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Zeranol induces COX-2 expression through TRPC-3 activation in the placental cells JEG-3

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ABSTRACT

Transient Receptor Potential Channels (TRPs) are commonly expressed in the reproductive tissues in human. Many female reproductive processes have been associated with these TRPs. The mycotoxin zeranol or α-zearalanol is derived from fungi in the *Fusarium* family. Limited exposure to zeranol appears to be safe. In North America, farmers are using synthetic zeranol to promote growth in livestock. As the health risks of exposure to residual zeranol have not been determined, this practice is disallowed in the European Community. In the present study the cellular calcium levels were elevated in JEG-3 cells treated with zeranol at or above 10 nM. Subsequent study indicated that expressions of TRP channels were induced. In response to the calcium flow, ERK, P38 and PKCβ were activated and COX-2 expression was increased. Specific TRP inhibitors were employed to establish the connection between the ion channel activity and COX-2 expression, and TRPC-3 appeared to be the triggering mechanism. Since the involvement of COX-2 is implicated in placental development and parturition, exposure to this mycotoxin poses a potential threat to pregnant women.

1. Introduction

Transient receptor potential channels (TRPs) are ion channels found on animal cell membrane. Some 28 TRPs are identified and classified into subfamilies: C, V, M, N, A, P, and ML, according to their structural resemblance (Benemei et al., 2015). These ion channel proteins may signal a vast number of stimuli, ranging from sensations to diseases. TRPs are widely expressed in female reproductive system. They are considered to be crucial in the motility of fertilized egg, implantation, placental development, transport of nutrients or metabolites, and laboring (Dorr and Fecher-Trost, 2011). Among the TRP isoforms, human placentae have been found to express C3, C4, C6, V5, V6, P2 as described previously (Dorr and Fecher-Trost, 2011).

Mycotoxins are chemicals produced by fungi that drive away competitive organisms in harvested crops (Richard, 2007). Among them, aflatoxins are considered to be the most toxic. Aflatoxin-contaminated maize was the culprit for the outbreak of mycotoxicosis in Kenya in 2004 (Azziz-Baumgartner et al., 2005). However, the mycotoxin zeranol or α-zearalanol is not as harmful and has been used in promoting cattle growth in North America (Bennett and Klich, 2003).

Many studies have been carried out to address the safety concern of this toxin. Since zeranol is estrogenic, it has similar effect as estrogen on tissues. The mycotoxin improves the vascular function (Zhen et al., 2011) and deters bone loss (Zong et al., 2012) in ovariectomized mice. It may also be antagonistic to estrogen. In breast xenografts grown in nude mice, estradiol and zeranol differentially regulate the expression of c-Myc, c-Fos, and EGFR (Deng et al., 2009). Unlike estrogen, prepubertal administration of zeranol does not promote *N*-methyl-*N*-nitrosourea-induced tumorigenesis in the mammary gland; however, it may interrupt normal ovarian functioning (Yuri et al., 2004). Other undesirable physiological functions have also been reported. It suppresses p53 expression in breast cancer cells (Ye et al., 2011a) and up-regulates aromatase expression in leptin-sensitized breast pre-adipocytes (Zhong et al., 2010). Its metabolites may cause oxidative DNA damage (Fleck et al., 2012), and facilitate the proliferation and transformation of MCF-10A breast cells (Ye et al., 2011b).

Our lab has previously shown that zeranol increases corticotropin-releasing hormone (CRH) expression (Wang et al., 2013b) and induces preterm birth in mice (Wang et al., 2013a). In the present study, effects of zeranol on TRP and the downstream gene expression were investigated in the placental cells JEG-3.

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2. Materials and methods

2.1. Chemicals

Zeranol and the TRP inhibitors ML204 (TRPC4 inhibitor), Pyrazole-3 (TRPC3 inhibitor), SKF96365 (TRPC6 inhibitor), Ruthenium Red (TRPV5 and TRPV6 inhibitor) were obtained from Sigma Chemical Co., St Louis, MO, USA. All chemicals, if not stated, were purchased from Sigma Chemical Co.

2.2. Cell culture

The epithelial choriocarcinoma cell line JEG-3 was a generous gift from Prof. Stephen Shiu, Department of Physiology, the University of Hong Kong, Hong Kong. The cells were maintained in RPMI medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technology, Rockville, MD), and incubated at 37 °C, 5% carbon dioxide. Cells were routinely subcultured when reaching 80% of confluency, and the passage number was kept below 30. Three days before experimentation, the cells were switched to phenol-red free RPMI medium (Invitrogen) with dextran-charcoal treated fetal bovine serum (Hyclone, Logan, UT, USA). JEG-3 cells were seeded in culture dishes at 5×10^2 cells/mm^2 at the first day of experiment. Allowing attachment for 1 day, the cells were treated with 0, 0.01, 0.1, 1, 10 and 100 nM zeranol for 24 h with DMSO as the carrier solvent. The final concentration of the solvent was 0.1% (vol/vol), and the control cultures (0 nM) received DMSO only.

2.3. Intracellular calcium determination

A colorimetric Calcium Detection Kit was employed for this assay (Abcam plc, cat no. ab102505). Briefly, JEG-3 cells were cultured and processed as described in the protocol. The cells were harvested by centrifugation. The cell pellets were re-suspended in Calcium Assay Buffer and sonicated on ice. The supernatant was transferred to a 96-well plate and mixed with Chromogenic Reagent and Calcium Assay Buffer. After a 10-min incubation, the calcium concentration was quantified by measuring the absorbance at 575 nm.

2.4. Quantitative real time RT-PCR assay

JEG-3 cells were cultured and treated as described above. After 24 h of treatment, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad CA USA). The concentration and purity of the isolated RNA were determined by the absorbance reading observed at 260 and 280 nm. 3 μg of total RNA, oligo-dT, and M-MLV Reverse Transcriptase (USB Corporation, Cleveland, Ohio, USA) were used for first strand synthesis. Target fragments were quantified by StepOne™ Real-Time PCR System (Applied Biosystems®, Life Technologies, Grand Island, NY, USA). Real-time PCR Master Mix Reagent kit was obtained from Applied Biosystems and PCR reactions were set up as described in the manual. A typical reaction contained 200 nmol/l of forward and reverse primer, 2 μl cDNA and the final reaction volume was 20 μl. The reaction was initiated by preheating at 50 °C for 2 min, followed by heating at 95 °C for 10 min. Subsequently, 45 amplification cycles were then carried out with 15 s denaturation at 95 °C and 1 min annealing and extension at 58 °C. The mRNA species were quantified by tailor-made Taqman® MGB probes for COX-2 (Cat. No. Hs00153133_m1, Assay-on-Demand®, Applied Biosystems, Foster City, CA, USA) and normalized by GAPDH expression (Cat No. Hs99999905_m1, Applied Biosystems). Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. Western blot analysis

Cells were washed once by PBS (pH 7.4) and harvested into a 1.5 ml microtube with 0.5 ml lysis buffer (PBS, 1%NP40, 0.5% sodium deoxycholate, 0.1% SDS). The lysis buffer contained protease inhibitors (40 mg/l PMSF, 0.5 mg/l aprotinin, 0.5 mg/l leupeptin, 1.1 mmol/l EDTA and 0.7 mg/l pepstatin) and phosphatase inhibitor cocktail (PhosphoSTOP tablet, Roche). The harvested cells were then lysed with a cell disruptor (Branson Ultrasonics Corp., Danbury, CT, U.S.A.) on ice for 30 s. The protein concentration of cell lysate was determined by Dc protein assay (BioRad, Richmond, CA, U.S.A.). 50 μg of lysate protein was loaded and separated on 10% SDS-PAGE and transferred onto an Immobilon PVDF membrane (Millipore, Bedford, MA, U.S.A.). Anti-COX-2 (Abcam, Cambridge, U.K.), anti-phospho-ERK-1/2, anti-phospho-JNK (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-phospho-PKCα/βII, anti-phospho-P38, anti-phospho-PKCβ(pan) (Cell Signaling Technology, Inc., Danvers, MA, U.S.A.) and anti-β-actin primary (Sigma Chem) and secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were used for protein detection. β-Actin served as the house-keeping protein. An ECL Detection Kit (Amersham, Arlington Heights, IL, U.S.A.) provided the chemiluminescence substrate for HRP, and the targeted protein was visualized by autochemiluminography. The optical density readings were determined by using the computer software ImageJ (National Institute of Mental Health, Bethesda MD, USA).

2.6. Transfection of TRPC3 expression plasmid

Cells were transfected with pcDNA6 TRPC3-expressing plasmids, which was cloned and sequence as described previously (Kwan et al., 2006), with Lipofectamine (Invitrogen). The culture medium was changed after 6 h and the cell protein were extracted after a 24-h incubation period as described above.

2.7. Statistical methods

A Prizm5® (GraphPad Software, Inc., CA, USA) software package was utilized for statistical analysis. The results were analyzed by one-way ANOVA followed by Dunnett's Test if significant differences ($p < 0.05$) were observed.

3. Results

3.1. Zeranol increased cellular calcium levels

The effect of zeranol on cellular calcium concentration was determined by an ELISA kit. The result indicated that zeranol at 10 nM or

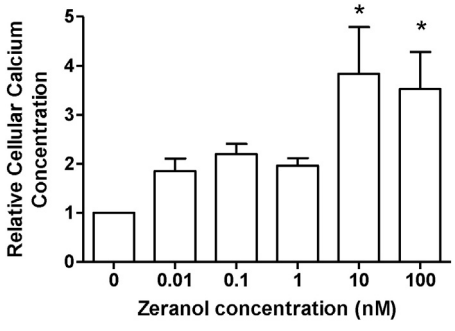

Fig. 1. Cellular calcium concentration in zeranol-treated JEG-3 cells. JEG-3 cells were seeded in 6-well plates and maintained in phenol-red free RPMI 1640 medium supplemented with 10% charcoal dextran-treated FBS. Cells were treated with zeranol for 24 h. The amount of intracellular calcium was determined by Calcium Detection Kit with independent experiments. Values are means ± SEM, n = 3. Means labeled with (*) are significantly ($p < 0.05$) different from control.

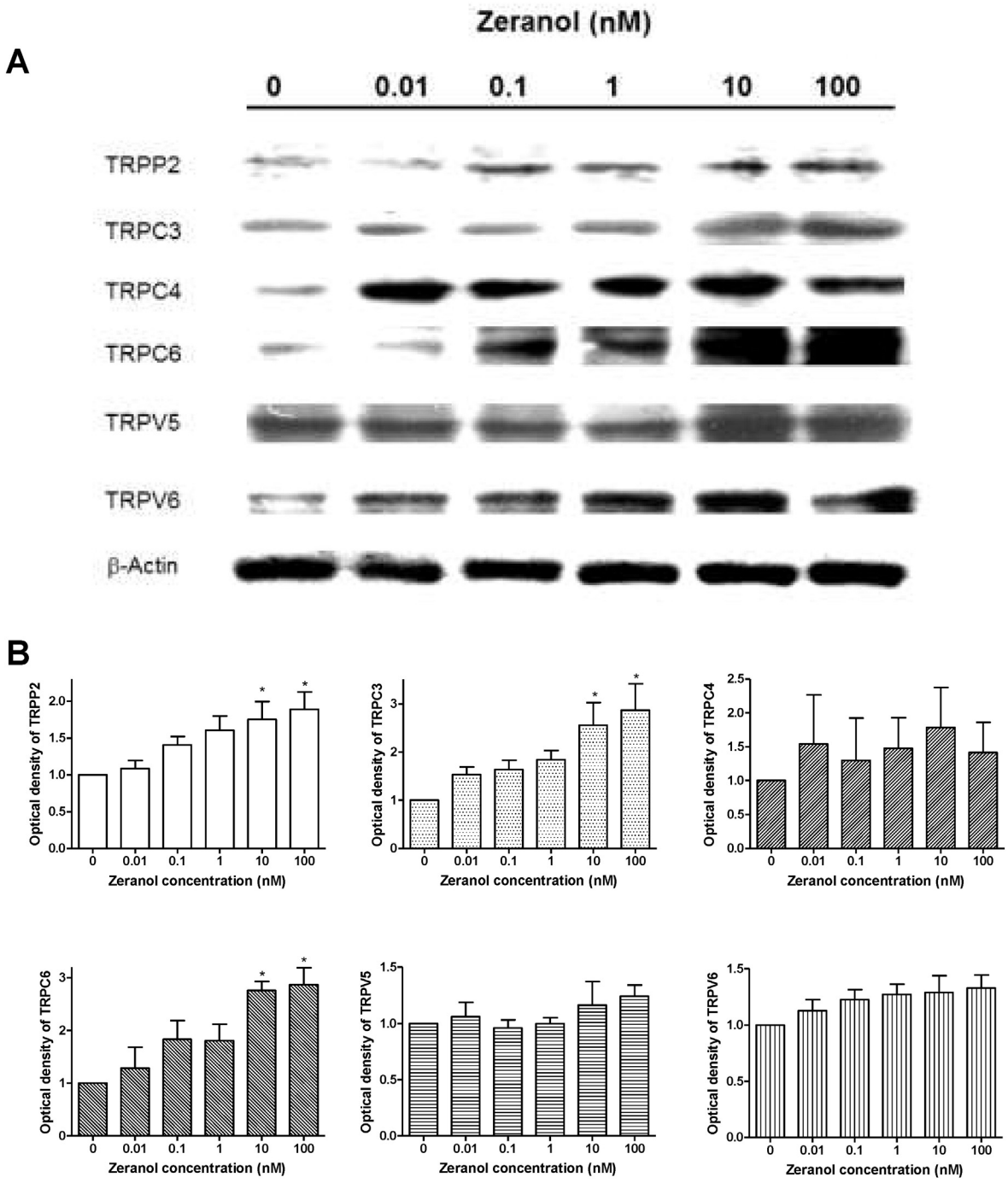

Fig. 2. Zeranol increased TRP expression. JEG-3 cells were seeded in 6-well plates and were treated with zeranol. TRP in the cell lysates was quantified by Western blot. The image (Fig. 2 A) is a representation of results from 3 independent experiments. The optical density of the images was analyzed and presented in Fig. 2 B. Values are means ± SEM, n = 3. Means labeled with (*) are significantly ($p < 0.05$) different from control.

above significantly ($p < 0.05$) increased intracellular calcium levels (Fig. 1).

3.2. TRP expression in zeranol-treated cell cultures

Since the cellular calcium concentration was augmented by zeranol treatment, we subsequently investigated the mycotoxin's effect on the expression of transient ion channels. Western blot results revealed that some TRP channel expressions were induced by zeranol treatment (Fig. 2A). Optical density measurements (Fig. 2B) indicated that TRPP2, TRPC3 and TRPC6 expressions in cultures treated with zeranol at or above 10 nM were significantly higher than those in the control (0 nM).

3.3. Status of COX-2, BCL-2 and p-PKC in zeranol-treated JEG-3 cells

As calcium inflow was observed from zeranol treatment, the statuses of proteins that are sensitive to intracellular calcium increase were determined. Our results showed that COX-2 and BCL-2 expression were dose-dependently increased by zeranol treatment (Fig. 3 A&B). No significant difference was observed for p-PKCα/βII in zeranol- treated samples, whereas p-PKCβ(pan) appeared to be activated from the

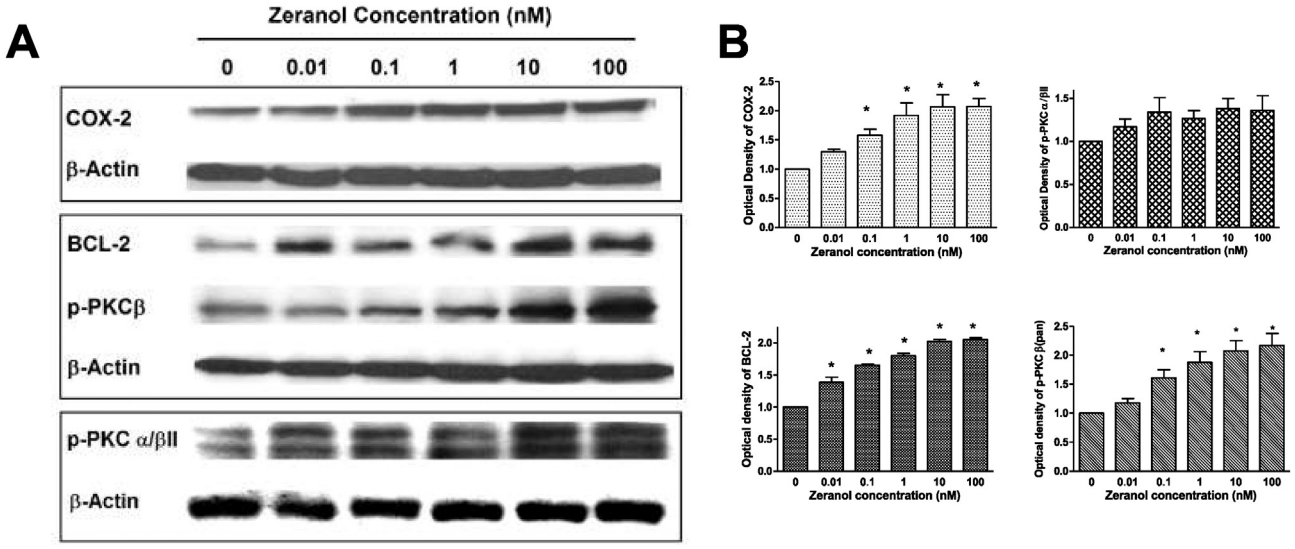

Fig. 3. Determining COX-2, p-PKCα/β, and BCL-2 in zeranol-treated samples. Cell lysate samples were prepared from zeranol-treated JEG-3 cells and immunoblotting was performed. Lanes labeled with 0, 0.1, 1, 10, 100 are samples treated with the respective zeranol concentration (nM). The image (Fig. 3A) represents one of at least 3 independent experiments with comparable results. The optical density readings for COX-2, p-PKCα/βII, p-PKCβ(pan) and BCL-2 proteins are shown in Fig. 3B. Values are means ± SEM, n = 3–4. Means labeled with (*) are significantly ($p < 0.05$) different from control.

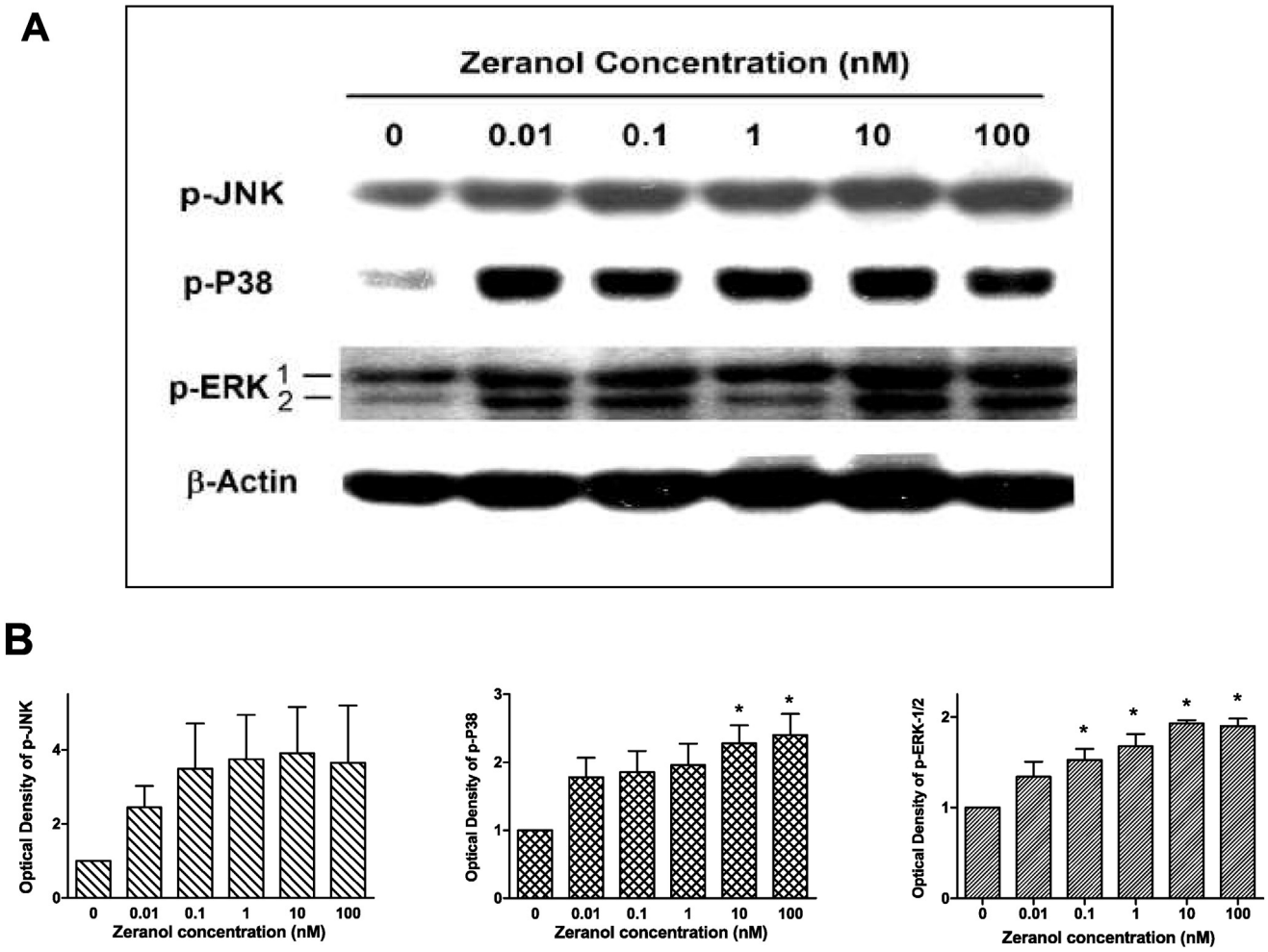

Fig. 4. Activation of MAP kinases in JEG-3 cells treated with zeranol. JEG-3 cells were seeded in 6-well culture dishes and treated with zeranol for 24 h. Amounts of p-ERK-1/2, p-P38, and p-JNK in cell lysates were determined by western blot analysis. The image shown in Fig. 4A is a representation of 4 independent experiments. The optical density readings for p-JNK, p-P38 and p-ERK proteins are shown in Fig. 4B. Values are means ± SEM, n = 4. Means labeled with (*) are significantly ($p < 0.05$) different from control.

optical density reading (Fig. 3B). This result suggested that the β-, not α- isoform of PKC was activated.

3.4. Effect of zeranol treatment on MAPK

Proteins of p-ERK-1/2, Rp-P38, and p-JNK in cell lysates were determined by western blot analysis. Increased amounts of phosphorylated ERK and P38 were found in zeranol-treated cultures as shown in Fig. 4. Optical density analysis (Fig. 4B) illustrated that cells treated with >0.1 or >10 nM zeranol displayed significant increases in p-ERK-1/2 and p-P38, respectively.

3.5. Effect of TRP inhibitors on COX-2

Since several TRPs were up-regulated, we employed specific inhibitors to examine the relationship between TRP and COX-2 expression. Inhibitor of TRPC-3 (Pyrazole-3) could reverse the increased COX-2 protein expression. TRPC-3 was likely the ion channel responsible for regulating the expression, since the TRPC-3 inhibitor consistently deterred COX-2 expression at the mRNA level (Fig. 5).

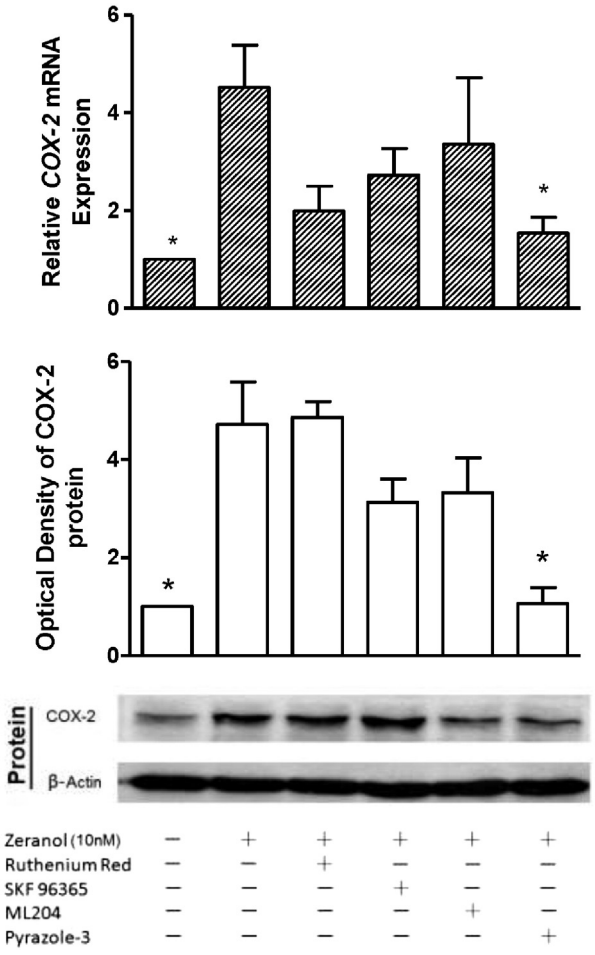

Fig. 5. TRPC inhibitor counteracted zeranol-induced COX-2 mRNA and protein expression in JEG-3 cells. JEG-3 cells were pre-treated with various TRP-specific inhibitors and cultured for 24 h. The TRP inhibitors were ML204 (TRPC4 inhibitor), Pyrazole-3 (TRPC3 inhibitor), SKF96365 (TRPC6 inhibitor), Ruthenium Red (TRPV5 and TRPV6 inhibitor). The mRNA and protein of COX-2 in the zeranol-treated cells was quantified by quantitative RT-PCR and Western blot. The mRNA and optical density values are means ± SEM, n = 8 and 4, respectively. Means labeled with (*) are significantly ($p < 0.05$) different than the samples treated with zeranol only. The Western blot image is a representation of 4 independent experiments with similar results.

3.6. Effect of TRP inhibitors on BCL-2, pERK, p-P38, p-PKCβ

As activations or upregulations in these proteins were observed, the involvement of TRPs was investigated. Pre-administration of ML204 (TRPC4 inhibitor) and Pyrazole-3 (TRPC3 inhibitor) in cells appeared to prevent the activation of ERK, and lesser amounts p-ERK were seen in samples treated with these two inhibitors (Fig. 6B). The MAPK ERK was the downstream signaling molecules of TRPC family ion channels. The MAPK p-P38 was also lessened in cultures pretreated with Ruthenium Red, ML-204 and Pyrazole-3, but the decreases were not significant ($p < 0.05$). Likewise, Pyrazole-3 also dampened the increased amount of p-PKCβ (Fig. 6B). Since BCL-2 expression was not changed under the influence of the TRP inhibitors, it was unlikely that TRPs regulated the gene's expression.

3.7. Verifying the expression of TRPC3 and COX-2 protein in JEG-3 cells

As no previous study has shown the expression of TRPC3 in JEG-3 cells and it appeared to be the determining factor in the findings of the current study, we carried out an additional immunoblotting experiment to verify the expression. The cells were treated with the COX-2 activator PMA or transfected with TRPC3-expressing plasmid. An increased amount of COX-2 or TRPC3 antibody-reactive band was seen in the respective blot in Fig. 7. These results indicated that TRPC3 and COX-2 were expressed in JEG-3 cells.

4. Discussion

In the present study, we showed that exposure to zeranol increased COX-2 expression through TRP signaling in JEG-3 cells. While many isoforms of TRP were increased in expression, TRPC-3 was likely the signaling ion channel involved in this upregulation. Our results also suggested that the increased TRPC-3 conveyed the signal to *COX-2* through PKCβ or P38 activation.

Human placentae express six isoforms of TRPs as abovementioned. TRPC, especially TRPC3, may control calcium influx and the contractility of the uterus at labor (Baggia et al., 1996). Similar to that in the uterus, placental TRPC expression increases near the end of gestation and peaks at labor. However, the physiological function of TRPC at labor is not known. Knockout mouse models illustrate that placental TRPV5 (Hoenderop et al., 2003) and V6 (Suzuki et al., 2008) transport calcium for fetal bone formation. TRPP2 appears to control ion transport in the placenta and may contribute to the fetal vessels and networks development in the labyrinth layer of the placenta (Allen et al., 2006). Consistent to the role in uterine contraction, TRPC3/4 could be responsible for controlling COX-2 expression in reproductive tissues.

The importance of COX-2 in female pregnancy has been illustrated by the infertility of *cox-2* knockout mice (Dinchuk et al., 1995). MAPKs and NFκB have been shown to be major signaling molecules triggering COX-2 expression at the uterus during decidualization in early pregnancy (Scherle et al., 2000), and p38 MAPK activation may contribute to the labor occurring at parturition (Takanami-Ohnishi et al., 2001). Ehrig at el. (Ehrig et al., 2015) have shown that a p38 MAPK inhibitor attenuates cardiotonic steroids-induced COX-2 expression in the cytotrophoblast cell line Sw-71. The importance of signal transduction processes in COX-2 expression during pregnancy has been suggested in these studies.

Zeranol is an estrogen-like compound and has been used for promoting growth of livestock. However, its estrogenic property might not be a factor in the present study as JEG-3 cells are not ER-responsive (O'Neil et al., 2001). The chemical is absorbed in the small intestine, and is found as its aglycone form or glucuronide and sulfate conjugates in blood (Pfeiffer et al., 2011). The chemical can be carried to the placenta through the circulation. The concentration of zeranol in human body fluid has been reported in several studies. The mean serum zeranol concentration in girls with precocious puberty was determined to be

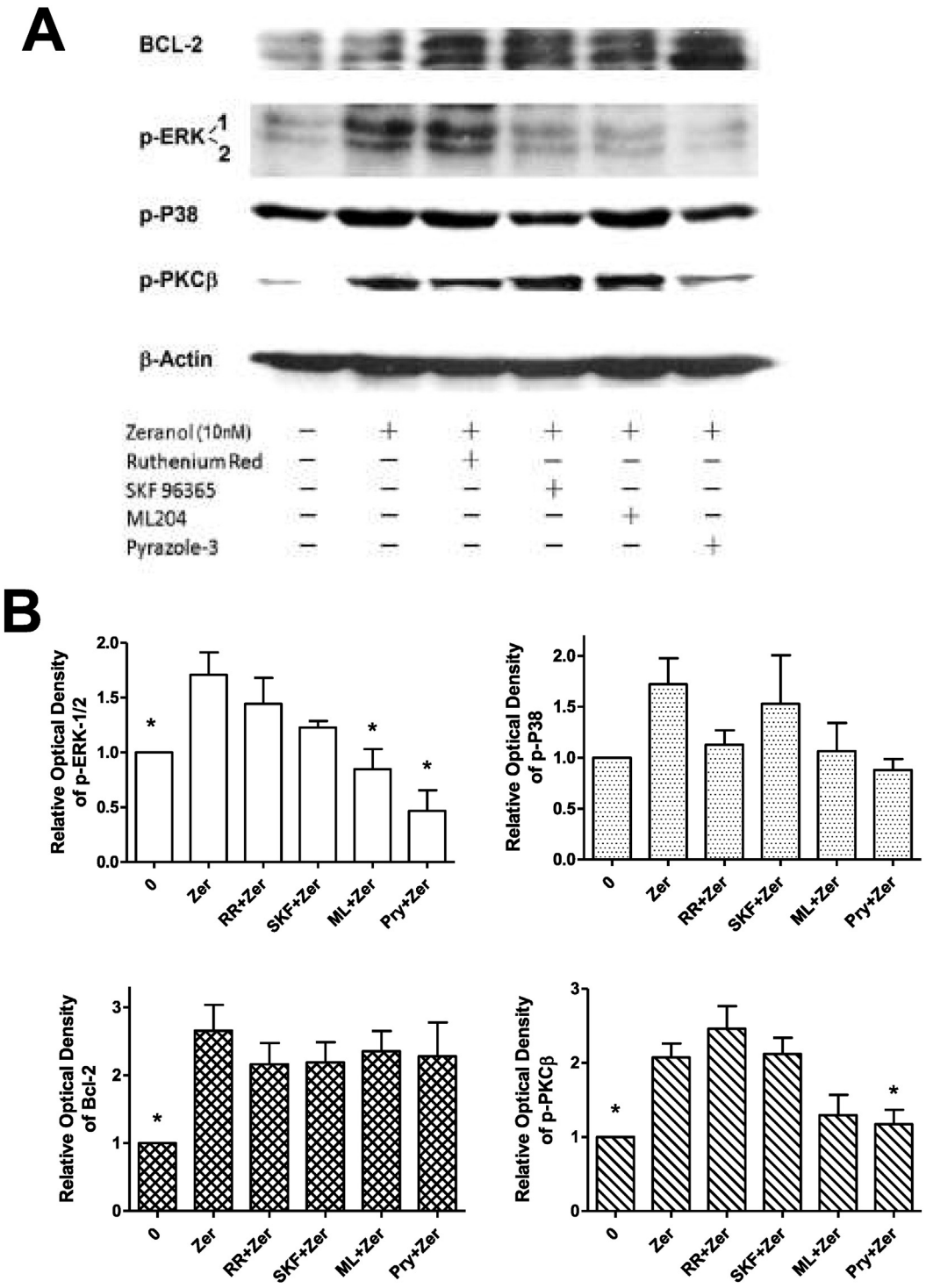

Fig. 6. Effect of TRP inhibitors on zeranol-induced MAPK, BCL-2, and p-PKCβ in JEG-3 cells. JEG-3 cells were pre-treated with various TRP-specific inhibitors and cultured for 24 h. The TRP inhibitors were ML204 (TRPC4 inhibitor), Pyrazole-3 (TRPC3 inhibitor), SKF96365 (TRPC6 inhibitor), Ruthenium Red (TRPV5 and TRPV6 inhibitor). The MAPKs, BCL-2, and PKCβ proteins in cell lysates were determined by immunoblotting. The Western blot image (Fig. 6A) is a representation of 4 independent experiments with similar results. The optical density values (Fig. 6B) are means ± SEM, n = 4. Means labeled with (*) are significantly ($p < 0.05$) different than the samples treated with zeranol only.

106.5 ng/l or 0.3 nM (Massart et al., 2008), and the mean urinary zeranol concentration is about 0.2 μg/l (Bandera et al., 2011). The mycotoxin concentrations ≤1 nM, which could increase COX-2 in the present study, should fall into the physiological range.

Since increased COX is associated with uterus contraction, COX inhibitors have been used attempting to treat preterm labor (Reinebrant et al., 2015). An upregulation of placental COX-2 expression as shown in the present study may lead to undesirable outcome on pregnancy.

The effect of zeranol on reproduction has not been fully addressed. Prenatal zeranol exposure may introduce testicular defects in mouse fetuses (Perez-Martinez et al., 1996). In addition, zeranol may potentially perturb the process of gestation as shown in the present investigation. Nevertheless, we have to exercise caution in the interpretation. Because of the limitation in replicates, *in vitro* studies may be precise but not accurate. The replicates in the present study were run in separate experiments, and this handling might minimize the weakness. Another

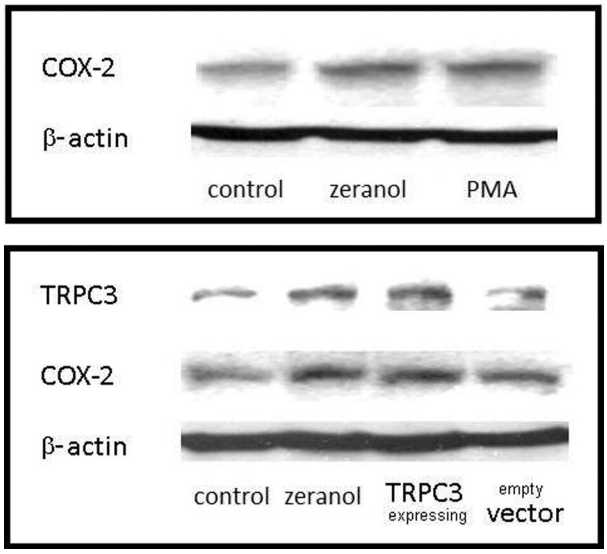

Fig. 7. Verifying TRPC3 and COX-2 expression in JEG-3 cells. JEG-3 cells were treated with 3 μM phorbol 12-myristate 13-acetate (PMA) 10 μM zeranol, or transfected with TRPC3-expressing plasmid and cultured for 24 h. TRPC3 and COX-2 in cell lysates were determined by immunoblotting.

problem could occur in the culture system itself. Since this culture model did not have a metabolic system in place, the half life and biological activity of zeranol could be mis-represented. In summary, this study demonstrated that zeranol upregulated COX-2 expression in JEG-3 cells. These findings might also help establishing TRPC's signaling pathways in placental cells.

Transparency document

The Transparency document associated with this article can be found, in online version.

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