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Original Communication

The effect of ovariectomy on the stimulation by phytoestrogens on creatine kinase specific activity in skeletal organs from female rats

Dalia Somjen[1,*], Batya Gayer[2], Fortune Kohen[2], Gary H. Posner[4], and Alvin M. Kaye[3,#]

[1]Institute of Endocrinology, Metabolism and Hypertension, Tel-Aviv Sourasky Medical Center and the Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 64239, [2]Department of Biological Regulation and [3]Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel. [4]Department of Chemistry, Johns Hopkins University, Baltimore, MD 21218, USA

ABSTRACT

Ovariectomy of immature female rats results in significant decrease in parameters in different organs. Previously we found that estradiol-17β (E_2) restored the different parameters of the different organs from ovariectomized female rats (Ovx) to values obtained in organs from intact immature female rats. E_2 stimulated creatine kinase (CK) specific activity, a hormonal-responsive marker in organs containing estrogen receptors and responsive to the hormones. In the present study, we compared the effects of E_2 with those of the phytoestrogens: quercertin (Qu), daidzein (D), genistein (G), biochainin A (BA) and their carboxy-derivatives cD, cG and cBA in immature and Ovx female rats, on CK in diaphyseal bone (Di) and epiphyseal cartilage (Ep) as well as uterus (Ut) and pituitary (Pi), when injected for 24 h with and without the SERM raloxifene (Ral), or with and without pre-treatment for 3 days with the less-calcemic vitamin D analog JK $1624F_2$-2 (JKF). Ovariectomy resulted in significant reduction in the basal levels of CK in all organs tested. All estrogenic compounds tested in both animal groups stimulated CK. Ral stimulated CK in all organs except Ut, but inhibited enzymatic stimulation by E_2 and some of the estrogenic compounds in a hormonal status- and organ-specific pattern. Pre-treatment with JKF increased CK response to E_2 and to some of the estrogenic compounds in a hormonal status- and organ-specific pattern. In summary, estrogenic target organs of female rats are modulated differently in a hormonal status- and organ- dependent manner in an unknown mechanism.

KEYWORDS: phytoestrogens, creatine kinase, diaphyseal bone, epiphyseal cartilage, uterus, pituitary

INTRODUCTION

Osteoblastic cells from different mammals express specific intracellular and membranal receptors for estrogens [1-3] and respond to different estrogens by a variety of parameters [4-8]. The age and hormonal status dependent-responses were studied by different researchers, but are still debatable in models of human osteoblastic cells in cell cultures *in vitro* [9-15].

We have analyzed the response of cultured female human derived osteoblasts (hObs) by different parameters to different estrogenic compounds,

*Address correspondence and reprint requests to:
Dr. D. Somjen, Institute of Endocrinology, Metabolism and Hypertension, Tel Aviv-Sourasky Medical Center, 6 Weizmann Street
Tel-Aviv 64239, Israel.
dalias@tasmc.health.gov.il
#Deceased

including estradiol-17β (E_2), the specific estrogen receptors ligands such as 2,3-bis (4-hydroxy-phenyl)-propionitrile (DPN; ERβ specific agonist) and 4,4',4''-[4-Propyl-(1H)-pyrazol-1,3,5-triyl] tris-phenol (PPT; ERα specific agonist), the SERM raloxifene (Ral) and a variety of phyto-estrogens derived mainly from soy as well as their synthetic carboxy-derivatives and protein-bound carboxy-derivatives. Whether these findings apply also to human female bone response to hormonal treatments in post-menopausal osteoporosis *in vivo* is not yet established [14-17].

We found that there is a higher expression of ERα mRNA and lower expression of ERβ mRNA in pre- than in post- menopausal hObs, with no significant difference in intracellular and membranal estrogen binding activities.

The non-hypercalcemic vitamin D analog JK $1624F_2$-2 (JKF) up-regulated the response to E_2 and to some phytoestrogens in hObs from both age groups to the same extent [18-21].

In the present review we summarize our findings using rat female skeletal organs derived from either immature or Ovx animals in comparison to uterus and pituitary on:

a. The effect of different estrogenic compounds on CK activity in rat female diaphyseal bone (Di), epiphyseal cartilage (Ep) compared to uterus (Ut) and pituitary (Pi) in both rat types.

b. Modulation by raloxifene (Ral) of the effect of different estrogenic compounds on CK response of rat organs in rat types.

c. Modulation by pretreatment with the less-calcemic vitamin D analog JK $1624F_2$-2 (JKF) of the effect of different estrogenic compounds on CK response of rat organs at both ages.

MATERIALS AND METHODS

Reagents

Chemicals

Estradiol-17β (E_2), creatine kinase (CK) assay kit and phytoestrogens used were purchased from Sigma Chemicals Co. (St. Louis, MO. USA). Raloxifene (Ral) was donated by Novartis Basel Switzerland. The carboxy-derivatives were synthesized by us [18, 22]. JK $1624F_2$-2 (JKF) was synthesized and provided by Dr. G. H. Posner, Johns Hopkins University Baltimore MD. USA [18, 20, 21]. All other reagents used were of analytical grade.

Hormonal treatment

Wistar-derived pre-pubertal female rats aged 25 days weighing 60 gr at the start of the experiment (immature) or 4 weeks post-ovariectomy (Ovx) were used. Immature or Ovx rats were injected for 24 h with either estradiol-17β (E_2, 5 μg/rat), raloxifene (Ral), genistein (G), carboxy-G (cG), biochainin A (BA), carboxy-BA (cBA), or daidzein (D); all free compounds at 500 μg/rat and all carboxy-derivatives at 50 μg/rat. In other experiments all hormones were injected together with Ral using the same concentrations as above.

For pre-treatment with the vitamin D less-calcemic analog, immature or Ovx female rats were injected daily for 3 days with JKF $1624F_2$-2 (JKF) (0.2 ng/gr BW) [20], and 24 h after the last analog injection, rats were injected with the different estrogenic compounds as described above. After the last injection rat organs were harvested for CK specific activity preparation and assay.

Creatine kinase specific activity preparation and assay

Rat organs: Di, Ep, Ut and Pi were collected and homogenized in cold isotonic extraction buffer using a Polytron homogenizer. Enzyme extracts were obtained by centrifugation of the homogenates at 14000 x g for 5 min at 4°C. CK specific activity was measured in a Kontron Model 922 Uvicon Spectrophotometer using a Sigma coupled assay kit and protein was assayed by Coomassie brilliant blue dye binding. Results are means ± SEM and are expressed as % of control of CK in hormone-treated compared to vehicle-treated control animals [5, 23, 24].

Statistical analysis

Data were calculated as % stimulation by the treatment relative to control rats for each experiment as previously described. Comparison between the control and various treatments were made by analysis of variance using ANOVA.

RESULTS

The specific activity of creatine kinase in skeletal organs from immature and Ovx female rats

CK specific activity in Di and Ep or Ut and Pi from immature female rats, were compared with these organs obtained from Ovx female rats. The results show that the level of enzyme specific activity in the organs from Ovx was significantly lower than the level of enzyme specific activity in these organs from immature female rats (Fig. 1). In Di and Pi there is ~ 80%, in Ep ~ 50% and in Ut ~ 30% higher CK in immature than in Ovx.

The effect of different estrogens on the specific activity of creatine kinase in Ep from immature and Ovx female rats

Injection of 500 µg Ral, 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA resulted in modulation of CK specific activity in Ep from immature or Ovx female rats (Fig. 2a). The stimulation by all compounds was similar in immature and in Ovx female rats (Fig. 2a), with slightly higher response to Ral and Qu and lower response to cG and D in immature female rats (Fig. 2a).

Modulation of age-dependent CK response of Ep from immature and Ovx female rats to estrogenic compounds by raloxifene (Ral)

Injection of 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA 5 µg E_2, 500 µg Qu alone or together with 500 µg Ral, increased CK in Ep from immature or Ovx female rats (Fig. 2a). Ral inhibited the stimulation of enzyme activity by all compounds in both immature and Ovx female rats, except cG in Ovx female rats (Fig. 2b).

Modulation of age-dependent CK response of Ep from immature and Ovx female rats to estrogenic compounds after pre-treatment with the vitamin D less-calcemic analog JKF

When intact or Ovx female rats were injected daily for 3 days with the less-calcemic vitamin D analog JKF at 0.2 ng/gr BW, CK was slightly modulated in Ep in both animal groups. Injection of 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA increased CK in Ep from immature and Ovx female rats (Fig. 2a). JKF did not modify the stimulation of CK in Ep in both age groups by E_2, but up-regulated the response to G, cG and to D in both age groups, whereas in immature female rats only the response to BA was up-regulated, while

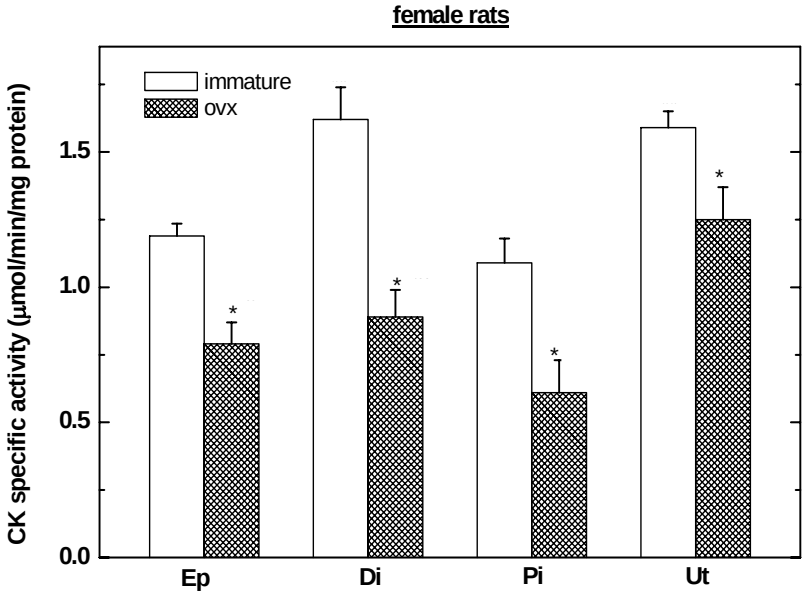

Fig. 1. CK specific activity rats in epiphyseal cartilage (Ep), diaphyseal bone (Di), pituitary (Pi) and uterus (Ut) from immature and Ovx female. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity means of Ovx control compared to immature control means: *, P <0.05.

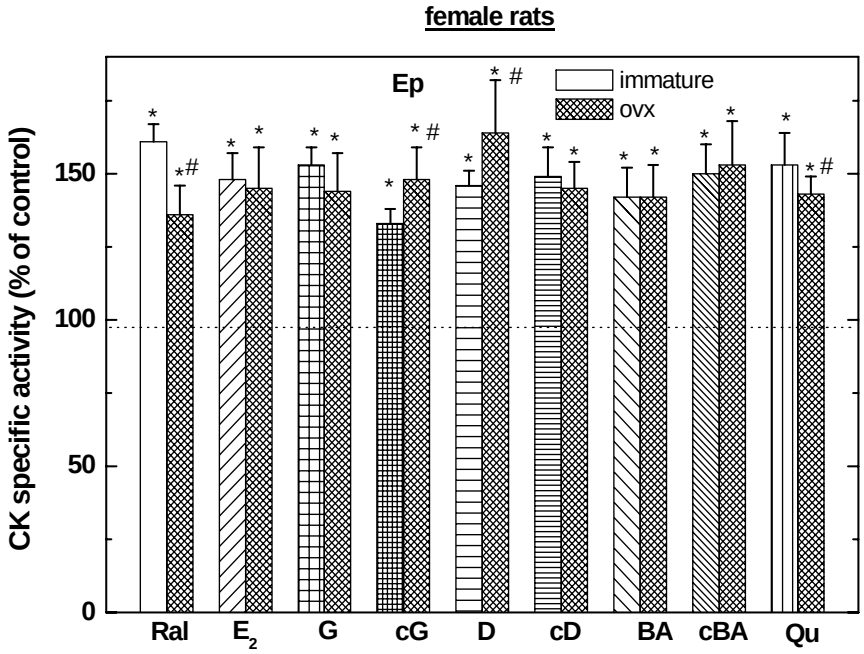

Fig. 2a. The effect of treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg Ral, G, D, BA and Qu or 50 µg cG, cD and cBA on CK specific activity in Ep. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05 and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

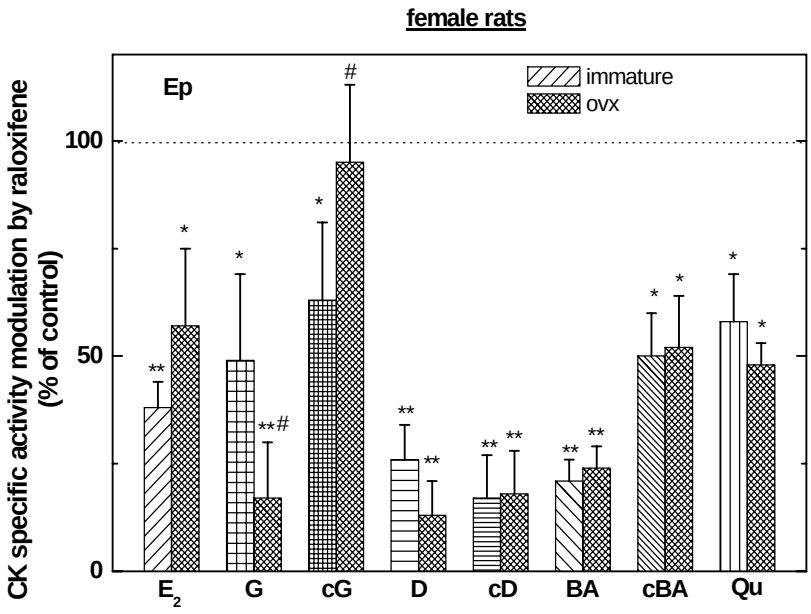

Fig. 2b. The effect of treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg Ral, G, D, BA and Qu or 50 µg cG, cD and cBA with and without Ral on CK specific activity in Ep. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05; ** P <0.01, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

the response to cBA was unchanged in both age groups and the responses to cD and Qu were down-regulated in both age groups (Fig. 2c).

The effect of different estrogens on the specific activity of creatine kinase in Di from immature and Ovx female rats

Injection of 500 µg Ral, 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA resulted in modulation of CK specific activity in Di from immature and Ovx female rats (Fig. 3a). In Di the stimulation by all compounds except by Ral, E_2, cG and D was similar in immature and in Ovx (Fig. 3a) female rats, with slightly higher response in immature to Ral, E_2, cG and D (Fig 3a).

Modulation of age-dependent CK response of Di from immature and Ovx female rats to estrogenic compounds by raloxifene (Ral)

Injection of 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA alone or together with 500 µg Ral increased CK in Di from immature and Ovx female rats (Fig. 3a). Ral inhibited the stimulation of CK activity by all compounds in both immature and in Ovx female rats (Fig. 3b). The inhibition by Ral of CK induced by E_2, G and D was higher in immature than in Ovx rats (Fig. 3b), whereas the inhibition of CK induced by all other compounds except cG was the same in both types of female rats (Fig. 3b).

Modulation of age-dependent CK response of Di from immature and Ovx female rats to estrogenic compounds after pre-treatment with the vitamin D less-calcemic analog JKF

When intact or Ovx female rats were injected daily for 3 days with the less-calcemic vitamin D analog JKF at 0.2 ng/gr BW, CK was slightly modulated in Di in both age groups. Injection of 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA increased CK in Di from immature and Ovx female rats (Fig. 3a). JKF did not modify the stimulation of CK in Di from both age groups by E_2 and G, but up-regulated the response to cG, cD,

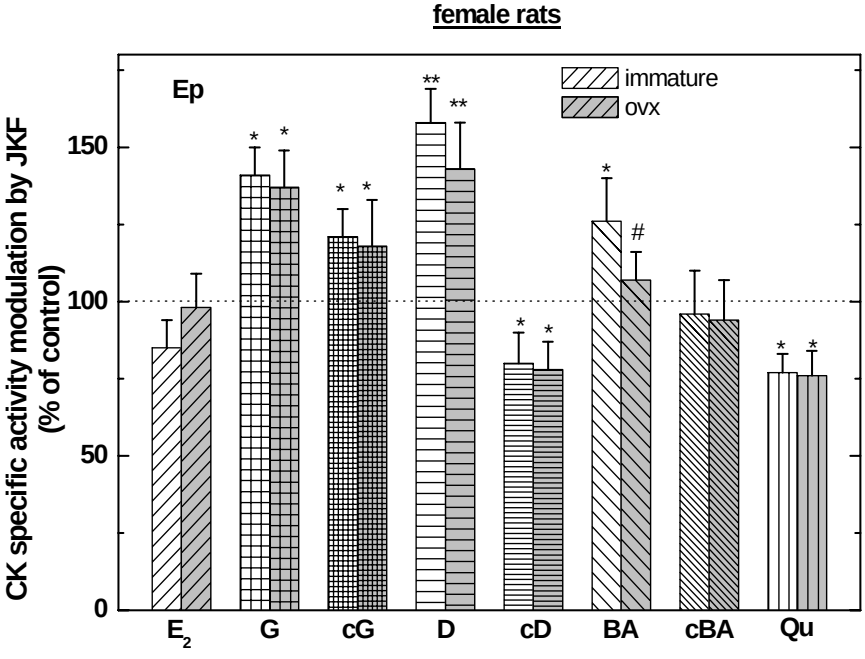

Fig. 2c. The effect of pre-treatment daily for 3 days with JKF (0.20 ng/gr BW) on the response to the different hormonal treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg G, D, BA and Qu or 50 µg cG, cD and cBA with and without Ral on CK specific activity in Ep. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05; ** P <0.01, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

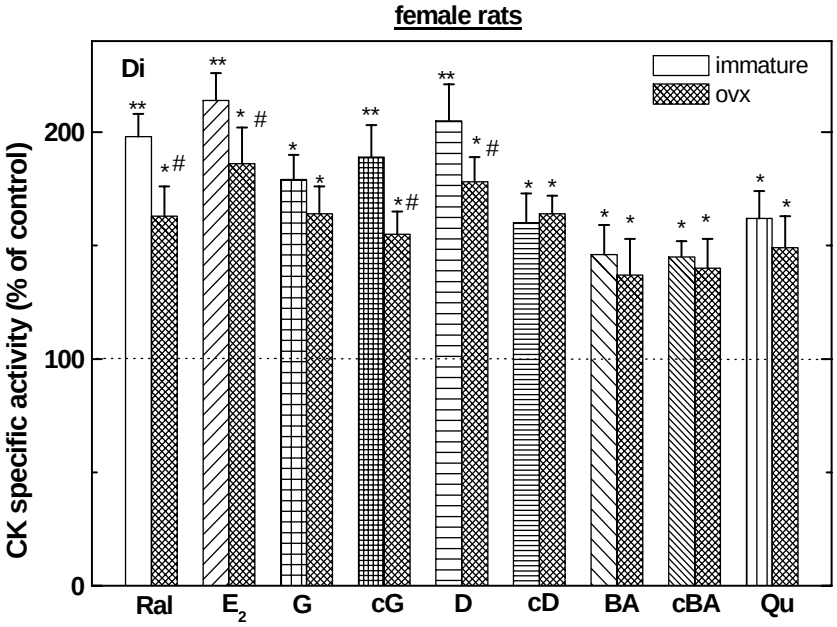

Fig. 3a. The effect of treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg Ral, G, D, BA and Qu or 50 µg cG, cD and cBA on CK specific activity in Di. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05, ** P <0.01, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

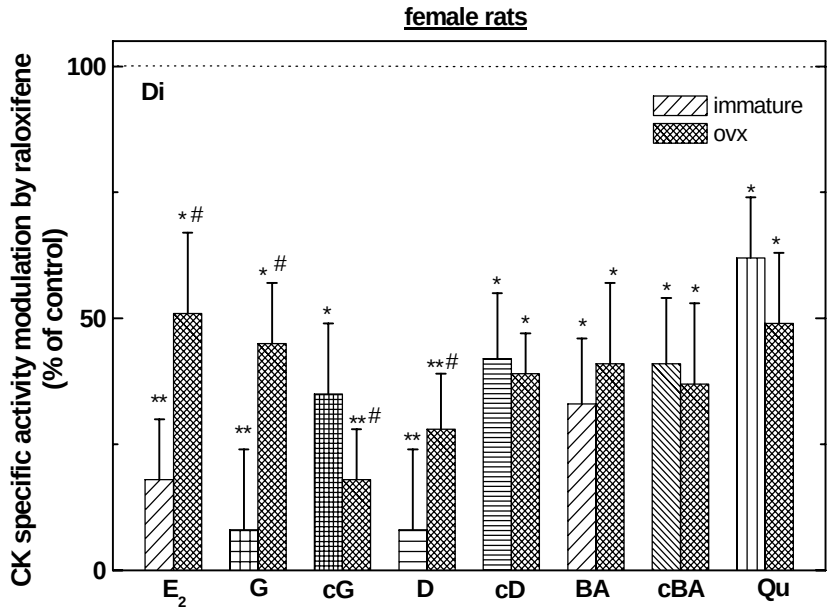

Fig. 3b. The effect of treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg Ral, G, D, BA and Qu or 50 µg cG, cD and cBA with and without Ral on CK specific activity in Di. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05; ** P <0.01, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

BA, cBA and Qu to higher extent in Ovx female rats and up-regulated the response to D to higher extent in immature female rats only (Fig. 3c).

The effect of different estrogens on the specific activity of creatine kinase in Ut from immature and Ovx female rats

Injection of 500 µg Ral, 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA resulted in modulation of CK specific activity in Ut from immature and Ovx female rats (Fig. 4a). In Ut the stimulation by all compounds was evident except by Ral. The response to G, cG, D and cD was higher in immature than in Ovx female rats (Fig. 4a), whereas the stimulation by E_2 and cBA was similar in both age groups and the stimulation by Qu was higher in Ovx than in immature female rats (Fig 4a).

Modulation of age-dependent CK response of Ut from immature and Ovx female rats to estrogenic compounds by raloxifene (Ral)

Injection of 5µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA alone or together with 500 µg Ral increased CK in Ut from immature and Ovx female rats (Fig. 4a). Ral inhibited the stimulation of enzyme activity by all compounds except by cBA in both immature and in Ovx female rats (Fig. 4b). The inhibition by Ral of CK induced by E_2 was higher in immature than in Ovx female rats (Fig. 4b), whereas the inhibition of CK induced by cG was higher in Ovx than in immature female rats (Fig. 4b).

Modulation of age-dependent CK response of Ut from immature and Ovx female rats to estrogenic compounds after pre-treatment with the vitamin D less-calcemic analog JKF

When intact or Ovx female rats were injected daily for 3 days with the less-calcemic vitamin D analog JKF at 0.2 ng/gr BW, CK was modulated in Ut in both animal groups. Injection of 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA increased CK in Ut from immature or Ovx female rats (Fig. 4a). JKF inhibited the stimulation of CK in Ut in both age groups, by E_2 and cD, but up-regulated the response to G and cG to a higher extent in Ovx, while up-regulating the response to

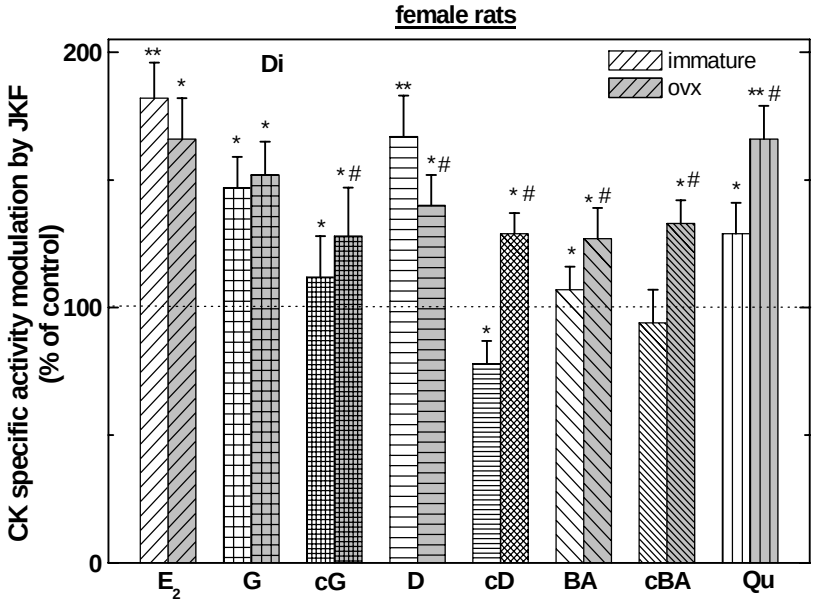

Fig. 3c. The effect of pre-treatment daily for 3 days with JKF (0.20 ng/gr BW) on the response to the different hormonal treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg G, D, BA and Qu or 50 µg cG, cD and cBA with and without Ral on CK specific activity in Di. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05; ** P <0.01, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

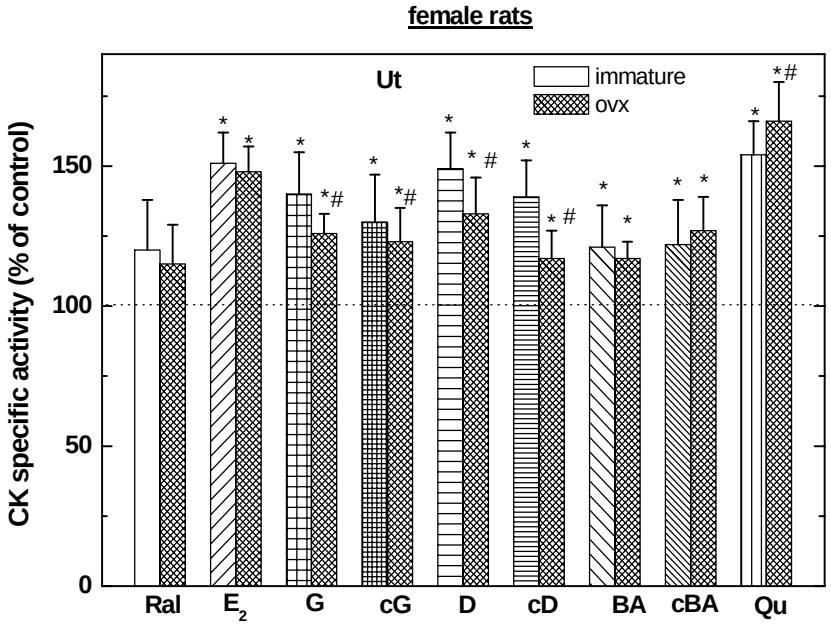

Fig. 4a. The effect of treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg Ral, G, D, BA and Qu or 50 µg cG, cD and cBA on CK specific activity in Ut. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

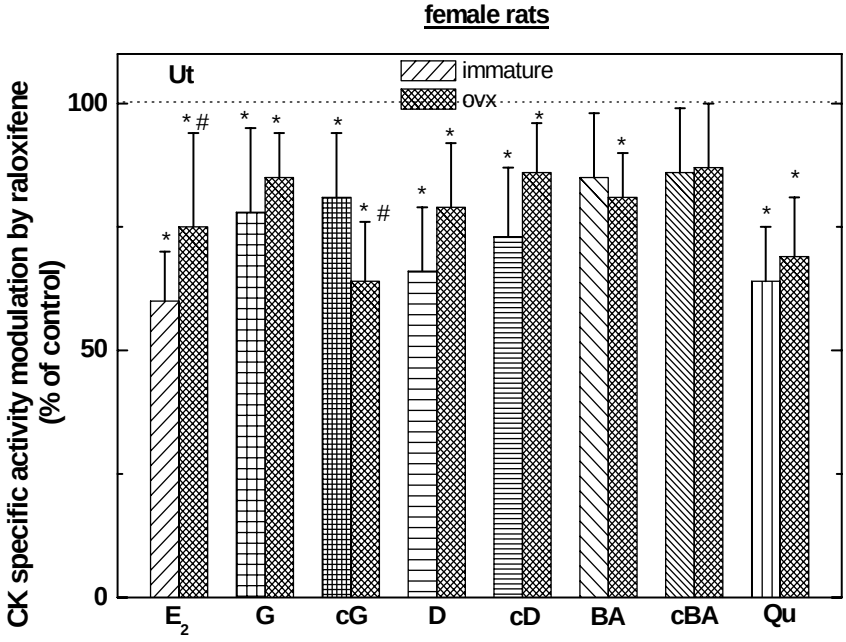

Fig. 4b. The effect of treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg Ral, G, D, BA and Qu or 50 µg cG, cD and cBA with and without Ral on CK specific activity in Ut. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05; and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

BA, cBA and Qu to higher extent in immature female rats, but the up-regulated response to D was similar in both animal groups (Fig. 4c).

The effect of different estrogens on the specific activity of creatine kinase in Pi from immature and Ovx female rats

Injection of 500 µg Ral, 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA resulted in modulation of CK specific activity in Pi from immature or Ovx female rats (Fig. 5a). The response to Ral, E_2 and G was higher in immature than in Ovx female rats (Fig. 5a), whereas the stimulation by all other hormones was similar in both animal groups (Fig 5a).

Modulation of age-dependent CK response of Pi from immature and Ovx female rats to estrogenic compounds by raloxifene (Ral)

Injection of 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA alone or together with 500 µg Ral increased CK in Pi from immature or Ovx female rats (Fig. 5a). Ral inhibited the stimulation of enzyme activity by all compounds in both immature and in Ovx female rats (Fig. 5b). The inhibition by Ral of CK induced by E_2 and D was lower in immature than in Ovx female rats (Fig. 5b), whereas the inhibition of CK induced by G was lower in Ovx female rats (Fig. 5b) and the stimulation by all other compounds was inhibited similarly in both animal groups.

Modulation of age-dependent CK response of Pi from immature and Ovx female rats to estrogenic compounds after pre-treatment with the vitamin D less-calcemic analog JKF

When intact or Ovx female rats were injected daily for 3 days with the less-calcemic vitamin D analog JKF at 0.2 ng/gr BW, CK was modulated in Pi in both animal groups. Injection of 5 µg E_2, 500 µg Qu, G, D, BA or 50 µg cG, cD or cBA increased CK in Pi from immature or Ovx female rats (Fig. 5a). JKF up-regulated the stimulation of CK in Pi in both animal groups by all hormones except by Qu. The up-regulated response to E_2, G and D was to a higher extent in immature female

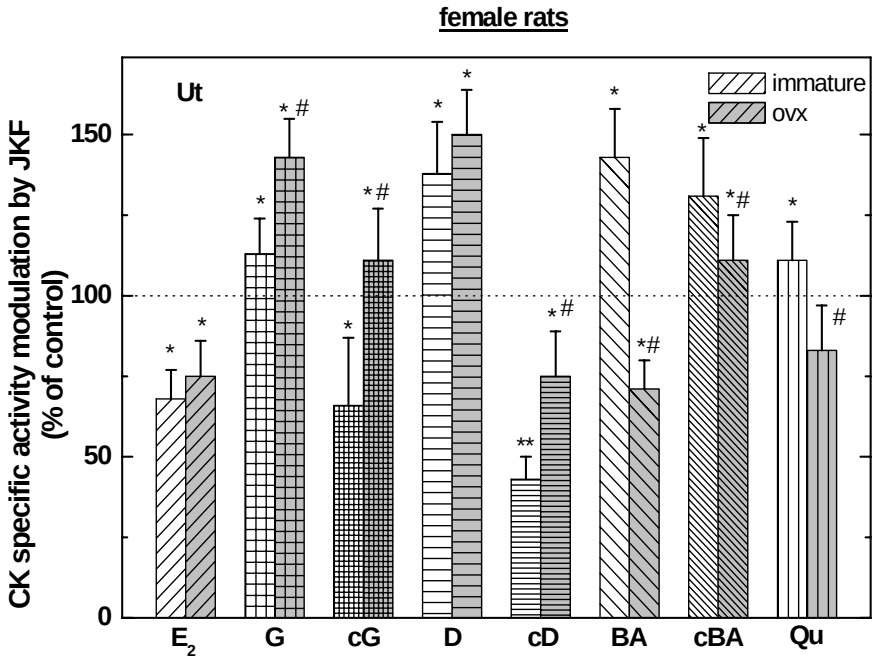

Fig. 4c. The effect of pre-treatment daily for 3 days with JKF (0.20 ng/gr BW) on the response to the different hormonal treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg G, D, BA and Qu or 50 µg cG, cD and cBA with and without Ral on CK specific activity in Ut. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

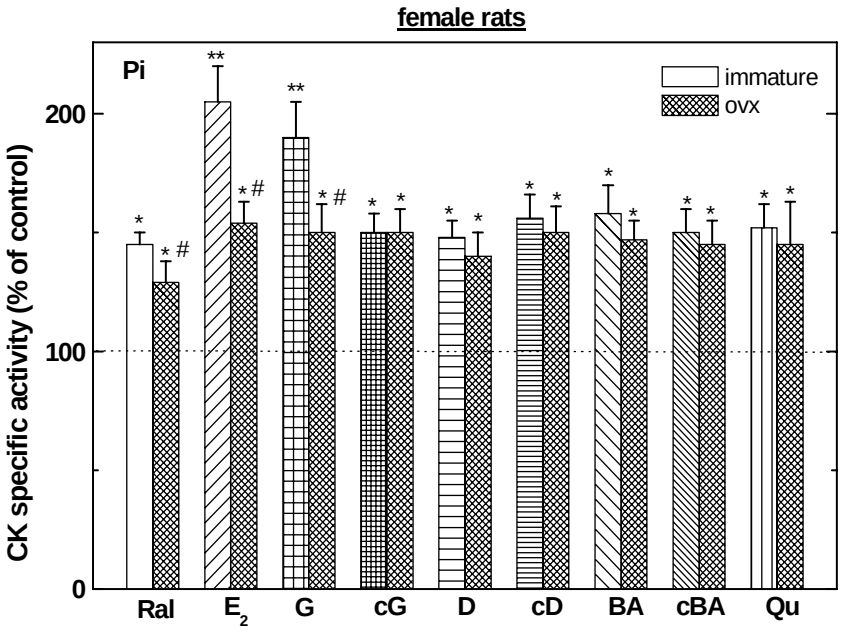

Fig. 5a. The effect of treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg Ral, G, D, BA and Qu or 50 µg cG, cD and cBA on CK specific activity in Pi. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05, ** P <0.01, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

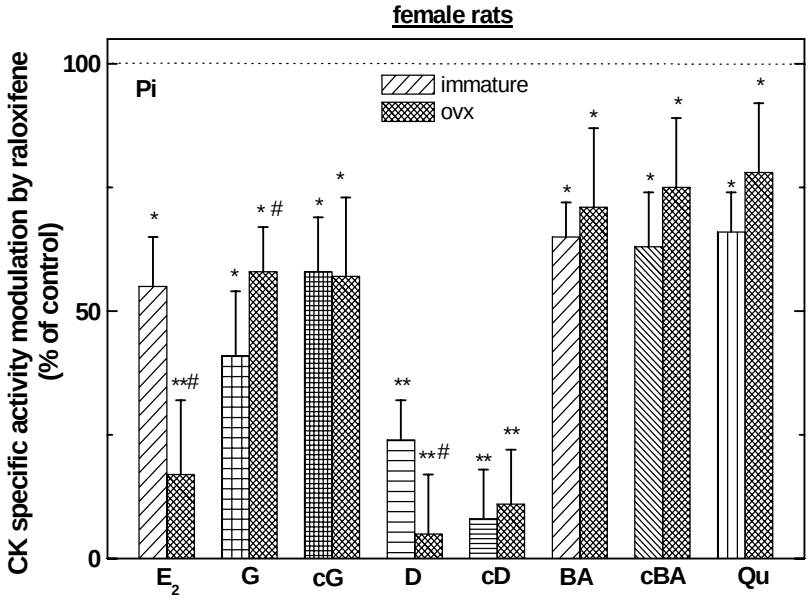

Fig. 5b. The effect of treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg Ral, G, D, BA and Qu or 50 µg cG, cD and cBA with and without Ral on CK specific activity in Pi. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05, ** P <0.01, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

rats while the up-regulated response to BA and cBA was to a higher extent in Ovx female rats, but the up-regulation of the response to cG was similar in both animal groups (Fig. 5c).

DISCUSSION

We have previously shown [22] age-dependent response of skeletal cells and organs to estrogenic compounds such as E_2, phytoestrogens from different sources and SERMs, exemplified by raloxifene (Ral), in rat bone *in vivo* and in human bone-derived cells in culture *in vitro*. We measured different intracellular effects such as cell proliferation determined as [^{3}H] thymidine incorporation into DNA and energy metabolism measured by the specific activity of creatine kinase BB as well as ERs mRNA expression, 1OHase mRNA expression and activity and LO mRNA expression and activity. In the present study we decided to examine whether the results obtained in cell cultures *in vitro* apply also to the animal models response of different rat organs from immature and Ovx rats to a variety of estrogenic compounds with and without Ral, as well as with and without pre-treatment with the vitamin D less-calcemic analog JKF. CK specific activity in all organs tested from immature female rats compared with those from Ovx female rats show that the level of enzyme specific activity in organs from Ovx is significantly lower than the level in organs from immature female rats. In Pi the stimulation by all compounds was higher in immature than in Ovx female rats, Ral inhibited the stimulation of enzyme activity by all compounds, but was higher in immature than in Ovx female rats, whereas in Ut, BA was inactive in both ages while all other compounds were slightly more active in immature rat Ut and Ral, although inactive by itself, inhibited almost all stimulated CK in immature and Ovx Ut. In Ep the stimulation by all compounds, was similar in immature and in Ovx female rats. Ral inhibited

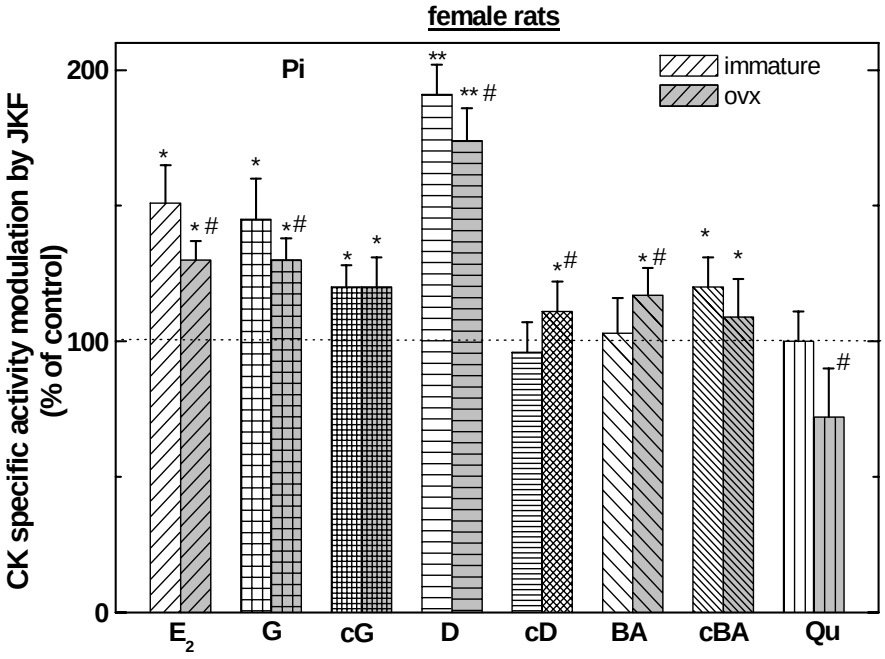

Fig. 5c. The effect of pre-treatment daily for 3 days with JKF (0.20 ng/gr BW) on the response to the different hormonal treatment for 24 h of immature and Ovx female rats with 5 µg E_2, 500 µg G, D, BA and Qu or 50 µg cG, cD and cBA with and without Ral on CK specific activity in Pi. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity of hormonal treated organs means compared to vehicle control means: *, P <0.05, ** P <0.01, and $^{\#}$, P <0.05 for values in Ovx compared to immature rats.

the stimulation of enzyme activity by all compounds in both types of female rats. In Di all compounds except the carboxy-derivatives were more active in immature compared to Ovx rat. Ral inhibited all stimulated CK except by cG in immature Di.

When intact or Ovx female rats were injected daily for 3 days with the less-calcemic vitamin D analog JKF at 0.2 ng/gr BW, CK was not significantly modulated in any of the organs in both age groups, and JKF did not increase CK in all organs tested by all hormones after the pre-treatment of either immature or Ovx female rats. This might be due to changes in the levels of the different types of ERs in the different organs and/or in their binding activity.

Of interest is the fact that the hormonal status-dependent response of bone *in vivo* to estrogenic compounds was only slightly non-significantly modified by manipulation of the endocrine environment by vitamin D compounds unlike in cultured human bone osteoblasts.

The key finding in the present study is that rat female organs derived from different hormonal status are still hormone-responsive but respond differently at different stages of development, to the estrogenic compounds similar to what we found previously [25, 26]. E_2 as well as some phytoestrogens and their carboxy-derivatives stimulate CK activity in rat tissues of both immature and Ovx similar to previous results [26]. The less-calcemic analog of vitamin D, JKF per se, did not increase significantly CK in rat tissues. Moreover pre-treatment with JKF for 3 days in both immature and Ovx female rats did not modify the response and the sensitivity to different estrogenic compounds except for E_2 similar to our previous results [13, 26].

In conclusion some of the rat organs from immature or Ovx female animals responded differently to estrogenic compounds in a yet unknown mechanism. Whether or not this applies also to human bone physiology *in vivo* is yet to be established.

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