Estrogen Receptor α Induction by Mitoxantrone Increases Abcg2 Expression in Placental Trophoblast Cells

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ABSTRACT: Substrate-induced upregulation of ATP-binding cassette subfamily G member 2 (ABCG2) has been well studied in cancer cells, but it is also important to understand whether ABCG2 is upregulated by its substrates in tissues in which it is constitutively expressed. In the present study, we aimed to clarify the regulatory mechanism of Abcg2 expression by its substrate, mitoxantrone, in placental cells. Abcg2 mRNA expression in rat placental TR-TBT 18d-1 cells treated with 10 μM mitoxantrone for 24 h was increased, compared with that in nontreated cells, whereas 10 μM pheophorbide-a had no effect. Methylated CpG level in the promoter region of the *Abcg2* gene was low and was not altered by mitoxantrone. On the contrary, mitoxantrone markedly increased the expression of estrogen receptor (ER) α and progesterone receptor (PR) B. Fulvestrant, an ER antagonist, attenuated the mitoxantrone-induced increase of Abcg2 mRNA expression, whereas mifepristone, a PR antagonist, had little effect. 17β-estradiol, an ER ligand, positively regulated the mitoxantrone-induced increase of Abcg2 expression. DNA demethylation by 5-aza-2-deoxycytidine treatment increased ERα expression, but mitoxantrone failed to facilitate the demethylation of ERα promoter in TR-TBT 18d-1 cells. In conclusion, Abcg2 expression is induced by mitoxantrone via the induction of ERα in TR-TBT 18d-1 cells. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:3364–3372, 2013

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INTRODUCTION

ATP-binding cassette subfamily G member 2 [ABCG2; also known as breast cancer resistance protein (BCRP) or mitoxantrone resistance-associated protein] was identified as an ABC-type efflux transporter associated with acquired multidrug resistance in cancer cells,[1,2] but it is also constitutively expressed in human placenta.[3] ABCG2 is localized in the apical membrane of placental syncytiotrophoblasts and plays a role in forming the placental barrier.[4,5] The fetal-to-maternal plasma concentration ratio of topotecan is increased in *Bcrp*1$^{-/-}$ fetuses compared with *Bcrp*1$^{+/+}$ fetuses.[6] The ATP-dependent uptake of mitoxantrone by microvillus membrane vesicles isolated from human placenta was inhibited by fumitremorgin C, an ABCG2 inhibitor, but not by verapamil, suggesting that ABCG2 primarily mediates the efflux of mitoxantrone from syncytiotrophoblasts.[7] Thus, it is generally accepted that ABCG2 plays a significant role in suppressing fetal transfer of its substrates, which include mitoxantrone, topotecan, and pheophorbide-a.

Several molecular mechanisms of substrate-induced upregulation of ABCG2 have been identified, mostly in drug-resistant cancer cells. It is well established that long-term exposure to mitoxantrone induces *ABCG2* gene expression in various types of cancer cells via gene amplification and chromosomal translocation.[2,8–10] Even short-term (within a single cell cycle) mitoxantrone exposure can eliminate ABCG2 promoter methylation, leading to

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transcriptional upregulation of ABCG2 in epigenetically silenced cancer cell lines.[11,12] On the contrary, it is not known whether short-term exposure to mitoxantrone activates ABCG2 transcription in cells that constitutively express ABCG2, such as placental syncytiotrophoblasts. This is of interest because it could influence the fetal disposition of ABCG2 substrate drugs.

The human *ABCG2* gene promoter contains nuclear receptor response elements such as estrogen response element, progesterone response element, aryl hydrocarbon receptor (AhR) responsive element, and hypoxia response element (HRE), which regulate the transcriptional activity of the ABCG2 promoter via binding with estrogen receptor (ER) α and β, progesterone receptor (PR) B, AhR, and hypoxia inducible factor 1 (HIF-1), respectively.[13] These nuclear receptor-mediated responses have been poorly characterized in the case of rodent Abcg2, except that HRE of mouse Abcg2 promoter was found to be involved in the increased expression of Abcg2 under hypoxia[14] and that 17β-estradiol, an ER ligand, is reported to induce Abcg2 mRNA expression in a rat brain capillary endothelial cell line.[15] In mouse placenta, the expression level of Abcg2 varies with gestational age and is significantly correlated with those of AhR, HIF-1α, and ERβ.[16] Moreover, mitoxantrone is reported to upregulate ERα transcription by mediating demethylation of the ERα promoter in breast cancer cells.[17] These observations led to the hypothesis that mitoxantrone induces enhanced expression of ABCG2 in placental cells by increasing the levels of nuclear receptors, even though the ABCG2 promoter is unmethylated.

TR-TBT 18d-1 cells have been established as a rat conditionally immortalized syncytiotrophoblast cell line,[18] which constitutively expresses Abcg2.[19] The aim of the present study is to clarify the regulatory mechanisms of Abcg2 expression by its substrates in TR-TBT 18d-1 cells. The effect of short-term exposure to mitoxantrone on the expression level of Abcg2 was studied in relation to epigenetic methylation status in the Abcg2 promoter region and activation of the transcriptional factors of Abcg2.

MATERIALS AND METHODS

Animals

Wistar female rats were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). All animal studies were performed in accordance with the guideline for the care and use of laboratory animals of Keio University.

Cell Culture

The rat placental TR-TBT 18d-1 cell line has been conditionally immortalized by introducing temperature-sensitive SV 40 large T-antigen. TR-TBT 18d-1 cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, St. Louis, Missouri), 100 U/mL benzylpenicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Invitrogen, Carlsbad, California) on six-well plates (BD Biosciences, Bedford, Massachussets) coated with porcine skin collagen type I (Nitta Gelatin Inc., Osaka, Japan) in a humidified atmosphere of 5% CO_2 in air. TR-TBT 18d-1 cells were grown to confluence at 33°C, the permissive temperature for T-antigen expression, and then further cultured for 4 days at 37°C, a nonpermissive temperature for T-antigen expression, to suppress the expression of SV40 T-antigen.[18] In the experiment to examine the effect of 17β-estradiol on the expression of Abcg2 mRNA, FBS for culture medium was predialyzed using 2 K molecular weight cutoff Slide-a-Lyzer dialysis cassettes (Pierce Chemical, Rockford, Illinois).

CpG Methylation Analysis by Bisulphite Pyrosequencing

Genomic DNAs were isolated using an Allprep DNA/RNA Mini Kit (Qiagen, Valencia, California). Conversion of unmethylated cytosines to uracils was performed with a Methyl Easy™ Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures, Sydney, Australia) according to the manufacturer's protocol. Predicted CpG islands in the promoter regions of rat Abcg2 (chr4:153,655,525-153,655,876) and ERα (chr1:42,667,165-42,667,290, 42,669,416-42,669,517, and 42,669,601-42,670,113) were amplified from the modified DNA by polymerase chain reaction (PCR) using one biotinylated primer and a regular primer. Amplicons were chemically denatured and the biotin-tagged primer-amplified chains were selectively picked up by using streptavidin-coated Sepharose beads. Selected single strands of DNA were annealed to sequencing primer by heating to 80°C for 2 min, followed by cooling to room temperature. Annealed samples were sequenced by using luciferin-based pyrosequencing technology (PyroMark Q24; Qiagen) according to the manufacturer's protocol. The degree of methylation was expressed for each DNA locus as percentage methylated cytosine with respect to the sum of methylated and unmethylated cytosine.

Quantitative Real-Time PCR Analysis

Total RNA was isolated with an Allprep DNA/RNA Mini Kit (Qiagen). RNA was reverse-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, California). Quantitative real-time PCR analysis was performed using an ABI PRISM 7300 Sequence Detector system (Applied Biosystems) with 2× SYBR Green PCR Master Mix (Applied Biosystems) and specific primers for rat