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Digoxin up-regulates multidrug resistance transporter (MDR1) mRNA and simultaneously down-regulates steroid xenobiotic receptor mRNA

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Abstract

A steroid xenobiotic receptor (SXR) is involved in the induction of MDR1/P-glycoprotein. MDR1 up-regulation by digoxin was previously demonstrated in human colon adenocarcinoma Caco-2 cells, but the participation of SXR remains unclear. Herein, the participation of SXR in MDR1 up-regulation was examined using reverse transcription-polymerase chain reaction in Caco-2 cells, and digoxin-tolerant cells (Caco/DX) as well as human colon carcinoma LS180 cells, which expressed SXR. MDR1 mRNA expression in Caco-2 or LS180 cells was increased by exposure to 1 μM digoxin for 24 h, in a concentration-dependent manner, but SXR mRNA decreased concentration-dependently and was undetectable or significantly lower at 1 μM digoxin, indicating antithetical changes in MDR1 and SXR mRNA expression. Moreover, the MDR1 mRNA level was higher in Caco/DX cells than Caco-2 cells, whereas the SXR mRNA level was lower in Caco/DX cells. Consequently, digoxin was demonstrated to up-regulate MDR1 mRNA and simultaneously down-regulate SXR mRNA expression.

Keywords: MDR1; P-glycoprotein; Steroid xenobiotic receptor; SXR; Digoxin; Caco-2 cells; LS180 cells

P-glycoprotein/MDR1 is a membrane-bound protein, which functions as an ATP-dependent transporter of xenobiotics from cells [1]. In 1976, its importance was first recognized because of its role in the development of multidrug resistance in Chinese hamster ovary cells [2]. It is now, however, well established that MDR1 is not only expressed in tumor cells, but also in normal tissues with excretory function, i.e., intestine, liver, kidney, and so on [3]. In addition, it is well accepted that MDR1 expressed throughout the body plays an important role in the pharmacokinetics of drugs that are MDR1 substrates, e.g., digoxin, cyclosporin, HIV protease inhibitors, and so on [1,3,4]. Therefore, the induction and inhibition of MDR1 are considered to be novel and important mechanisms for drug interactions in humans [4], and thus many studies on the expression and function of MDR1 have been performed.

Several studies had shown that transcriptional regulation of the *MDR1* gene was unexpectedly complex and was far from being completely understood. However, some factors affecting MDR1 expression have been identified [5], and the *MDR1* promoter is found to contain a GC-box for Sp1, an inverted CCAAT element (Y-box) for YB-1 and NF-Y, a p53 element, an AP-1 element, a CAAT element for a complex of NF-κB and c-*fos* proteins, a C/EBP element for NF-IL-6, a heat-shock element (HSE) for heat-shock transcription factor (HSF), and a steroid xenobiotic receptor (SXR) element for orphan nuclear receptor SXR (also known as PXR, PAR, PRR, or NR1I2). Among them, SXR is involved in the expression of both MDR1 and major drug metabolizing enzyme CYP3A4 [6], and may coordinate drug efflux via MDR1 and drug metabolism via CYP3A4. In addition, SXR was also suggested to regulate multidrug resistance in SXR-expressing tumors [6]. Recently, it was demonstrated that SXR was activated by many steroids [7], rifampicin [8], hyperforin (one of

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the constituents of St. John's Wort) [9], and HIV protease inhibitors [10]. Therefore, it is important for controlling the MDR1 expression to evaluate the transcriptional regulation of MDR1 via SXR.

Previously, we demonstrated that a transient exposure (24 h) to digoxin up-regulates MDR1 mRNA expression in human colon adenocarcinoma Caco-2 cells using a reverse transcription-polymerase chain reaction (RT-PCR) [11]. Moreover, continuous exposure to digoxin was found to up-regulate the function and expression of MDR1 in Caco-2 cells [12]. Because digoxin has a steroid structure in its molecular structure, it may be able to interact with SXR, which is activated by many steroids [7]. However, it is not yet clear whether SXR participates in the up-regulation of MDR1 expression caused by exposure to digoxin. In this study, we evaluated the participation of SXR in the up-regulation by examining the effects of digoxin on MDR1 and SXR mRNA expression in Caco-2 and digoxin-tolerant Caco-2 cells (Caco/DX) [12] as well as human colon carcinoma LS180 cells, in which the expression of SXR is confirmed [6].

Materials and methods

Chemicals. Digoxin was purchased from Sigma–Aldrich (St. Louis, MO). All other agents were obtained commercially and were of analytical grade requiring no further purification.

Cells and cell culture. Caco-2 cells (47–52 passages) were grown in complete culture medium consisting of Dulbecco's modified Eagle's medium (DMEM; Cat. No. 12800-017, Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Lot. No. 41K2300, Sigma–Aldrich), 100 U/mL penicillin G, 100 μg/L streptomycin sulfate, and 0.1 mM non-essential amino acids (Invitrogen). Cells were seeded at a density of 2×10^6 cells/100-mm dish (in diameter) in 10 mL of complete DMEM and were subcultured every 3 or 4 days with 0.02% EDTA and 0.05% trypsin (Invitrogen).

Caco/DX cells were established for digoxin tolerance by the continuous exposure of Caco-2 cells to 100 nM (about 80 ng/mL) digoxin [12]. Briefly, cells were cultured in the complete DMEM including 100 nM digoxin for ca. 3 months and a subline that could tolerate digoxin was cloned. Caco/DX cells were maintained in the complete DMEM containing 100 nM digoxin and were subcultured in a manner similar to Caco-2 cells. Digoxin at this concentration was moderately cytotoxic for Caco-2 cells and induced MDR1 mRNA expression after 24 h [11].

LS180 cells (50 passage) were grown in complete culture medium consisting of α-minimal essential medium (α-MEM, Cat. No. M4655, Sigma–Aldrich) supplemented with 10% heat-inactivated FBS (Lot. No. 41K2300, Sigma–Aldrich), 100 U/mL penicillin G, 100 μg/L streptomycin sulfate, and 0.1 mM non-essential amino acids (Invitrogen). Cells were seeded at a density of 2×10^6 cells/100-mm dish (in diameter) in 10 mL of complete α-MEM and were subcultured every 3 or 4 days in the same way as Caco-2 cells.

RT-PCR analysis of MDR1 and SXR mRNAs. RT-PCR analysis was performed as described previously [11–13]. Caco-2 or LS180 cells (1×10^6 cells, respectively) were seeded on plastic culture dishes (60 mm in diameter) in 5 mL of the respective complete culture medium. The culture medium was exchanged every 2 days for fresh complete culture medium without any drugs. Both cells were precultured for 10 days in a humidified atmosphere of 5% CO_2–95% air at 37 °C. Then, the culture medium was exchanged for that without or with the indicated concentrations of digoxin and further incubated for 24 h in a humidified atmosphere of 5% CO_2–95% air at 37 °C. In the case of Caco/DX cells, cells were seeded as above, precultured for 10 days following a change every 2 days with fresh complete DMEM containing 100 nM digoxin, and further incubated for 24 h.

Total RNA was isolated from the cells using a GenElute Mammalian Total RNA Kit (Sigma–Aldrich) and aliquots (150 ng) of total RNA were used for reverse transcription with an RNA PCR Kit (AMV) ver. 2.1 (Takara Shuzo, Shiga, Japan). The RT reaction was conducted in 15 μL of RT reaction mixture and this was incubated at 30 °C for 10 min and subsequently at 42 °C for 15 min in a thermal cycler (iCycler Thermal Cycler, Bio-Rad Laboratories, CA). The RT reaction was terminated by heating at 99 °C for 5 min, followed by cooling at 5 °C for 5 min in a thermal cycler (Bio-Rad), giving the RT product.

The PCR primers for amplification of SXR cDNA were originally designed by the primer design software Primer3 [14] (available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). The PCR primers for amplification of MDR1 cDNA, SXR cDNA, and β2-microglobulin (B2M) cDNA were synthesized by Proligo K. K. Japan (Kyoto, Japan) and their sequences were as follows [11,12]: MDR1 (ABCB1, Accession No. AF016535) forward primer 5′-CCC ATC ATT GCA ATA GCA GG-3′ and reverse primer 5′-GTT CAA ACT TCT GCT CCT GA-3′ (2712–2868 bp); SXR (NR1I2, Accession No. AY091855) forward primer 5′-TCC GGA AAG ATC TGT GCT CT-3′ and reverse primer 5′-CAC TCC CAG GTT CCA GTC TC-3′ (1187–1484 bp); and B2M (Accession No. AB021288) forward primer 5′-ACC CCC ACT GAA AAA GAT GA-3′ and reverse primer 5′-ATC TTC AAA CCT CCA TGA TG-3′ (284–397 bp). PCR amplification of cDNA was performed in a total reaction volume of 25 μL including 5 μL RT product using an RNA PCR Kit (AMV) ver. 2.1 (Takara). PCR amplification was initiated by one cycle of 94 °C for 2 min followed by 28 (MDR1 and SXR) or 22 (B2M) sequential cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 45 s in a thermal cycler (Bio-Rad), giving the PCR product. PCR products were separated on Tris–acetate–EDTA 3% agarose gels containing 100 ng/mL ethidium bromide and photographed under ultraviolet illumination at 312 nm with a Polaroid camera. Band densities were measured using the computer program NIH Image ver. 1.62 (National Institutes of Health, Bethesda, MD), and the ratio of band density (MDR1 or SXR/B2M) was calculated.

Statistical analysis. Comparisons between two and among more than three groups were performed by unpaired Student's *t* test and repeated one-way analysis of variance (ANOVA) followed by Dunnett's test, respectively; *P* values of less than 0.05 (two-tailed) were considered significant.

Results

Expression of SXR mRNA in Caco-2 and LS180 cells

The SXR mRNA expression in Caco-2 cells was compared with that in LS180 cells by RT-PCR (Fig. 1). The PCR product (298 bp) derived from SXR mRNA was little detected in Caco-2 cells, but was readily detectable in LS180 cells, in which the expression of SXR is confirmed [6].

Concentration-dependent effect of digoxin on MDR1 and SXR mRNA expression in Caco-2 cells

The MDR1 mRNA expression in Caco-2 cells was not affected by the exposure to 1 nM digoxin for 24 h, but a significant increase of ca. 4-fold was observed in the case of 1 μM digoxin (Fig. 2A). The SXR mRNA

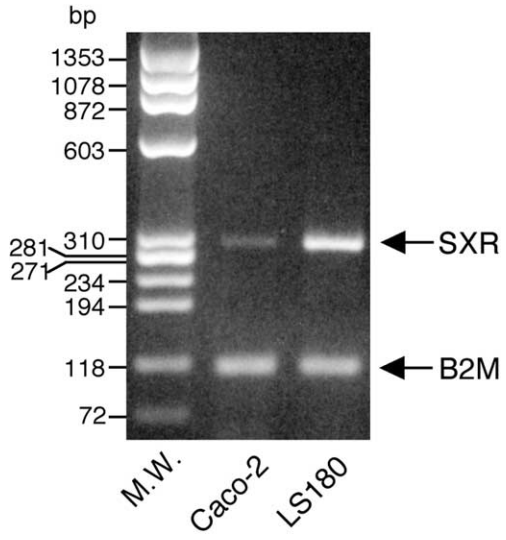

Fig. 1. Expression of SXR mRNA in Caco-2 and LS180 cells. Total RNA was extracted from Caco-2 and LS180 cells, and the SXR and B2M mRNA expression was measured by RT-PCR. A representative electrophoretogram of SXR and B2M is presented. MW, molecular weight marker (ϕX174-*Hae*III digest).

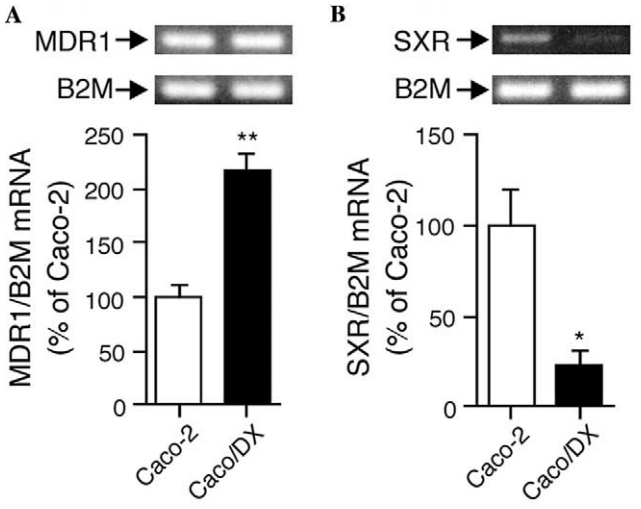

Fig. 3. Expression of MDR1 (A) and SXR (B) mRNAs in Caco-2 and Caco/DX cells. Representative electrophoretograms of MDR1 (A), SXR (B), and B2M are presented. Data were obtained after densitometric analysis of three independent electrophoretograms. Results are expressed as percentages of the ratio of MDR1 (A) or SXR (B) to B2M mRNA levels in Caco-2 cells. Each bar represents the mean ± SE of three independent experiments. * and ** $P < 0.05$ and 0.01 significantly different from Caco-2 cells, respectively. Caco/DX, the digoxin-tolerant Caco-2 cells.

expression in Caco-2 cells was little affected by exposure to digoxin at up to 10 nM, but exposure to 1 μM digoxin made the expression to drop to a level below the limit of detection (Fig. 2B). The expression of both MDR1 and SXR mRNAs was digoxin concentration-dependent, but the trend for change in the SXR mRNA expression on exposure to digoxin was completely opposite to the change in MDR1 mRNA expression.

Expression of MDR1 and SXR mRNAs in Caco-2 and Caco/DX cells

The MDR1 mRNA expression was about 2-fold higher in Caco/DX cells than Caco-2 cells (Fig. 3A). In contrast, the SXR mRNA expression was significantly lower in Caco/DX cells than Caco-2 cells, and antithetical changes in MDR1 and SXR mRNA expression were found (Fig. 3B).

Concentration-dependent effect of digoxin on MDR1 and SXR mRNA expression in LS180 cells

The MDR1 mRNA expression in LS180 cells increased in a digoxin concentration-dependent manner (Fig. 4A). The MDR1 mRNA expression in LS180 cells

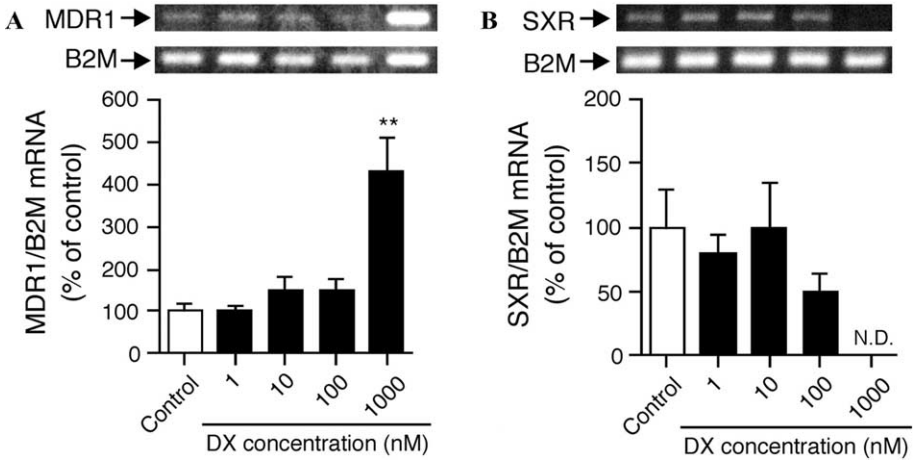

Fig. 2. Concentration-dependent effect of digoxin on MDR1 (A) and SXR (B) mRNA expression in Caco-2 cells. Representative electrophoretograms of MDR1 (A), SXR (B) and B2M are presented. Data were obtained after densitometric analysis of three independent electrophoretograms. Results are expressed as percentages of the ratio of MDR1 (A) or SXR (B) to B2M mRNA levels in the control. Each bar represents the mean ± SE of three independent experiments. ** $P < 0.01$ significantly different from the respective control. DX, digoxin, ND, not detected.

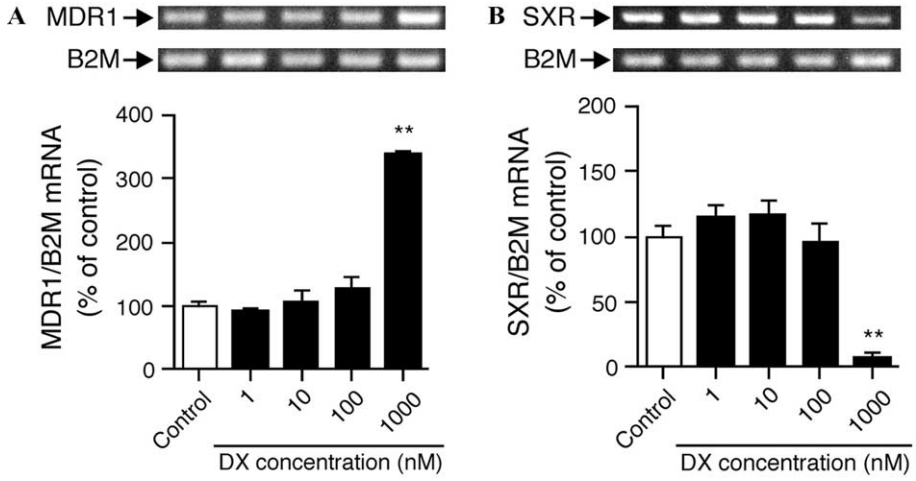

Fig. 4. Concentration-dependent effect of digoxin on MDR1 (A) and SXR (B) mRNA expression in LS180 cells. Representative electrophoretograms of MDR1 (A), SXR (B), and B2M are presented. Data were obtained after densitometric analysis of three independent electrophoretograms. Results are expressed as percentages of the ratio of MDR1 (A) or SXR (B) to B2M mRNA levels in the control. Each bar represents the mean ± SE of three independent experiments. $^{**}P < 0.01$ significantly different from the respective control. DX, digoxin.

was not significantly affected by exposure to up to 100 nM digoxin for 24 h, but a significant increase of ca. 3-fold was observed at 1 μM digoxin. The SXR mRNA expression in LS180 cells was not changed by exposure to up to 100 nM, but decreased significantly on exposure to 1 μM digoxin (Fig. 4B). These antithetical changes in MDR1 and SXR mRNA expression were similar to those in Caco-2 cells.

Discussion

The orphan nuclear receptor SXR has been reported to bind the −7852 to −7837 sequence in the MDR1 promoter, which is an upstream enhancer containing a motif related to broad specificity xenobiotic sensitivity [8]. The binding of SXR to this sequence results in the activation of MDR1 and CYP3A4 transcription in response to several xenobiotic inducers, and thus SXR is involved in the regulation of drug clearance pathways, i.e., drug efflux via MDR1 and drug metabolism via CYP3A4 [6]. Therefore, the evaluation of the transcriptional regulation of MDR1 via SXR is very important for controlling the MDR1 expression. The purpose of this study was to examine whether SXR participates in the up-regulation of MDR1 expression caused by exposure to digoxin.

First, we compared the SXR mRNA expression in Caco-2 cells with that in LS180 cells by RT-PCR (Fig. 1). SXR mRNA was expressed in Caco-2 cells as well as LS180 cells, in which the expression of SXR was previously confirmed [6]. In addition, the SXR mRNA level in Caco-2 cells was detectable but low as compared with that in LS180 cells. Thummel et al. [15] reported that the pregnane X receptor (PXR) mRNA was undetectable in Caco-2 cells, but readily detectable in LS180 cells by RT-PCR[15]. The difference in the SXR mRNA expression between Caco-2 cells in our laboratory and theirs might be derived from the culture conditions, because the gene expression in Caco-2 cells was suggested to be susceptible to culture conditions [16]. However, marked differences in SXR mRNA expression between Caco-2 and LS180 cells were observed in both the present and previous studies [15]. Thus, using both Caco-2 as SXR-poor cells and LS180 as SXR-rich cells, the effects of exposure to digoxin on MDR1 and SXR mRNA expression were examined.

The MDR1 mRNA expression in Caco-2 cells dramatically increased with a significant dependence on the exposure to 1 μM digoxin for 24 h (Fig. 2A), but the SXR mRNA was undetectable following exposure to the same concentration (Fig. 2B). So, it was found that the trend for change in the SXR mRNA expression caused by exposure to digoxin was the opposite to the change in MDR1 mRNA expression. Moreover, the level of MDR1 mRNA was ca. 2-fold higher in Caco/DX cells than Caco-2 cells (Fig. 3A), whereas the SXR mRNA expression was significantly lower in Caco/DX cells (Fig. 3B), indicating that there was an antithetical relationship in the changes in MDR1 and SXR mRNA expression between Caco-2 and the tolerant cells. Collectively, it was demonstrated that the exposure to digoxin up-regulated MDR1 mRNA expression and simultaneously down-regulated SXR mRNA expression, suggesting some participation of SXR in the MDR1 up-regulation caused by exposure to digoxin in Caco-2 cells.

In addition, antithetical changes in MDR1 and SXR mRNA expression were also observed in LS180 cells (Fig. 4), similar to Caco-2 cells. Accordingly, it was suggested that the phenomena observed on exposure to digoxin were not specific to Caco-2 cells and occurred

via a non-cell-specific mechanism. In the present study, we report for the first time that the down-regulation of SXR was followed by the up-regulation of MDR1.

Using pharmacological and genetic approaches, SXR was previously found to be activated by some xenobiotic inducers and this activation resulted in MDR1 mRNA and MDR1/P-glycoprotein expression [6–10]. These results were apparently inconsistent with our findings, but might be partly explained by differences in the experimental methods. The previous studies indirectly examined the activation of SXR using a reporter assay, which gave results on the gene transcription of SXR [6–10], whereas we evaluated the expression of the SXR mRNA itself as the total of gene transcription and post-transcription using RT-PCR. In addition, the possibility exists that the down-regulation of SXR mRNA expression was caused by the decline in SXR mRNA stability, not the decrease in gene transcription. However, there is no firm evidence, so we consider an understanding of the present and previous findings essential.

Regrettably, the significance of the down-regulation of SXR mRNA expression remains unclear, but we speculate that it plays an important role in the feedback regulation of MDR1 expression. This speculation might be supported by the finding that the expression of MDR1 mRNA in Caco/DX cells is kept at a particular level and does not continue increasing (unpublished personal findings). Further, if it is the feedback regulation, control of the selective down-regulation of SXR at the basal level may help us to predict MDR1-related pharmacokinetics, to evade MDR1-mediated drug–drug interaction, and to reverse the multidrug resistance in cancer chemotherapy.

In summary, we demonstrated for the first time that exposure to digoxin up-regulated MDR1 mRNA and simultaneously down-regulated SXR mRNA expression. These results suggested that SXR participates in the MDR1 up-regulation caused by exposure to digoxin, and furthermore the down-regulation of SXR plays an important role in the feedback regulation for the up-regulation.

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