Bone Metabolism and Tenofovir: Evidence of Direct Effect on Calcium-Sensing Receptor

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Background

Low bone mineral density has been reported in numerous studies involving HIV-infected individuals, and an increased susceptibility to fracture has been documented. The pathogenesis of reduced bone mineral density is multifactorial, and the use of certain antiretroviral agents has been associated to bone metabolism derangements. In particular, tenofovir disoproxil fumarate (TDF) has been associated with a decrease in bone mineral density and increased bone turnover. The pathogenesis of this bone toxicity remains to be characterized, although impaired phosphate balance and altered vitamin D metabolism have been suggested as likely mechanisms. Nevertheless, available information is controversial.

Recent studies showed a link between the use of TDF in antiretroviral regimen and the development of hyperparathyroidism. Early changes in parathyroid hormone (PTH) serum concentration have been documented in HIV-patient initiating antiretroviral treatment with a TDF-containing regimen. The raise of PTH concentration was exacerbated by vitamin D deficiency, but it occurred also in vitamin D repleted patients, and the use of TDF was found to be an independent predictor of elevated PTH concentration.

The secretion of PTH by the parathyroid glands is finely regulated according to the extracellular calcium concentration, which is sensed by a specific receptor (calcium-sensing receptor, CaR). Hypocalcemia promotes a rapid release of PTH, while restoration of normocalcemia causes levels of PTH to decrease. CaR activation causes a significant stimulation of extracellular signal-regulated kinase (ERK1/2) activity in human parathyroid cells, leading to the inhibition of PTH secretion (Figure 1).

Although calcium is the natural CaR binder, there are other substances that are able to bind the receptor, stimulating (calcimimetic agents) or inhibiting (calcilytic agents) its activity (Figure 2). The rapid raise of PTH concentration after initiation of antiviral treatment with TDF, prompted us to hypothesize a direct effect of TDF on CaR. In particular, we postulated an inhibitory effect of the antiretroviral agent on CaR. To test this hypothesis we conducted in vitro experiments using specific assays and testing the activation of the receptor in presence or absence of TDF.

Methods

Human embryonic kidney cells transfected with CaR wild-type gene (HEK-293-CaR WT) were used (figure 3). Cells were grown in standard conditions (37° C, 5%CO2) and the activity of CaR was assessed after stimulation with different concentrations of CaCl2 (0.5mM-1mM-3mM-5mM) and TDF (100nM-1μM) (kindly provided by Gilead Sciences, Inc). Analysis of ERK1/2 activation – as a marker of CaR activity – was performed by Western blotting with 1:1000 and 1:2000 dilution polyclonal anti-p44/42 ERK and anti-phospho-p44/42ERK antibodies, respectively (Cell Signaling Technology, Beverly, MA). The band intensity, corresponding to the levels of protein expression, was measured by Image J software. Experiments were repeated at least three times.

Figure 1. CaR activation causes a stimulation of extracellular signal-regulated kinase (ERK1/2) activity in human parathyroid cells, leading to the inhibition of PTH secretion.

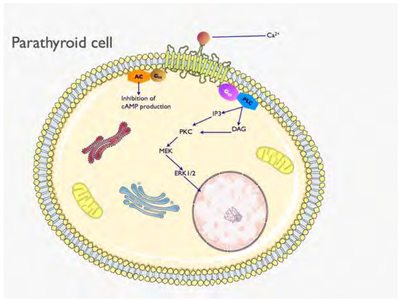


Figure 2. Calcimimetic agents stimulate the activity of CaR while calcilytic agents would mimic a state of hypocalcemia and promote PTH secretion.

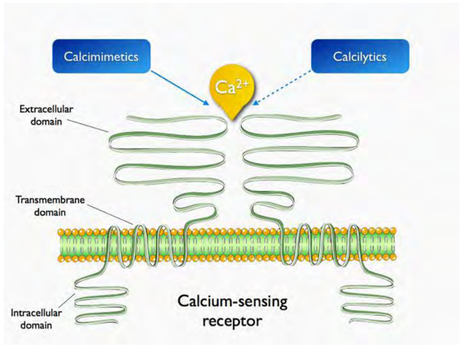
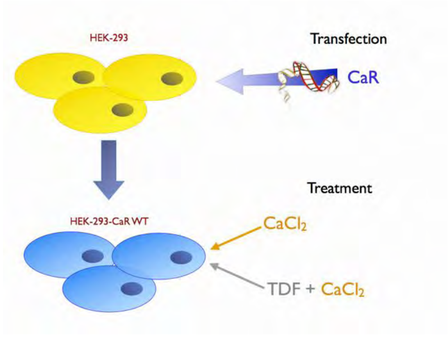


Figure 3. Experimental model



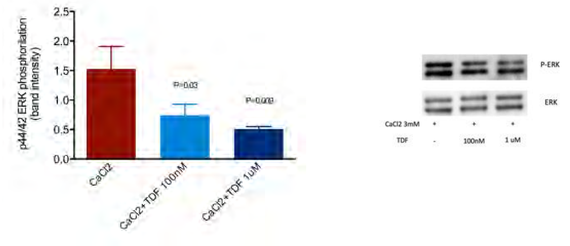
Results

We tested CaR activation using different concentrations of CaCl2, and we obtained the maximum activation of the receptor at the concentration of 3 mM. We therefore employed this concentration for the subsequent sets of experiments.

Initial experiments showed that the stimulation of stable transfected HEK-293-CaR WT with CaCl2 and TDF at 10uM led to cellular damage and reduced viability, not allowing completing the entire procedure. The 10 uM concentration was therefore not used.

We observed a marked reduction of ERK activation by adding TDF to cell cultures (Figure 4) in a dose-dependent manner. The addition of 100 nM of TDF reduced ERK activation by 53%, while adding 1 uM TDF to cell culture reduced ERK activity by 80%. (The percent activation was 46±2 and 20±4 at 100nM and 1uM TDF, respectively).

Figure 4. Effect of increasing TDF concentration on p44/42 ERK phosphorylation



Conclusions

The addition of TDF to cell cultures markedly reduced CaR activity, indicating a direct effect of TDF on the receptor. Similarly to the effect of other calcilytic compounds, the CaR inhibitory effect of TDF was dose-dependent. Higher concentration of TDF added to the medium induced a more profound inhibition of the receptor. Our data support the hypothesis of a direct effect of TDF on CaR that may be responsible for the metabolic alterations observed in patients receiving the drug as part of their antiretroviral treatment. The elevation of PTH serum concentration recorded in HIV-infected patients receiving TDF may thus be sustained by an inhibited activity of CaR. The effect of TDF on bone metabolism leading to a reduction of bone mineral density may thus not be only due to altered phosphate homeostasis or kidney dysfunction, as hypothesized in some studies.