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Doxorubicin modulates P-glycoprotein transcription differently during Caco-2 differentiation

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ABSTRACT

P-glycoprotein (P-gp) is an efflux transporter protecting cells from various chemical threats. Upregulation of P-gp expression, mediated by reactive oxygen species (ROS), has been reported in doxorubicin-treated cells. However, during cell differentiation cellular redox status may vary. This study investigated the influence of cell differentiation on doxorubicin-mediated P-gp expression in Caco-2 cells, and its relation to cellular redox status. During 21-day culture, Caco-2 cells were divided into pre-, during-, and post-differentiation, based on total cell numbers, percentage of G_0/G_1 cells, and alkaline phosphatase activity. Our study demonstrated that Caco-2 cells were in a more reduced state on differentiation. At low concentrations (<5–10 μM), doxorubicin increased *ABCB1* mRNA levels in all differentiation states. The inductive effect vanished on increasing doxorubicin concentrations to 50–100 μM. Moreover, *N*-acetylcysteine inhibited doxorubicin-mediated *up-regulation of ABCB1 mRNA* in the pre-differentiated and differentiating cells, but not in the post-differentiated cells. Our findings suggested that Caco-2 differentiation states affect doxorubicin-mediated *ABCB1* transcription and intracellular ROS level. Cellular redox status may contribute to the *ABCB1* transcription through ROS-dependent mechanisms in the pre- and during-differentiation phases, but not in the post-differentiation phase. Furthermore, the differentiation status should be concern for P-gp induction study in Caco-2 cell model.

Keywords: Caco-2 cells, cellular redox status, differentiation, doxorubicin, P-glycoprotein, reactive oxygen species

INTRODUCTION

P-glycoprotein (P-gp; encoded from *ABCB1* gene) is an efflux drug transporter in the ATP-binding cassette superfamily. This transporter is expressed in several tissues including the brush border membrane of small intestine as well as cancerous tissues. P-gp serves as a cellular protector by limiting uptake of various chemical threats into the cells.[1] Changing in *ABCB1* transcription involves several signal transduction pathways, particularly those activated by reactive oxygen species (ROS) and other stress signals.[2-6] For example, doxorubicin increased *ABCB1* transcription through ROS-related mechanisms in HepG2 and MCF-7 cells.[3-6]

In general, glutathione in its reduced form (GSH) is a major protective ROS scavenger inside the cells.[7,8] The glutathione redox ratio (GSH:GSSG) indicates cellular redox status, as well as the degree of oxidative stress. Higher GSH/GSSG ratio reflects more reduced redox state with lesser degree of oxidative stress, and vice versa.[7] It was demonstrated that the doxorubicin-resistant MCF-7 cells with highly expressed P-gp had lower baseline GSH:GSSG ratio than the sensitive MCF-7 cells.[6] In addition, GSH depletion was linked to significant upregulation of P-gp expression in rat brain microvessel endothelial cells.[9,10] It is likely that changes in the cellular redox status affects potential endogenous ROS generation, leading to modulation of P-gp expression.

Doxorubicin is a known P-gp inducer with ROS generating capability. Recently, it was reported that doxorubicin rapidly upregulated P-gp expression level in the confluent Caco-2 cells.[5] As known, Caco-2 cells, a human colon adenocarcinoma with intrinsic expression of P-gp, are widely used for studying intestinal permeability and drug transporters. After confluence under specific condition, the cells undergo spontaneous differentiation into enterocyte-like cells expressing several drug transporters (e.g., P-gp) and brush border enzymes (e.g., alkaline phosphatase [ALP] and sucrose).[11,12] In addition, GSH level gradually decreases during Caco-2 differentiation whereas GSSG level remains unchanged, suggesting alteration

of cellular redox status.[13] Hence, it can be anticipated that ROS-mediated Caco-2 responses including P-gp up-regulation in the pre-differentiated and differentiated states may vary depending on cellular redox status. Nevertheless, there is no evidence suggesting influence of Caco-2 differentiation states and cellular redox status on P-gp induction through ROS-mediated mechanisms.

Therefore, this study investigated the effect of doxorubicin on *ABCB1* transcription in three differentiation states of Caco-2 cells. During the 21-day culturing periods, the cells were divided into the pre-, during-, and post-differentiation phases based on percentage of cells in G_0/G_1, total cell numbers, and ALP activity. The influence of differentiation states toward doxorubicin treatment on *ABCB1* transcription was determined and correlated to cellular redox status.

MATERIALS AND METHODS

Materials

CellROX® deep red reagent, TRIzol® reagent, and TURBO DNA-free™ kit were obtained from Thermo Fisher Scientific Inc. (MA, USA). Doxorubicin HCl was purchased from Merck Millipore (Darmstadt, Germany). Dulbecco's modified Eagle medium (DMEM) and L-glutamine were purchased from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Biochrom AG (Berlin, Germany). Improm-II™ reverse transcription system was purchased from Promega Corporation (WI, USA). MEM non-essential amino acid, menadione sodium bisulfite, *N*-acetylcysteine (NAC), penicillin G potassium salt, *p*-nitrophenol, *p*-nitrophenyl phosphate, propidium iodide, streptomycin sulfate, and sulfosalicylic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). SsoFast™ EvaGreen® Supermix was purchased from Bio-Rad Laboratories Inc. (CA, USA).

Cell Culture and Treatment

The Caco-2 cells (human colorectal adenocarcinoma cells, HTB-37™) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acid, 1% penicillin G/streptomycin, and 2 mM L-glutamine at 37°C in humidified atmospheric condition of 95% air and 5% CO_2. The cells were routinely passaged every 3–4 days (at approximately 70% confluency).

In each experiment, Caco-2 cells (20,000 cells/cm^2) were treated with doxorubicin at the non-cytotoxic concentrations for 24 h. The non-cytotoxic concentrations in this study were the concentrations which had no effect on either cell viability (viable cells ≥80%) or cell differentiation. In some experiments, the cells were pre-incubated with NAC (5 mM) for 30 min before addition of doxorubicin. At the end of treatment periods, the cells were washed 3 times with phosphate-buffered saline (PBS), and collected for further measurements.

Cell Cycle Analysis

After fixing the cells with 70% ethanol, the fixed cells were incubated with propidium iodide (20 µg/ml) at 37°C for 15 min. Then, the fluorescent intensity was measured at 488 and 585/42 nm (excitation and emission wavelengths), using a BD FACSCalibur flow cytometer (BD biosciences, CA, USA). The percentage of cells in each cell cycle phase was gated from 10,000 count events.

Cell Counting

The cells were stained with 0.04% Trypan blue solution and counted for both the living and dead cells on hemocytometer under the microscope (Axiovert® 135, Carl Zeiss AG, Germany).

ALP Activity Assay

The cell homogenate was prepared in 10 mM Tris/150 mM NaCl buffer, pH 8.0. A 250-µl aliquot of homogenate was incubated with a 750-µl of *p*-nitrophenyl phosphate (2.5 mg/ml) at 37°C for 5 min, followed by addition of a 50-µl of 0.5 M NaOH.[14] The formation of *p*-nitrophenol was measured spectrophotometrically at 405 nm (Wallac 1420, Perkin Elmer Inc., MA, USA).

Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR)

The cellular DNA-free RNA fraction was extracted and collected with TRIzol reagent, followed by residual DNA digestion using TURBO DNA-free kit. Total RNA (250 ng) was converted to cDNA using an oligo $(dT)_{15}$ primer and Improm-II reverse transcription kit. The qPCR reactions were performed using CFX™ 96 RT-PCR Detection System (Bio-Rad Laboratories Inc., CA, USA) from SsoFast™ EvaGreen® Supermix containing 1 µl of cDNA. The PCR reaction was run at 95°C 5 s and 60°C 5 s using either *ABCB1* primers (Forward: 5′-CCC ATC ATT GCA ATA GCA GG-3′, Reverse: 5′-TGT TCA AAC TTC TGC TCC TGA-3′; 400 nM) or *GAPDH* primers (Forward: 5′-TGC ACC ACC AAC TGC TTA GC-3′, Reverse: 5′-GGC ATG GAC TGT GGT CAT GAG-3′; 300 nM) for 40 cycles.[15,16] The specificity of amplified products was determined by melting curve analysis. Relative quantification of *ABCB1* expression was performed using Pfaffl's method.[17]

Glutathione Enzymatic Recycling Assay

The GSH/GSSG ratio was determined by the enzymatic recycling assay.[18] The cells were homogenized in potassium phosphate-EDTA buffer containing 0.1% Triton X-100 and 0.6% sulfosalicylic acid. For measurement of total GSH, cell lysates were incubated with glutathione reductase enzyme (3.33 units/ml) and DTNB (1.62 mM) at 25°C to generate GS-TNB adduct. Then, *β*-NADPH (0.8 mM) was added to activate the reductase activity. Glutathione reductase catalyzed a conversion of GS-TNB to GSH, and released TNB from adduct. The absorbance of TNB was measured by spectrophotometry at 405 nm (Wallac 1420, Perkin Elmer Inc., MA, USA). In another set of experiment, the amount of GSSG was determined by incubating cell lysates with 2-vinylpyridine (0.9 M) at room temperature for 1 h. Then, triethanolamine (1.25 M) was added into the assay mixture to neutralize excess 2-vinylpyridine for 10 min. After incubation period, the amount of GSSG was measured by the method as described above. The GSH/GSSG ratio was calculated by the following formula: GSH/GSSG ratio = (Total glutathione – GSSG)/GSSG.

Intracellular ROS Measurement

Intracellular ROS level was determined by CellROX® deep red kit. Briefly, the cells were treated with CellROX® deep red (5 μM) at 37°C for 30 min, and fixed in 4% formaldehyde/PBS buffer. The fluorescent intensity was measured at 635 and 661/16 nm (excitation and emission wavelengths) using a BD FACSCalibur flow cytometer (BD biosciences, CA, USA).

Data Analysis

Data were expressed as mean ± standard error of the mean (SEM) from at least three independent experiments ($n \geq 3$). The statistical analysis was performed either by Mann–Whitney U-test or Kruskal–Wallis test, followed by Mann–Whitney U-test with Bonferroni correction, where appropriate. Statistical significance was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Differentiation States of Caco-2 Cells

Cell differentiation is the multi-step process which transitions Caco-2 cells from colonic to enterocytic-like cells.[12] The differentiation timeline of Caco-2 cells can be influenced by several factors such as seeding density, passage numbers, culturing condition, media compositions, and material of cell culture surface.[19-21] In our culture condition, the growth states of 21-day Caco-2 cells were divided to the pre-, during-, and post-differentiation based on three differentiation markers; that is, percentages of G_0/G_1 cells, total cell numbers, and ALP activity [Figure 1]. Our cells reached their confluence at day 3 after seeding. The cell numbers and cell population in G_0/G_1 phase increased in time-dependent manner, reaching the plateau phase around day 7 [Figure 1a and b]. The ALP activity increased gradually and reached the plateau phase at day 11 after seeding [Figure 1c]. Taken together, the differentiation states of Caco-2 cultured for 21 days could be split into three states including the pre-, during-, and post-differentiation states with the proposed timeline shown in Figure 2.

Effects of Doxorubicin on *ABCB1* mRNA Level in Caco-2 Cells

Doxorubicin was able to affect *ABCB1*/P-gp expression at transcription level in Caco-2 cells. We further demonstrated that the modulating effect of doxorubicin on *ABCB1* mRNA levels depended on drug concentrations as well as cell differentiation states. At low concentrations (up to 10 μM in the pre- and post-differentiated cells; up to 5 μM in the differentiating cells), doxorubicin enhanced transcription process of *ABCB1* gene [Figure 3]. In addition, its upregulation effect was at the highest magnitude when the Caco-2 cells were in the pre-differentiation state. On increasing doxorubicin concentrations to 50–100 μM, the effect of doxorubicin on *ABCB1* upregulation apparently vanished. Exclusively in the differentiating cells, doxorubicin at the high concentration (100 μM) downregulated *ABCB1* mRNA level significantly. As known, doxorubicin has a potential to inhibit biosynthesis of various macromolecules (e.g., DNA, RNA, and proteins).[22] At the transcription level, conformation of DNA sequences flanking to the promoters of human *c-myc*, *c-fos*, *and hsp70* genes was distorted on doxorubicin treatment, leading to transcriptional inhibition of these genes.[23] In this regard, doxorubicin at high concentrations might be able to inhibit the *ABCB1* transcription. Nevertheless, the mechanisms underlying doxorubicin-mediated *ABCB1* downregulation in the differentiating cells remain to be clarified.

Involvement of Cellular Redox Status and ROS in Doxorubicin-mediated *ABCB1* Transcription

Several studies suggested that ROS involved with inductive mechanisms of doxorubicin on *ABCB1*/P-gp expression in various cancer cell lines including Caco-2, HepG2, and MCF-7 cells.[24-26] It can be anticipated that cellular redox status takes part in doxorubicin-induced *ABCB1* expression. In this study, we demonstrated that the GSH/GSSG ratio shifted toward more reduced state when Caco-2 cells underwent into differentiation [Figure 4]. However, it was reported that cellular redox status of the differentiated Caco-2 cells was in a more oxidized state, as compared to that of the proliferating cells.[13] This inconsistent finding might arise from the differences in culturing condition and other biological factors. It is worth noting that Caco-2 cell line exhibits highly inter-laboratory variations in gene expression and cellular characteristics.[19-21]

Nevertheless, the GSH/GSSS ratios observed in this study were well correlated with levels of intracellular ROS generated from either menadione (a positive ROS generator) or doxorubicin treatments. Both compounds significantly increased intracellular ROS levels in the pre-differentiated cells, but neither in the differentiating cells nor the post-differentiated cells [Figure 5]. In addition, both doxorubicin-/and menadione-mediated increases of intracellular ROS were abolished by NAC (5 mM), a precursor of GSH and ROS scavenger [Figure 6].

Involvement of ROS in the doxorubicin-mediated, *ABCB1* alteration was evaluated further in Caco-2 cells at each differentiation state. As shown in Figure 7, NAC significantly inhibited increases of *ABCB1* mRNA levels induced by doxorubicin (5 μM) in the pre-differentiated cells and the differentiating cells. Although rising of ROS level in the differentiating cells was not observed, it is likely that ROS was responsible for doxorubicin-mediated upregulation of *ABCB1* gene in the pre- and during- differentiation phases. Taken that cellular redox status of the differentiated cells was apparently in the more reduced state than those pre-differentiated cells, doxorubicin might generate small amount of ROS that was below limit of detection of CellROX® deep red assay.

ROS-independent mechanisms in gene modulating action of doxorubicin could not be excluded.[27,28] We demonstrated that NAC had no suppressive effect on doxorubicin-mediated *ABCB1* upregulation in the post-differentiated cells [Figure 7]. Thus, it was likely that doxorubicin interfered with *ABCB1* transcription in the post-differentiated cells through ROS-independent mechanisms such as direct interaction with an aryl hydrocarbon receptor, the orphan nuclear receptor known to enhance *ABCB1* gene transcription.[29] We hypothesized that doxorubicin was able to upregulate ABCB1 expression through ROS-dependent and ROS-independent mechanisms. In the fully differentiated Caco-2, the intracellular ROS level might

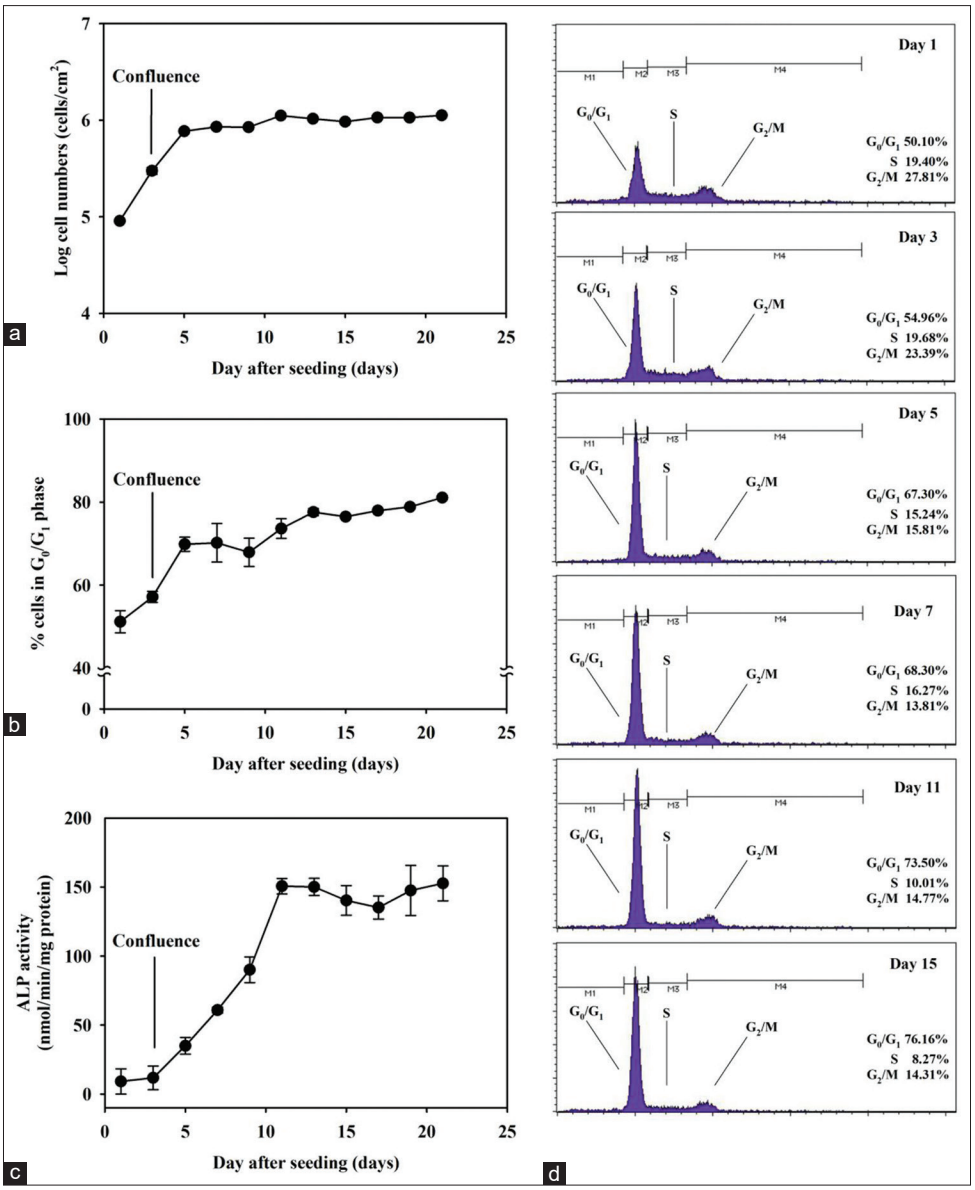

Figure 1: Differentiation markers of Caco-2 cells during 21-day culturing period. (a) Total cell numbers. (b) Percentage of cells in G0/G1 phase. (c) Alkaline phosphatase activity. (d) Representative fixed-scale chromatograms of cell cycle analysis. Data are expressed as mean ± SEM (n = 3, duplicates)

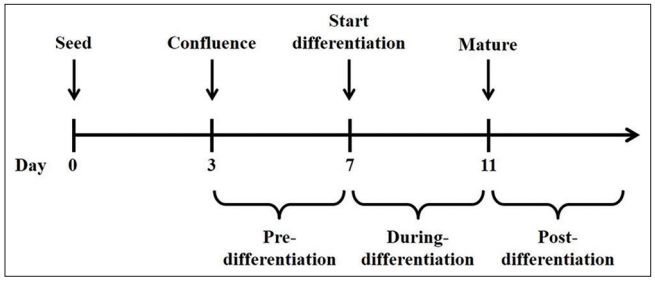

Figure 2: Proposed time course of Caco-2 differentiation phases

decrease, and the upregulation pathways related to ROS might be subsided under a reduced redox environment. However, those ROS-independent mechanisms in doxorubicin-mediated *ABCB1* transcription remained to be investigated further.

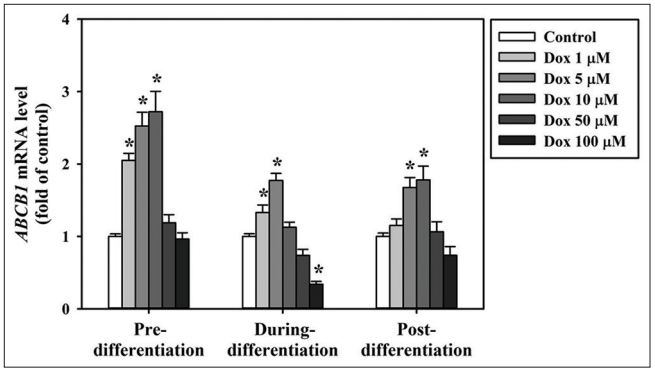

Figure 3: Effect of doxorubicin (Dox) on *ABCB1* mRNA levels in Caco-2 cells at different differentiation phases. *P < 0.05 compared with control (n = 3, triplicates)

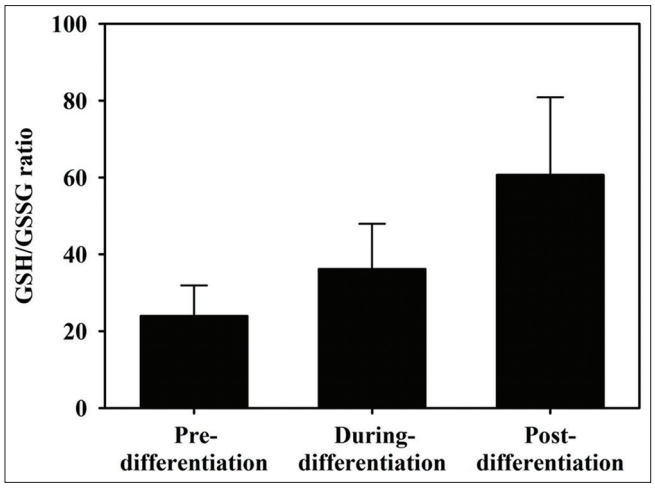

Figure 4: The glutathione redox ratio (GSH/GSSG) in Caco-2 cells at different differentiation phases (n = 5)

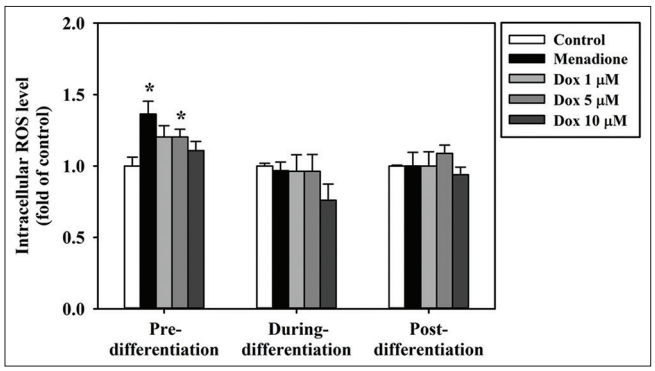

Figure 5: Intracellular reactive oxygen species levels in Caco-2 cells at different differentiation phases after 24-h treatment with either doxorubicin (Dox; 1–10 μM) or menadione (10 μM). *P < 0.05 compared with control (n = 3, duplicates)

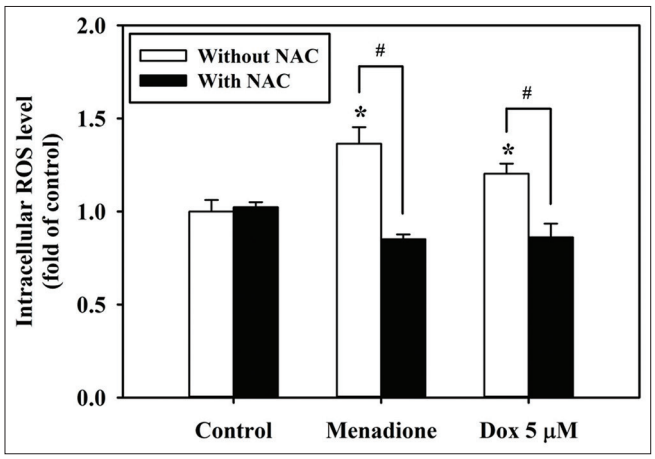

Figure 6: Effect of NAC (5 mM) on intracellular ROS levels in the pre-differentiated Caco-2 cells after 24-h treatment with either doxorubicin (Dox; 5 μM) or menadione (10 μM). Data are expressed as mean ± SEM (n = 3, duplicates). *P < 0.05 compared with control. #P < 0.05 compared between groups with NAC (solid bar) and without NAC (open bar)

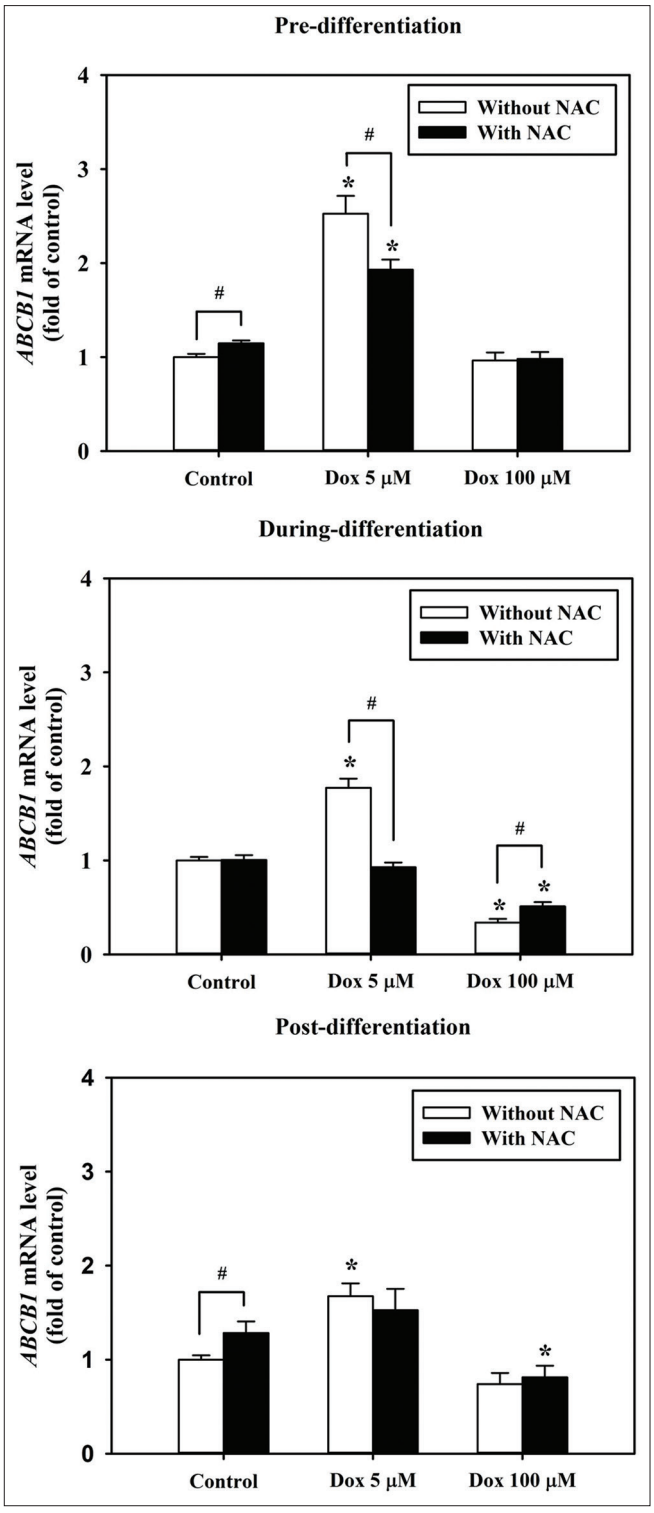

Figure 7: Effect of doxorubicin (Dox) on *ABCB1* mRNA in the cells at different differentiation phases in the absence or presence of 5 mM NAC. Data are expressed as mean ± SEM (n = 3, triplicates). *P < 0.05 compared with control. #P < 0.05 compared between groups with NAC (solid bar) and without NAC (open bar)

Caco-2 cells have also been used as an alternative model to investigate P-gp expression, since there is currently no established model for P-gp induction.[30] The P-gp inductive studies in Caco-2 were reportedly conducted in varied differentiation states, for example,, in pre-differentiated,[5,31-33] and fully differentiated cells.[34-37] However, this is the first

study to demonstrate the influence of Caco-2 differentiation process on *ABCB1* transcription induced by chemical threats. Our findings clearly suggested that the differentiation states of Caco-2 cells should be critical concern in P-gp induction study. Moreover, it should be aware that data from different states of Caco-2 differentiation should not be extrapolated or substituted for one another.

CONCLUSIONS

This study demonstrated for the first time that the differentiation states of Caco-2 cells strongly affects doxorubicin-mediated *ABCB1* mRNA transcription. The cellular redox status may contribute to the upregulation of *ABCB1* gene through ROS-dependent mechanisms in the pre- and during- differentiation phases. In addition, doxorubicin may also modulate *ABCB1* transcription through ROS-independent mechanisms. Moreover, the differentiation status should be another critical concern for P-gp induction study, when the Caco-2 cells are used as a cell model.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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