FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 51, No. 1, 2013
pp. 18–24

ORIGINAL STUDY

VIA MEDICA

Monobutyl phthalate induces the expression change of G-Protein-Coupled Receptor 30 in rat testicular Sertoli cells

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Abstract: The aim of the study was to explore whether G-Protein-Coupled Receptor 30 (GPR30) was expressed in rat testicular Sertoli cells and to assess the impact of monobutyl phthalate (MBP) on the expression of GPR30 in Sertoli cells. By using RT-PCR, Western-Blot and immunofluorescent microscopy, the expression of GPR30 in rat Sertoli cells was found at both gene and protein level. Cultures of Sertoli cells were exposed to MBP (10––1000 μM) or a vehicle. The results indicated that the expression of GPR30 increased at gene and protein levels in Sertoli cells following administration of MBP even at a relatively low concentration. We suggest that changes of GPR30 expression may play an important role in the effects of the xenoestrogen MBP on Sertoli cell function. *(Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 1, 18–24)*

Key words: monobutyl phthalate, GPR30, Sertoli cell, xenoestrogen, nongenomic action, rat

Introduction

Phthalates belong to chemicals widely used in industry. They include endocrine disrupting chemicals (EDCs) acting as hormone-like agents that may interfere with the synthesis, secretion, transport and elimination of natural hormones in the body [1]. Di(*n*-butyl) phthalate (DBP) is a weak estrogenic compound extensively applied in the cosmetics industry and medical products [2]. Data suggested that xenoestrogens, such as DBP, exert adverse effects on the development of male reproductive system. For example, DBP treatment in pregnant rats could cause a set of disorders in male offspring, including Leydig cell aggregation and hyperplasia, the suppression of intratesticular testosterone level, an increased number of gonocytes, and the presence of multinucleated gonocytes in fetal testis [3–5]. In addition, DBP exposure *in utero* led to hypospadias, cryptorchidism, prostate, epididymis and vas deferent agenesis, decreased anogenital distance and delayed preputial separation [6] which resemble the symptoms of testicular dysgenesis syndrome related to diminished testosterone action in the fetal period of life [7].

It was shown in previous studies that the majority of xenoestrogens exerted their estrogenic effects primarily by binding to classical estrogen receptors (ERs). However, it has been demonstrated that all the xenoestrogens possess a very weak affinity for ERs [8]. For example, DBP is approximately six to seven orders of magnitude less potent than 17β-estradiol [2]. The mechanisms by which xenoestrogens might affect the male reproductive system at a relatively low concentration remain unknown. Besides the classical genomic action, increasing evidence indicates that estrogen can also cause rapid, nongenomic action by binding to a specific membrane estrogen receptor [8]. The G-protein-coupled non-classical membrane ER (GPCR), also known as GPR30, has been widely rec-

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ognized as a membrane ER which plays an important role in nongenomic actions. It was demonstrated by many authors that binding of estrogen to GPR30 could trigger the up-regulation of intracellular Ca^{2+} and cAMP, and then activate the mitogen activated protein kinase (MAPK) pathway [9]. Based on similar chemical structure, xenoestrogens may also mimic such nongenomic actions of estrogens.

Monobutyl phthalate (MBP), the active monoester metabolite of DBP hydrolyzed by intestinal hydrolases upon ingestion and absorption in the gut [10, 11], could inhibit fetal testosterone synthesis, resulting in the anti-androgenic effect of DBP [12]. Additionally, MBP inhibited testicular descent in prenatal rats after administering to pregnant rats from 15th to 17th gestational day [13]. In bovine adrenal chromaffin cells and human neuroblastoma SH-SY5Y cells, DBP and MBP suppressed the increase of the epibatidine-induced Ca^{2+} concentration [14]. MBP was reported to have estrogenic activity in several kinds of animals and cell types, however its mechanism of action needs to be determined.

Sertoli cells (SCs), located within the seminiferous epithelium, are involved in the formation of blood-testis barrier. SCs exert an important function in nourishing and supporting germ cells and play an essential role in normal onset of spermatogenesis. It was reported that exposure to MBP can destroy junctional structures between Sertoli cells [15], and it has been suggested that specific impairment of Sertoli cell microenvironment can induce a dysfunction in sperm production.

The aim of our study was to investigate whether GPR30 is expressed in Sertoli cells and examine the possible effect of MBP on the expression of GPR30 in Sertoli cells in rat testis. In the current study we have demonstrated that GPR30 participated in the response of MBP-treated Sertoli cells.

Material and methods

Primary culture of rat testicular Sertoli cells. Sprague-Dawley rats were purchased from Nanjing Medical University and kept in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Sertoli cells were isolated from the testes of 28-day-old rats in accordance with the method of Li with some modifications [16]. Testes were removed and decapsulated, then washed twice in phosphate-buffered saline (PBS). The seminiferous tubules were dispersed gently using ophthalmic forceps and then transferred into 50 mL plastic tubes. The seminiferous tubules were digested in 0.25% trypsin solution in a rocking incubator (35°C, 50 rpm, 7 min) to remove Leydig cells and other interstitial tissue, followed by 0.1% collagenase I treatment (35°C, 200 rpm, 25 min) to remove the peritubular cells. The homogenate was filtered through a 100-mesh (100 sieve meshes per square inch) stainless steel filter, and collected by centrifugation at 1200 rpm for 7 min. Cells were washed three times in PBS. Isolated cells were placed on cell culture dishes at a density of 1.5×10^6 cells/ml. Cells were grown initially in DMED/F-12 medium with 5% fetal bovine serum (FBS), containing sodium bicarbonate (2.4 mg/L), HEPES (15 mM/L), penicillin (100 IU/mL), and streptomycin (100 IU/mL). Cells were incubated in a humidified atmosphere of 95% air, 5%CO_2 at 35°C. After 2 days of culture, Sertoli cells with tiny protruding dendrites were attached to the bottom of the dishes, while most of germ cells were suspended in the medium. After the medium was changed the purity of the cultured Sertoli cells was more than 95%.

Reverse Transcription-PCR assay. RT-PCR assay was used to demonstrate the mRNA expression of GRP30 in Sertoli cells. By using Column Animal RNAout kit (TIANDZ, Beijing, China), total RNA was extracted from cultured Sertoli cells *in vitro* according to the manufacturer's protocol. Purity of the total RNA was determined by the ratio of absorbance readings at 260 nm and 280 nm, the result of 260/280 nm fell in the range of 1.8-2.0. After diluting the samples to obtain the same concentration (0.05 μg/μL), the total RNA was reverse-transcribed using the EasyScript First-Strand cDNA Synthesis SuperMix (TranGen Biotech Co., Beijing, China). The resulting cDNA was used for PCR. The PCR master mix contained 12.5 μL 2×Taq master mix (BOERDI, Nanjing, China), 1 μL cDNA, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM) and 9.5 μL nuclease-free water. Primers specific for our target genes were designed as showed in Table 1. 18S rRNA was chosen as a housekeeping gene. Compared with beta-actin and glyceraldehyde-3-phosphate dehydrogenase, 18S rRNA was the most stable housekeeping gene and superior for normalization in comparative analyses [17]. Reactions were run in the following conditions: 1×95°C 2min, 27× [95°C 30s, 56°C 30s and 72°C 30s]. PCR products were separated on a 1% agarose gel, stained with ethidium bromide and viewed under a UV transilluminator (Junyi-Dongfang Co., Beijing, China)

RT-PCR was also used to observe whether MBP exposure *in vitro* alters the expression of GPR30 in Sertoli cells. Purified Sertoli cells were plated into 6-well culture plates at 1.5×10^5 cells/mL in 2 mL culture medium. After 24h incubation cells were treated with 17β-estradiol (E2) (0.1 nM) as positive control and MBP (0, 10, 100, 1000 μM), and incubated at 35°C for 5, 10, 15, 30 min and 24 hours. GPR30 expression in the treated Sertoli cells was measured by RT-PCR assay performed as described above.

Western blotting. Cells were washed twice with cold PBS and lysed in a lysis buffer (50 mMTris–HCl pH 7.4, 150 mM

Table 1. Primers used in RT-PCR experiment

GENE	Primer information		
	Primer	**Sequence (5'–3')**	**Length**
GPR30	Forward	GGACGAGCAGTATTACGATA	460bp
	Reverse	CCAGCAGATGAAGAAGACA	
18S	Forward	TGCCTTCCTTGGATGTAG	124bp
	Reverse	CGTCTGCCCTATCAACTTTCG	

NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100 and 0.1% SDS) for 30 min on ice. The lysate was centrifuged at 12,000×*g* for 15 min, the supernatants were collected and protein concentration was determined by Bradford protein assay. Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Roche, Mannheim, Germany) by standard procedures. Transferred blots were incubated sequentially with blocking agent (5% non-fat milk in PBS-tween), primary anti-GPR30 antibodies (Abcam Co., Hong Kong, China) and peroxidase conjugated secondary antibodies (Boster Company, Wuhan, China). Signal detection was performed with an enhanced chemiluminescence detection kit. Band density was measured by Bio-Rad Quantity One software.

Immunofluorescence study. Sertoli cells cultured on polylysine-coated coverslips for 24 h were fixed in 4% (wt/vol) paraformaldehyde for 20 min and then permeabilized for 5 min with 0.25% (vol/vol) Triton X-100 at room temperature. After rinsing with PBS three times, cells were blocked with 3% bovine serum albumin for 30 min. After washing, cells were incubated with primary anti-GPR30 antibodies (1:50 dilution) overnight at 4°C. Alexa Fluor 594 donkey anti mouse IgG (Invitrogen, USA) was applied for 30 min at 37°C to visualize staining. After three times washing, cells were incubated with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) for 5 min to stain cell nuclei. The coverslips were attached to glass slides using 90% glycerin and visualized under a confocal scanning laser microscope.

Immunofluorescence was used to evaluate whether MBP exposure *in vitro* alters the expression or location of GPR30 in Sertoli cells. Sertoli cells were cultured on polylysine-coated coverslips for 24 h. Cells were treated with MBP (0, 10, 100, 1000 μM) and incubated at 35°C for 5, 15, 30 and 60 min. After incubation, immunofluorescence was used to detect the distribution of GPR30 according to the protocol described above.

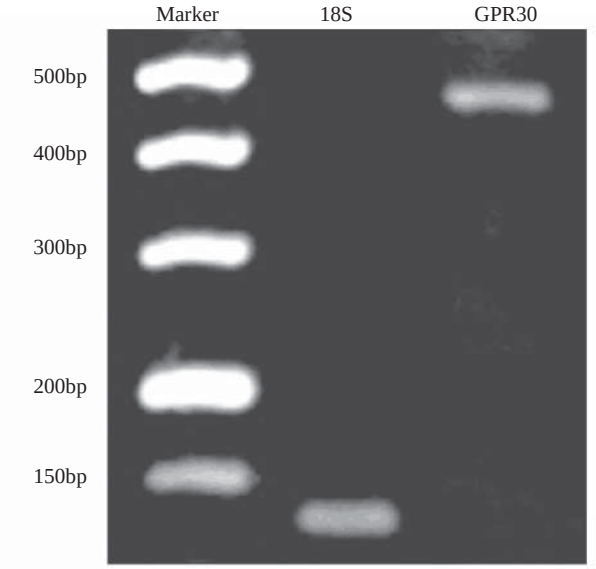

Figure 1. GPR30 mRNA expression in Sertoli cells as determined by RT-PCR. Figure shows only gel electrophoresis of the PCR products and their respective molecular weights (EtBr staining)

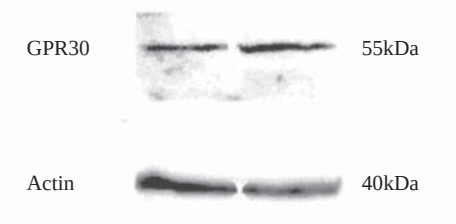

Figure 2. GPR30 protein expression in Sertoli cells as determined by Western blot method

Results

The expression of GPR30 in Sertoli cells

The expression of GPR30 in Sertoli cells was demonstrated at mRNA level (Figure 1), and at protein level by immunoblotting and cell immunofluorescence analysis. Affinity-purified GPR30 antibody detected a single band with an apparent molecular weight of 55 kDa in the immunoblot analysis (Figure 2). As shown in Figure 3, GPR30 was mainly distributed on cell membrane and not in cell nuclei.

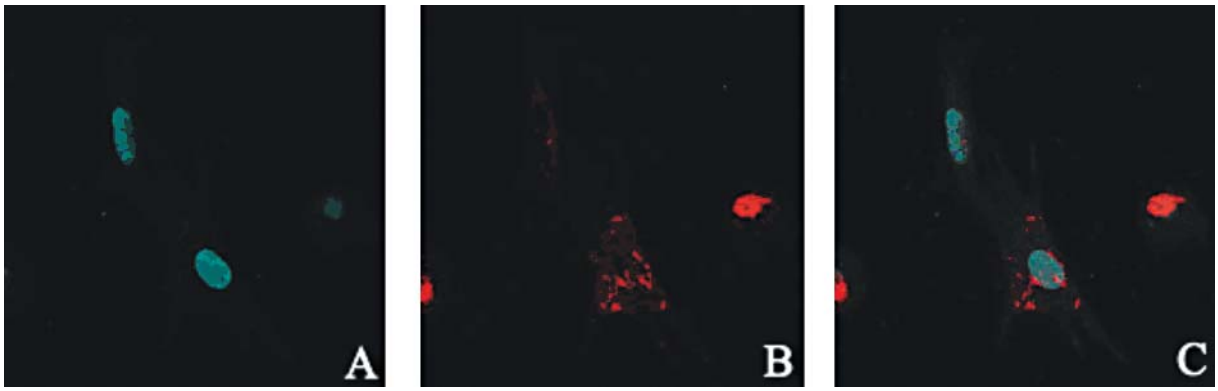

Figure 3. The distribution of GPR30 in untreated Sertoli cells. **A**: Nuclei of Sertoli cells were visualized by DAPI staining; **B**: GPR30 protein presence in Sertoli cells was detected by Alexa Fluor 594 immunofluorescence; **C**: merged image

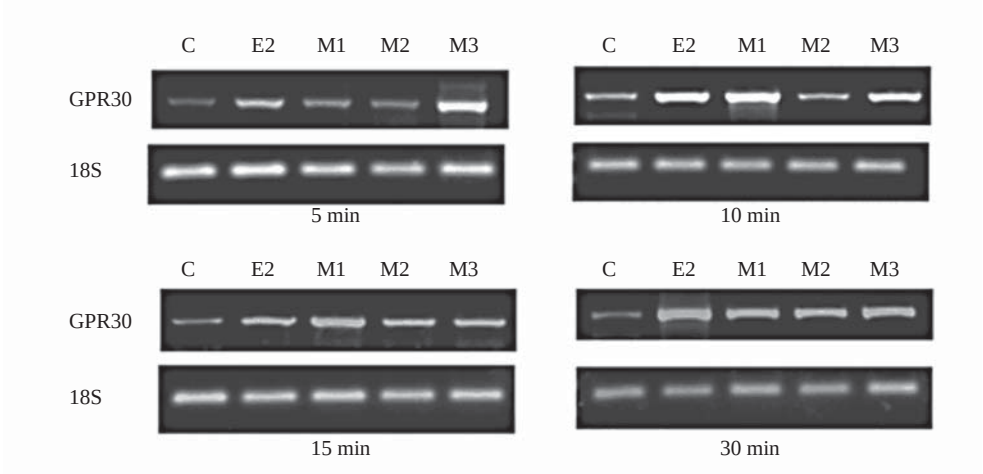

Figure 4. Effects of MBP on gene expression of GPR30 in Sertoli cells. Cells were treated with MBP at various concentrations for 5, 10, 15 and 30 min. C: control group; E_2: 17β-estradiol group; M1: 10 μM MBP; M2: 100 μM MBP; M3: 1000 μM MBP. The experiments were repeated three times

The effect of MBP on the expression of GPR30 in Sertoli cells

Sertoli cells were treated with 17β-estradiol (E2) and MBP at different concentrations and collected at different time points (5, 10, 15, 30 min and 24 h). The results are presented in Figures 4 and 5. In the positive control group (E2 group), the level of GPR30 mRNA was gradually up-regulated from 5 to 15 min. At low (10 μM) and high (1000 μM) concentrations of MBP, there was a similar trend in GPR30 mRNA expression with E2 group. However, after exposure to MBP for 24 h, the data showed a downward trend in the mRNA level of GPR30 (Figure 5). Protein distribution of GPR30 was mostly unchanged after exposure to MBP for 30 and 60 min (Figure 6).

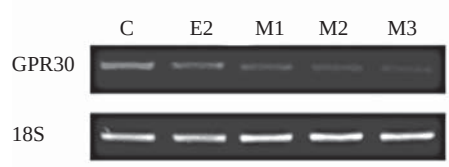

Figure 5. Effects of MBP on gene expression of GPR30 in Sertoli cells treated with MBP at various concentrations for 24 h. C: control group; E_2: 17β-estradiol group; M1: 10 μM MBP; M2: 100 μM MBP; M3: 1000 μM MBP. The experiments were repeated three times

Discussion

In many animal and cell models it was shown that environmental estrogens could induce rapid, cell-sur-

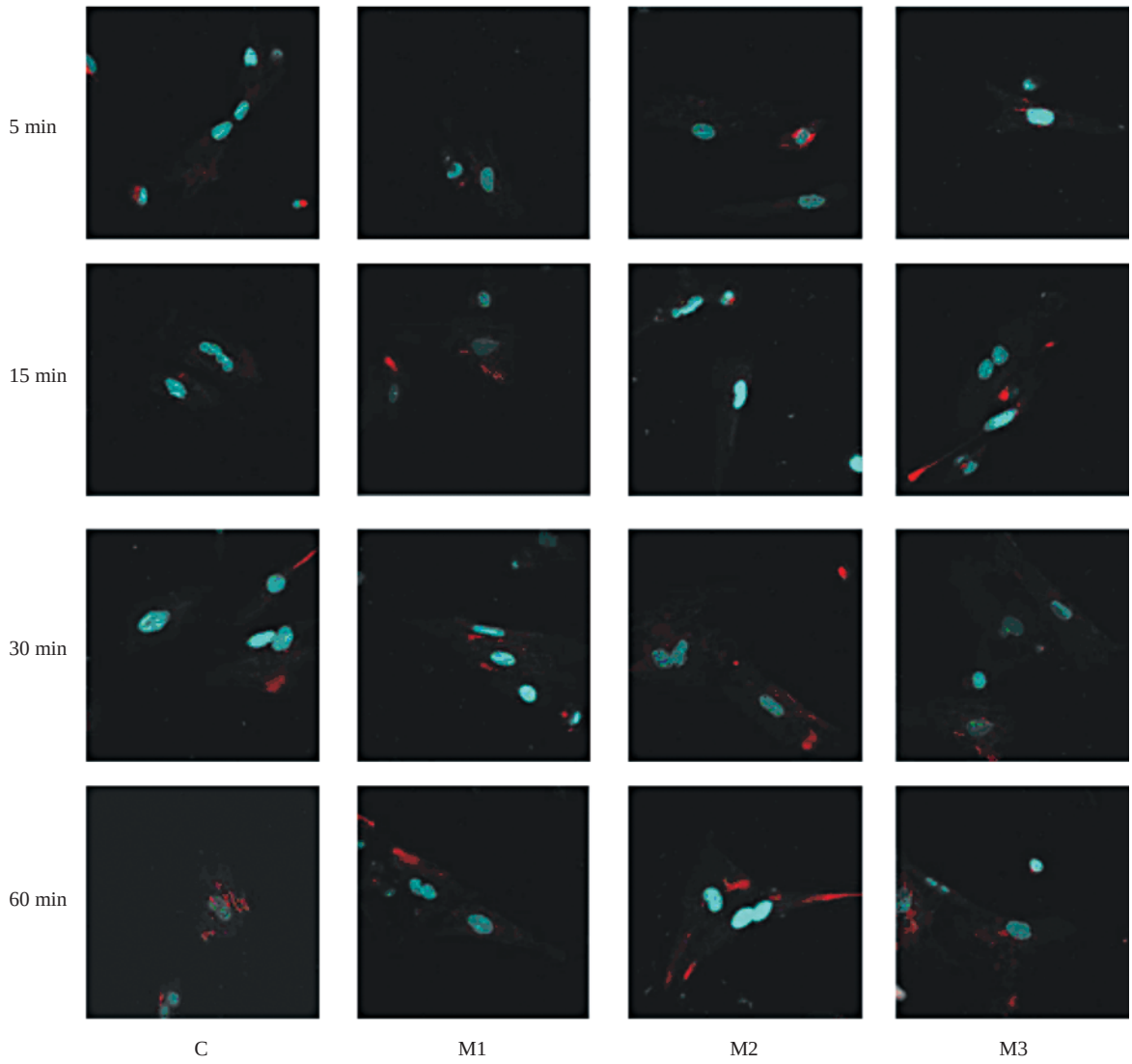

Figure 6. Effects of MBP on changes in the expression of GPR30 in Sertoli cells. Cells were exposed to MBP at various concentrations for 5, 15, 30 and 60 min. The expression of GPR30 was detected by immunofluorescence. C: control group; E_2: 17β-estradiol group; M1: 10 μM MBP; M2: 100 μM MBP; M3: 1000 μM

face initiated nongenomic action [18, 19]. However, there were few studies concerning whether GPR30 can be expressed in Sertoli cells and whether xenoestrogens can affect the expression of GPR30. The results of the present study provide the first clear evidence that estrogen membrane receptor GPR30 is expressed in Sertoli cells in rat testes. The treatment of Sertoli cells with MBP could up-regulate mRNA expression level of GPR30 after short exposure, however, the level was down-regulated after cell exposure to MBP for 24 hours. These results illustrate that GPR30 may be regarded as a sensitive biochemical marker for observing the rapid response of Sertoli cells to MBP.

In the present *in vitro* study, we focused on the effect of relatively low concentrations of MBP on GPR30 expression. The selected doses of MBP could not induce cytotoxicity to Sertoli cells. Our previous investigations had shown that EC_{50} of MBP for inhibiting the viability of Sertoli cells was 16mM [16], suggesting that the doses used in our study did not affect cell viability. It needs to be emphasized that the concentrations at a micro-molar level used in our study are rarely encountered in the environment, which, however, does not exclude toxicological implications of the current study. MBP is present in a variety of foods and can be accumulated to a high level in human body through food intake, while it is not easily degraded [20].

In our study, we speculate that GPR30 is the binding site in the plasma membrane for MBP. As a member of the rhodopsin-like family of G-protein-coupled receptors spanning the membrane seven times, GPR30 is an integral membrane protein that localizes to the endoplasmic reticulum and plasma membrane. GPR30 has a high affinity to estrogens, and their binding results in intracellular calcium mobilization and synthesis of phosphatidylinositol (3,4,5)--trisphosphatein the nucleus. This protein therefore plays a role in the rapid nongenomic signaling events widely observed following stimulation of cells and tissues with estrogen-like chemicals [21, 22]. It is interesting that a wide variety of xenoestrogens with diverse structures have strong binding affinities for GPR30. For example, Bisphenol A has the binding affinity to GPR30 as much as 8–50 times higher than that to ERs. Nonylphenol and Kepone have the binding affinity for GPR30 3–4-fold higher than that for the ERs. In contrast, the binding affinity of mycotoxin for ERα is 8–20-fold higher than its affinity for GPR30 [23–25]. The above data imply that GPR30 might mediate estrogen actions induced by xenoestrogens [9]. Treatment of GPR30-positive cells with estrogens could activate the intracellular signaling pathways. 17β-estradiol has been shown to activate Erk-1/2 kinase via transactivation of the epidermal growth factor (EGF) receptor by the release of proHB-EGF from the cell surface [26].

In addition, xenoestrogens can activate the second messenger system by binding to GPR30 [27]. Downstream of G-protein, estrogen-like chemicals lead to activation of a SRC-like tyrosine kinase, phosphorylation of the adaptor protein SHC, and through the activation of a metalloprotease, the extracellular release of heparin-bound epidermal growth factor (HB-EGF) [9].The release of HB-EGF activates the EGF receptor resulting in the induction of the mitogen-activated protein kinase (MAPK) pathway. There can be also other pathways involved. Phosphoinositide 3-kinase (PI3K) pathway also could be activated via GPR30. As a result of the accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), the anti-apoptotic and proliferative kinase AKT becomes activated [28, 29]. All of these signaling events happen within seconds and minutes, furthermore, these signals elicit changes in the gene expression of a whole network of transcription factors including SRF, CREB, and members of the ETS family which promotes the expression of a second wave of transcription factors such as FOS, JUN, EGR1, ATF3, C/EBPd, and NR4A2. Cells become literally reprogrammed under the effect of this network of transcription factors [30].

Our results indicate that MBP stimulates the gene expression of GPR30 within 5–30 min, which corresponds to the effects of 17β-estradiol. We speculate that MBP caused a rapid, nongenomic effect through binding with GPR30 in the cell membrane, then converted into specific signaling pathway downstream and mediated the mRNA expression of GPR30. We suggest that after 24 hours GPR30 protein formed negative feedback to inhibit the mRNA level of GPR30 as the dose of MBP rose. The present results showed that MBP potentially interfered with the estrogen action through GPR30 in Sertoli cells. Since xenoestrogens could potentially cause estrogen-like action through GPR30 in a wide range of tissues, extensive studies on the biological function of GPR30 are needed to determine the action induced by xenoestrogens.

In summary, we showed that GPR30 was expressed in rat Sertoli cells at both gene and protein level. The exposure of Sertoli cells to MBP influenced the expression of GPR30. This might indicate that GPR30 may be a potential mediator of the action of MBP on Sertoli cells. Future studies should demonstrate whether MBP has the ability to trigger the nongenomic effects.

Acknowledgment

This work was supported by National Natural Science Foundation of China (30970530, 31200401), the National Basic Research Program of China (973 program 2010CB945103), Natural Science Foundation of Jiangsu Province of China (BK2012307), and Open Research Fund of State Key Laboratory of Bioelectronics, Southeast University.

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Submitted: 11 July, 2012
Accepted after reviews: 27 February, 2013