Less calcemic Vitamin D analogs enhance creatine kinase specific activity and modulate responsiveness to gonadal steroids in the vasculature

Dalia Somjen a, b, Gary H. Posner c, Naftali Stern a, b, *  

a Institute of Endocrinology, Metabolism and Hypertension, Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel  
b The Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel  
c Department of Chemistry, The Johns Hopkins University, Baltimore, MD, USA  

Received 13 December 2005; accepted 19 June 2006

Abstract

Vitamin D receptors are widely expressed in the cardiovascular system, in which Vitamin D and its metabolites exert a variety of biological activities such as regulation of cellular proliferation and differentiation, cell calcium transients and cell energy metabolism in vitro. The latter is mediated through the control of the brain type creatine kinase specific activity (CK), which serves to provide a readily available reservoir for ATP generation under increased work-load. In the present study we undertook to assess the role of Vitamin D on energy metabolism in the rat heart and aorta in vivo by using CK, which is a key energy metabolizing enzyme and compare Vitamin D depleted and repleted animals.

Vascular tissues from female or male Vitamin D-depleted rats showed 61–80% lower CK activity in the aorta (Ao) and left ventricle of the heart (Lv) than control, Vitamin D-replete rats. Moreover, neither estradiol-17β (E2) nor dihydrotestosterone (DHT), which increases CK specific activity in Ao and Lv of intact female or male rats, respectively, were able to stimulate CK in Vitamin D-depleted rats.

Treatment of intact female rats for 2 weeks or 2 months with the less-calcemic Vitamin D analogs JKF 1624F2-2 (JKF) or QW 1624F2-2 (QW) (Fig. 1), did not significantly affect CK specific activity. However, after pretreatment with these analogs, there was an up regulation of the E2-induced CK response in Ao and Lv. In intact female rats, all Vitamin D analogs also potentiated the in vivo CK response to the SERMs raloxifene (Ral) and tamoxifen (TAM) in Ao and Lv. However the inhibitory effect of Ral or TAM on E2-induced CK activity was lost after pretreatment with Vitamin D analogs. The non-calcemic analog CB 1093 (CB) induced a significant increase in estradiol receptor α (ERα) protein in both myocardial and aortic tissue from intact and from ovariectomized female rats. Collectively, these results indicate that Vitamin D analogs modulate cell energy homeostasis in vascular tissues through induction of CK and up regulation of the response and sensitivity of CK in vascular tissues to E2 and to SERMs, possibly through via an increase in ERα protein in female derived organs. These results corroborate our previous in vitro studies in human vascular cells and further suggest that the Vitamin D system plays an important physiological role in maintaining normal cell energy reservoir in the vasculature.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Vitamin D; Vitamin D analogs; Heart; Aorta; Creatine kinase

1. Introduction

There is growing evidence that Vitamin D exerts multiple direct effects in the cardiovascular system by affecting the transcription rate of such genes as the vascular endothelial growth factor, matrix metalloproteinase type 9, myosin, and structural proteins, such as elastin and type I collagen [1–5]. On the other hand, the overall role of Vitamin D derivatives in the vasculature in vivo has been difficult to discern. An array of reported effects has often complicated attempts to offer a comprehensive assessment of the expected cardiovascular health impact of Vitamin D. Not only is the separation of the direct tissue effect of Vitamin D from its calcemic load...
difficult \textit{in vivo}, but observations that Vitamin D induced calcifications in vascular smooth muscle cells \cite{6} on the one hand and induction of PG\textsubscript{2} secretion in vascular smooth muscle cells (VSMC) \cite{7} and suppression of renin secretion on the other hand \cite{8} make the prediction of its overall effect rather difficult. Since in previous studies we have investigated the independent effects and the interaction between non-calcemic analogues of Vitamin D and estradiol-17\textbeta\ (E\textsubscript{2}) in VSMC \textit{in vitro} \cite{8,9}, we have now turned to assess these effects in vascular organs \textit{in vivo}. In preliminary studies we found that single injection of E\textsubscript{2} increased the brain type isozyme of creatine kinase (CK) specific activity in aorta (Ao) and the left ventricle of the heart (Lv) from rat females \cite{10}.

In the present study we extended these preliminary findings \cite{10}, using CK, a marker of the genomic action of E\textsubscript{2}, formerly known as “estradiol-inducible protein”, to assess the effects of Vitamin D depletion as well as of several less- and non-calcemic Vitamin D analogs on the rat heart and aorta, as a function of the rat’s gonadal status. The results indicate that Vitamin D analogs exert gonadal-steroid -independent biological activity in vascular tissues acutely, and function as enhancers of the response and sensitivity of vascular tissues to E\textsubscript{2} and to SERMs in association with, and presumably through increased ER\textalpha\ protein in the female derived organs.

2. Materials and methods

2.1. Reagents

All reagents used were analytical grade. Chemicals, 17\beta-estradiol (E\textsubscript{2}), dihydrotestosterone (DHT), Tamoxifen (TAM) and the creatine kinase assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO). Raloxifene (Ral) was the gift of Dr. B. Fournier (Ciba-Geigy, Basel, Switzerland). JKF and QW, were synthesized by us \cite{11–15} and CB 1093 was the gift of Leo Pharmaceutical Products, Ballerup, Denmark. All other reagents were of analytical grade.

2.2. Animals

1. Wistar-derived, locally bred rats were used at initial age of 25 days. The rats were maintained at 23°C, on a 14 h light, 10 h dark schedule and fed pelleted food and water ad libidum. Female or male rats were used either as intact, or after bilaterally ovariectomy (Ovx) or castration (Cast) respectively, and the treatments started 2 weeks post-surgery. Rats were sacrificed 24 h after the last injection, by cervical dislocation and organs were removed for biochemical measurements. Experiments were carried out according to the regulations of the Committee on Experimental Animals of the Tel-Aviv Sourasky Medical Center and the NIH guidelines.

2. Wistar-derived rats were obtained as described previously, at the age of 4 weeks, the rats were made D-depleted by switching them to grow in the dark on a Vitamin D-deficient diet supplemented with Ca (0.75%) and phosphate (0.55%) \cite{16}.

2.3. Hormonal treatment

Rats were injected daily for 1, 2 weeks or 2 months (8 weeks) with CB, JKF or QW at different doses (0.01–0.2 ng/gr BW) or with a single dose as indicated. Twenty- four hours after the last Vitamin D analog injection, rats were injected with E\textsubscript{2} at 0.5 or 5 \textmu g/rat, raloxifene (Ral) or TAM at 500 \textmu g or both or 50 \textmu g DHT.

2.4. Creatine kinase specific activity

Twenty four hours after the last injection, rat organs were collected in cold isotonic extraction buffer \cite{10}, homogenized using a Polytron homogenizer (Kinematica A.G., Littau, Switzerland), and enzyme extracts obtained by centrifugation of homogenates at 14,000 × g for 5 min at 4°C in an Eppendorf microcentrifuge. CK activity was measured in a Kontron Model 922 Uvicon Spectrophotometer using a Sigma coupled assay kit (UV-47). Protein was assayed by Coomassie brilliant blue dye binding \cite{17}.

2.5. Western blot analysis

After the treatment with the Vitamin D analogs rat organ extracts were obtained and analyzed as was described before \cite{19} for ER\textalpha\ using specific antibodies (Santa Cruz) and enhanced chemiluminescence’s reagents from Amersham. Bands were quantified by densitometry in at least three different experiments.

2.6. Statistical analysis

The significance of differences between experimental and control means was evaluated using Student’s \textit{t}-test or ANOVA, in which \( n = 5 \) represented the number of animals per group.
3. Results

3.1. Effects of Vitamin D depletion on CK activity in the rat heart and aorta

CK constitutive specific activity was lower by about 60% in the left ventricle (Lv) and aorta (Ao) of both female and male Vitamin D deficient rats (Fig. 2). As shown in Fig. 2, acute administration of E2 (5 µg/rat) in Vitamin D-replete female rats and dihydrotestosterone (DHT 50 µg/rat) in male rats elicited a significant increase in CK activity in left ventricular (∼50%) and aortic (∼100–150%) homogenates. In contrast, the induced effects of E2 and DHT on CK in the female and male corresponding organs of Vitamin D-depleted rats was either markedly attenuated (female and male aorta) or entirely absent (female and male left ventricle; Fig. 2)

3.2. Effect of chronic administration of the Vitamin D analogs JKF and QW on E2-induced myocardial and aortic CK activity

Chronic administration of the Vitamin D analogs JKF and QW for 1, 2 or 8 weeks did not affect CK specific activity in myocardial (Lv) and aortic (Ao) CK in intact female rats.

While such chronic treatment with each of these analogs at a dose of 0.1 ng/gr body weight was, in itself, devoid of direct effect, the left ventricular and aortic response to a single injection of E2 was consistently higher in intact female rats pretreated with these Vitamin D analogs for the corresponding periods of 1, 2 or 8 weeks (Fig. 3).

3.3. Effect of E2 on myocardial and aortic CK activity in intact female rats after chronic pre-treatment with the Vitamin D analogs JKF and QW

Two-week treatment of intact female rats with JKF or QW at doses ranging between 0.05 and 0.2 ng/g had no discernible effect on CK activity (data not shown). However, as shown in Fig. 4, pretreatment with the Vitamin D analog QW or JKF for 2 weeks, increased left ventricular and aortic response to a single injection of E2 in agonist, tissue and dose-related
manner. For example, although both JKF and QW induced quantitatively similar potentiation of the CK response to E2, there was a difference between these Vitamin D analogs in terms of the tissue sensitivity to their effect, such that left ventricular CK was apparently more sensitive to lower concentrations of JKF relative to QW.

We also examined whether or not differences exist among Vitamin D analogs in terms of the response to varying concentrations of E2 (Fig. 5). Overall, pre-treatment with JKF elicited the largest CK responses compared with other analogs. Further, JKF but not QW increased CK responses even at a low E2 concentration, thus suggesting lager tissue sensitivity to JKF relative to QW.

3.4. Vitamin D analogs modify the effect of raloxifene on myocardial and aortic CK activity

Pretreatment with each one of the Vitamin D analogs JKF, CB and QW in intact female rats for 2 weeks, significantly potentiated the CK response to Ral in both the left ventricular and aortic tissue. Notably, however, Ral which inhibited the stimulatory effect of E2 on CK at both sites in rats not treated with Vitamin D analogs, was not able to block E2’s stimulatory effect on left ventricular and aortic CK in rats receiving pre-treatment with any of the three Vitamin D analogs tested (Fig. 6).

3.5. Effect of gonadal status on E2- or DHT-induced CK activity in Vitamin D analog—pretreated rats

Ovariectomy per se had no effect on either aortic or left ventricular CK activity in female rats. Likewise, two weeks of pretreatment with CB per se had no effect on CK activity at these sites. In ovariectomized (Ovx) female rats E2 (at 5 μg/rat), induced a twofold increase in left ventricular and aortic CK. In parallel to the findings in intact rats, pre-treatment with the Vitamin D analog CB potentiated the effect of E2 on Lv and aortic CK (Fig. 7). In contrast to the lack of pretreatment with CB per se had no effect on CK activity at these sites. In ovariectomized (Ovx) female rats E2 (at 5 μg/rat), induced a twofold increase in left ventricular and aortic CK. In parallel to the findings in intact rats, pre-treatment with the Vitamin D analog CB potentiated the effect of E2 on Lv and aortic CK (Fig. 7). In contrast to the lack of pretreatment with CB per se had no effect on CK activity at these sites. In ovariectomized (Ovx) female rats E2 (at 5 μg/rat), induced a twofold increase in left ventricular and aortic CK. In parallel to the findings in intact rats, pretreatment with the Vitamin D analog CB potentiated the effect of E2 on Lv and aortic CK (Fig. 7). In contrast to the lack of pretreatment with CB per se had no effect on CK activity at these sites. In ovariectomized (Ovx) female rats E2 (at 5 μg/rat), induced a twofold increase in left ventricular and aortic CK. In parallel to the findings in intact rats, pretreatment with the Vitamin D analog CB potentiated the effect of E2 on Lv and aortic CK (Fig. 7). In contrast to the lack of pretreatment with CB per se had no effect on CK activity at these sites. In ovariectomized (Ovx) female rats E2 (at 5 μg/rat), induced a twofold increase in left ventricular and aortic CK. In parallel to the findings in intact rats, pretreatment with the Vitamin D analog CB potentiated the effect of E2 on Lv and aortic CK (Fig. 7). In contrast to the lack
of effect of CB per se on CK in ovariecomized female rats, castrated rat males pretreated with CB showed a significant reduction in CK activity of both aortic and left ventricular CK (Fig. 7). Nevertheless, CB pretreatment augmented the relative response of both aortic and myocardial CK to DHT by a factor of ∼two folds, which was seen in intact and castrated male rats alike.

Additionally, pretreatment with the Vitamin D analog CB affected the interaction of tamoxifen with E$_2$ in Ovx female rats. Both E$_2$ (at 5 μg/rat) and tamoxifen (TAM at 500 μg/rat) induced a twofold increase in left ventricular and aortic CK (Fig. 8). However, when E$_2$ was given along with TAM, the increase in CK activity could no longer be elicited in either the myocardial or the aortic tissue. Pre-treatment with the Vitamin D analog CB for 2 weeks modified this response pattern. First, CB-pretreated rats had a larger CK response to both E$_2$ and TAM in at both organs. Second, in CB-pretreated rat, TAM did not block E$_2$-induced CK activity. Indeed, in CB-pretreated ovariecomized female rats receiving both E$_2$ and TAM, the observed CK activity was roughly equivalent to that observed in rats receiving either E$_2$ or TAM, regardless of whether activity was measured in the left ventricle or the aorta.

The question of how chronic pretreatment with a Vitamin D analog affects tissue response to E$_2$ was also reassessed with a longer pretreatment period of 8 weeks, and the use of JKF and QW. As shown in Fig. 9, ovariecomized female rats pretreated with JKF or QW had CK activity that were roughly similar to control level, except for a somewhat lower aortic CK in QW-treated rats. In parallel to the observations with a 2-week pretreatment protocol, pretreatment with either JKF or QW for 8 weeks in Ovx female rats, resulted in an augmented CK response to E$_2$ at both organs (Fig. 9).

3.6. Effect of the Vitamin D analog CB on estrogen receptor α (ERα) protein expression

Fig. 9 depicts the densitometric analysis of Western blots performed on protein extracts from rat organs after 2 weeks of treatment with the analog CB. In intact female rats, pre-treatment with CB resulted in a substantial increase in ERα protein in the aorta with a small increase in the left ventricle. In ovariecomized female rats, CB induced a sizable increase in ERα protein expression in both organs (Fig. 10).
4. Discussion

The present study suggests that Vitamin D plays a role in normal myocardial and vascular cell metabolism in vivo, and that it apparently has both direct and indirect effects, the latter operating through modulation of the influence of gonadal steroids on CK at these sites. The possibility that Vitamin D is directly and positively involved in the maintenance of normal cell energy balance in the heart and aorta is supported by the finding that normal CK levels in these organs were markedly reduced in Vitamin D deficient rat. Further, Vitamin D-depleted rats had impaired E2- and DHT-dependent induction in myocardial and aortic CK. The results of these in vivo experiments per se would be difficult to separate from those of multiple inevitable confounding factors present in Vitamin D-depleted animals, such as calcium depletion and secondary hyperparathyroidism. Nevertheless, when combined with previous in vitro studies showing direct effects of Vitamin D analogs on CK in cultured VSMC [8], the present results lend further support to the concept that Vitamin D acts as a physiologic modulator of CK in cardiovascular tissues. Because the CK reaction is important for rapid re-synthesis of ATP when the heart increases its work, decrease of CK activity may impair the ability to deliver ATP to energy-consuming cellular systems [18].

Besides this type of functional derangement in energy homeostasis, there is also previous evidence that Vitamin D depletion induces structural and functional changes in the cardiac muscle, namely, increase in muscle size relative to body weight in association with significant decrease in myofibrillar area and increase in extra cellular space and augmented myocardial contractility, none of which could not be reversed by normalization of hypocalcemia [19]. In another report, rats maintained on calcium -supplemented Vitamin D-deficient diet not only displayed increased heart weight, but had evidence of myocardial cell hyperplasia [20]. The latter finding supports the concept that Vitamin D also prevents excessive cell growth in the cardiovascular system. In this context, we have recently observed that Vitamin D elicits a reciprocal dose-dependent increase in CK activity and suppression of DNA synthesis in human VSMC [21].

The second key finding in this study is that Vitamin D analogs potentiate the in vivo responsiveness and sensitivity of vascular tissue CK to gonadal steroids. These results corroborate a previous report from our laboratory that the Vitamin D analog JKF markedly amplified the CK response to E2 and to DHT in cultured human vascular smooth muscle cells as well as in and human endothelial (E304) cells in vitro [8]. It is also in agreement with our observation in E304 cells, where Vitamin D analogs increased DNA response to gonadal steroids by four- to five-fold [8].

The observations in this study that the Vitamin D analog CB increases the expression of ERα protein, provide one potential mechanism by which pretreatment with Vitamin D analogs might increase vascular tissue sensitivity to estrogenic compounds. This finding also complements our previous findings in human vascular smooth muscle cells in vitro, in which pretreatment with the Vitamin D JKF increased both ERα mRNA and protein expression [8]. These results also resemble observations in cultured human bone cells, in which pretreatment with JKF or CB significantly up regulated the response to E2 in all female-derived cells and to DHT in mature male-derived cells [22-23]. Vitamin D was also reported to induce ERα expression in two prostate cancer cell lines [24].

These findings, however, should not be generalized to other tissue or cell types, nor to other estrogenic effects in the vasculature. For example, in MCF-7 cells 1,25(OH)2D3 and several Vitamin D analogs reduced ERα expression in association with potent anti proliferative effects [25,26]. In human VSMC, Vitamin D analogs did not amplify the stimulatory effects exerted on DNA synthesis by low E2 concentration and, indeed, prevented the suppression of DNA synthesis exerted by high concentrations of E2 and DHT [8].

Finally, we observed that not only the action of E2 but also that of SERMs such as tamoxifen and raloxifene on CK was potentiated by pretreatment with Vitamin D analogs in vivo. These results should prompt examination of the possibility that the effects of estrogen or raloxifene therapy in post menopausal women, can be augmented by Vitamin D or its analogs, presumably via modulation of ER expression.

References


