Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1307–1313
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway

Christophe Prouillet[a,b,1], Jean-Claude Mazière[b], Cécile Mazière[b], Alice Wattel[a], Michel Brazier[a], Saïd Kamel[a,*]

[a]*Laboratoire de Biologie et pharmacie cliniques, Unité de recherche sur les mécanismes de la résorption osseuse (UMRO), UPRES EA 2086, Faculté de Pharmacie, Université de Picardie Jules Verne, 80038 Amiens Cedex 01, France*
[b]*Laboratoire de Biochimie, JE 23 29, CHRU d'Amiens, Hôpital Nord, 80054 Amiens Cedex 01, France*

Received 16 September 2003; accepted 18 November 2003

Abstract

Many plant-derived substances have estrogenic activities. Due to their ability to bind the estrogen receptor (ER), these compounds have the potential to counteract the deleterious effects of estrogen deficiency on bone. In this study, we investigated the *in vitro* effect of two widespread flavonols, quercetin and kaempferol, on alkaline phosphatase (ALP) activity in MG-63 cultured human osteoblasts. We found that both flavonols significantly increased ALP activity. This effect was markedly reduced by PD 98059, an inhibitor of the extracellular regulated kinase (ERK) pathway, and by ICI 182780, an antagonist of ERs. Western blot studies confirmed that ERK is rapidly activated in cells treated by both flavonols. Finally, ICI 182780 markedly inhibits the flavonol-induced ERK activation. The data presented in this study support the conclusion that, in MG-63 osteoblasts (i) the increase in ALP activity by flavonols involves a rapid stimulation of ERK activation but also involves the ER, and that (ii) the activation of ERK by flavonols occurs most likely downstream of the ERs activation. Taken together, these results suggest that flavonols derivatives as quercetin and kaempferol can stimulate osteoblastic activity. Such compounds may represent new pharmacological tools for the treatment of osteoporosis.

Keywords: Osteoblasts; Alkaline phosphatase; ERK; Flavonols; Estrogen receptors

1. Introduction

Osteoporosis, is a disease characterized by a reduced bone mass resulting in increased bone fragility and fracture risk [1]. Due to the increase in the lifespan of the population, osteoporosis became a major and growing health problem in developed countries. Estrogen deficiency which occurs at menopause plays a major role in the development of osteoporosis in post-menopausal women [2,3]. In this pathological situation, there is an abnormally high bone turnover due to both an increase in osteoclastic bone resorption and a decrease in osteoblastic bone formation. In order to prevent adverse effects of estrogen deficiency on bone [4], estrogen replacement therapy has been proposed. A more recently developed pharmacological approach consisted on the use of selective estrogen receptor modulator (SERM) a class of compounds which can reproduce the estrogen effects on bone [5]. Many investigators have shown that a class of plant-derived substances had estrogenic activities, they are so-called ''phytoestrogen''. These include the flavonoids family comprising isoflavones and flavonols derivatives. Due to their ability to bind ER, these naturally compounds could have positive effect against bone loss. Indeed, isoflavones such as daidzein and genistein, which are found in abundance in soybeans and their derivative foods, have been shown to

* Corresponding author. Tel.: +33-3-22-82-77-90; fax: +33-3-22-82-74-69.
E-mail addresses: christophe.prouillet@sa.u-picardie.fr (C. Prouillet), said.kamel@sa.u-picardie.fr (S. Kamel).
[1] Co-corresponding author.
Abbreviations: ALP, alkaline phosphatase; ERK, extracellular regulated kinase; ER, estrogen receptor; MAPK, mitogen activated protein kinase.

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.11.009

reduce the occurrence of osteoporosis in various experimental models [6,7] and in a recent clinical study [8]. Moreover, the low incidence of osteoporosis in Asian women has been attributed to a diet rich in isoflavones [9,10]. The hypothesis that flavonols could be also bioactive molecules able to counteract the deleterious effect of estrogen deficiency which occurs at menopause, has been recently addressed by Horcajada-Molteni *et al.* [11], who demonstrated that rutin, a glycoside derivative of quercetin, one of the main flavonol, inhibits ovariectomy-induced osteopenia in female rats. While *in vitro* isoflavones effects have been extensively investigated, at the present time very few studies have been concerned with the effect of flavonols on cultured bone cells. Recently, we demonstrated that flavonols as quercetin and kaempferol decrease osteoclastic bone resorption *in vitro* by targeting directly the mature osteoclast by a mechanism involving, at least in part, the ER [12]. Miyake *et al.* reported the promoting effect of kaempferol on the differentiation and mineralization of murine pre-osteoblatic cell line [13]. Herein, we extend these results by investigating the effect of quercetin and kaempferol, two of the most widespread flavonols, on the ALP activity of MG-63 human osteoblastic cells and we attempted to clarify the mechanism involved. We show that the studied flavonols increase the ALP activity, and that this effect is dependent upon both the ERK pathway and the ER activation.

2. Materials and methods

2.1. Chemicals

Quercetin, kaempferol, EGF, 17-β estradiol, BSA, *para*-nitrophenyl phosphate and Triton X-100 were obtained from SIGMA. The MEK inhibitor PD 98059 was obtained from Calbiochem, and ICI 182780 from Tocris Cookson Ltd. PBS (pH 7.4) was from Life Technologies. The anti-phospho ERK and anti-ERK antibodies were supplied by Cell Signaling.

2.2. Cell culture

The human osteoblastic cell strain MG-63 was purchased from ATCC. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), and 1% penicillin/streptomycin supplemented with 10% fetal calf serum (Biowhittakker). For experiments, DMEM devoid of Phenol Red was used, because of the previously reported estrogen-like activity of the later compound. Twenty four hours after seeding in this medium supplemented with 10% FCS and antibiotics, the cells were shifted to DMEM devoid of Phenol Red and fetal calf serum, but supplemented with 0.1% BSA and antibiotics, in the absence or in the presence of the studied effectors. Depending on the parameter studied (ALP activity or ERK activation), the cells were seeded either in six-well plates (Corning B.V.) or in 60 mm Petri dishes (Nunc), respectively.

2.3. Measurement of alkaline phosphatase activity

After incubation with effectors, the cells were washed twice with PBS (Life Technologies), harvested with rubber policemen, and resuspended in PBS supplemented with PBS 1%, Triton X-100. The cell lysate was then sonicated for 1 min. ALP activity was assayed by a spectrophotometric method using *para*-nitrophenyl phosphate as substrate [14]. The OD was measured at 405 nm with a Bio-Rad microplate reader Model 550. The results, normalized on a protein basis, were expressed as percentages of control. Protein determination was carried out with the Bio-Rad protein assay dye kit (Bio-Rad Laboratories).

2.4. MTT proliferation assay

Cells were cultured in absence or in presence of both flavonols (50 μM) during a period of 24 or 48 hr. After incubation with flavonols, the cells were washed twice with PBS. Then, cells were treated with MTT solution (5 mg/mL in PBS diluted 1/10 in DMEM devoid of Phenol Red) for 1 hr. Cells were then lysed by SDS 10% (w/v) and optical density was measured at 560 nm [15].

2.5. Immunoblot analysis

After treatment, cells were harvested in PBS by scrapping and then centrifuged. The pellet was lysed in Tris 20 mM pH 7.9, NaCl 400 mM, EDTA 1 mM, Glycerol 20%, dithiothreitol 1 mM, PMSF 1 mM, leupeptin and aprotinin 5 μg/mL, and Na_3VO_4 0.1 mM, Triton X-100 1% then clarified by centrifugation. Fifty microgram of supernatant was boiled 95° for 2 min in Laemli buffer (bromophenol blue 0.1%, β mercapto-ethanol 5%, SDS 2.3%, glycerol 10%, Tris base 62.5 mM) and then submitted to 8% SDS–PAGE. After transfer of proteins onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech), equal loading was checked by visualization with the Ponceau Red dye (Sebia). After incubation with the appropriated antibody the blots were revealed with the enhanced chemiluminescence ECL kit from Amersham Pharmacia Biotech and analyzed by densitometry (Personal Laser Densitometer, Molecular Dynamics).

2.6. Statistical analysis

All experiments were performed at least three times in duplicate. A one-way ANOVA for multiple comparison was used for statistical analysis. A difference between experimental groups was considered to be significant when the probability value was less than 5%.

3. Results

3.1. Quercetin and kaempferol increase ALP activity via protein synthesis

Data in Fig. 1 show that treatment of MG-63 cells with quercetin or kaempferol increased ALP activity in a dose and time-dependent manner (Fig. 1a and b). At 50 μM, an about 1.5- and 1.7-fold increase in activity was observed after 48 hr of treatment with quercetin and kaempferol, respectively, as compared to controls. Figure 1c shows the effects of both flavonols on MG-63 cell proliferation assessed by MTT assay. Neither kaempferol nor quercetin did show any significant cytotoxic effect at the concentration used (50 μM) after 24 or 48 hr of treatment.

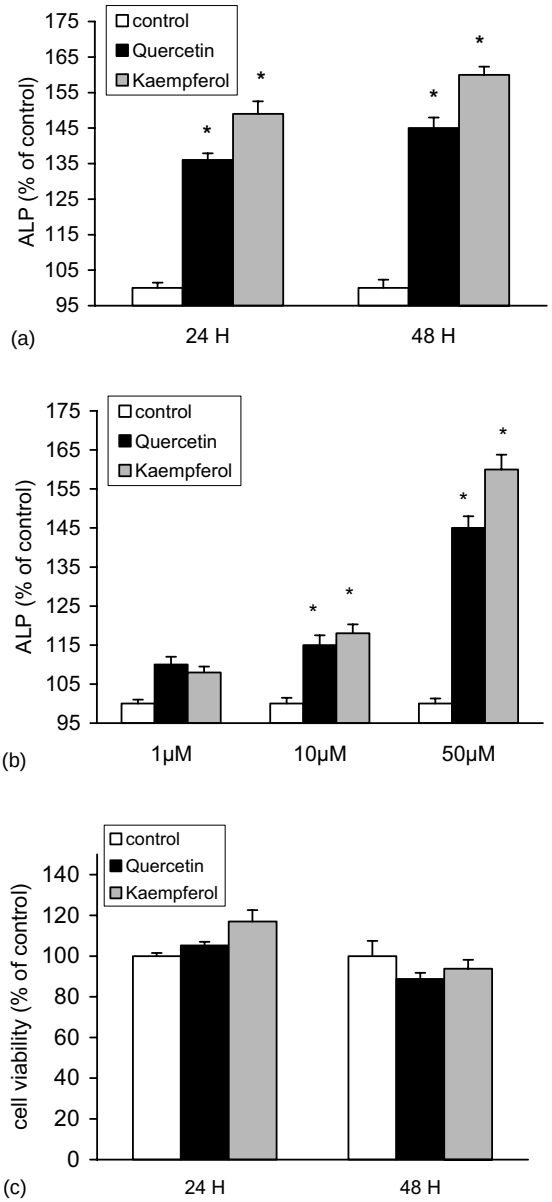

Fig. 1. Quercetin and kaempferol increase ALP activity in MG-63 human osteoblasts and do not affect cell viability. (a) Cells were treated with flavonols 50 μM for 24 or 48 hr in DMEM medium devoid of Phenol Red and supplemented with 0.1% BSA. ALP activity was then measured as described in Section 2. (b) Cells were treated for 48 hr with flavonols 1, 10 or 50 μM in the same medium. Results are expressed as percentage of controls (100% = untreated cells; ALP activity = 20 ± 4.8 nmol/mg protein/min). (c) Cells were treated with flavonols 50 μM for 24 or 48 hr in DMEM medium devoid of Phenol Red and supplemented with 0.1% BSA and MTT proliferation assay was processed as described in Section 2. Each point is the mean of nine experimental values ±SEM. $^{*}P < 0.001$ compared with control values (ANOVA test).

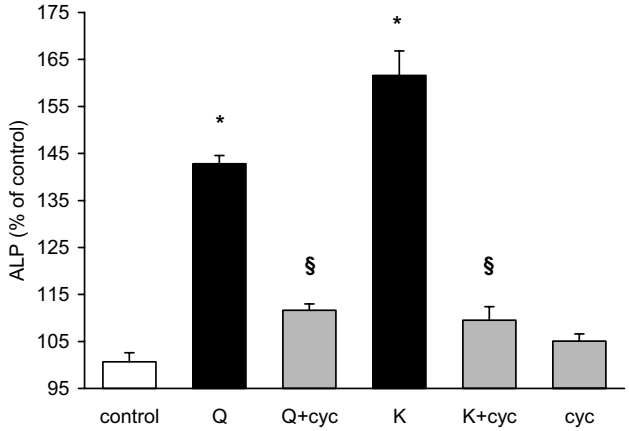

Fig. 2. Inhibitory effect of cycloheximide on quercetin and kaempferol-induced increase in ALP activity in MG-63 human osteoblasts. Cells were pretreated for 24 hr with cycloheximide 10 μg/mL. After this time period, cell culture medium was changed and cells were then further incubated for 48 hr with 50 μM of quercetin or kaempferol. ALP activity was then measured as described in Section 2. Q: quercetin; K: kaempferol; cyc: cycloheximide. Results are expressed as percentage of controls (each point is the mean of six experimental values ±SEM; 100% = untreated cells). $^{*}P < 0.001$ compared with control values (ANOVA test); $^{\S}P < 0.001$ compared with flavonols treatment alone (ANOVA test).

The flavonol-induced increase in ALP is almost completely prevented by a 24 hr pre-treatment of MG-63 cells with the protein synthesis inhibitor cycloheximide (Fig. 2).

3.2. The increase in ALP activity by quercetin or kaempferol involves ERK activation and the ER

Since the mitogen activated protein kinase (MAPK) pathway has recently been suggested to be involved in the regulation of ALP activity in other osteoblastic cells [16], we checked the effect of PD 98059, an inhibitor of the ERK pathway, on the flavonol-induced increase in ALP activity in MG-63 cells. As can be shown in Fig. 3a, when cells were incubated with flavonols in the presence of PD 98059, the stimulatory effect of quercetin or kaempferol on ALP activity was markedly reduced. We also investigated the effect of ICI 182780, a "pure" anti-estrogen [17], on the quercetin and kaempferol-induced increase in ALP activity. Figure 3b demonstrates that, under the same conditions, ICI 182780 also markedly reduces the stimulatory action of the studied flavonols on ALP activity. Taken together, these results suggest that ERK and the ER(s) are both required for the observed effect.

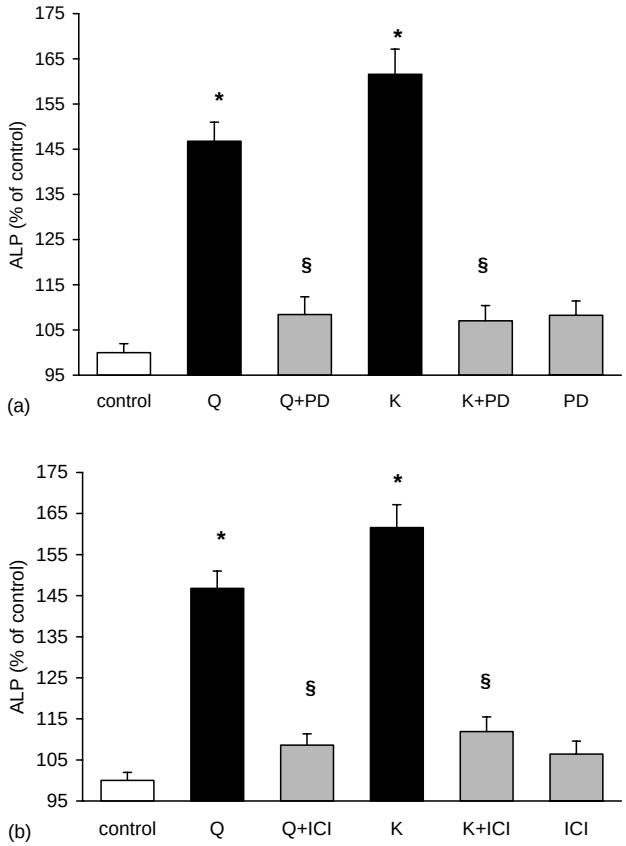

Fig. 3. (a) PD 98059 (PD) and (b) ICI 182780 (ICI) inhibit the flavonol-induced increase in ALP activity. MG-63 cells were pretreated for 2 hr with 10 μM PD 98059 or 1 μM ICI 182780 and then further incubated for 48 hr with 50 μM of flavonols. ALP activity was then measured as described in Section 2. Q: quercetin; K: kaempferol. Results are expressed as percentage of controls (each point is the mean of six experimental values ±SEM; 100% = untreated cells). $^{*}P < 0.001$ compared with control values (ANOVA test); $^{§}P < 0.001$ compared with flavonols treatment alone (ANOVA test).

3.3. Direct evidence for a rapid activation of ERK by flavonols

Involvement of the ERK pathway in the increase in ALP activity by flavonols was suggested by the marked inhibitory effect of PD 98059. So, we assessed the effect of flavonols on ERK phosphorylation by Western blotting. MG-63 cells were treated with both compounds for 2, 5, 15, 30, and 60 min. The stimulation of ERK phosphorylation was increased within 2 min after flavonols treatment, reached maximum within 5 min incubation and was decreased toward the basal level after 60 min (Fig. 4a and b). This result suggests that the stimulatory effect of flavonols on ERK phosphorylation is an early event in the cellular action of these molecules.

3.4. EGF and 17-β estradiol also increase ALP activity and rapidly activate ERK phosphorylation

The fact that the flavonol-induced increase in ALP activity was prevented by either PD 98059 or ICI

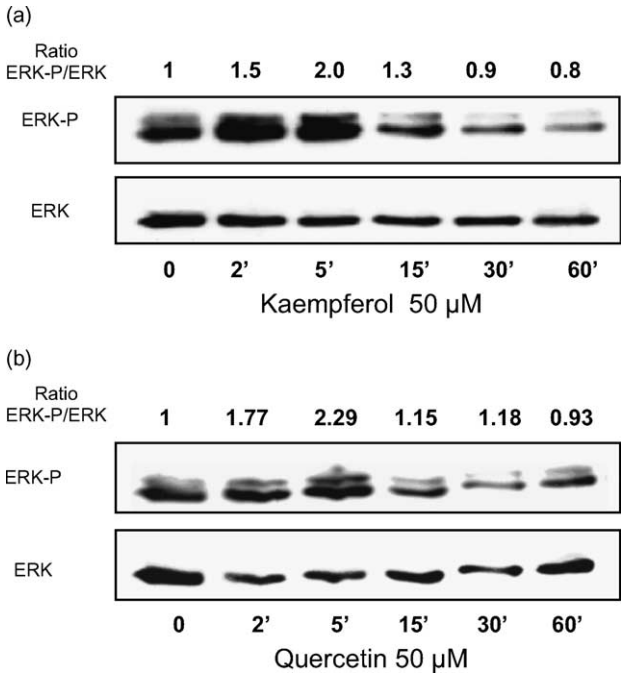

Fig. 4. Western blot analysis of ERK phosphorylation in MG-63 cells treated with 50 μM kaempferol (a) or quercetin (b) in time ranged from 2 to 60 min. ERK-P: phosphorylated ERK; ERK: unphosphorylated ERK. Results of a typical experiment.

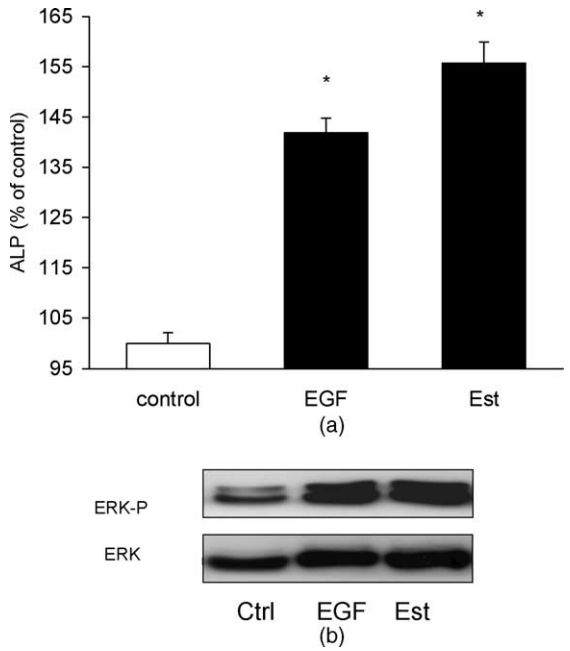

Fig. 5. Effect of EGF and 17-β estradiol on ALP activity (a) and ERK phosphorylation (b) in MG-63 human osteoblasts. (a) Cells were treated for 48 hr with EGF 30 ng/mL or 17-β estradiol 10^{-8} M, then ALP activity was measured as described in Section 2. Results are expressed as percentage of controls (means of four experimental values ±SEM). $^{*}P < 0.001$ compared with control values (ANOVA test). (b) MG-63 cells were treated for 5 min with EGF 30 ng/mL or 17-β estradiol 10^{-8} M, then ERK phosphorylation was assessed by Western blot analysis. Results are of a typical experiment. $^{*}P < 0.001$ compared with control values (ANOVA test).

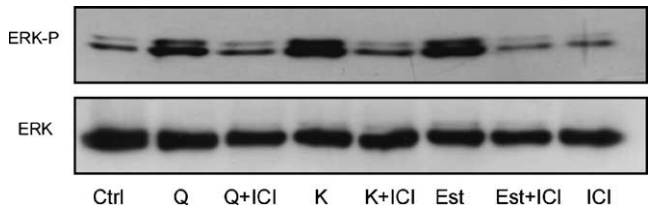

Fig. 6. ICI 182780 prevents the flavonol- and the 17-β estradiol-induced activation of ERK. Cells were treated for 5 min with quercetin or kaempferol 50 μM, or with 17-β estradiol 10^{-8} M. ICI 182780 1 μM was added 15 min before cell treatment with flavonols or 17-β estradiol. Q: quercetin; K: kaempferol; Est: 17-β estradiol. Results are of a typical experiment.

182780 suggests that other activators of the ERK pathway or of the ER could also influence the ALP activity in MG-63 osteoblasts. Indeed, as shown in Fig. 5a, either EGF or 17-β estradiol induced a significant increase in ALP activity, close to that observed with quercetin, and slightly less marked than that of kaempferol. Both compounds also rapidly (within 5 min) activate ERK phosphorylation (Fig. 5b), which is in favor of tight cross talks between ERK pathway and the ER in the control of ALP activity in osteoblasts.

3.5. ERK activation by flavonols occurs mainly downstream of the ER activation

Crosstalks between the ER(s) and the MAPK signaling pathways have been described in various experimental models [18]. In order to investigate whether the flavonol-induced ERK activation occurs (i) downstream of the ER activation, or (ii) upstream of the ER activation, or (iii) if the two pathways are independently stimulated by the flavonols, we studied the effect of the pure ER antagonist ICI 182780 on ERK phosphorylation in MG-63 cells treated with quercetin or kaempferol. Results in Fig. 6 show that ICI 182780 markedly inhibits the ERK activation induced by the two flavonols as well as by 17-β estradiol. This suggests that ERK activation mainly takes place downstream of the ER(s) activation.

4. Discussion

In this study we clearly show that the two studied flavonols, quercetin and kaempferol, investigated in the range of 1–50 μM, increase the activity of ALP in MG-63 human osteoblasts without any significant cytotoxic effect on the cells. The maximal stimulatory effect on ALP was observed after 48 hr of treatment with 50 μM flavonols. We therefore used this condition for further studies. We have demonstrated that cycloheximide, a well known protein synthesis inhibitor, prevented the flavonol-induced ALP activation, indicating that *de novo* protein synthesis is essential for this response. Moreover, an activation of the ERK pathway is required for the observed phenomenon, because of the markedly reduction of the enhancing action of flavonols by MEK inhibitor PD 98059. The fact that ICI 182780, an antagonist of the ER, also prevents the flavonol-induced increase in ALP activity, indicates that this receptor is directly involved in the effect of flavonols. Therefore, it can be hypothesized that ERK activation and ER binding are both required for the induction of ALP activity by flavonols in MG-63 cells. Estrogen receptors exist as two subtypes ERα and ERβ and osteoblasts express both receptors [19]. The interaction between flavonols and flavonoids with ERα and ERβ is well documented [20,21]. Kuiper *et al.* [20], who studied the estrogenic potency of several phytoestrogens, showed that kaempferol, and to a lesser extent quercetin, have the capacity to bind both ER subtypes. It has also been demonstrated [21] that kaempferol has a greater binding affinity for ERβ than daïdzein, a compound well known to reproduce some effects of estrogen on osteoblastic cells [22]. Flavonols can be thus considered to have the so-called ''phytoestrogenic properties'' and one of the mechanisms involved in the increase in ALP activity by flavonols in our experimental model could therefore be the activation of the ER. Indeed, as discussed above, the inhibitory effect of ICI 182780 on the flavonol-induced increase in ALP activity, strongly suggests that quercetin and kaempferol stimulate ALP activity by an ER-dependent pathway. On the other hand, the increase in ALP activity by flavonols is almost completely prevented by the MEK inhibitor PD 98059, which demonstrates an involvement of the ERK pathway in the stimulatory effect of quercetin or kaempferol on ALP activity. This result is in accordance with a recent report of Lai *et al.* [16] who showed that the expression of a ERK1 dominant negative protein markedly inhibits ALP activity in human osteoblastic cells. It is of note that Migliaccio *et al.* [23] studying the mitogenic effect of estradiol, observed in an other experimental system (MCF-7 human cancer cells), that estradiol rapidly (within minutes) activates the MAPK pathway, an effect which is prevented by the anti-estrogen ICI 182780. These authors also pointed at a rapid interaction of the estradiol-receptor complex with the tyrosine kinase Src. Src can phosphorylate Shc, an adaptor protein which in turn can associate with the Grb2/Sos complex, leading to $p21^{ras}$ activation [23], and therefore to a stimulation of the ERK pathway. A similar mechanism has also been reported in osteoblasts [24,25] as well as in osteoclasts [26]. According to this point of view, the two studied flavonols, by their ability to bind the ERs, could secondarily activate the ERK pathway, an event required for the induction of ALP activity as suggested by the effect of PD 98059. Because of the rapidity with which MAPkinase activation occurs, a pending question is that of the ''identity'' of the concerned ER(s). Are they the ''classical'' ERs, or the ''membrane'' ERs, the existence of which being supported by growing lines of evidence [27–30]? Indeed, several studies have suggested that ERs located in the plasma membrane could be involved in the activation of

the ERK pathway. In this regard, it could be conceived that flavonols, as estradiol, could rapidly activate the ERK pathway *via* their interaction with a subtype of ERs located in the plasma membrane. The mechanism whereby a rapid effect on ERK pathway, which takes place within minutes, induces a stimulatory effect on ALP remains to be specified. MAP kinase activation *via* a non genomic action of ER can lead to downstream modulation of several transcription factor such as ELK1 [31], AP-1 [32], CREB and C/EBP β [33] and it has been recently suggested that the promoter of human ALP gene contain an AP-1 binding site [34]. It is thus possible to assume that flavonol-induced activation of some transcription factor such as AP-1 could be an important downstream target of ERK-activation mediated by ER in osteoblast cells, providing a rational and a possible link between a rapid ERK activation and an increase in ALP activity. Interestingly, recent works of Kousteni *et al.* have shown that non genomic action of sex steroids is essential for their bone protective effect [35]. In this regard, the fact that natural flavonols can reproduce the non genomic effects of estradiol may have some importance in the regulation of bone metabolism.

In conclusion, this study demonstrates that flavonols have a stimulatory effect on ALP activity and can rapidly activate ERK in osteoblasts by an ER dependent pathway. These results with those previously reported by us [12] and by others [13] suggest that flavonols are substances which can both decrease osteoclastic activity and stimulate osteoblastic activity. Such compounds may represent new pharmacological tools for the treatment of osteoporosis.

Acknowledgments

C.P. thanks the Agence pour la recherche et l'information en fruits et légumes frais (APRIFEL) for a grant; J.C.M. (JE 23 29), M.B. and S.K. (UPRESA 2086) thank the DRED, the Ligue Nationale Contre le Cancer, Comité de la Somme, and the Université de Picardie Jules Verne for financial support.

References

[1] Consensus Development Conference V. Diagnosis prophylaxis and treatment of osteoporosis. Am J Med 1993;90:646–50.
[2] Manolagas SC, Jilka RL. Bone marrow, cytokines, and bone remodelling. Emerging insights into the pathophysiology of osteoporosis. N Engl J Med 1995;332:305–11.
[3] Dempster DW, Lindsay R. Pathogenesis of osteoporosis. Lancet 1993;341:797–805.
[4] Christiansen C, Lindsay R. Estrogens bone loss and preservation. Osteoporos Int 1990;1:7–13.
[5] Pinkerton JV, Santen R. Alternatives to the use of estrogen in postmenopausal women. Endocr Rev 1999;20:308–20.
[6] Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewicz-Sapuntzakis M, Guo P, Kukreja SC. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 1996;126:161–7.
[7] Arjmandi BH, Birnbaum R, Goyal NV, Getlinger MJ, Juma S, Alekel L, Hasler CM, Drum ML, Hollis BW, Kukreja SC. Bone-sparing effect of soy protein in ovarian-hormone-deficient rats is related to its isoflavone content. Am J Clin Nutr 1998;68(Suppl):1364S–8S.
[8] Morabito N, Crisafulli A, Vergara C, Gaudio A, Lasco A, Frisina N, D'Anna R, Corrado F, Pizzoleo MA, Cincotta M, Altavilla D, Ientile R, Squadrito F. Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: a randomized double-blind placebo-controlled study. J Bone Miner Res 2002;10:1904–12.
[9] Ross PD, Norimatsu H, Davis JW, Yano K, Wasnich RD, Fujiwara S, Hosoda Y, Melton III LJ. A comparison of hip fracture incidence among native Japanese, Japanese Americans, and American Caucasians. Am J Epidemiol 1991;133:801–9.
[10] Fujita T, Fukase M. Comparison of osteoporosis and calcium intake between Japan and the United States. Proc Soc Exp Biol Med 1992; 200:149–52.
[11] Horcajada-Molteni MN, Crespy V, Coxam V, Davicco MJ, Remesy C, Barlet JP. Rutin inhibits ovariectomy-induced osteopenia in rats. J Bone Miner Res 2000;15:2251–8.
[12] Wattel A, Kamel S, Mentaverri R, Lorget F, Prouillet C, Petit JP, Fardelonne P, Brazier M. Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. Biochem Pharmacol 2003;65:34–42.
[13] Miyake M, Arai N, Ushio S, Iwaki K, Ikeda M, Kurimoto M. Promoting effect of kaempferol on the differentiation and mineralization of murine pre-osteoblastic cell line MC3T3-E1. Biosci Biotechnol Biochem 2003;67:1199–205.
[14] Sabokbar A, Millet PJ, Myet B, Rushton N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. Bone Miner 1994;27:57–67.
[15] Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;89: 271–7.
[16] Lai CF, Chaudhary L, Fausto A, Halstead LR, Ory DS, Avioli LV, Cheng SL. Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. J Biol Chem 2001;276:14443–50.
[17] Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. Cancer Res 1991;51:3867–73.
[18] Coleman KM, Smith CL. Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. Front Biosci 2001;6:1379–91.
[19] Arts J, Kuiper GG, Janssen JM, Gustafsson JA, Lowik CW, Pols HA, van Leeuwen JP. Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 1997;138:5067–70.
[20] Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with receptor beta. Endocrinology 1998;139:4252–63.
[21] Harris HA, Bapat AR, Gonder DS, Frail DE. The ligand binding profiles of estrogen receptors alpha and beta are species dependent. Steroids 2002;67:379–84.
[22] Sugimoto E, Yamaguchi M. Stimulatory effect of Daidzein in osteoblastic MC3T3-E1 cells. Biochem Pharmacol 2000;59:471–5.
[23] Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, Auricchio F. Tyrosine kinase/p21^{ras}/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 1996;15:1292–300.
[24] Endoh H, Sasaki H, Maruyama K, Takeyama KI, Waga I, Shimizu T, Kato S, Kawashima H. Rapid activation of MAP kinase by estrogen in the bone cell line. Biochem Biophys Res Commun 1997;235: 99–102.
[25] Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS,

Roberson PK, Weinstein RS, Jilka RL, Manolagas SC. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 2001;104:719–30.

[26] Brubaker KD, Gay CV. Estrogen stimulates protein tyrosine phosphorylation and Src kinase activity in avian osteoclasts. J Cell Biochem 1999;76:206–16.

[27] Pappas TC, Gametchu B, Watson CS. Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J 1995;9:404–10.

[28] Razandi M, Pedram A, Greene GL, Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ER alpha and ER beta expressed in Chinese hamster ovary cells. Mol Endocrinol 1999;13:307–19.

[29] Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc Natl Acad Sci USA 2000;97:5930–5.

[30] Zhang Z, Maier B, Santen RJ, Song RX. Membrane association of estrogen receptor alpha mediates estrogen effect on MAPK activation. Biochem Biophys Res Commun 2002;28:926–33.

[31] Duan R, Xie W, Burghardt RC, Safe S. Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. J Biol Chem 2001;276: 11590–8.

[32] Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 2000;74:311–7.

[33] Kousteni S, Han L, Chen JR, Plotkin L, Bellido T, O'Brien CA, Jilka RL, Manolagas SC. Non genotropic regulation of CREB, C/EBPβ, as well as Elk-1 and AP-1-mediated transcription by estrogens: downstream effects of ERK and JNK kinase modulation required for antiapoptosis. J Bone Miner Res 2002;17:S169.

[34] Peverali FA, Basdra EK, Papavassiliou AG. Stretch-mediated activation of selective MAPK subtypes and potentiation of AP-1 binding in human osteoblastic cells. Mol Med 2001;7:68–78.

[35] Kousteni S, Chen JR, Bellido T, Han L, Ali AA, O'Brien CA, Plotkin L, Fu Q, Mancino AT, Wen Y, Vertino AM, Powers CC, Stewart SA, Ebert R, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC. Reversal of bone loss in mice by nongenotropic signaling of sex steroids. Science 2002;298:843–6.