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Involvement of ERK1/2 and JNK Pathways in 17β-estradiol Induced Kir6.2 and SK2 Upregulation in Rat Osteoblast-like Cells

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The functional expression of potassium (K^+) channels has electrophysiologically been studied in bone cells from several species, however, their identity and regulation of gene expressions in bone cells are not well known. In the present study, to investigate how K^+ channel expressions are regulated by estrogen, we measured changes of transcript levels of various Ca^{2+}-activated (K_{Ca}) and ATP-sensitive K^+ channels in rat osteoblastic ROS 17/2.8 cells after treatment with estrogen. Application of 17β-estradiol (E_2) for 24 h and 48 h increased mRNA and protein expressions of inwardly rectifying K^+ channel (Kir) 6.2 and type 2 small conductance K_{Ca} channel (SK2), respectively. Combined treatment of cells with 17β-E_2 and ICI 182,780, a pure antiestrogen, suppressed 17β-E2-induced alterations of SK2 and Kir6.2 mRNA levels. In addition, treatment of cells with U0126, a specific inhibitor of extracellular receptor kinases (ERK)1/2, and SP600125, a specific inhibitor of c-jun N-terminal kinase (JNK) blocked the enhancing effects of 17β-E_2 on SK2 and Kir6.2 protein expressions. On the other hand, blocking of p38 mitogen-activated protein kinase had no effect. Taken together, these results indicate that 17β-E_2 modulates SK2 and Kir6.2 expressions through the estrogen receptor, involving ERK1/2 and JNK activations.

Key Words: Estrogen, Osteoblastic cell, Kir6.2, SK2, ERK1/2, JNK

INTRODUCTION

Bone cells are known to respond to external stimuli through changes of second messenger system and ion channel activities (Somjen et al, 1987; Chesnoy-Marchais & Fritsh, 1989; Abou-Samra et al 1992; Yukihiro et al, 1994). However, many studies to date have focused on the role of calcium (Ca^{2+}) channels. Therefore, relatively little is known about identity or physiological relevances of other channels in bone cells. Potassium (K^+) channels and currents have been demonstrated in rat, chick, and human osteoblastic cells (Dixon et al, 1984; Ypey et al, 1988; Ravesloot et al, 1990; Chesnoy-Marchais & Fritsch, 1993; Davidson, 1993; Moreau et al, 1996; Moreau et al, 1997; Yellowley et al, 1998). In MG-63 and SaOS-2 human osteoblast-like cells, Moreau and collaborators (1996) found that parathyroid hormone and prostaglandin E_2 regulate BK activity possibly via the opening of voltage-gated Ca^{2+} channels. Furthermore, they (1997) demonstrated that large conductance Ca^{2+}-activated potassium (K_{Ca}) channels (BK) and ATP-sensitive potassium (K_{ATP}) channels are involved in the modulation of osteocalcin secretion. They, therefore, proposed that K^+ channels could serve as part of a signal transduction in bone cells. In addition, BK has been suggested as a trigger in the signaling cascade induced by mechanical strains in human osteoblasts (Rezzonico et al, 2003). Despite this body of work, less is known concerning their contributions under pathophysiological conditions in bone cells. Moreover, in osteoblastic cells, the molecular identity of K_{Ca} and K_{ATP} channels and the alterations of their expression by hormones involved in bone homeostasis have not yet been throughly investigated.

Estrogen distinctly inhibits bone loss and bone turnover (Rodan & Martin, 2000), and it also increases bone mineral density (Rodan & Martin, 2000). Therefore, estrogen replacement therapy has long been considered as the first line remedy for preventing osteoporosis in peri-menopausal women (Rodan & Martin, 2000). Okabe et al (2000) reported that 17β-estradiol (E_2) inhibits inwardly rectifying potassium (Kir) currents of osteoclast via nongenomic mechanisms, thereby protecting bone loss possibly through the regulation of intracellular Ca^{2+} concentration. Estrogen has also been known to increase various local factors or protein productions which are involved in signal transduction (Rickard et al, 1999; Manolagas et al, 2002). Up to date, however, only a few studies on estrogen-mediated modulation of K^+ channel activities in osteoblasts have been reported. Moreover, molecular mechanisms of estrogen action on bone are not fully understood.

In the present study, we examined the effect of 17β-estradiol (E_2) on the expressions of K_{Ca} and K_{ATP} channel sub-

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ABBREVIATIONS: E_2, 17β-estradiol; K_{Ca} channel, Ca^{2+}-activated potassium channel; SK, small conductance Ca^{2+}-activated potassium channel; Kir, inwardly rectifying K^+ channel; ERK1/2, extracellular receptor kinases1/2; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinase.

types. We also investigated possible signal pathways involved in E_2 effects on these channels in rat osteoblastic ROS17/2.8 cells. The results showed that 17β-E_2 enhances mRNA and protein expressions of type 2 small conductance K_{Ca} channel (SK2) and Kir6.2, the pore-forming K_{ATP} channel core, through cascades of extracellular receptor kinases (ERK) 1/2 and c-jun N-terminal kinase (JNK).

METHODS

Cell culture

Rat osteosarcoma ROS 17/2.8 cells were cultured in Dublecco's modified eagles medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics-antimyotics (100 units/ml penicillin, 100 μg/μl streptomycin, and 0.25 μg/μl amphotericin B) at 37°C in a humidified chamber containing 5% CO_2. Culture medium and supplements were obtained from Gibco BRL (Gland Island, USA). 17β-E_2 and ICI 182,780, a pure antiestrogen, were obtained from Sigma (St. Louis, USA) and Tocoris (Bristol, UK), respectively. Highly selective inhibitors of the mitogen activated protein kinase (MAPK) family members were purchased from Sigma and Calbiochem (San Diego, USA).

For measurements of the mRNA and protein expressions, cells were seeded at a density of 1×10^6 cells into 100 mm dishes and at a density of 25×10^4 cells in 60 mm dishes. The cells were grown for 24 h in phenol red free DMEM supplemented with 10% FBS, starved in serum-free medium for 24 h, and then cultured in DMEM containing 2.5% FBS and appropriate reagents for an indicated time period. To elucidate whether 17β-E_2 acts through receptor-dependent signaling pathways, cells were treated with ICI 182,780 or MAPK family inhibitors alone or in combination with 17β-E_2 for a time period indicated.

Reverse transcription-polymerase chain reaction (RT- PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA), and cDNA was synthesized with M-MLV reverse transcriptase (Promega, Medison, USA). Using Taq polymerase (Finnzyme, Espoo, Finland), cDNA amplifications were performed in DNA thermal cycler (MJ research, Watertown, USA). Cycling parameters for sulfonylurea receptor (SUR)1 and SUR2A/2B were 35 cycles of 1 min at 95°C, 45 sec at 59°C, and 1 min at 72°C. Cycling parameters for other primers were 30 cycles of 1 min at 95°C, 45 sec at an annealing temperature, and 1 min 72°C. Annealing temperatures were 53°C for SK2, 55°C for BK, SK3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 57°C for intermediate conductance K_{Ca} channels (IK) and Kir 6.2, 60°C for Kir6.1, and 63°C for SK1. One-tenth of PCR products was resolved on a 1% agarose gel containing 0.5 μg/ml ethidium bromide, viewed under ultraviolet light, and quantified using Quantity One 1-D image analysis software (Biorad, Hercules, USA). Primers for IK, Kir6.2, and GAPDH were designed using Primer 3 software (Rozen & Skaletsky, 2000). A single PCR product of the expected size, as denoted in Table 1, was detected in each sample. The scale of each band was expressed as a ratio of the optical intensity of each band to that of the GAPDH band.

Western blot analysis

After appropriate treatments, cells were lysed with lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40) containing protease inhibior cocktail (Roche). Forty μg of protein from each sample was size-fractionized by 10% SDS-polyacrylamide gel electrophoresis, electrotransferred onto a nitrocellulose membrane, and incubated overnight at 4°C with primary antibodies (1 : 500) for SK2 (Alomone Labs, Jerusalem, Israel) and Kir6.2 (Alomone Labs). After incubation and washing, membranes were in-

Table 1. Primer sequences for the polymerase chain reaction (PCR)

Gene	Primer sequence (5'→3')	Product size (bp)	Reference
BK	5'-GGCTGGAAGTGAATTCTGTAG-3' 5'-TGAGTAAGTAGACACATTCCC-3'	312	Gu et al, 2001
IK	5'-CTTGGGTGCTGTCTGTGG-3' 5'-GTGTTTCTCCGCCTTGTTG-3'	233	AF149250
SK1	5'-CAGGCCCAGCAGGAGGAGTT-3' 5'-GGCGGCTGTGGTCAGGTG-3'	159	Gu et al, 2001
SK2	5'-TCCGACTTAAATGAAAGGAG-3' 5'-GCTCAGCATTGTAGGTGA-3'	190	Gu et al, 2001
SK3	5'-GTGCACAACTTCATGATGGA-3' 5'-TTGACACCCCTCAGTTGG-3'	182	Gu et al, 2001
Kir6.1	5'-AAAGGAAGATGCTGGCCAGGAA-3' 5'-CCGTGATGCCTTTCTCCATGTA-3'	338	Wulfsen et al, 2000
Kir6.2	5'-GGCAGATGAAAAGGAGTGGA-3' 5'-CACAAGAACATCCGAGAGCA-3'	225	U44897
SUR1	5'-TGGGGAACGGGGCATCAACT-3' 5'-TGGCTCTGGGGCTTTTCTC-3	387	Wulfsen et al, 2000
SUR2A/2B	5'-GACAGCCTTTGCGGATCG-3' 5'-GCATCGAGACACAGGTGCTG-3'	2A 387 2B 211	Masayuki et al, 2000
GAPDH	5'-TGGAAGATGGTGATGGGTTT-3' 5'-AAGATGGTGAAGGTCGGTGT-3'	230	NM_017008

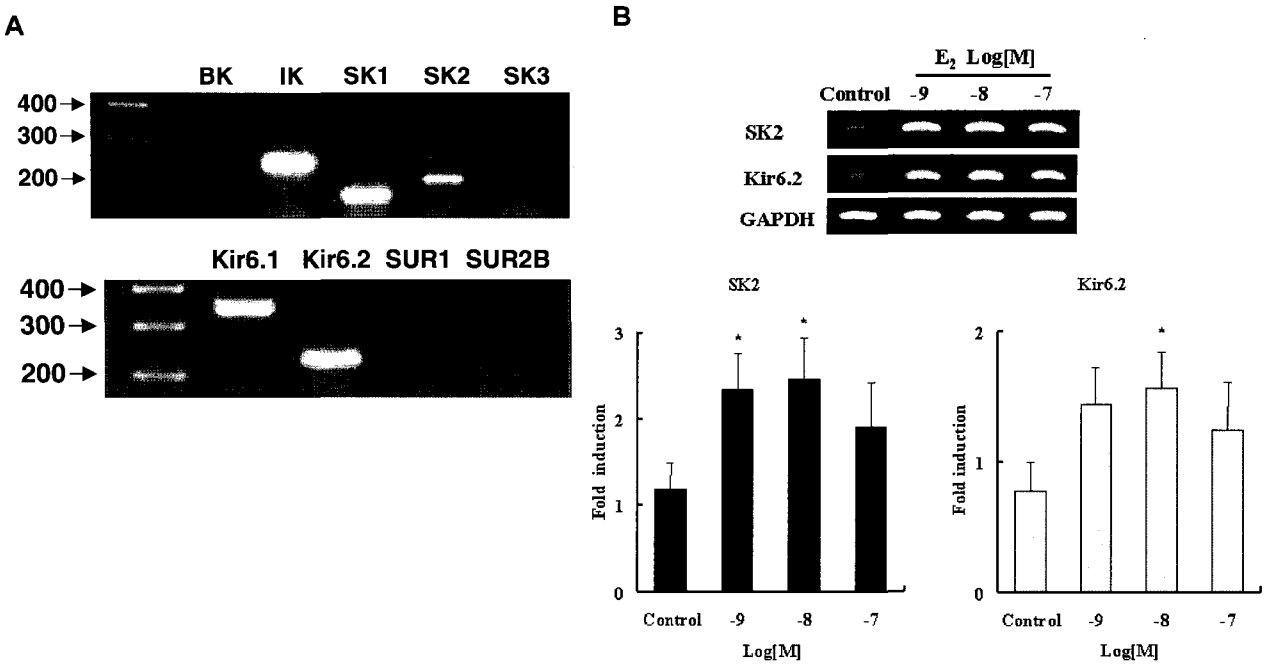

Fig. 1. (A) Ethidium bromide-stained gel of PCR products for K_{Ca} and K_{ATP} channel subunit mRNAs in osteoblastic ROS 17/2.8 cells. (B) Effect of treatment with 17β-estradiol (E_2) for 24 h on SK2 and Kir6.2 mRNA expressions in ROS 17/2.8 cells. In each sample, the expression of mRNAs was corrected with GAPDH and represented as fold induction relative to the control level of 1. Values represent means±SEM (n=6). Asterisks denote statistical significance ($p<0.05$) compared with control.

cubated with a secondary antibody conjugated with peroxidase (1 : 2,000), and immunoreactive signals were detected by enhanced chemiluminescence substrate (Amersham Biosciences, Buckinghamshire, England). Signals were visualized on autoradiography film and quantified by molecular imager using Quantity One 1-D analysis software (Biorad, Hercules, USA). Equal protein loading was confirmed by subsequent probing with the mouse monoclonal antibody against β-actin (1 : 5,000) in each experiment.

Statistical analysis

Data are presented as mean±SEM. In each sample, expressions of mRNA and proteins were corrected for GAPDH and β-actin, respectively, and represented as fold induction relative to the control level of 1. Differences were evaluated using the Student-t, Tukey multiple comparison or Kruskal-Wallis 1-way ANOVA on ranks test with $p<0.05$ considered significant.

RESULTS

In ROS 17/2.8 cells, IK, SK1, SK2, Kir6.1 and SUR2B mRNAs were highly expressed, whereas transcript signals for the BK and SUR1 were hardly detected in these cells (Fig. 1A). The effect of estrogen on the K_{Ca} and K_{ATP} genes and protein expressions was investigated. Application of 17β-estradiol (E_2) for 24 h dose-independently enhanced the Kir6.2 and SK2 mRNA levels by about 1.5- and 2.4-fold, respectively, compared with vehicle treated controls (Fig. 1B), whereas it could not induce any changes of transcript signals of IK, SK1, SK3, Kir6.1, and SUR2B (data not shown). The protein expressions of Kir6.2 and SK2 after 48 and 72 h of 10^{-8} M 17β-E_2 application were approximately 1.4- and 2-7 fold higher than those of untreated controls (Fig. 2).

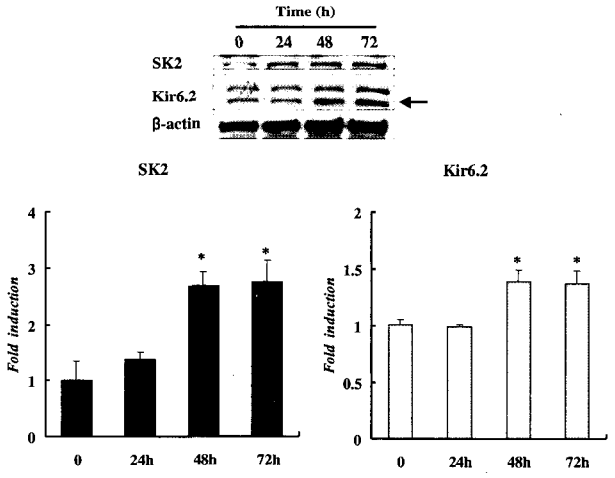

Fig. 2. Effect of estrogen on SK2 and Kir6.2 protein expressions in ROS 17/2.8 cells. Cells were incubated with 10 nM 17β-E_2 for 24, 48, and 72 h. In each sample, the expression of proteins was corrected with β-actin and represented as fold induction relative to the control level of 1. Values represent means±SEM (n=4). Asterisks denote statistical significance ($p<0.05$) compared with 0 time.

Treatment of ROS 17/2.8 cells with ICI182780 (1 μM, 24

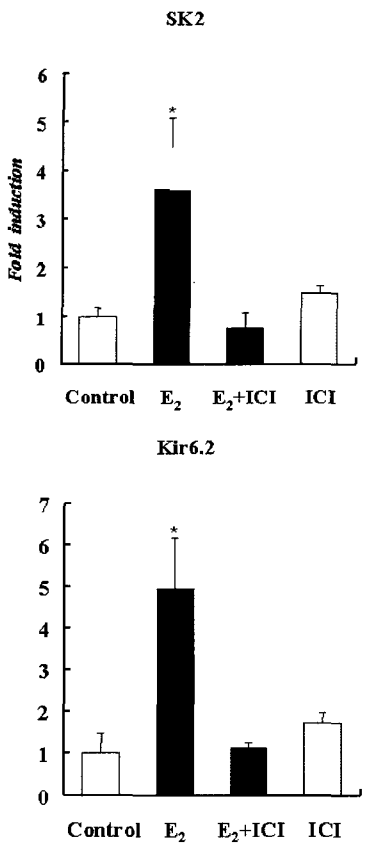

Fig. 3. Effect of estrogen receptor antagonist, ICI 182,780, on SK2 and Kir6.2 mRNA expressions in ROS 17/2.8 cells. Cells were treated with 1 μM ICI 182,780 alone or in combination with 10 nM 17β-E_2 for 24h. Values represent means±SEM (n=4). Asterisks denote statistical significance ($p<0.05$) compared with control.

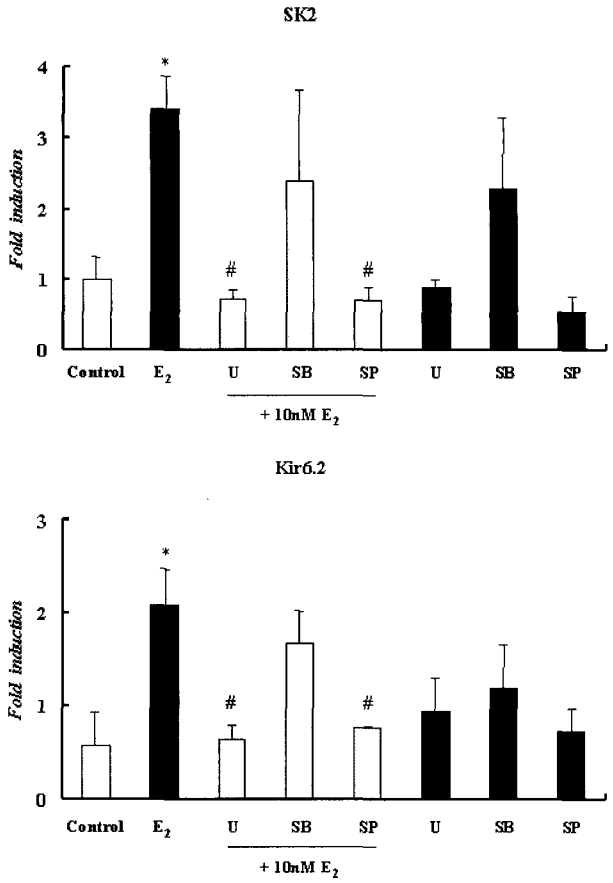

Fig. 4. Effect of selective inhibitors of the major downstream mitogen-activated protein kinase (MAPK) on SK2 and Kir6.2 mRNA expressions in ROS 172.8 cells. Cells were treated with 10 μM MAPK family inhibitors, U0126 (U), SB203580 (SB), and SP600125 (SP), alone or in combination with 10 nM 17β-E_2 for 24 h. In each sample, the expression of mRNAs was corrected with GAPDH and represented as fold induction relative to the control level of 1. Values represent means±SEM (n=3). Asterisks denote statistical significance ($p<0.05$) compared with control. Sharps represent statistical significance ($p<0.05$) compared with 17β-E_2 treatment alone.

h) alone did not alter the Kir6.2 and SK2 transcript signals. However, treatment of the cells with ICI182780 and 10 nM 17β-E_2 together for 24h significantly suppressed the enhancing effects of 17β-E_2 on Kir6.2 and SK2 gene expressions (Fig. 3).

To determine whether the MAPK signaling cascades are involved in the 17β-E_2-induced alterations of SK2 and Kir6.2 expressions, the highly selective inhibitors of MAPK family members were applied alone or in combination with 17β-E_2. Treatment of the cells with inhibitor (10 μM, 24 h) alone did not alter the basal expressions of Kir6.2 and SK2 mRNAs. However, among the three selective inhibitors tested, 10 μM U0126, a specific inhibitor of ERK1/2, and 10 μM SP600125, a specific inhibitor of JNK, almost completely blocked the stimulatory activity of 17β-E_2 on the mRNA expressions of SK2 (E_2 alone, 3.4±0.47, n=3; E_2+U0126, 0.74±0.12, n=3; E_2+SP600125, 0.71±0.18, n=3) and Kir6.2 (E_2 alone, 2.1±0.40, n=3; E_2+U0126, 0.64±0.15, n=3; E_2+SP600125, 0.76±0.02, n=3) (Fig. 4). Enhanced protein expressions of SK2 (E_2 alone, 1.7±0.10, n=4; E_2+U0126, 1.0±0.19, n=4; E_2+SP600125, 0.59±0.17, n=4) and Kir6.2 (E_2 alone, 2.1±0.40, n=4; E_2+U0126, 0.90±0.20, n=4; E_2+SP600125, 1.0±0.11, n=4) induced by 17β-E_2 were also significantly suppressed by co-administrations of U0126 and SP600125 (Fig. 5). On the other hand, SB203580, a selective p38 MAPK inhibitor, had no effect on the 17β-E_2-induced changes of SK2 and Kir6.2 expressions (Figs. 4, 5).

DISCUSSION

In the current study, we found that Kir6.1, Kir6.2, and SUR2B mRNAs are present in ROS 17/2.8 cells, whereas SUR1 and SUR2A transcripts are not. K_{ATP} channel in these cells therefore appears to consist of either Kir6.1 or Kir6.2 as the pore-forming core and SUR2B as the regulatory subunit. Cell-type specific expression of different combinations of Kir6.x and SUR subunits contributes to the diversity in functional properties (Cole & Clement-Chomienne, 2003). As for K_{Ca} transcripts, RT-PCR revealed the presence of IK and three types of SK in rat osteoblastic cells. In contrast to our findings, others have shown that charybdotoxin sensitive BK currents are present in human

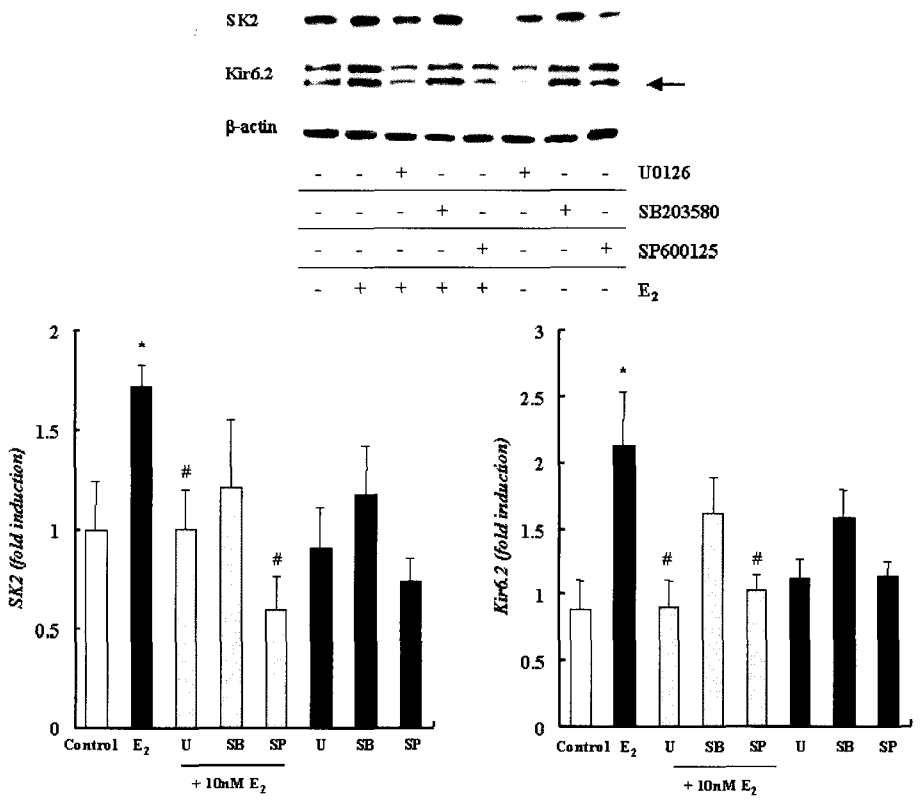

Fig. 5. Effect of selective inhibitors of the major downstream mitogen-activated protein kinase (MAPK) on SK2 and Kir6.2 protein expressions in ROS 172.8 cells. Cells were treated with 10 μM MAPK family inhibitors, U0126 (U), SB203580 (SB), and SP600125 (SP), alone or in combination with 10 nM 17β-E_2 for 24 h. In each sample, the expression of proteins was corrected with β-actin and represented as fold induction relative to the control level of 1. Values represent means±SEM (n=4). Asterisks denote statistical significance ($p<0.05$) compared with control. Sharps represent statistical significance ($p<0.05$) compared with 17β-E_2 treatment alone.

osteoblasts (Moreau et al,1997; Weskamp et al, 2000; Rezzonico et al, 2003). Gu et al (2001) confirmed the presence of messages for SK1, SK2, and SUR2 in mouse osteocyte-like MLO-Y4 cells. In accordance with our results, they were not able to validate the existence of BK and SUR1 mRNAs in these cells. Collectively, these results suggest that the bone cells might have K_{Ca} channel types different from species to species.

In the present study, treatment of rat osteoblast like cells with 17β-E_2 was found to increase mRNA and protein expressions of Kir6.2 and SK2. There are many examples of activation or inhibition of K^+ channel activity by estrogen in a variety cell types via non-genomic mechanisms. Bosch et al (2002) found that 17β-E_2 elevates SK3 transcript signals in guinea pig brain, and Ranki et al (2002) showed in cardiac cells that 17β-E_2 increases levels of SUR2A subunit, consequently enhancing K_{ATP} channel formation. Additionally, in vascular smooth muscle cells, estrogen can activate BK via its direct interaction with the regulatory-subunit (Valverde et al, 1999). On the other hand, estrogen has been demonstrated to downregulate K_{ATP} channel in pancreatic β-cells (Nadal et al, 1998; Ropero et al, 1999) and to suppress Kir currents in osteoclast (Okabe et al, 2000). These results together with our present findings strongly suggest that modulation of K_{Ca} and K_{ATP} channel expressions by estrogen is different, depending on their region to function.

Estrogen has been known to exert beneficial effects on nonreproductive tissues such as cardiovascular, central nervous, and skeletal systems (Manolagas & Kousteni 2001). Signaling via the estrogen receptor (ER) occurs through two mechanisms; those involving modulation of ER-responsive genes and those involving rapid activation of signaling pathways (Cooke et al, 1998; Rickard et al, 1999). In various estrogen target cells, including endothelial cells, osteoblasts, and endometrial cells, estrogen has earlier been shown to be able to stimulate the MAPK signaling pathways through ER (Kousteni et al, 2001; Prouillet et al, 2004; Simoncini et al, 2004; Seval et al, 2006). Therefore, we proposed that the MAPK signaling cascades are involved in the 17β-E_2-induced alterations of SK2 and Kir6.2 expressions, and attempted to elucidate their involvement and found herein that both ERK1/2 inhibitor U0126 and JNK inhibitor SP600125 completely abrogated the stimulatory effect of 17β-E_2 on the Kir6.2 and SK2 mRNA and protein expressions. These findings, therefore, suggest that 17β-E_2 regulates both K^+ channel expressions through receptor-dependent MAPK pathways.

Although the mechanism by which MAPK is linked to 17β-E_2 induced SK2 and Kir6.2 up-regulation in rat os-

teoblasts remains largely unclear, the previous studies (Ghanshani et al, 2000; Chae & Dryer, 2005) suggest the possibility that MAPK cascades mediate estrogen effects on SK2 and Kir6.2 expressions through either transcriptional modulation of their promoter or regulation of their trafficking (Chae & Dryer, 2005). Further studies, however, are required to clearly elucidate underlying mechanism that might link MAPK to upregulation of SK2 and Kir6.2 in rat osteoblasts.

K^+ channel plays a key role in the regulation of membrane potential. Although physiological relevance of membrane hyperpolarization response in bone cells is not obvious, Kawase et al (1996) reported that activation of K_{ATP} channels by calcitonin gene-related peptide (CGRP)) induces membrane hyperpolarization in response to mechanical stimuli and inhibits calcium uptake, and thus may be involved in bone remodeling in rat osteoblastic cell lines. Salter et al (2000) also demonstrated that activation of SK by interleukin-1β results in the same consequences as in human bone cells. In addition, Kawase & Burns (1998) strongly suggested that membrane hyperpolarization in rat osteosarcoma UMR106 cells as a result of CGRP-stimulated K^+ efflux via K_{ATP} channels induces rapid changes in cell morphology and produces cell shrinkage. Nevertheless, further work is required to clarify physiological importance of our findings in regulation of other molecular and biochemical responses of osteoblasts to mechanical stimulation.

In conclusion, our current findings suggest that 7β-E_2 increases Kir6.2 and SK2 expressions through receptor-dependent MAPK pathways, thereby controlling activities of K_{ATP} channel and SK2 in rat osteoblast-like ROS17/2.8 cells.

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