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Original Communication

Ovariectomy decreases the responsiveness to estrogenic compounds of creatine kinase specific activity in aorta and left myocardial ventricle

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ABSTRACT

Ovariectomy of female rats decreased metabolic activities in various organs which are restored by estradiol-17β (E_2) and phytoestrogens. We now compared the effects of E_2 with those of the phytoestrogens namely quercertin (Qu), daidzein (D), genistein (G), biochainin A (BA) and their carboxy-derivatives cD, cG and cBA on aorta (Ao) and left myocardial ventricle (Lv) in immature (Imm) and ovariectomized (Ovx) female rats, on creatine kinase (CK) specific activity, when injected for 24h with and without the SERM raloxifene (Ral) or with and without pre-treatment for 3 days with the less-calcemic vitamin D analog JKF 1624 F_2-2 (JKF). We found: 1) Ovariectomy significantly reduced basal CK in Ao by 47% and in Lv by 36%. 2) All estrogens stimulated CK in both types of rats. 3) In Ao and in Lv some of the estrogens were more effective in Imm than in Ovx. 4) The SERM Ral inhibited estrogenic stimulated CK slightly less effectively in Ovx than in Imm in both organs. 5) In Ao, pre-treatment with JKF decreased CK response to different estrogens whereas in Lv, JKF up-regulated CK stimulation only by E_2, G and D in both animals. The modulation of response by Ral or JKF might be due to effects on the level and activity of estrogen receptors leading to altered responsiveness. In summary, the estrogenic response of vascular female rat organs are modulated by ovariectomy in an organ- and substrate-dependent manner correlated with modulation of the level and/or binding activity of the different receptor(s).

KEYWORDS: phytoestrogens, creatine kinase, aorta, left myocardial ventricle

INTRODUCTION

Mammalian vascular cells express specific intracellular and membranal receptors for estrogens (ERs) [1, 2] and respond to different estrogenic compounds by many parameters [3-5]. The age dependent-responses were studied in many organs by different researchers but are still debatable in models of vascular cells in cell cultures *in vitro* [4-6] and in model rats *in vivo* [7].

We have analyzed the response of human derived vascular smooth muscle cells (VSMC) in culture by different parameters to different estrogenic compounds, including estradiol-17β (E_2), the specific estrogen receptors ligands 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN; ERβ specific

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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 phytoestrogens derived mainly from soy including their synthetic carboxy-derivatives and protein-bound carboxy-derivatives. Whether these findings apply also to *in vivo* models of vascular organs in the female rats as well as in post-menopausal human subjects is not yet established [8].

We found that there is a higher expression of ERβ mRNA and lower expression of ERα mRNA in VSMC as well as intracellular and membranal estrogen binding activities [8-9].

The less-calcemic vitamin D analog JK $1624F_2$-2 (JKF) modulated the response to E_2 and some phytoestrogens in VSMC to some extent [9].

In the present study, we summarize our findings using female rat vascular organs derived from either immature or Ovx animals carried out in our laboratory on:

a. The effect of different estrogenic compounds on CK specific activity in rat female aorta (Ao) and left myocardial ventricle (Lv) in both types of animals.

b. Modulation by Raloxifene (Ral) of the effect of different estrogenic compounds on CK specific activity response of Ao and Lv in both types of animals.

c. Modulation by pre-treatment with the less-calcemic vitamin D analog JKF $1624F_2$-2 (JKF) of the effect of different estrogenic compounds on CK specific activity response of Ao and Lv in both types of animals.

MATERIALS AND METHODS

Chemicals

Estradiol-17β, creatine kinase assay kit and phytoestrogens used, were purchased from Sigma Chemicals Co. (St. Louis, MO. USA). Raloxifene was donated by Novartis Basel Switzerland. The carboxy-derivatives of the phytoestrogens were synthesized by us [10]. JK $1624F_2$-2 (JKF) was synthesized and provided by Dr. G.H. Posner, Johns Hopkins University Baltimore MD. USA [11]. All other reagents used were of analytical grade.

Hormonal treatment

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

For pre-treatment with the vitamin D less-calcemic analog, immature or Ovx female rats were injected daily for 3 days with JK $1624F_2$-2 (JKF) (0.2ng/rat) [7], and 24h after the last analog injection, rats were injected with the different estrogenic compounds as described above for extra 24h. After the last injection, rat organs were harvested for creatine kinase (CK) specific activity extraction and assay [12, 13].

Creatine kinase specific activity preparation and assay

Rat organs were obtained, 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 [12, 13].

Statistical analysis

Data were calculated as % stimulation by the treatment relative to control rats (vehicle-treated) 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 aorta and left myocardial ventricle from immature and Ovx female rats

CK specific activity in Ao and Lv from immature female rats compared to the same organs 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 Ao there is 47% higher CK in immature than in Ovx, and in Lv there is 36% higher CK in immature than in Ovx (Fig. 1).

Estrogenic modulation of CK response of aorta and left myocardial ventricle from immature and Ovx female rats

Injection of 5µg E_2, 500µg Qu, Ral, G, D, BA or 50µg cG, cD or cBA increased CK in Ao and in Lv by all compounds (Fig. 2a, 2b). In Ao the stimulation by all compounds except Ral, cG, cD and Qu was higher in immature (Fig. 2a) than in Ovx female rats by 30-300%. In Lv all compounds were active in both ages. Only G was more active in immature compared to Ovx rat Lv (Fig. 2b), whereas cG, cD, BA, cBA and Qu were more active in Ovx compared to immature rat Lv (Fig. 2b). All other compounds, E_2, Ral and D were similarly active in Lv from both types of animals (Fig. 2b).

Modulation of estrogenic CK response of rat female aorta and left myocardial ventricle by Ral

Injection of 5µg E_2, 500µg Qu, G, D, BA or 50µg cG, cD or cBA or Ral (500µg) alone or the hormones with Ral, increased CK in Ao and in Lv by all hormones (Fig. 2a, 2b). Ral inhibited CK stimulation by all hormones in Ao (Fig 3a) and in Lv (Fig. 3b). Ral inhibition was similar in both organs in both immature and Ovx female rats (Fig. 3a, 3b).

Modulation of estrogenic CK response of rat female aorta and left myocardial ventricle by 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 0.2ng/rat, CK was slightly modulated in Ao and Lv in both age groups (Fig. 4a, 4b). Injection of 5µg E_2, 500µg Qu, G, D, or BA or 50µg cG, cD or cBA and JKF 0.2ng alone, or after JKF pre-treatment modulated increased CK in both organs by all hormones (Fig. 4a and 4b). In Ao the stimulation only by cG was higher in immature (Fig. 4a) but in Ovx (Fig. 4b) female rats cG, cD and Qu responses were up-regulated by JKF. In Lv, JKF up-regulated the response to E_2, G and D only in both animal groups (Fig. 4b).

DISCUSSION

We have previously shown the response of vascular cells and organs to estrogenic compounds

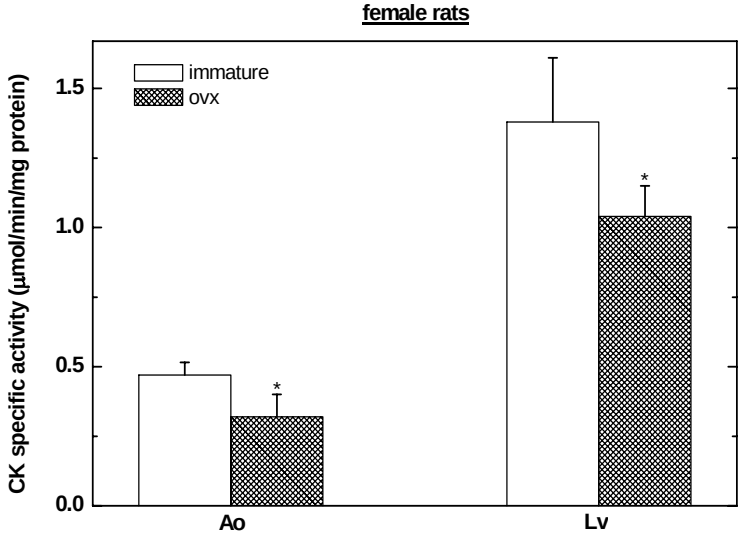

Fig. 1. Creatine kinase specific activity of aorta (Ao) and left ventricle of the heart (Lv) in immature and Ovx female rats. Organs were obtained and assayed as described. Results are means ± SEM for 5 animals/group. Enzyme specific activity means of Ovx control compared to immature control means: *, *P<0.05*.

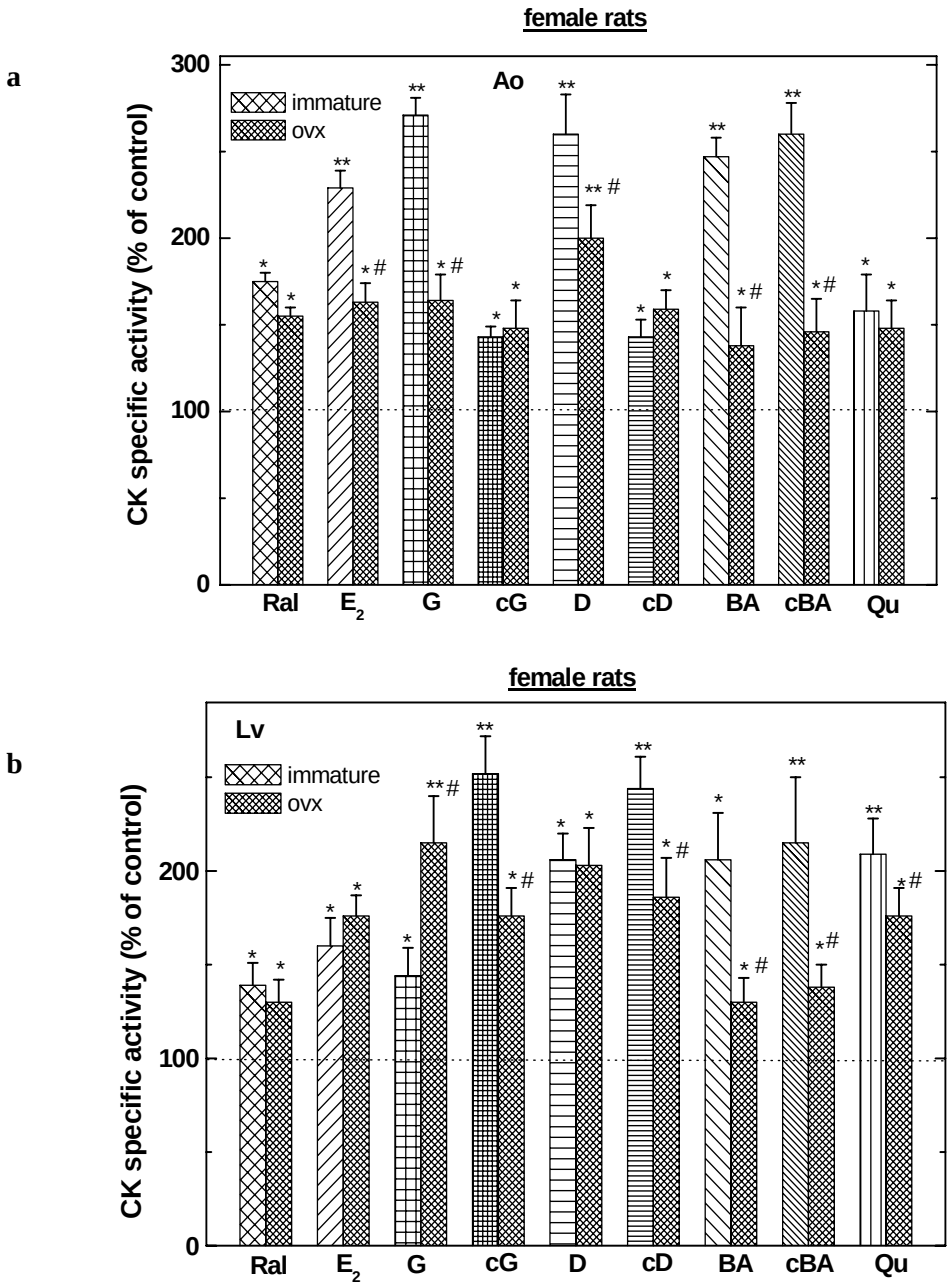

Fig. 2 a. The effect of treatment for 24h of immature and Ovx female rats with E_2 (5µg), quercertin (Qu; 500µg), raloxifene (ral; 500µg), genistein (G), daidzein (D), biochainin A (BA), carboxy G (cG), carboxy D (cD) and carboxy BA (cBA) all free compounds at 500µg and the carboxy-derivatives at 50µg, on CK specific activity in Ao. 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: *, $P<0.05$, **, $P<0.01$, #, $P<0.05$ for the difference in the response between Ovx and immature derived organs. **b.** The effect of treatment for 24h of immature and Ovx female rats with E_2 (5µg), quercertin (Qu; 500µg), raloxifene (ral; 500µg), genistein (G), daidzein (D), biochainin A (BA), carboxy G (cG), carboxy D (cD) and carboxy BA (cBA) all free compounds at 500µg and the carboxy-derivatives at 50µg, on CK specific activity in Lv. 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: *, $P<0.05$, **, $P<0.01$, #, $P<0.05$ for the difference in the response between Ovx and immature derived organs.

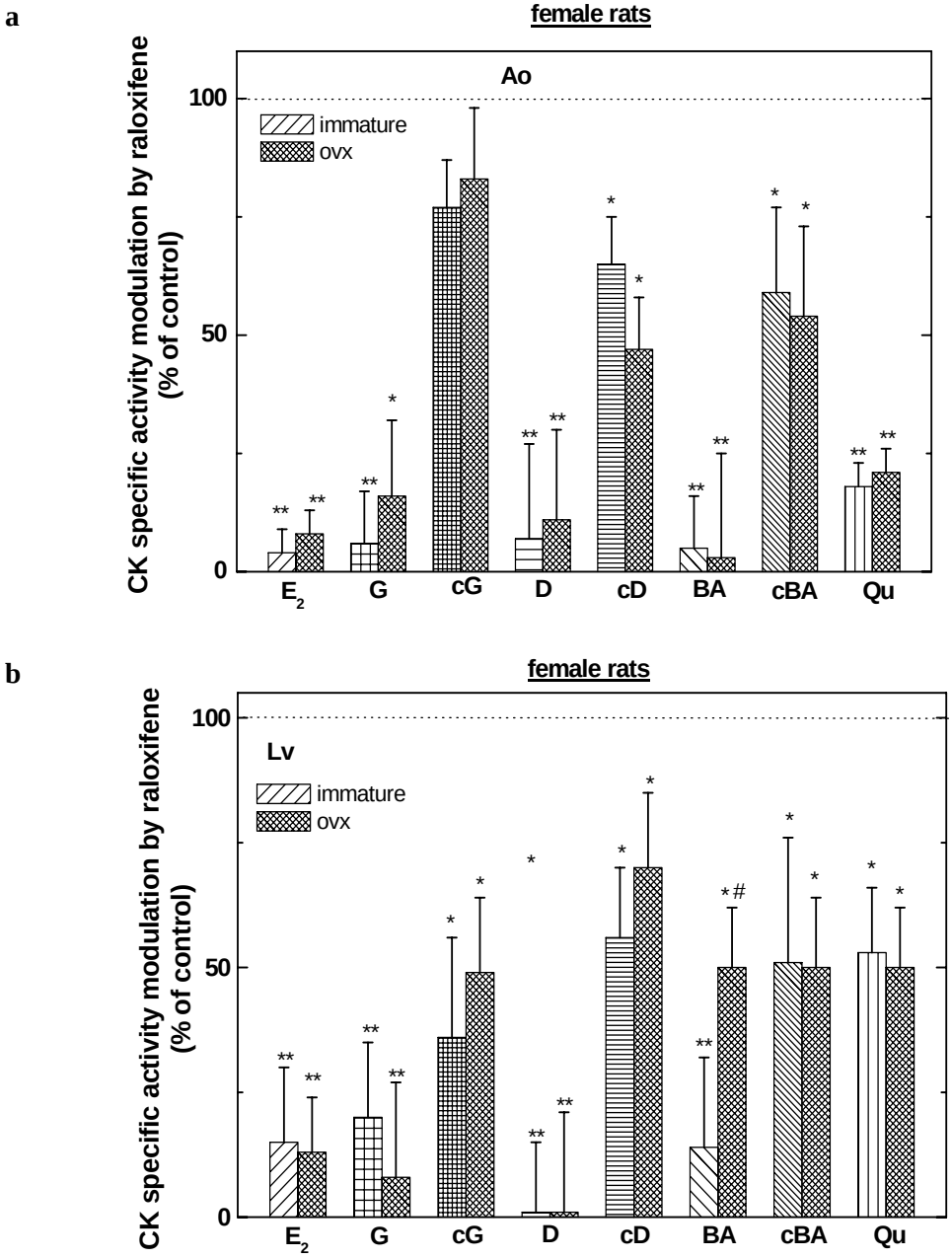

Fig. 3 a. The effect of treatment for 24h of immature and Ovx female rats with E_2 (5µg), quercertin (Qu; 500µg), genistein (G), daidzein (D), biochainin A (BA), carboxy G (cG), carboxy D (cD) and carboxy BA (cBA) all free compounds at 500µg and the carboxy-derivatives at 50µg, raloxifene (ral; 500µg) or hormones together with Ral on CK specific activity in Ao. 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*. **b.** The effect of treatment for 24h of immature and Ovx female rats with E_2 (5µg), quercertin (Qu; 500µg), genistein (G), daidzein (D), biochainin A (BA), carboxy G (cG), carboxy D (cD) and carboxy BA (cBA); all free compounds at 500µg and the carboxy-derivatives at 50µg, raloxifene (ral; 500µg) or hormones together with Ral on CK specific activity in Lv. 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*, #, *P<0.05* for the difference in the response between Ovx and immature derived organs.

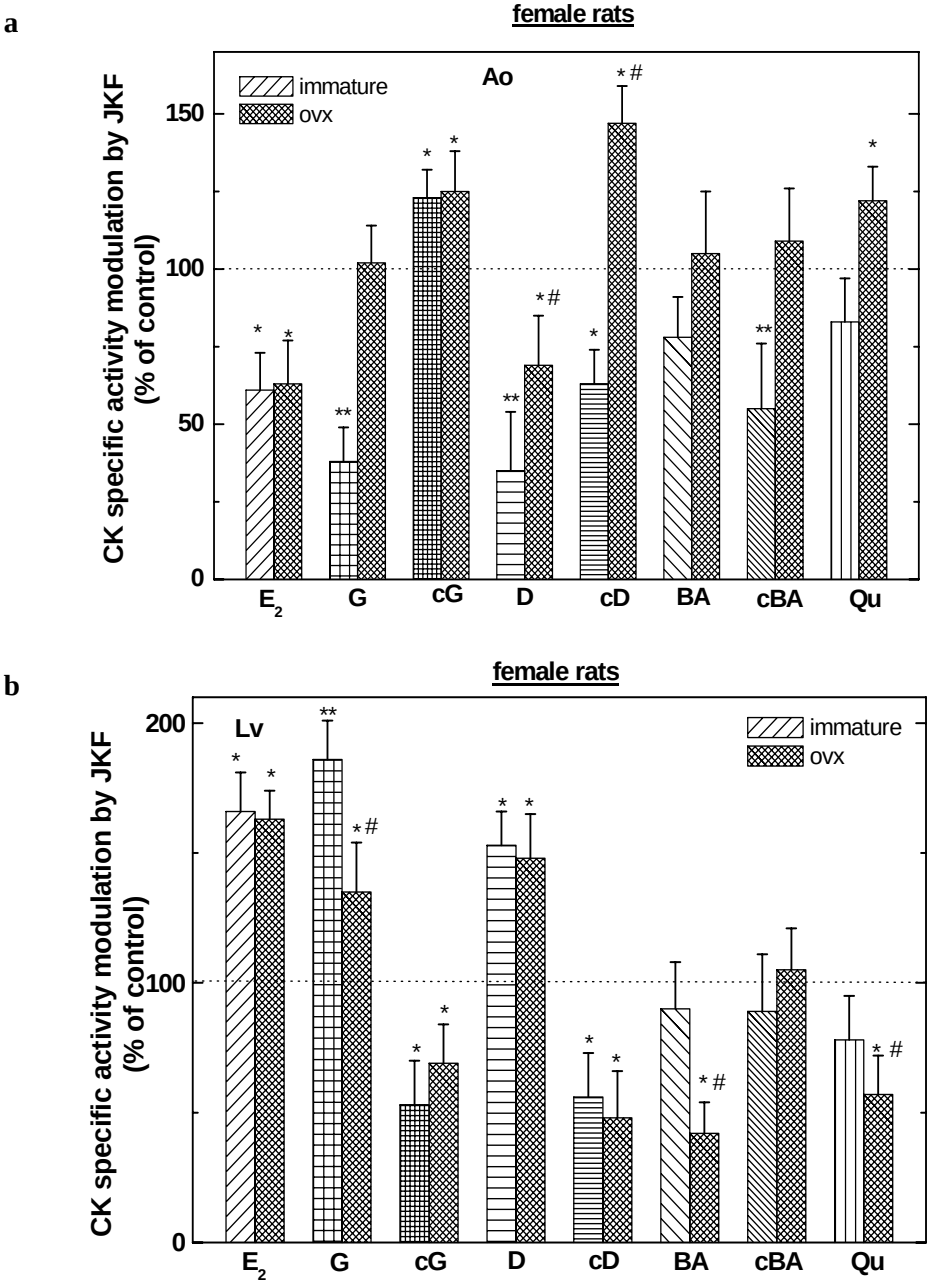

Fig. 4 a. The effect of treatment for 24h of immature and Ovx female rats with E_2 (5µg), quercertin (Qu; 500µg), genistein (G), daidzein (D), biochainin A (BA), carboxy G (cG), carboxy D (cD) and carboxy BA (cBA) all free compounds at 500µg and the carboxy-derivatives at 50µg, after pretreatment with JKF (0.2ng/gr) on CK specific activity in Ao. 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: *, $P<0.05$, **, $P<0.01$, #, $P<0.05$ for the difference in the response between Ovx and immature derived organs. **b.** The effect of treatment for 24h of immature and Ovx female rats with E_2 (5µg), quercertin (Qu; 500µg), genistein (G), daidzein (D), biochainin A (BA), carboxy G (cG), carboxy D (cD) and carboxy BA (cBA) all free compounds at 500µg and the carboxy-derivatives at 50µg, after pretreatment with JKF (0.2ng/gr) on CK specific activity in Lv. 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: *, $P<0.05$, **, $P<0.01$, #, $P<0.05$ for the difference in the response between Ovx and immature derived organs.

such as E_2, phytoestrogens from different sources and SERMs, exemplified by raloxifene (Ral), in rat Ao and Lv *in vivo* and in human vascular smooth muscle cells in culture *in vitro* [2, 3, 5]. We measured *in vitro* 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 set to examine whether the results obtained with cell culture *in vitro* apply also *in vivo* to the age-dependent response of vascular rat organs such as aorta (Ao) and left myocardial ventricle (Lv) from immature and Ovx rats to different estrogenic compounds with and without Ral or with and without pre-treatment with the vitamin D less-calcemic analog JKF. CK specific activity in Ao and Lv from immature female rats was higher compared with those from Ovx female rats. In Ao the stimulation by all compounds, except Ral, cG, cD and Qu was higher in immature than in Ovx female rats. In Lv all compounds except E_2, Ral and D were more active in immature female rats. Only G was more active in Ovx rat Lv. Ral inhibited the stimulation by all compounds tested in both Ao and Lv from both immature and Ovx female rats.

When intact or Ovx female rats were injected daily for 3 days with the less-calcemic vitamin D JKF at 0.2ng/rat, CK was not significantly modulated in vascular organs from both age groups. In Ao of immature female rats, JKF did not up-regulate CK stimulation by E_2, G, D, BA, cBA and Qu. In Ao from Ovx female rats JKF up-regulated the response to cG, cD and Qu. In Lv, on the other hand, JKF up-regulated the response in both age groups to E_2, G and D only. Whether this is correlated with changes in estrogen receptors expression and/or activity is still to be analyzed.

In conclusion, rat female vascular organs in both immature and Ovx rats are hormone-responsive and respond differently at their different stages of development, to a variety of estrogenic compounds, similar to other rat organs tested. Moreover, they also respond to vitamin D analog slightly, but the estrogenic responses to some compounds were modulated in both organs after this pre-treatment. Whether or not this applies also to human bone physiology *in vivo* is yet to be established.

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