ARTICLE
Journal of Cellular Biochemistry 112:625–632 (2011)

Journal of Cellular Biochemistry

The Effects of Estrogen Receptors α- and β-Specific Agonists and Antagonists on Cell Proliferation and Energy Metabolism in Human Bone Cell line

D. Somjen,* S. Katzburg, O. Sharon, M. Grafi-Cohen, E. Knoll, and N. Stern

Institute of Endocrinology, Metabolism and Hypertension, Tel-Aviv Sourasky Medical Center and the Sackler Faculty of Medicine, Tel-Aviv 64239, Israel

ABSTRACT

In cultured human osteoblasts estradiol-17β (E2) modulated DNA synthesis, the specific activity of creatine kinase BB (CK), 12 and 15 lipoxygenase (LO) mRNA expression and formation of 12- and 15-hydroxyeicosatetraenoic acid (HETE). We now investigate the response of human bone cell line (SaOS2) to phytoestrogens and estrogen receptors (ER)-specific agonists and antagonists. Treatment of SaSO2 with E2, 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN; ERβ-specific agonist), 4,4′,4″-[4-propyl-(1H)-pyrazol-1,3,5-triyl] tris-phenol (PPT; ERα-specific agonist), biochainin A (BA), daidzein (D), genistein (G) and raloxifene (Ral) showed increased DNA synthesis and CK. Ral inhibited completely all stimulations except DPN and to some extent D. The ERα-specific antagonist methyl-piperidino-pyrazole (MPP) and the ERβ-specific antagonist 4-[2-phenyl-5,7-bis (tri-fluoro-methyl) pyrazolo [1,5-a]pyrimidin-3-yl] phenol (PTHPP) inhibited DNA synthesis, CK and reactive oxygen species (ROS) formation induced by estrogens according to their receptors affinity. The LO inhibitor baicaleine inhibited only E2, DPN and G's effects. E2 and Ral unlike all other compounds had no effect on ERα mRNA expression, while ERβ mRNA expression was stimulated by all compounds. All compounds modulated the expression of 12LO and 15LO mRNA, except E2, PPT and Ral for 12LO, and 12- and 15-HETE productions and stimulated ROS formation which was inhibited by NADPH oxidase inhibitors diphenyleneiodonium chloride (DPI) and *N*-acetyl cysteine and the estrogen inhibitor ICI. DPI did not affect hormonal-induced DNA and CK. In conclusion, we provide evidence for the separation of mediation via ERα and ERβ pathways in the effects of estrogenic compounds on osteoblasts, but the role of LO/HETE/ROS is unclear. J. Cell. Biochem. 112: 625–632, 2011.

KEY WORDS: ESTRADIOL-17β; DPN; PPT; MPP; PTHPP; SASO2; LIPOXYGENASE; HETE; DNA; CK

INTRODUCTION

Estrogenic compounds appear to confer multiple skeletal protective effects in vitro (Kaye et al., 1990; Somjen et al., 1991; Fournier et al., 1996; Katzburg et al., 2001) and in vivo (Gallagher, 1988; Somjen et al., 1989; Brzezinski and Debi, 1999), and to positively affect skeleton outcome in prospective trials in post-menopausal women (Consensus Opinion, 2000; Delmas, 2000; Manolagas et al., 2002). We found that estradiol-17β as well as other estrogenic compounds and other anabolic compounds such as parathyroid hormone (PTH) stimulate DNA synthesis and creatine kinase (CK)-specific activity as well as LO mRNA and hydroxyeicosatetraenoic acid (HETE) formation (Somjen et al., 2008, 2010b). We also found that vitamin D metabolites and analogues stimulate DNA synthesis and CK-specific activity as well as LO mRNA and HETE formation (Somjen et al., 2010c). These stimulations of DNA and CK are partially inhibited by the HETE formation inhibitor baicaleine (Baic; Somjen et al., 2010c). These compounds also transiently increased reactive oxygen species (ROS) formation which is inhibited by the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI), which also almost completely inhibited DNA and CK stimulation (Somjen et al., 2010c).

In the present study, we analyzed the expression and regulation of lipoxygenase (LO) enzymes by estrogenic compounds in cultured human bone cell line; SaOS2. We focused on these enzymes in osteoblastic cell line since LO products were shown to modulate MAPK activity (Tong et al., 2002; Tunc et al., 2008) and proliferation or survival (Kim et al., 2006) in a number of cell types and could thus play a role in promoting cell growth, either independently or through estrogenic action. Additionally, there is recent strong circumstantial evidence linking LO expression to bone mineral content (Mody et al., 2001; Klein et al., 2004). We hypothesized that

*Correspondence to: Dr. D. Somjen, PhD, Tel-Aviv Sourasky Medical Centre and The Sackler Faculty of Medicine, Institute of Endocrinology, Metabolism and Hypertension, Tel-Aviv University, Tel-Aviv 64239, Israel.
E-mail: dalias@tasmc.health.gov.il

Received 7 October 2010; Accepted 9 November 2010 • DOI 10.1002/jcb.22959 • © 2010 Wiley-Liss, Inc.
Published online 22 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

the growth promoting effects of estrogens in human osteoblastic cell line may be associated with accelerated production of LO metabolites, whose putative action may explain some of the newly found links between LO and bone density.

We also set to determine whether or not estrogenic compounds affect ROS production in SaOS2 similar to our previous data on the effects of vitamin D compounds in primary cultured human female bone cells (Somjen et al., 2010b) and in SaOS2 cells (Somjen et al., 2010c).

We set to determine using human bone cell line SaSO2: (1) Whether or not estrogenic compounds affect ROS production with and without NADPH oxidase inhibitors: DPI or *N*-acetyl cystein (NAc). (2) Whether or not SERM and the estrogen inhibitor ICI modulate ROS production by estrogenic compounds. (3) Whether or not the antagonists methyl-piperidino-pyrazole (MPP) or 4-[2-phenyl-5,7-bis (tri-fluoro-methyl) pyrazolo [1,5-a]pyrimidin-3-yl] phenol (PTHPP) inhibit estrogenic compounds stimulation of DNA or CK or ROS. (4) Whether or not the LO inhibitor Baic contribute to estrogenic modulation of DNA and CK stimulations. (5) Whether or not interfering with estrogenic compounds-induced ROS formation by DPI abolished the estrogenic stimulation of DNA and CK.

We found that: (1) E2 as well as 4,4′,4″-[4-propyl-(1H)-pyrazol-1,3,5-triyl] tris-phenol (PPT), 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN), biochainin A (BA), daidzein (D), genistein (G) and raloxifene (Ral) increases CK and DNA which were inhibited specifically by their specific antagonists and the SERM Ral. (2) E2 as well as PPT, DPN, BA, D, G and Ral increases ROS formation as detected by nitroblue tetrazolium (NBT) colorimetric method or using 2′,7′-dichlorofluorescein diacetate (DCF) for fluorescent microscopy. (3) E2 stimulated ROS dose and time dependently. (4) All estrogens tested up-regulate both 12LO (except E2, DPN and Ral) and 15LO expression and activity measured by the production of 12- and 15-HETE. (5) Baic which inhibits 12- and 15-HETE formation through blockade of 12LO and 15LO inhibited DNA and CK by E2, DPN and G but not by PPT, BA, D and Ral. (6) Interfering with estrogens-induced ROS formation by DPI abolished only very slightly the stimulation by all estrogenic compounds of DNA synthesis and CK-specific activity.

In conclusion, our data suggest that different estrogenic compounds, as well as vitamin D metabolites and analogues, increase oxidative stress in bone cells, in part via induction of LO mRNA and LO enzymatic activity. The role of ROS production in the physiology of estrogenic activity in SaSO2 and probably other models of human bone cells is not yet clear. This previously unrecognized feature of estrogenic compounds appears to be shared by multiple members of the estrogens family and is not exclusively linked to ERα or ERβ and/or membranal binding sites.

MATERIALS AND METHODS

CHEMICALS

Nitroblue tetrazolium (NBT), DPI, BA, D, G, Ral and CK assay kit were from Sigma Chemicals Co. (St. Louis, MO). DCF was from Molecular Probes (Eugene, OR). DPN and PPT and ERα-specific antagonist the MPP and the ERβ-specific antagonist PTHPP were from Tocris biosciences (Bristol, UK). All other reagents used were of analytical grade.

CELL CULTURES

Human bone cell line SaOS2 was obtained from ATCC (Manassas, VA) and grown according to their instructions.

HORMONAL TREATMENT

Sub-confluent cells were treated daily with vehicle, E2 at 30 nM,or at other different concentrations, DPN at 420 nM, PPT at 390 nM, BA or d or G or Ral all at 3,000 nM, or in the presence of ERα antagonist MPP at 100 nM and ERβ antagonist PTHPP at 150 nM:

a. for 3 daily additions for mRNA of 12LO and 15LO type 1 expression or estrogen receptor α (ERα) and estrogen receptor β (ERβ) were determined as previously described (Somjen et al., 2002) or;
b. for 1 h with serum-free medium, followed by the addition of vehicle or estrogenic compounds at the concentrations mentioned above for 10 min and HETE were extracted and assayed as previously described (Somjen et al., 2008) or;
c. for 1 h or at other time points, by the addition of vehicle or estrogenic compounds with and without the inhibitors DPI or NAc or ICI at 1 μM, at the concentrations mentioned above for ROS assay as previously described both chemically and using fluorescent microscopy (Somjen et al., 2010b,c) or;
d. for 24 h with the addition of vehicle or estrogenic compounds at the concentrations mentioned above for DNA synthesis and for CK-specific activity as previously described (Somjen et al., 2005).

DETERMINATION OF MRNA FOR ERα AND ERβ BY RT-PCR

RNA was extracted and expression of ERα and ERβ was carried out by RT-PCR as described previously (Somjen et al., 2002).

DETERMINATION OF MRNA FOR 12LO AND 15LO BY RT-PCR

RNA was extracted and expression of 12LO and 15LO enzymes was carried out by RT-PCR as described previously (Somjen et al., 2008).

DETERMINATION OF THE LEVELS OF 12- AND 15-HETE BY HPLC

Cells and medium were extracted for HETE and analyzed by HPLC as described previously (Somjen et al., 2008).

DETERMINATION OF ROS FORMATION

Cells were treated for 1 h with vehicle or estrogenic compounds for ROS formation using NBT quantitative colorimetric method (Tunc et al., 2008; Somjen et al., 2010b) or by the qualitative fluoro-microscopy using DCF with 20× magnification, as described (Somjen et al., 2010c).

ASSESSMENT OF DNA SYNTHESIS

Twenty-two hours after hormonal treatment, ^{3}[H] thymidine was added for 2 h and its incorporation into DNA was determined as described (Katzburg et al., 1999).

CREATINE KINASE EXTRACTION AND ASSAY

Twenty-four hours after hormonal treatment, CK was extracted and assayed as described (Somjen et al., 2002, 2005).

STATISTICAL ANALYSIS

The significance of differences between experimental and control means was evaluated using Student's *t*-test or ANOVA, in which n = 5–8 number of cultures. $P < 0.05$ was considered significant.

RESULTS

MODULATION OF ERα AND ERβ MRNA EXPRESSION IN SAOS2 BONE CELL LINE BY ESTROGENIC COMPOUNDS

Both ERα and ERβ mRNA are expressed in human bone cell line (SaOS2) with higher abundance of ERα (Table I). Daily treatment for 3 days E2 at 30 nM, DPN at 420 nM, PPT at 390 nM or BA or D or G or Ral all at 3,000 nM to bone cells modulated the expression of mRNA for ERβ and ERα in SaOS2 (Fig. 1). All hormones increased the expression of mRNA for ERβ and only all except E2 and Ral increased ERα expression in the cells (Fig. 1a).

MODULATION OF LO MRNA EXPRESSION SAOS2 BONE CELL LINE BY ESTROGENIC COMPOUNDS

Daily treatment for 3 days E2 at 30 nM, DPN at 420 nM, PPT at 390 nM or BA or D or G or Ral all at 3,000 nM to bone cells modulated the expression of mRNA for 12LO and 15LO mRNA. All hormones increased the expression of mRNA for 15LO and only DPN, BA, D and G increased 12LO mRNA expression in these cells (Fig. 1b).

THE EFFECT OF ESTROGENIC COMPOUNDS ON DNA SYNTHESIS AND CK-SPECIFIC ACTIVITY IN SAOS2 BONE CELL LINE

Addition of E2 at 30 nM, DPN at 420 nM, PPT at 390 nM or BA or D or G or Ral all at 3,000 nM for 24 h induced DNA synthesis (Fig. 2a, upper panel) and CK-specific activity (Fig. 2a, lower panel) in SaOS2. E2, DPN and D were slightly more active than the other compounds in stimulation of DNA synthesis.

THE EFFECT OF ESTROGENIC COMPOUNDS ON DNA SYNTHESIS AND CK-SPECIFIC ACTIVITY IN SAOS2 BONE CELL LINE IN THE PRESENCE OF RALOXIFENE

Addition of Ral which is specific ERα antagonist at 3,000 nM together with E2 at 30 nM or DPN at 420 nM or PPT at 390 nM or BA or D or G or Ral all at 3,000 nM to bone cells modulated the induction of DNA synthesis (Fig. 2b, upper panel) and CK-specific activity (Fig. 2b, lower panel) in SaOS2. Ral inhibited the stimulations of both parameters by all compounds but did not affect the stimulatory effects of DPN, and only slightly affected the modulations by D (Fig. 2b).

TABLE I. The Expressions of ERα and ERβ mRNA in SaOS2 Human Bone Cell Line

SaOS2		
ERα ($2^{-\Delta}$ct)	ERβ ($2^{-\Delta}$ct)	ERα/ERβ
0.0035 ± 0.0004	0.0013 ± 0.0002	1:0.37

Details are given in the experimental section.

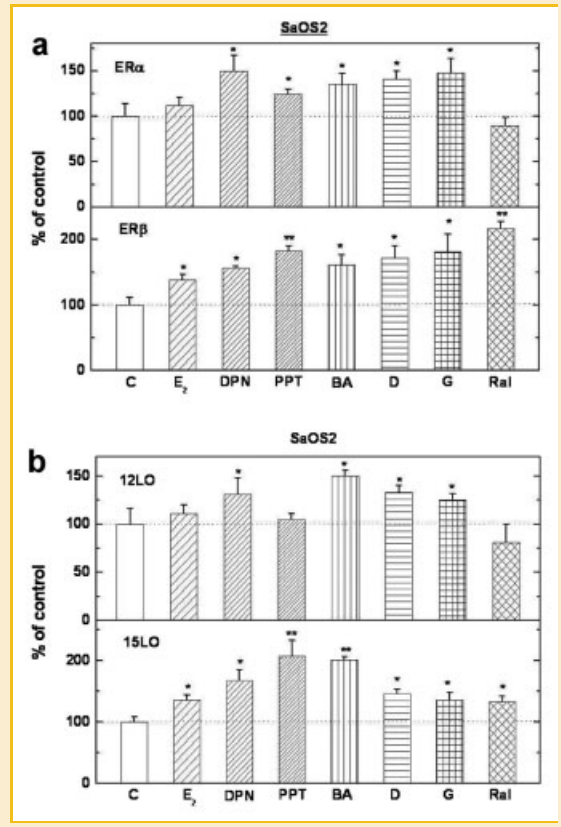

Fig. 1. a: The effects of E2, DPN, PPT, BA, D, G and Ral on the expression of ERα (upper panel) and ERβ (lower panel) mRNA in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$. b: The effects E2, DPN, PPT, BA, D, G and Ral on the expression of 12LO (upper panel) and 15LO (lower panel) mRNA in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$.

THE EFFECT OF ESTROGENIC COMPOUNDS ON DNA SYNTHESIS AND CK-SPECIFIC ACTIVITY IN SAOS2 BONE CELL LINE IN THE PRESENCE OF ERα AND ERβ ANTAGONISTS

Addition of E2 at 30 nM, DPN at 420 nM, PPT at 390 nM or BA or D or G all at 3,000 nM alone or in the presence of ERα antagonist MPP at 100 nM or ERβ antagonist PTHPP at 150 nM; modulated the induction of DNA synthesis (Fig. 4b, upper panel) and CK-specific activity (Fig. 2c, lower panel) in SaOS2. The antagonists alone did not affect DNA or CK in the cells, but MPP inhibited the stimulations of both parameters by all compounds without affecting the stimulations by DPN and slightly the effects of D (Fig. 2c). On the other hand, the ERβ antagonist PTHPP inhibited all stimulations of both parameters by all compounds but did not affect the stimulations by PPT or G (Fig. 2c).

MODULATION OF HETE FORMATION IN SAOS2 BONE CELL LINE BY ESTROGENIC COMPOUNDS

Addition of E2 at 30 nM, DPN at 420 nM, PPT at 390 nM or BA or D or G or Ral all at 3,000 nM modulated the formation of 12 (Fig. 3,

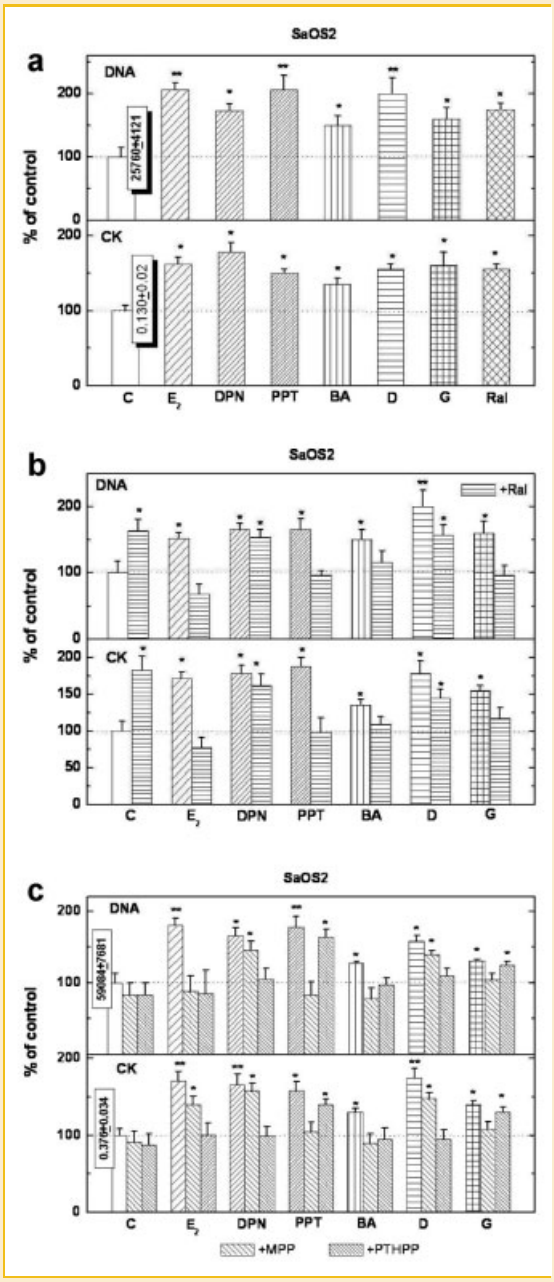

Fig. 2. a: The effects of E2, DPN, PPT, BA, D, G and Ral on DNA synthesis (upper panel) and on CK-specific activity (lower panel) in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$. In brackets are given the basal values of DNA and CK. b: The effects of E2, DPN, PPT, BA, D and G in the presence of Ral on DNA synthesis (upper panel) and on CK-specific activity (lower panel) in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$. c: The effects of E2, DPN, PPT, BA, D and G in the presence of ERα antagonist (MPP) or ERβ antagonist (PTHPP) on DNA synthesis (upper panel) and on CK-specific activity (lower panel) in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$.

upper panel) and 15-HETE (Fig. 3, lower panel) in SaOS2 cells. All estrogens tested stimulated both 12- and 15-HETE formation in these cells (Fig. 3). Since LOs mRNA expression was also stimulated by all compounds, it suggests that there are also changes in enzyme activity and not only its synthesis.

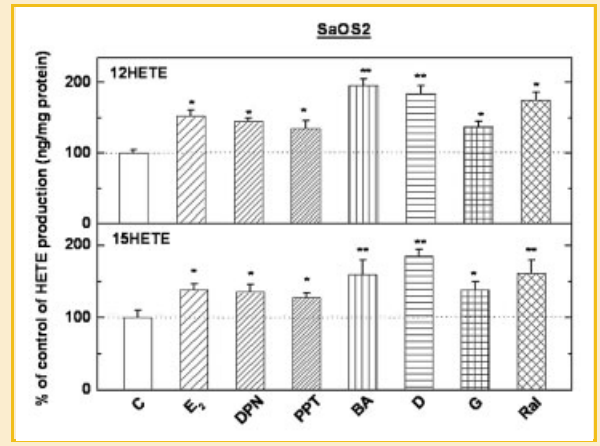

Fig. 3. The effects of E2, DPN, PPT, BA, D, G and Ral on the formation of 12-HETE and 15-HETE production in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$.

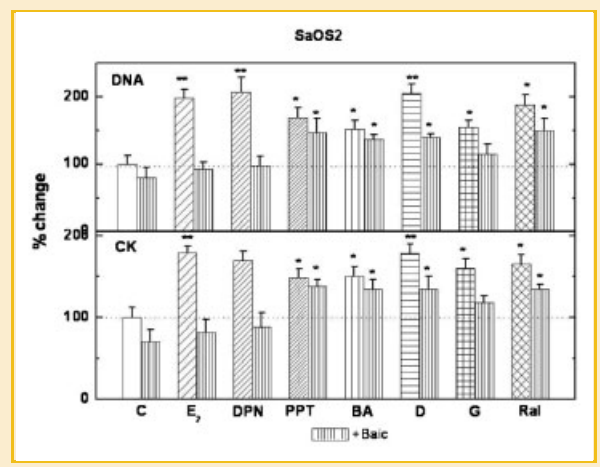

Fig. 4. The effects of E2, DPN, PPT, BA, D, G and Ral in the presence of baic on DNA synthesis (upper panel) and on CK-specific activity (lower panel) in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$.

THE EFFECT OF ESTROGENIC COMPOUNDS ON DNA SYNTHESIS AND ON CK-SPECIFIC ACTIVITY IN SAOS2 BONE CELL LINE WITH AND WITHOUT BAICALEINE

Treatment of SaOS2 cells for 24 h with E2 at 30 nM, DPN at 420 nM, PPT at 390 nM or BA or D or G or Ral all at 3,000 nM resulted in increased DNA synthesis (Fig. 4, upper panel) and CK-specific activity (Fig. 4, lower panel). Baic, the inhibitor of 12- and 15-HETE synthesis, abolished the E2- and DPN- and G- but not all other estrogenic compounds-stimulated DNA synthesis and CK-specific activity in these cells (Fig. 4), suggesting that ERβ might be more involved in LO activation and HETE formation.

THE EFFECT OF ESTROGENIC COMPOUNDS ON ROS FORMATION IN SAOS2 BONE CELL LINE WITH AND WITHOUT NADPH OXYDASE INHIBITORS

Treatment of SaOS2 bone cells for 1 h with estrogenic compounds; E2 at 30 nM or DPN at 420 nM and PPT at 390 nM or BA or D or G or Ral all at 3,000 nM followed by incubation for 60 min with NBT resulted in stimulated ROS formation by all compounds (Fig. 5a). The NADPH oxidase inhibitor DPI inhibited completely ROS formation by all compounds tested (Fig. 5a). Treatment of SaOS2 bone cells for 1 h with estrogenic compounds; E2 at 30 nM or BA or D or G all at 3,000 nM followed by incubation for 60 min with NBT resulted in stimulated ROS formation by all compounds (Fig. 5b). The NADPH oxidase inhibitor NAc inhibited completely ROS formation by all compounds tested (Fig. 5b). Treatment of SaOS2 bone cells for 1 h with estrogenic compounds; E2 at 30 nM or BA or D or G all at 3,000 nM followed by incubation for 60 min with NBT resulted in stimulated ROS formation by all compounds (Fig. 5c). The estrogenic activity inhibitor ICI inhibited completely ROS formation by all compounds tested (Fig. 5c).

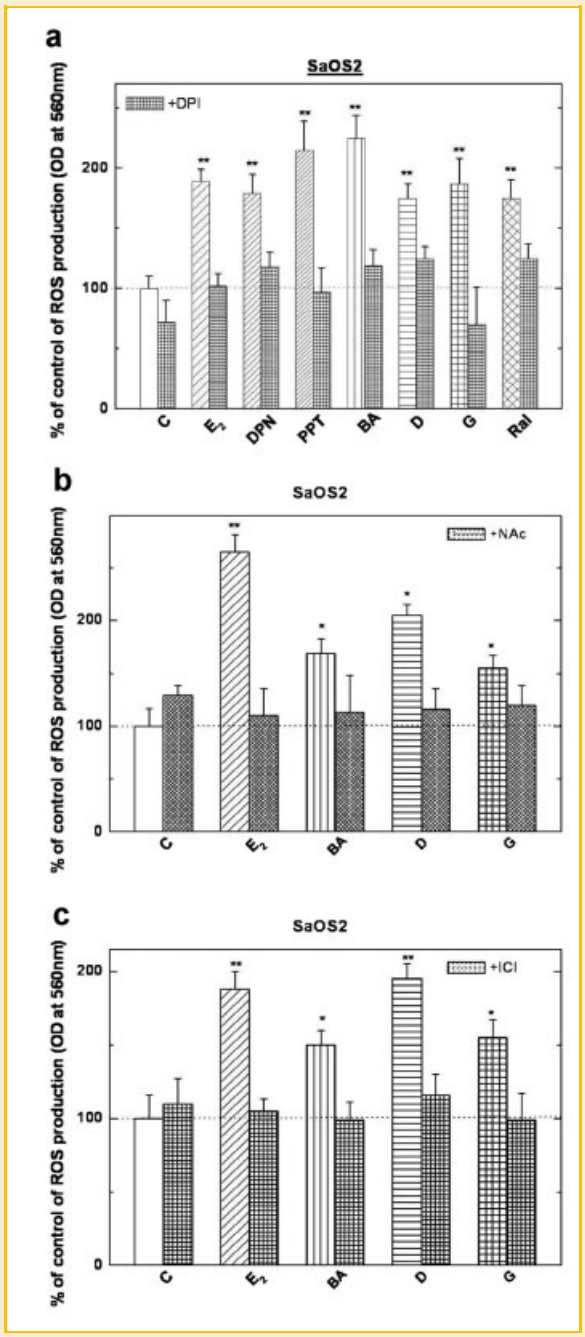

Fig. 5. a: The effects of E2, DPN, PPT, BA, D, G and Ral in the presence of DPI on ROS formation in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$. b: The effects of E2, BA, D or G in the presence of NAc on ROS formation in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$. c: The effects of E2, BA, D or G in the presence of ICI on ROS formation in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$.

THE EFFECT OF E2 AT DIFFERENT CONCENTRATIONS AND FOR DIFFERENT TIME PERIODS ON ROS FORMATION IN SAOS2 BONE CELL LINE WITH AND WITHOUT DPI OR ICI

Treatment of SaOS2 bone cells for different time periods with E2 at 30 nM followed by incubation for 60 min with NBT resulted in stimulated ROS formation by all compounds (Fig. 6a). Maximum

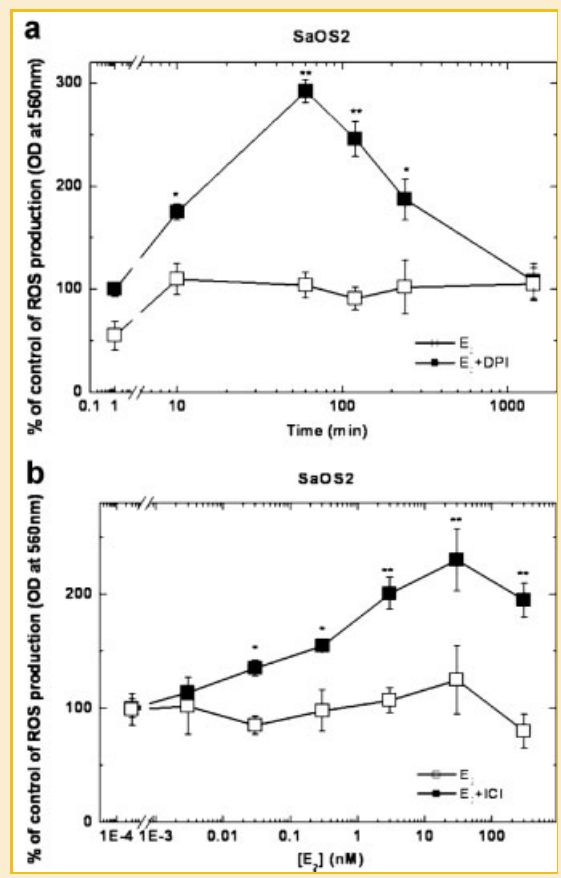

Fig. 6. a: Time-dependent stimulation by E2 in the presence of DPI of ROS formation in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$. b: Dose-dependent stimulation by E2 in the presence of ICI of ROS formation in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$.

increase was at 60 min, followed by decline with basal levels at 24 h. ROS formation was inhibited by DPI at all time periods (Fig. 6a). The estrogenic activity inhibitor ICI inhibited completely ROS formation by E2, used at different doses ranging from 0.03 to 300 nM for 60 min with maximal increase at 30 nM (Fig. 6b).

THE EFFECT OF ESTROGENIC COMPOUNDS ON DNA SYNTHESIS AND ON CK-SPECIFIC ACTIVITY IN SAOS2 BONE CELL LINE WITH AND WITHOUT DPI

Treatment of SaOS2 cells for 24 h with estrogenic compounds; E2 at 30 nM or DPN at 420 nM and PPT at 390 nM or BA or D or G or Ral all at 3,000 nM resulted in increased DNA synthesis (Fig. 7, upper panel) and CK-specific activity (Fig. 7, lower panel). Addition of the ROS formation inhibitor DPI together with the different estrogens inhibited only slightly and partially the stimulation of DNA synthesis and CK-specific activity by E2, DPN and D but not by all other compounds tested (Fig. 7).

THE EFFECT OF ESTROGENIC COMPOUNDS ON ROS FORMATION IN SAOS2 BONE CELL LINE WITH AND WITHOUT DPI

Treatment of SaOS2 cells for 1 h with E2 at 30 nM or DPN at 420 nM or PPT at 390 nM followed by incubation with DFC for 1 h resulted in increased ROS production as measured by qualitative fluorescent microscopy using 20× magnification (Fig. 8a,b). The addition of the NADPH oxidase inhibitor DPI inhibited completely ROS formation in these cells by all estrogenic compounds (data not shown).

THE EFFECT OF ESTROGENIC COMPOUNDS ON ROS FORMATION IN SAOS2 BONE CELL LINE WITH AND WITHOUT ERα AND ERβ ANTAGONISTS

Treatment of SaOS2 cells for 1 h with E2 at 30 nM or DPN at 420 nM and PPT at 390 nM or in the presence of ERα antagonist MPP at 100 nM and ERβ antagonist PTHPP at 150 nM; followed by incubation with DFC for 1 h resulted in increased ROS production as measured by qualitative fluorescent microscopy using 20× magnification (Fig. 8c–f). MPP or PTHPP inhibited completely ROS formation in these cells induced by E2. MPP inhibited also ROS formation induced by PPT, whereas PTHPP inhibited also ROS formation by DPN (Fig. 8c–f). The addition of the NADPH oxidase inhibitor DPI inhibited completely ROS formation in these cells by all estrogenic compounds (data not shown).

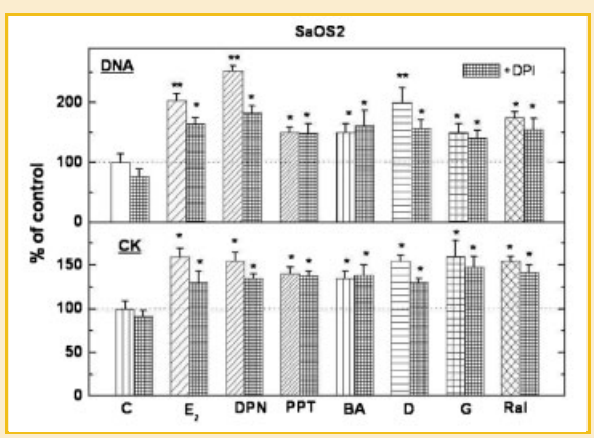

Fig. 7. The effects of E2, DPN, PPT, BA, D, G and Ral in the presence of DPI on DNA synthesis (upper panel) and on CK-specific activity (lower panel) in SaOS2 human bone cell line. Details are given in the experimental section, $^{*}P < 0.05$, $^{**}P < 0.01$.

THE EFFECT OF ESTROGENIC COMPOUNDS ON ROS FORMATION IN SAOS2 BONE CELL LINE WITH AND WITHOUT DPI

Treatment of SaOS2 cells for 1 h with BA or D or G or Ral at 3,000 nM followed by incubation with DFC for 1 h resulted in increased ROS production as measured by qualitative fluorescent microscopy using 20× magnification (Fig. 8g). The addition of the NADPH oxidase inhibitor DPI inhibited completely ROS formation in these cells by all estrogenic compounds (data not shown).

DISCUSSION

The effects of estrogenic compounds both the ERα-specific agonist PPT and the ERβ-specific agonist DPN as well as E2 itself and the different phytoestrogens; BA, D or G or the SERM Ral on ROS formation complements our findings in human skeletal cells in vitro, where estrogenic compounds stimulate cell proliferation, energy metabolism as well as other responses mediated by membranal binding sites (Somjen et al., 1991, 2005). Although reports suggest that 12- and 15-HETE interact with multiple signals promoting cell replication, their effects are probably not redundant, since inhibition of their production hinders normal cell growth in a variety of cell types (Kim et al., 2006). Recent publications linked also 12/15LO or 15LO and 12LO (platelet type) to bone density (Klein et al., 2004; Ichikawa et al., 2006). In this study, we provide more direct evidence for the expression and biological role of LOs in bone cell biology. We found that cultured human bone cells and lines express mRNA of three types of Los, that is, the platelet type 12LO, 15LO type 1 and 15LO type 2 (Somjen et al., 2008). Here, we show that the expression of the LOs in these cells is modulated not only by vitamin D metabolites and analogues (Somjen et al., 2010b,c) or PTH (Somjen et al., 2008), but also by different estrogenic compounds as well; all of which induce cell proliferation and energy metabolism. It was demonstrated previously that there is a possible intimate role of 15LO in breast tumor in human in which ERα is dominant (Jiang et al., 2006). In this study, we found that the PPT the ERα agonist and Ral increased the expression of 15LOmRNA which might lead similarly to ROS production. The expression of these enzymes results in the ability of bone cells to secrete 12-HETE and 15-HETE, the products of LOs. Also it was shown that LOs are able to produce free radicals species which intermediates oxidation of synthetic estrogens (Nunez-Delicado et al., 1997). Moreover, the generation of HETE which is driven by the estrogenic compounds is linked to osteoblastic growth induced by some of these hormones; E2, DPN and G and only to some extent D. It might be that these effects are linked to the compounds activating ERβ rather than ERα although the difference in their expression is not dramatically different. We therefore can see that the production of ROS is ER-dependent and it is dependent on both ERα and ERβ. The HETE raise local oxidative

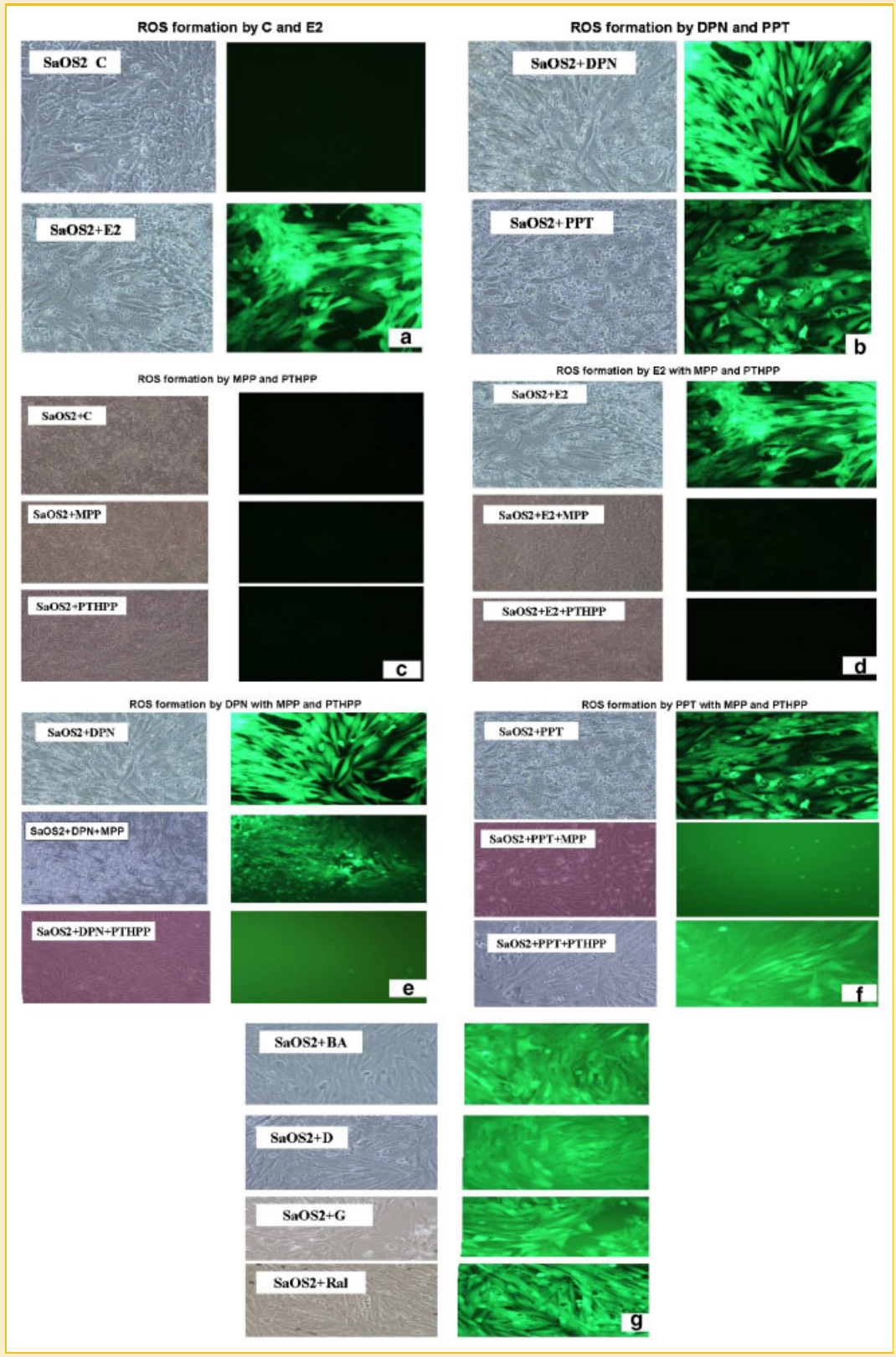

Fig. 8. The effects of E2 (a), DPN or PPT (b) and ERα antagonist (MPP) or ERβ antagonist (PTHPP) on ROS production in SaOS2 human bone cell line as measured by fluorescent microscopy at 20× magnification, after treatment with DFC (c). The effects of E2 (d); DPN (e) or PPT (f) in the presence of ERα antagonist (MPP) or ERβ antagonist (PTHPP). The effects of BA, D, G and Ral (g) on ROS production in SaOS2 human bone cell line as measured by fluorescent microscopy at 20× magnification, after treatment with DFC. Details are given in the experimental section.

stress as was shown here by measurements of ROS formation induced by all estrogenic compounds. But addition of DPI, the ROS production inhibitor, did not block the stimulation of DNA and CK by all compounds. This might be explained by its transient effect as shown here for E2 and as was shown before for other hormones (Somjen et al., 2008, 2010c). Because oxidative stress, in turn, may lead to inhibition of differentiation of bone osteoblasts-like cells (Mody et al., 2001) and acceleration of osteoclast differentiation (Klein et al., 2004), its induction may also result in the release of oxidizing fatty acids which unfavourably affect overall bone osteoblast/osteoclast homeostasis through enhanced oxidative stress. Presently, however, these potential secondary sequels of estrogens-stimulated LO activities in bone remain entirely conjectural and are the subject of future investigation.

This previously unrecognized feature of estrogenic compounds appears to be shared by multiple members of the estrogens family and is not exclusively linked to ERα or ERβ and/or membranal binding sites.

REFERENCES

Brzezinski A, Debi A. 1999. Phytoestrogens: The ''natural'' selective estrogen receptor modulators? J Obstet Gynecol Reprod Biol 85:47–51.

Consensus Opinion. 2000. Effects of menopause and estrogen replacement therapy or hormone replacement therapy in women with diabetes mellitus: Consensus Opinion of The North American Menopause Society. Menopause 7:87–95.

Delmas PD. 2000. Treatment of postmenopausal osteoporosis. Lancet 359:2018–2026.

Fournier B, Haring S, Kaye AM, Somjen D. 1996. Stimulation of creatine kinase specific activity in human osteoblast and endometrial cells by estrogens and anti-estrogens and its modulation by calciotropic hormones. J Endocrinol 150:275–285.

Gallagher JC. 1988. Drug therapy of osteoporosis: Calcium, estrogen and vitamin D. In: Riggs BL, Melton LJ III, editors. Osteoporosis: Etiology, diagnosis and management, Vol. 1. New York: Raven Press. pp 389–401.

Ichikawa S, Koller DL, Johnson ML, Lai D, Xuei X, Edenberg HJ, Klein RF, Orwoll ES, Hui SL, Foroud TM, Peacock M, Econs MJ. 2006. Human ALOX12, but not ALOX15, is associated with BMD in white men and women. J Bone Miner Res 21:556–564.

Jiang WG, Watkins G, Douglas-Jones A, Mansel RE. 2006. Reduction of isoforms of 15-lipoxygenase (15-LOX)-1 and 15-LOX-2 in human breast cancer. Prostaglandins Leukot Essent Fatty Acids 74:235–245.

Katzburg S, Lieberherr M, Ornoy A, Klein BY, Hendel D, Somjen D. 1999. Isolation and hormonal responsiveness of primary cultures of human bone-derived cells: Gender and age differences. Bone 25:667–673.

Katzburg S, Ornoy A, Hendel D, Lieberherr M, Kaye AM, Somjen D. 2001. Age and gender specific stimulation of creatine kinase specific activity by gonadal steroids in human bone-derived cells in culture. J Endocrine Invest 24:166–172.

Kaye AM, Weisman Y, Harell A, Somjen D. 1990. Hormonal stimulation of bone cell proliferation. J Steroid Biochem Mol Biol 37:431–435.

Kim HJ, Chang EJ, Kim HM, Lee SB, Kim HD, Su Kim G, Kim HH. 2006. Antioxidant α-lipoic acid inhibits osteoclast differentiation by reducing nuclear factor-κB DNA binding and prevents *in vivo* bone resorption induced by receptor activator of nuclear factor-κB ligand and tumor necrosis factor-α. Free Rad Biol Med 40:1483–1493.

Klein RF, Allard J, Avnur Z, Nikolcheva T, Rotstein D, Carlo AS, Shea M, Waters RV, Belknap JK, Peltz G, Orwoll ES. 2004. Regulation of bone mass in mice by the lipoxygenase gene ALOx15. Science 303:229–232.

Manolagas SC, Kousteni S, Jilka RL. 2002. Sex steroids and bone. Recent Progr Horm Res 57:385–409.

Mody N, Parhami F, Sarafian TA, Demer LL. 2001. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. Free Rad Biol Med 31:509–519.

Nunez-Delicado E, Sanchez-Ferrer A, Garcia-Carmona F. 1997. Hydroperoxidative oxidation of diethylstilbestrol by lipoxygenase. Arch Biochem Biophys 348:411–414.

Somjen D, Weisman Y, Harell A, Berger E, Kaye AM. 1989. Direct and sex specific stimulation by sex steroids of creatine kinase activity and DNA synthesis in rat bone. Proc Natl Acad Sci USA 86:3361–3363.

Somjen D, Weisman Y, Mor Z, Harell A, Kaye AM. 1991. Regulation of proliferation of rat cartilage and bone by sex steroid hormones. J Steroid Biochem Mol Biol 40:717–723.

Somjen D, Zaltsman Y, Gayer B, Kulik T, Knoll E, Stern N, Lu LJW, Toldo L, Kohen F. 2002. 6-Carboxymethyl genistein: A novel selective oestrogen receptor modulator (SERM) with unique differential effects on the vasculature, bone and uterus. J Endocrinol 173:415–427.

Somjen D, Kohen F, Lieberherr M, Gayer B, Schejter E, Katzburg S, Limor R, Sharon O, Knoll E, Posner GH, Kaye AM, Stern N. 2005. Membranal effects of phytoestrogens and carboxy derivatives of phytoestrogens on human vascular and bone cells: New insights based on studies with carboxybiochanin A. J Steroid Biochem Mol Biol 93:293–303.

Somjen D, Tordjman K, Katzburg S, Knoll E, Sharon O, Limor R, Naidich M, Naor Z, Hendel D, Stern N. 2008. Lipoxygenase metabolites are mediators of PTH-dependent human osteoblast growth. Bone 42:491–497.

Somjen D, Katzburg S, Knoll E, Sharon O, Posner GH, Stern N. 2010b. Vitamin D analogs induce lipoxygenase mRNA expression and activity as well as reactive oxygen species (ROS) production in human bone cells. J Steroid Biochem Mol Biol 121:265–267.

Somjen D, Katzburg S, Grafi-Cohen M, Knoll E, Sharon O, Posner GH. 2010c. Vitamin D metabolites and analogs induce lipoxygenase mRNA expression and activity as well as reactive oxygen species (ROS) production in human bone cell line. J Steroid Biochem Mol Biol (in press).

Tong WG, Ding XZ, Adrian TE. 2002. The mechanisms of lipoxygenase inhibitor-induced apoptosis in human breast cancer cells. Biochem Biophys Res Commun 296:942–948.

Tunc O, Thompson J, Tremellen K. 2008. Development of the NBT assay as a marker of sperm oxidative stress. Inter J Androl 33:13–21.