Short-term androgen deprivation does not alter CaR and VDR mRNA expression in duodenal mucosa in male rats

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Androgen deprivation is associated with decline in intestinal calcium absorption. The effect of androgen on CaR and VDR intestinal mucosa has not yet been studied. Calcium homeostasis, arc bone mineral density (aBMD, dual energy X-ray absorptiometry) and expression of CaR and VDR mRNA in duodenal mucosa of orchidectomized (ORX) and sham operated (Sham) adult Sprague Dawley rats at 4 week have been studied. There was no significant difference in serum calcium, alkaline phosphatase, calcidiol and calcitriol levels between both the groups. Serum testosterone (T) (ng/dl) and inorganic phosphorous (iP) (mg/dl) levels were significantly lower in ORX rats. As compared to Sham rats, ORX rats had significant decline in in-vitro aBMD at proximal, middle and distal tibia, proximal, mid and distal femur and femoral neck (P<0.05). Northern blot analysis revealed no significant alteration in the CaR and VDR mRNA expression in duodenal mucosa in ORX rats. CaR and VDR mRNA expression in duodenal mucosa is therefore, not affected by physiological concentrations of testosterone in rats.

Hypogonadism is an important cause of male osteoporosis. It results in increased bone turnover and loss of cancellous and cortical bone. Longitudinal studies have reported age-related decline in serum total testosterone (T) in elderly men. It is firmly established that orchidectomy results in decreased bone mass in experimental animals and men. Replacement therapy with testosterone in post-pubertal and elderly hypogonadal males results in increased bone mineral density (BMD).

Androgen deficiency results in decreased net intestinal calcium absorption, which in turn, is determined primarily by vitamin D receptor (VDR) in both sexes and estrogen receptor (ER) in females. Estrogen increases the calcium absorption directly and through up regulating VDR and post-receptor events in intestinal mucosa. The interaction of androgen and VDR expression in the rat intestine has not been explored.

Role of calcium sensing receptor (CaR), which is expressed along the entire length of the gastrointestinal tract, in the intestinal calcium absorption remains unclear. No report exists regarding influence of androgen deficiency on CaR expression in intestine. Since active transcellular calcium absorption occurs mainly in duodenal mucosa, effect of androgen deprivation on CaR and VDR mRNA can be best studied in duodenum.

The present study has been carried out to study the effect of orchidectomy on mRNA levels of CaR and VDR in duodenal mucosa of male adult rats.

Materials and Methods

Sprague Dawley adult male rats (250-290 g and 1 year of age) were maintained in separate cages at 12:12 hr L:D cycles and had free access to chow diet and tap water. They were divided randomly into 2 groups (a) orchidectomized rats (ORX; n=6); orchidectomy was performed through single ventral incision under parenteral ketamine and open mask ether anesthesia; (b) sham operated rats (Sham; n=6). All the rats were given intramuscular antibiotics (cefotaxim) and analogesics (tramadol hydrochloride) postsoperatively. Institutional Ethics Committee for Animal Experiments approved the experiment protocol. The care of experimental animals conformed to Guidelines on the Handling and Training of Laboratory Animals (www.frame-UK.demon.co.UK/guidelines.htm).

Blood was collected from orbital vein in chilled tubes at baseline and 4 weeks. Serum was separated after centrifugation at 4°C and stored at 70°C till the time of assay. The animals were sacrificed at 4 weeks by decapitation. The duodenum was harvested.

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washed with ice-cold saline and phosphate buffered saline, laid open and the mucosa was gently scraped off. The tissue was frozen in liquid nitrogen and stored at -70°C for RNA extraction. In vivo BMD measurements were performed at baseline and at 4 weeks before sacrifice. The femora and tibia were dissected out for in vitro BMD measurements after sacrifice.

Biochemical and hormonal estimations — The biochemical and hormonal measurements were performed at baseline and at 4 weeks. Serum total calcium (Ca), inorganic phosphorus (IP) and alkaline phosphatase (ALP) were estimated by commercially available kits (Sigma Diagnostics, MO, USA). Serum calcidiol (RIA, Diasorin, MN, USA), calcitriol (RIA, Diasorin, MN, USA) and T (RIA, Diagnostic Systems Lab, Texas, USA) levels were measured. The sensitivity of serum calcidiol, calcitriol and T assays were 1.5 ng/ml, 4 pg/ml and 4 ng/dl, respectively.

Areal bone mineral density (aBMD) and bone mineral content (BMC) quantitation — In vivo BMC and aBMD measurements were obtained by Dual Energy X-ray Absorptiometry using small animal software (DXA; Hologic QDR 4500A, Waltham, MA, USA) at baseline and at 4 weeks of study before sacrifice. Rats were anaesthetized by open mask diethyl ether for in vivo measurements. The anaesthetized rats were placed in supine position, the appendages were secured flat to the table by a thin tape and the whole body scan of the rats was obtained. In vivo BMC and aBMD measurements were analyzed in whole body (WB), lumbar spine L1-L4 (LS), whole femur (WF) and whole tibia (WT).

For in vitro BMD measurements, after sacrifice femora and tibia were dissected out, attached soft tissue was scraped off using a scalpel and adherent fatty tissue was removed by placing bones in ethanol and diethyl ether. Individual bone was placed in water and in vitro aBMD and BMC measurements were obtained. All the measurements were obtained on the same day. Sub-regional analyses of femur were performed at femoral neck, proximal, mid and distal 1/3rd regions of femur. Similarly, the sub-regional analyses of tibia were performed at proximal, mid and distal 1/3rd regions. To assess the short-term drift of DXA measurements, two rats and two femora were measured 5 times on same day and co-efficient of variation (%CV) for in vivo and in vitro measurements, respectively, were determined. The co-efficient of variation of in vivo and in vitro measurements were 0.56 and 0.61%, respectively. DXA machine was calibrated with small animal step phantom daily to correct for long-term drift. The BMC and aBMD results are expressed in g and g/cm² respectively.

RNA Isolation — All chemicals and reagents were purchased from Sigma (St Louis, MO, USA) unless specified. Total RNA was extracted from duodenal mucosa according to a protocol for single step RNA isolation based on acid guanidium thiocyanate-phenol-chloroform extraction. Purity of total RNA was confirmed by measuring the absorbance at 260-280 nm using spectrophotometer (Pharmacia, Sweden). The 260/280 ratio of total RNA ranged from 1.9 to 2.0 indicating pure RNA.

Northern blot procedure — Total RNA sample (30μg) was denatured and size fractionated in 1% agarose-2.2M formaldehyde denaturing gel and transferred to nylon membrane (Hybond, Amersham, Inc. UK). Gel purified VDR and β-actin cDNA probe fragments were labeled by random primed synthesis with 32P-labeled deoxy-CTP to a specific activity of greater than 10⁸ cpm/μg. CaR-cDNA probe complementary to the nucleotides 779-1355 of the rat kidney CaR gene was synthesized by TransProbe T kit (Pharmacia, Piscataway, NJ) using T3 polymerase and 32P-UTP.

Pre-hybridization of blots was carried out in a solution containing 5X SSPE, 5% SDS, 5X Denhardt’s reagent, sonicated salmon sperm DNA (100 μg/ml) and 50% formamide at 42°C for 2 hr. Subsequently, the solution was replaced with fresh solution containing the radiolabeled probe (10⁸ cpm/ml) and hybridization was carried out with continuous shaking at 42°C for 18 hr. Washing for CaR, probed with cRNA probe was carried out at high stringency (0.1X SSC, 0.1% SDS at 65°C) for 30 min as described before. Blot probed with VDR and β-actin cDNA probes was washed in 2X SSPE, 0.1% SDS and in 1X SSPE, 0.1% SDS twice for 10 min at room temperature and further twice in 0.1X SSPE, 0.1% SDS at room temperature for 10 min each as described before.

Statistical analysis — The data are expressed as mean±SD. All the biochemical, hormonal and densitometric parameters were normally distributed. These parameters in ORX group were compared with sham group using Student’s t test at baseline and at 4 weeks. The difference was considered statistically significant at P<0.05.
Results

Baseline
There was no significant difference in weights, serum Ca, ALP, calcidiol, calcitriol, iP, and T in the two groups (P > 0.05). In vivo aBMD and BMC of WB, LS, WT and WF were similar in both the groups (P > 0.05).

At 4 weeks

General and biochemical—There was no change in weight and behavior in both the groups. ORX group had significantly lower mean serum T levels at 4 weeks as compared to baseline (P < 0.005) while there was no significant change in mean LS-aBMD in sham rats. There was a significant decline in mean aBMD at WF and WT in ORX groups as compared to the sham groups (P < 0.05; Table 2).

In vivo bone mineral density measurements—At 4 weeks, mean aBMD was significantly lower at proximal, mid and distal 1/3rd regions of isolated femur and tibia and also in femoral neck in ORX group as compared to sham groups (P < 0.005; Table 2).

CaR mRNA and VDR mRNA expression—There was no change in mRNA expression of CaR (Fig. 1a) and VDR (Fig. 1b) in duodenal mucosa in ORX group, as evidenced by northern blot analysis and densitometric quantification, normalized to β-Actin mRNA expression.

Discussion
The results demonstrate that androgen deficiency for 4 weeks in rat resulted in significant decline in mean aBMD at lumbar spine in-vivo, and femur (proximal, mid and distal 1/3rd regions and femoral neck) and tibia (proximal and middle 1/3 regions) as compared to that at baseline (P < 0.005) while there was no significant change in mean LS-aBMD in sham rats. There was a significant decline in mean aBMD at WF and WT in ORX groups as compared to the sham groups (P < 0.05; Table 2).

<table>
<thead>
<tr>
<th>Time</th>
<th>Sham-operated group Baseline</th>
<th>4 weeks</th>
<th>Orchidectomized group Baseline</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>240 ± 36</td>
<td>283 ± 38</td>
<td>220 ± 22</td>
<td>297 ± 36</td>
</tr>
<tr>
<td>S Ca (mg/dl)</td>
<td>7.7 ± 0.68</td>
<td>9.6 ± 2.20</td>
<td>7.31 ± 0.03</td>
<td>7.66 ± 1.03</td>
</tr>
<tr>
<td>S iP (mg/dl)</td>
<td>7.05 ± 0.098</td>
<td>8.78 ± 1.2</td>
<td>6.65 ± 0.62</td>
<td>6.47 ± 0.5*</td>
</tr>
<tr>
<td>S ALP (IU/L)</td>
<td>17.6 ± 4.1</td>
<td>20.3 ± 2.7</td>
<td>19.4 ± 4.2</td>
<td>21.9 ± 5.0</td>
</tr>
<tr>
<td>S Calcidiol (ng/ml)</td>
<td>2.8 ± 0.8</td>
<td>2.5 ± 0.19</td>
<td>2.8 ± 0.04</td>
<td>2.5 ± 0.16</td>
</tr>
<tr>
<td>S Calcitriol (pg/ml)</td>
<td>105 ± 14</td>
<td>109 ± 10</td>
<td>115 ± 12</td>
<td>117 ± 11</td>
</tr>
<tr>
<td>S T (ng/dl)</td>
<td>135 ± 19</td>
<td>129 ± 20</td>
<td>138.4 ± 18</td>
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Abbreviations: Ca: total calcium, iP: inorganic phosphorus, ALP: alkaline phosphatase, T: testosterone
* P<0.05 (Student’s t test)

Table 2—In vivo areal bone mineral density (aBMD, g/cm²) by DEXA in various experimental groups of rats
[Values are mean ± SD]

<table>
<thead>
<tr>
<th>aBMD</th>
<th>Sham-operated group Baseline</th>
<th>4 weeks</th>
<th>Orchidectomized group Baseline</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Body</td>
<td>0.124 ± 0.015</td>
<td>0.133 ± 0.012</td>
<td>0.120 ± 0.019</td>
<td>0.130 ± 0.012</td>
</tr>
<tr>
<td>Lumbar Spine</td>
<td>0.124 ± 0.008</td>
<td>0.121 ± 0.01</td>
<td>0.132 ± 0.010</td>
<td>0.121 ± 0.012</td>
</tr>
<tr>
<td>Whole Femur</td>
<td>0.122 ± 0.012</td>
<td>0.191 ± 0.001</td>
<td>0.133 ± 0.020</td>
<td>0.175 ± 0.013*</td>
</tr>
<tr>
<td>Whole Tibia</td>
<td>0.0873 ± 0.006</td>
<td>0.172 ± 0.07</td>
<td>0.081 ± 0.044</td>
<td>0.144 ± 0.010*</td>
</tr>
</tbody>
</table>

*P<0.05 (Student’s t test)

Table 1—Biochemical profile of experimental rats at baseline and 4 weeks
[Values are mean ± SD]

<table>
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Abbreviations: Ca: total calcium, iP: inorganic phosphorus, ALP: alkaline phosphatase, T: testosterone
* P<0.05 (Student’s t test)

Data at baseline and at 4 weeks of orchidectomized group has been compared to corresponding data of sham operated group.

Data at baseline and at 4 weeks of orchidectomized group has been compared to corresponding data in sham operated group.
Table 3—*In vitro* areal BMD (g/cm²) and BMC (gm) measurements in dissected bones in experimental rats at 4 weeks
[Values are mean SD]

<table>
<thead>
<tr>
<th>Bone Site</th>
<th>Sham-operated group</th>
<th></th>
<th></th>
<th>Orchiectomized group</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aBMD</td>
<td>BMC</td>
<td></td>
<td>aBMD</td>
<td>BMC</td>
<td></td>
</tr>
<tr>
<td>Prox 1/3³ femur</td>
<td>0.209±0.003</td>
<td>0.0956±0.002</td>
<td></td>
<td>0.179±0.01*</td>
<td>0.0869±0.011</td>
<td></td>
</tr>
<tr>
<td>Mid 1/3³ femur</td>
<td>0.189±0.002</td>
<td>0.0459±0.0007</td>
<td></td>
<td>0.158±0.01*</td>
<td>0.0379±0.0037*</td>
<td></td>
</tr>
<tr>
<td>Distal 1/3³ femur</td>
<td>0.159±0.008</td>
<td>0.0557±0.008</td>
<td></td>
<td>0.128±0.004*</td>
<td>0.0353±0.003*</td>
<td></td>
</tr>
<tr>
<td>Femoral Neck</td>
<td>0.1418±0.07</td>
<td>0.004±0.0002</td>
<td></td>
<td>0.124±0.061*</td>
<td>0.0038±0.002</td>
<td></td>
</tr>
<tr>
<td>Prox 1/3³ tibia</td>
<td>0.169±0.016</td>
<td>0.0266±0.0031</td>
<td></td>
<td>0.140±0.01*</td>
<td>0.0205±0.003*</td>
<td></td>
</tr>
<tr>
<td>Mid 1/3³ tibia</td>
<td>0.169±0.008</td>
<td>0.02450.002</td>
<td></td>
<td>0.146±0.01*</td>
<td>0.0197±0.003*</td>
<td></td>
</tr>
<tr>
<td>Distal 1/3³ tibia</td>
<td>0.183±0.01</td>
<td>0.0279±0.005</td>
<td></td>
<td>0.158±0.006*</td>
<td>0.0382±0.005*</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Prox: Proximal, Mid: middle.
*P<0.05 (student's t test)
Data at baseline and at 4 weeks in ORX group has been compared to corresponding data in sham group.

*In vitro.* Effect of androgen deficiency on VDR and CaR mRNA expression in duodenal mucosa was not observed.

Significant decline in cancellous bone at lumbar spine, femur and tibia was observed even at 4 weeks. In contrast, Vanderschueren *et al.* observed decline in cancellous bone at lumbar vertebra and proximal metaphysical bone of tibia and cortical width at femur only at 4 months and not at 5 weeks following orchidectomy in mature (13 months old) rats. No attempt was made to measure cortical width at the middle 1/3³ tibia and femur, sites of predominantly cortical bone. The decline in aBMD, at these sites suggests loss of cortical bone. Other workers have observed thinning of cortical width at 1 month following orchidectomy in adult rats.

Significant decline in mean serum phosphorus in ORX rats was observed when compared with sham rats. Decline in serum IP has been observed in androgen deficient male rats. No significant change in serum calcidiol and calcitriol was observed. Other studies have reported no change or decreased serum 1,25(OH)₂D₃ levels in hypogonadism.

Androgen deprivation results in decline in intestinal calcium absorption in maturing rats and in human, though reports to the contrary exist. Androgen receptors have been identified in mucosa of bovine small intestine. It is possible that androgen receptor is involved in active intestinal calcium absorption. There is no study of expression of androgen receptor in intestine in androgen derivational state. Interestingly, studies have shown stimulatory effect of estrogen on intestinal absorption in male castrated rats. Since P450 aromatase activity has been demonstrated in intestinal mucosa, it seems plausible that circulating androgens increase intestinal calcium absorption after aromatization.
VDRs are present in the intestinal mucosa and play a critical role in the intestinal calcium absorption. Testosterone significantly increases the affinity of intestinal VDR to calcitriol in chicken intestine. We observed no change in VDR mRNA expression in duodenal mucosa following ORX, which implies that VDR expression is not affected by androgen status in rats. It seems plausible that androgen may affect the post-receptor signaling of CaR cannot be ruled out. Further studies are required to study the role of CaR in the intestinal calcium absorption.

For the first time present study demonstrated no alteration in the expression of CaR mRNA in the duodenal mucosa in ORX rats as compared to sham rats. However, the possibility that androgen may influence the post-receptor signaling of CaR cannot be ruled out. Further studies are required to study the role of CaR in the intestinal calcium absorption.

References


23. Reinahardt R T & Horst R L, Parathyroid hormone down regulates 1,25-dihydroxy D receptor (VDR) and VDR messenger ribonucleic acid in vitro and blocks homologous up-regulation of VDR in vivo, Endocrinology, 127 (1990) 912.