrificed 15 days after right nephrectomy. As compared to the group on the HPD for 15 days, the parathyroid gland weight in rats on the phosphorus restricted diet was much less than the parathyroid gland weight in rats sacrificed after 15 and 30 days of the HPD. Moreover, parathyroid gland weight in rats on the phosphorus restricted diet was not different from that in sham-operated rats on a NPD. Thus, the assumption is that the phosphorus restricted diet reduced the weight of the parathyroid gland and parathyroid gland weight approached that in normal rats. To our knowledge, only one other study has shown that dietary phosphorus restriction reduces parathyroid gland weight in azotemic rats with established hyperparathyroidism after 2 weeks of a HPD [17]. In that study, the reduction in parathyroid gland weight was less than in our study probably because the dietary phosphorus content (0.2%) was greater than the <0.05% dietary phosphorus content used in our study.

The VLPD used in our study resulted in a marked reduction in PCNA positive cells, a marker of parathyroid cell proliferation. Because apoptosis was not studied, it is difficult to know whether the reduced weight of the parathyroid gland indicates a decrease in the number of parathyroid cells. It could be that cell hypertrophy and increased vascularity and interstitial fluid could have contributed more to the parathyroid weight at 15 days than in more chronic enlargement. If such were the case, then it might be possible to decrease the weight of the parathyroid gland without specifically invoking apoptosis as the cause. Thus, how 15 days of marked dietary phosphorus restriction resulted in a marked reduction of parathyroid gland weight deserves further study. Finally, in the setting of dietary phosphorus restriction, it would be difficult to determine whether bosentan had any additional inhibitory effect because of the marked suppressive effect of dietary phosphorus restriction.

In developing hyperparathyroidism, bosentan reduced the increase in parathyroid gland weight in azotemic rats given a NPD for 15 days. However, there was no reduction in PTH values. Moreover, the difference in PTH values among the different dietary phosphorus groups was much more marked than the differences in parathyroid gland weight. The increase in parathyroid gland weight of the group given a NPD for the first 15 days was similar to that in the group on a HPD even though PTH values were 50% less in the group on the NPD. Similarly, the reduction in PTH values in the groups on the...
phosphorus restricted diet as compared to the HPD, was much greater than the observed difference in parathyroid gland weight. Thus, other factors such as hypo- and hypercalcaemia and the marked differences in serum phosphorus values were presumably important modifiers of PTH values.

In summary, we have shown in 5/6 NPX rats that bosentan does reduce ET-1 expression in the parathyroid gland whether rats are on a high, normal, or low PD. In developing hyperparathyroidism, bosentan reduced the increase in parathyroid gland weight but not PTH values in rats on a NPD. In studies of developing and established hyperparathyroidism in rats on a HPD, bosentan did not reduce the increase in parathyroid gland weight or PTH values. In conclusion, ET-1 blockade with bosentan did not prevent parathyroid gland growth in the azotemic rat.

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Conflict of interest statement. The authors have no conflicts of interest.

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