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Tumor Necrosis Factor Alpha Increases P-glycoprotein Expression in a BME-UV *In Vitro* Model of Mammary Epithelial Cells

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Abstract

P-glycoprotein is an efflux pump belonging to the ATP-binding cassette super-family that influences the bioavailability and disposition of many drugs. Mammary epithelial cells express various drug transporters including P-glycoprotein, albeit at low level during lactation. During inflammatory reactions, which can be associated with changes in epithelial barrier functions, pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) are elevated in milk and serum. In this study, the role of TNF-α in the regulation of P-glycoprotein was determined in cultured BME-UV cells, an immortalized bovine mammary epithelial cell line. The protein production of P-glycoprotein and mRNA expression of *bABCB1*, the gene encoding P-glycoprotein, were increased after 24 hours of TNF-α exposure. The highest observed effects for TNF-α on the regulation of P-glycoprotein was after 72 hours of exposure. Protein and mRNA expression also significantly increased after 120 hours of TNF-α exposure, but it was lower than the level that observed in the cells exposed to TNF-α for 72 hours. The apical to basolateral flux of digoxin, a P-glycoprotein substrate, was decreased in the TNF-α-exposed epithelium. This effect was reversed when verapamil or ketoconazole, compounds known to interact with P-glycoprotein, were added together with digoxin into the donor compartment. Probenecid, a compound known to interact with organic anion transporters, but not P-glycoprotein, did not increase the flux of digoxin. This model has important implications for understanding the barrier function of the mammary epithelium and provides insight into the role of P-glycoprotein in the accumulation and/or removal of xenobiotics from milk and/or plasma.

Keywords

Mammary epithelium; P-glycoprotein; Pharmaco-/toxicokinetic; TNF-α

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Introduction

The body of complex animals is separated into different compartments by biological barriers that generate and modify the volume and composition of these compartments by including or excluding substances. The functional concept of biological barriers has shifted from that of a static barrier to a complex of transport systems that include various membrane transporters. Efflux pumps are a family of ATP-dependent transport proteins that are reportedly expressed in several major physiological barriers including the blood-brain barrier (BBB), kidney, intestine, and placenta [1–3]. The mammary epithelium, the most likely rate-limiting barrier between plasma and milk, expresses various drug transporters including P-glycoprotein [1, 4, 5].

P-glycoprotein (the *ABCB1* gene product, which was formally known as MDR1) is an ATP-hydrolyzing efflux pump belonging to the ATP binding cassette super-family (ABC family) [6, 7]. P-glycoprotein plays an important role in xenobiotic absorption, distribution, and elimination as a result of its tissue distribution and cellular localization. For example, P-glycoprotein is expressed at the brush borders of epithelial cells in the intestinal tract, limiting absorption after oral administration and facilitating secretion into the intestinal lumen [8]. In the liver, P-glycoprotein is expressed at the canalicular (apical) surface of hepatocytes, facilitating the excretion of drug molecules, toxins and their metabolites into the bile [9]. In the kidney, P-glycoprotein is expressed on the apical surface of epithelial cells lining the proximal tubules where it mediates the elimination of endogenous metabolic waste products and xenobiotic metabolites [10]. At biological barriers such as the BBB, P-glycoprotein is expressed on the luminal plasma membrane of the capillary endothelium, where it extrudes drugs or toxins back into the capillaries so that they do not reach the brain [11].

P-glycoprotein expression can be modulated by different factors, including drugs, toxic agents, heat shock, inflammation, pregnancy and lactation [1, 12, 13]. Several *in vitro* and *in vivo* investigations have shown that inflammation and pro-inflammatory cytokines such as TNF-α alter the expression of P-glycoprotein. The effects of these cytokines appear at different levels of regulation including transcriptional, translational, post-translational [13–15].

During inflammatory reactions, which can be associated with changes in mammary epithelial barrier functions, pro-inflammatory cytokines such as TNF-α are elevated in serum and milk. TNF-α is significantly increased in milk and serum in coliform mastitis and *Escherichia coli*-derived lipopolysaccharide-infused mammary gland [16–18]. Furthermore, exposure of bovine peripheral blood mononuclear cells to *Staphylococcal aureus* enterotoxins for 3 hours resulted in the rapid production of TNF-α that reached maximal levels by 48 hours then declined by 96 hours after initial exposure [19]. Influences of the pro-inflammatory cytokine TNF-α on the regulation of efflux pump in the mammary epithelial cells has not yet been reported.

Whole-animal models used to study the effects of pro-inflammatory cytokines on the expression and activity of P-glycoprotein provide information on the overall effects of inflammation on the regulation of P-glycoprotein. However, the effects of specific cytokines on the regulation of P-glycoprotein are complicated by the release of a complex series of endogenous cytokines. Thus, tractable *in vitro* models provide answers to elucidate the specific roles of inflammatory modulators. An immortalized bovine mammary epithelial cell line (BME-UV) was used in the present study. This cell line was selected as a model for the blood-milk barrier due to its ability to express transport proteins [4], establish a cell polarity with well-formed tight junctions between adjacent cells, exhibit a transepithelial electrical

resistance, and its responsiveness to steroid hormones [20, 21]. The aim of the present study was to determine the effects of cytokine TNF-α on the expression and activity of P-glycoprotein in BME-UV cells.

Materials and Methods

Modulatory effects of TNF-α on gene expression in cultured BME-UV cells were determined by measuring mRNA and protein levels following exposure to TNF-α for selected durations of time. Effects on the permeation of epithelial barrier were examined by performing diffusion studies using compounds known to interact with P-glycoprotein.

Cell Culture

The BME-UV cells were cultured under conditions similar to those described previously in detail [20, 21]. Briefly, the BME-UV cells were grown to 65–75% confluency in 25 cm^2 plastic culture flasks (Corning, Corning, NY). Dissociated and dispersed cells were seeded on permeable polyester supports, (Transwell, Corning) and fed with asymmetrical media. Typical bovine medium (TBM), which contains little lactose and has concentrations of electrolytes that closely mirror serum, bathed the basolateral aspect of the cells throughout all experiments. The apical aspect was exposed to apical bovine medium (ApM) of low electrolyte-high lactose composition that resembles the ionic composition of milk. Composition of TBM and ApM were reported previously [20, 21]. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Media on both the apical and basolateral aspects of the cells were refreshed daily. Cells were maintained in culture on permeable supports for 2 weeks to form a confluent, polarized and electrically tight monolayer prior to all experiments.

Incubation Conditions with Pro-Inflammatory Cytokine (TNF-α)

Cells were grown to confluence and exposed to pathophysiologically relevant concentrations (200 ng/ml) of recombinant bovine TNF-α (Thermo Scientific, Rockford, IL) for 24, 72, and 120 hours prior to harvesting the cells for RNA or protein isolation, or using them in transport studies. TNF-α concentration within this range have been reported in dairy cows with mastitis [16]. Paired cell monolayers were maintained without exposure to TNF-α (unexposed).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) RNA Extraction—RNA was isolated from confluent monolayers of BME-UV cells using RNeasy Mini kit (QIAGEN, Foster City, CA) according to the manufacturer's directions. RNA concentrations were determined by the measurement of optical density at 260 nm (NanoDrop 8000, Thermo Scientific). Isolated RNA quality was verified by an OD_{260}/OD_{280} absorption ratio greater than 1.8 and less than 2.0. Non-degraded and sufficiently concentrated RNA (0.5 to 1.0μg/μl) was used in RT-PCR assays.

Primer Design for RT-PCR—Bovine sequences of mRNA for each gene were obtained from the National Center for Biotechnology Information Genebank [22]. Primer pairs (GGAGCCCATTCTGTTTGACT and TCCTTTGTCCCCTACTCTGG) to detect mRNA coding for bovine P-glycoprotein (*bABCB1*) and (AAGATTGTCAGCAATGCC and ACAGACACGTTGGGAG) to detect mRNA coding for bovine GAPDH (*bGAPDH*) were chosen to give amplification product between 150 and 300 base pairs using Primer3 software [23], a web-based primer design program. Both primer pairs were custom synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA).

RT-PCR—The expression of *bABCB1* in mammary epithelial cells was determined by RT-PCR (iCycler, Bio-Rad Laboratories, Hercules, CA) with the use of a commercial kit (Quantitect SYBR Green RT-PCR kit and reagents, Qiagen). Reactions were performed in a final volume of 25 μL containing 100 ng of template RNA, 0.5 μM forward and reverse primers, 0.25 μL Quantitect RT Mix, 12.5 μL Quantitect SYBR Green (1x) and 8.25 μL RNase-free Water. Triplicate determinations were performed for each sample (three samples total) of each target gene (*bABCB1* and *bGAPDH*) in a 96-well optical plate. This experiment was repeated four times on separate days. RT-PCR assay was conducted in the manner where samples of bovine kidney, no-template reaction and no reverse transcriptase were included in the assay.

RT-PCR Protocol—The RT-PCR protocol consisted of reverse transcription (50°C for 30 minutes), PCR initial activation (95°C for 15 minutes), followed by 40 cycles of denaturing at 94°C for 15s, annealing at 58°C for 30s, and extension at 72°C for 30s. A melt curve analysis from 65°C–95°C at 0.5°C/s was conducted immediately following PCR. Relative gene expression in different treatments relative to the average expression in untreated cultures was calculated using the $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen [24].

Western Blot

The immunoreactive bands for P-glycoprotein were measured in plasma membrane fractions isolated from both untreated and TNF-α treated BME-UV cells on western blots by modifying previously described method [25]. Briefly, protein content was determined using a bicinchoninic assay (Thermo Scientific) and 30 μg of protein were resolved on 4–20% SDS-prepoured polyacrylamide gels (Thermo Scientific) and transferred to PVDF transfer membrane (Thermo Scientific). Equal protein loading and integrity of the transfer was verified with Ponceau red staining before antibody labeling. The membrane was blocked for 1 hour at room temperature with 5% powdered non-fat dry milk (Santa Cruz Biotechnology, Santa Cruz, CA) in phosphate buffered saline containing 0.1% Tween 20 (PBS-T). Washed membranes were incubated overnight at 4°C with the monoclonal antibody, C219 (Covance, Denver, PA). Then, washed membranes were incubated for 1 hour at room temperature with horse-radish peroxidase-conjugated goat anti-mouse IgG (1:1500) in PBS-T containing 5% non-fat dry milk powder. P-glycoprotein immunorecativity was visualized using Femto-chemiluminescence substrate (Thermo Scientific). Gels were scanned (Kodak Image Station 4000R, Rochester, NY) and the intensity of the immunoreactive bands was quantified (Molecular Imaging software, Rochester, NY).

Transport Studies

Flow-Through Diffusion Cell System—Confluent monolayers of BME-UV cells on permeable supports were mounted as a barrier in a flow-through diffusion cell system (Permegear, Bethlehem, PA). The system has automated receptor fluid collection and a high precision multichannel dispenser (Ismatec SA, Switzerland). The flow rate of receptor fluid through the receiver compartment of each diffusion chamber was controlled at 4 ml/h. Temperature was maintained at 37°C by circulating water through the diffusion chambers holder. Donor compartments of diffusion chambers were capped directly after adding test solutions. Sequential samples were collected from the receiver side at regular time intervals (15 min) over a period of 120 min. Samples of either [^{14}C]- or [^{3}H]-radiolabeled test compounds were quantified by dual labeled β-scintillation counting with quench correction (Beckman LS6500, Beckman Coulter, Inc., CA). Automatic calculation of disintegrations per minute (DPM) was used for each sample.

Digoxin Transport Studies—[^{3}H]-digoxin ([^{3}H]-Digx), (American Radiolabeled Chemicals, Saint Louis, MO), known P-glycoprotein substrate, was prepared in ApM

together with [^{14}C]-mannitol ([^{14}C]-Mnt), (American Radiolabeled Chemicals). [^{14}C]-Mnt was used as a marker for paracellular movement [26].

Study 1—The apical to basolateral (Ap-to-BL) flux of digoxin across BME-UV monolayers that were exposed to pro-inflammatory cytokine TNF-α for 24, 72, and 120 hours was determined and compared to unexposed cells. Transport experiments were initiated by adding 1 mL of test solution containing 0.05 μM of [^{3}H]-Digx to the upper compartment (donor) together with 2μM [^{14}C]-Mnt with or without verapamil (Verp, 50 μM), (Sigma-Aldrich, St. Louis, MO), a compound known to interact with P-glycoprotein [6].

Study 2—The Ap-to-BL flux of digoxin across BME-UV monolayers that were exposed to pro-inflammatory cytokine TNF-α for 72 hours was investigated and compared to unexposed cells. This study was performed in the presence or absence of ketoconazole (Keto, 200 μM), (Sigma-Aldrich), which is also known to interact with P-glycoprotein [27, 28] or probenecid (Prob, 200 μM), (Sigma-Aldrich), a compound known to interact with organic anion transporters (OAT) [29, 30].

Data Analysis

The digoxin flux (μmol/cm^2 per hour) was calculated based on the amounts of digoxin recovered in the receiver compartment per unit time and then divided by mannitol flux at each measured time point to account for changes in overall epithelial integrity. Fluxes were normalized by the effective diffusion area (cm^2) of the cell culture insert.

Statistical Analysis

All statistical analyses were performed using commercially available software (SIGMASTAT, Systat Software Inc., San Jose, CA). Group comparisons were made by ANOVA. When the ANOVA showed significant differences among treatments, the Holm-Sidak posttest (versus control) was performed to determine the specific treatment that is statistically significant different from the control (unexposed). The level of significance for all tests was set at $P < 0.05$.

Results

Influence of TNF-α on Monolayer Integrity

The flux of mannitol across BME-UV monolayer increased significantly after 24 and 72 hours of TNF-α exposure (Table 1). However, the flux of mannitol decreased to values indistinguishable from untreated after exposure to TNF-α for a longer time (120 hours), which may indicate the epithelial monolayers were able to restore their integrity. The lowest flux value of mannitol was in the unexposed monolayers ($6.5 \pm 0.7 \times 10^{-6}$ μmol/cm^2 per hour), while the highest value was in the TNF-α exposed monolayers for 24 hours ($15.5 \pm 0.8 \times 10^{-6}$ μmol/cm^2 per hour).

Influence of TNF-α on ABCB1 mRNA Expression

The results from RT-PCR analysis demonstrate a significant increase in the *bABCB1* mRNA expression after 24 hours of TNF-α exposure (Fig. 1). The mRNA levels were further upregulated after 72 hours of TNF-α exposure where it reached the maximum increase in mRNA expression (27 ± 2 %) compared to unexposed cells during the experiment. After 120 hours treatment with TNF-α, the increase in mRNA expression was 21 ± 6 %.

Influence of TNF-α on the Expression of P-glycoprotein

BME-UV cells exhibited different immunodetectable levels of P-glycoprotein after exposure to TNF-α for different periods of time as shown in Figs 2. A significant increase in P-glycoprotein immunoreactive level was observed after 24 hours of TNF-α exposure. Longer exposure to TNF-α led to a further increase in immunoreactive protein levels that reached the maximum observed effect after 72 hours of TNF-α exposure. After 120 hours exposure of TNF-α, the immunoreactive protein levels was increased significantly compared to the level of unexposed cells but it was lower than the level that was observed in the cells exposed to TNF-α for 72 hours.

Influence of TNF-α on the Ap-to-BL flux of digoxin

The apical to basolateral flux/time curves of digoxin across BME-UV cells treated with TNF-α for different durations are shown in the Fig. 3. The Ap-to-BL flux of digoxin across the epithelial monolayer was significantly decreased after 24 hours of TNF-α exposure. Similar consequences were observed after longer exposure to TNF-α (72 hours) where the flux of digoxin was decreased 43 ± 3 % compared to unexposed monolayers. The Ap-to-BL flux of digoxin after 120 hours of TNF-α exposure was decreased by 18 ± 8 %, which was not significantly different from digoxin flux across the unexposed cells (Fig. 3).

The normalized amount of digoxin that crossed unexposed BME-UV monolayers was nearly double the amount that crossed TNF-α exposed BME-UV monolayers. The highest intracellular content of digoxin was detected in the unexposed BME-UV cells ($6.3 \pm 2.6 \times 10^{-4}$ nmol), while a lower intracellular content was observed in the TNF-α exposed cells for 24 hours ($4.6 \pm 1.1 \times 10^{-4}$ nmol) and the lowest level was observed after exposing the BME-UV cells with TNF-α for 72 hrs ($3.0 \pm 0.2 \times 10^{-4}$ nmol). The recovery of digoxin from the entire system at the conclusion of experiment was 96 ± 2 %.

Addition of verapamil to the donor compartment increased digoxin flux across TNF-α exposed BME-UV monolayers to the extent that there were no observable differences between exposed and unexposed cells (Fig. 4). Ketoconazole also increased digoxin flux across BME-UV monolayers exposed to TNF-α for 72 hours. Probenecid (a compound that is known unaffected by p-glycoprotein, but is known to interact with OAT) had no observable effect on digoxin flux across the BME-UV monolayers, regardless of TNF-α exposure (Figures 5).

Rapid steady state conditions were attained quickly in less than 15 minutes. The whole system exhibits steady state activities over the experimental period of 2 hours. The results provide consistent evidence that the observations are related to the effect of TNF-α on P-glycoprotein expression. The correlation between digoxin flux and mRNA expression or P-glycoprotein immunoreactivity were both consistent as supported by regression (r^2= 0.71 and 0.65, respectively).

Discussion

Cytokine TNF-α exposure induced the expression of *ABCB1* mRNA and increased the amount of P-glycoprotein present in BME-UV cells. A significant decrease in the Ap-to-BL flux of digoxin across the TNF-α-exposed BME-UV monolayer was also observed. This latter observation can be attributed to the upregulation of P-glycoprotein in mammary epithelial cells, resulting in a greater efflux of digoxin back into the donor fluid. *bABCB1* mRNA and P-glycoprotein levels increased depending on the duration of exposure to TNF-α, with maximum levels at 72 hours. The observed changes in the expression level of both mRNA and P-glycoprotein suggests that the effect of cytokine TNF-α on P-glycoprotein expression is at the transcription and/or translation level.

Mannitol flux increased across the BME-UV monolayer after exposure to TNF-α, suggesting that barrier function was compromised leading to an increase in paracellular solute flux. Similar results were observed by Quesnell et al [21], who studied the effects of pro-inflammatory cytokines on the integrity of mammary epithelial monolayer (BME-UV) via analysis of transepithelial electrical resistance (R_{te}). They observed a 30% decrease in R_{te} value after exposure to TNF-α for 8 hours. By adding mannitol together with digoxin, we could account for this decrease in monolayer integrity by normalizing digoxin to mannitol flux. The resulting observations were therefore indicative of TNF-α associated changes in transcellular digoxin flux.

Digoxin flux across the BME-UV monolayer may involve more than one transport system (e.g. P-glycoprotein and Oatp2). Verapamil and ketoconazole, both compounds known to interact with P-glycoprotein, decrease the effect of TNF-α by either competing with digoxin or inhibiting the P-glycoprotein pump directly [6, 31]. Probenecid, which does not interact P-glycoprotein but is known to affect the activity of other drug transporters including OATs and Oatp2, had no effect on the flux of digoxin across the BME-UV monolayer. These results support our conclusion that the decrease in apical to basolateral flux of digoxin is attributable to increased P-glycoprotein expression rather than decreased Oatp2 expression.

Enhanced P-glycoprotein expression can explain the significant decrease in transcellular digoxin flux across the TNF-α-exposed BME-UV monolayers. The rate of digoxin efflux into the apical solution is proportioned to P-glycoprotein expression and/or activity, and the appearance of digoxin in the receiver compartment is inversely proportioned to P-glycoprotein activity. The differences in the intracellular contents of digoxin at the conclusion of experiment among different treatments were consistent with the other results in the current study. Enhanced P-glycoprotein expression in TNF-α-exposed mammary epithelial cells led to pump more digoxin molecules out of the cells. Similarly, higher expression of P-glycoprotein resulted in lower percentages of the administered digoxin dose crossing the BME-UV monolayer by the transcellular route, which is consistent with the observations in this study.

Two molecular mechanisms account for the changes in P-glycoprotein transport activity and expression induced by TNF-α; changes in the dynamics of transporter trafficking, release from the internalized inactive state (vesicle), or increased synthesis of transporter protein [32]. Furthermore, the expressed protein can be in active or inactive states. Thus, understanding the molecular mechanisms of TNF-α induced effects may help us in explaining the inconsistency between the expression and function of P-glycoprotein in BME-UV cells.

A significant increase (different levels) in *ABCB1* mRNA or protein expression of P-glycoprotein or both were observed in different cell culture models when cells were exposed to TNF-α for different periods of time. For example, immortalized human brain capillary endothelial cells [25], primary porcine brain capillary endothelial cells [33], and primary rat hepatocytes [34]. These results agree with our observations that the effect of TNF-α on the regulation of P-glycoprotein is dependent on the duration of TNF-α exposure.

There are also studies that show opposing results. A decrease in *ABCB1* mRNA and protein levels under the influence of TNF-α was reported in isolated primary placental trophoblasts [35] and human colon carcinoma cell lines, HCT15 and HCT116 [36]. These observations may be explained by the inter-species differences, the cell type in which transporter regulation was studied, as well as dose and exposure time of TNF-α [37]. Another important factor is the differences in the distribution of cytokines receptors among different types of cells [38].

Conclusion

The results from the current study indicate that TNF-α induced *bABCB1* mRNA and protein level of P-glycoprotein and decreased significantly the flux of digoxin across BME-UV cells. The regulation of P-glycoprotein in the mammary epithelium has important implications for understanding the barrier function of the mammary epithelium and provides insight into the role of P-glycoprotein in the accumulation and/or removal of specific substrates from milk and/or plasma. P-glycoprotein levels may affect the mammary epithelial cells' exposure to xenobiotics as they accumulate on the apical side of the epithelial barrier. This exposure may lead to risky consequences because mammary tissue and milk possess enzymes capable of activating drugs and xenobiotic agents that may contribute to breast cancer and other diseases through formation of genotoxic agents in breast tissue and milk.

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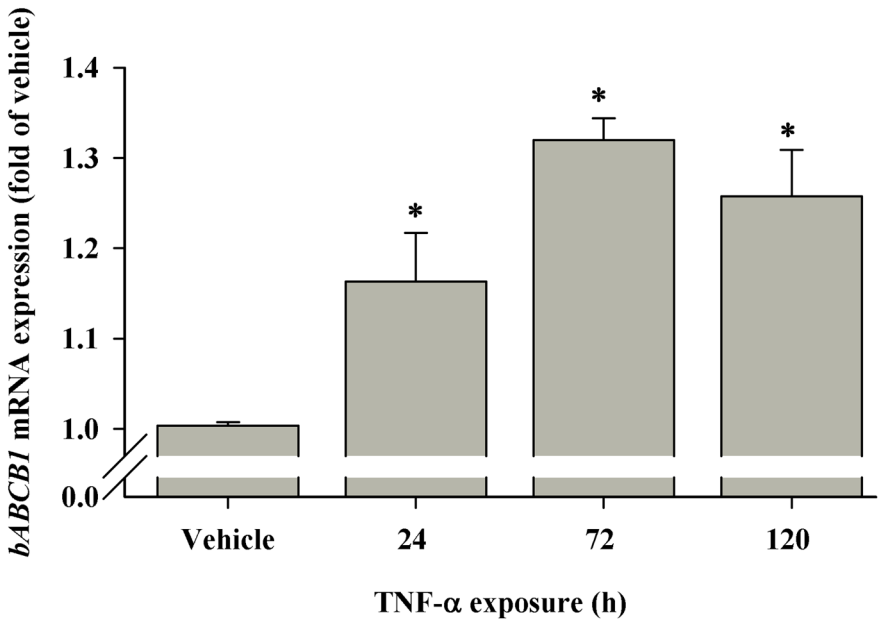

Figure 1.

TNF-α exposure increases *bABCB1* mRNA expression in confluent BME-UV monolayers. Monolayers were exposed to 200 ng/ml of TNF-α for 24, 72, and 120 hours before cells were harvested for RNA isolation. Star indicates the outcomes are significantly different from the vehicle. Data represents mean ± SE for n = 4, ($P < 0.05$).

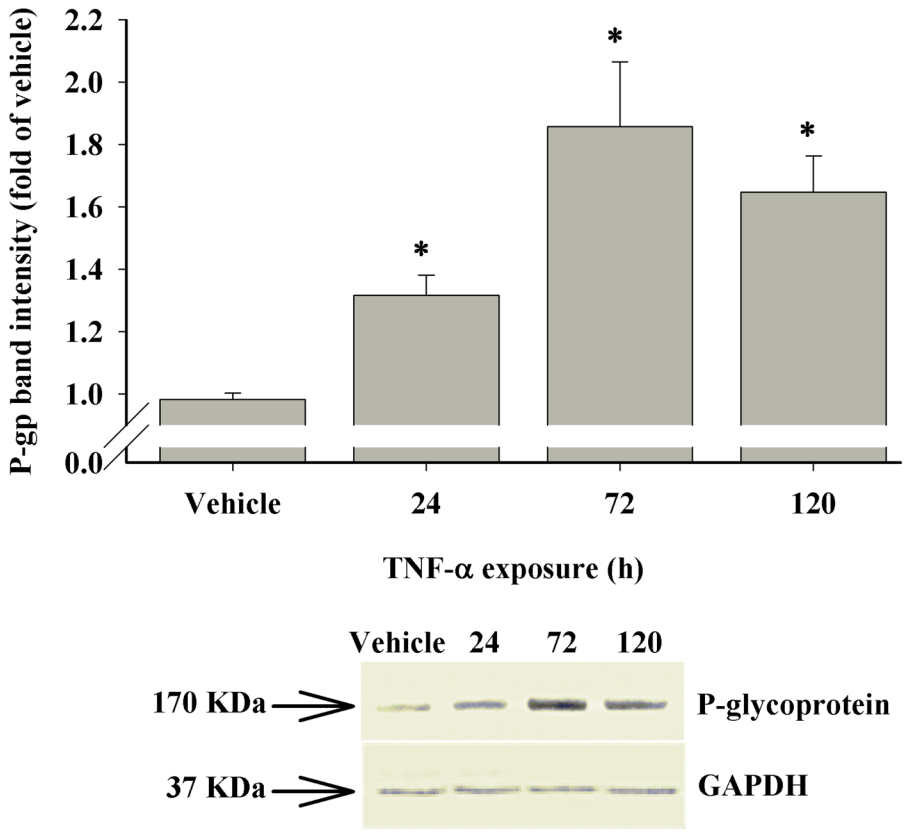

Figure 2.

Western blot analysis of total protein lysates identified significant changes in immunoreactive bands intensity of P-glycoprotein due to the exposure to TNF-α. Confluent BME-UV monolayers were exposed to TNF-α for the indicated durations before cells were harvested. Star indicates the outcomes are statistically significant different from the vehicle (4). Data represent mean ± SE for n = 5, ($P < 0.05$).

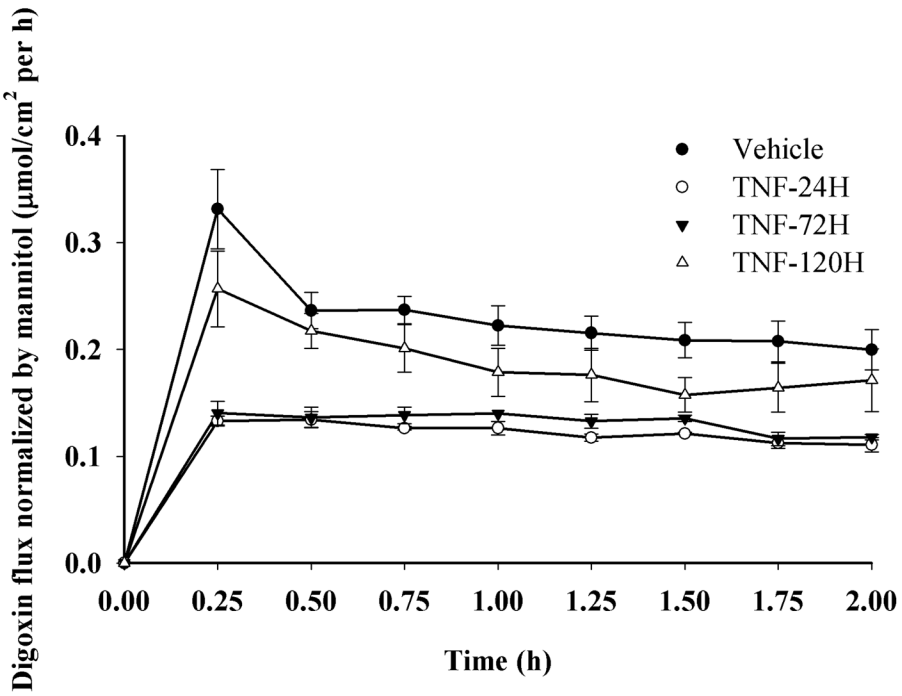

Figure 3.

TNF-α exposure, for 24 and 72 h, significantly ($P < 0.05$) decreases the mannitol-normalized flux of digoxin across BME-UV monolayers. Digoxin flux across the BME-UV monolayer from Ap-to-BL side after exposing BME-UV cells to TNF-α for 24 (TNF-24H); 72 (TNF-72H) and 120 hours (TNF-120H) prior to performing the transport study. Data represent mean ± SE for n = 3, ($P < 0.05$).

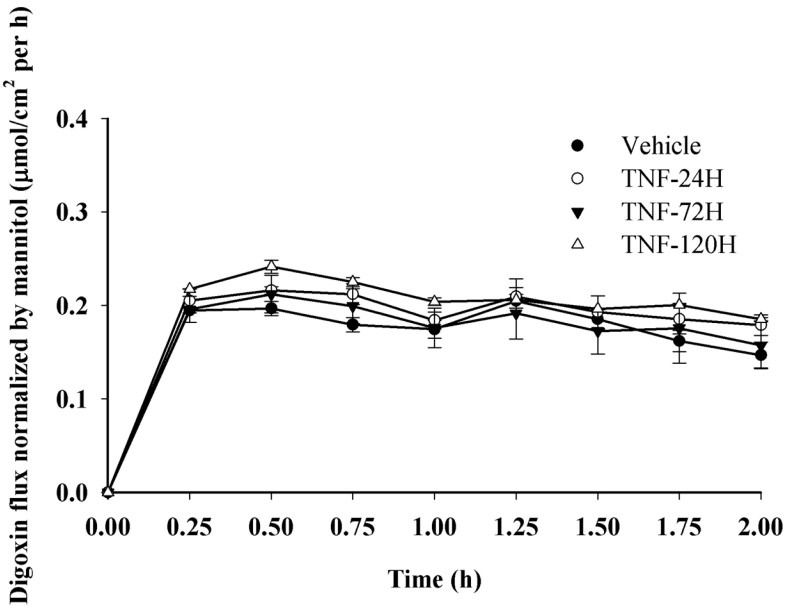

Figure 4.

Following exposure to TNF-α for the indicated durations, there were no significant differences in digoxin flux across the BME-UV monolayers in the presence of verapamil, a compound known to interact with P-glycoprotein. BME-UV cells were exposed to TNF-α for 24 (TNF-24H); 72 (TNF-72H) and 120 hours (TNF-120H) prior to performing the transport study. Data represents mean ± SE for n = 3, ($P < 0.05$).

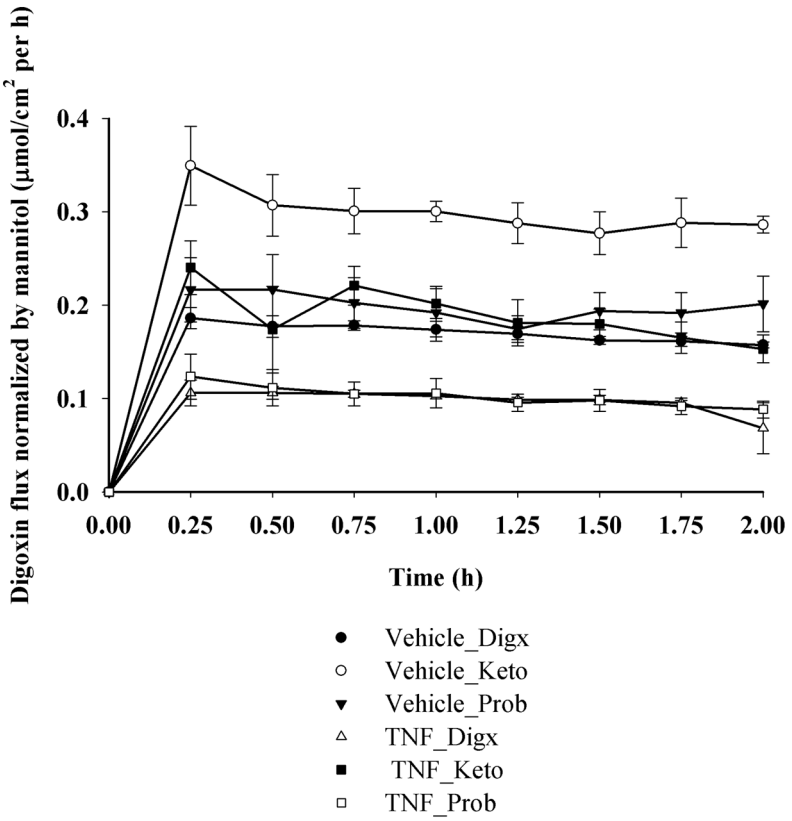

Figure 5.

Ketoconazole but not probenecid increased significantly ($P < 0.05$) the mannitol-normalized flux of digoxin across the TNF-α-exposed and unexposed BME-UV monolayers. Mannitol-normalized flux of digoxin (TNF_Digx or Vehicle_Digx) across the BME-UV monolayer from Ap-to-BL side. BME-UV cells were exposed to TNF-α for 72 hours prior to performing the transport study in the presence or absence of ketoconazole (TNF_Keto or Vehicle_Keto) or probenecid (TNF_Prob or Vehicle_Prob). Data represent mean ± SE for n = 3, ($P < 0.05$).

Table 1

Mannitol flux across BME-UV monolayer from Ap-to-BL side

	Treatments[1]			
	Untreated	**TNF-24H**	**TNF-72H**	**TNF-120H**
Flux, nmol/cm^2/hr	$6.5 \times 10^{-3}a$	$15.5 \times 10^{-3}b$	$14.1 \times 10^{-3}b$	$9.6 \times 10^{-3}a$
SE[2]	0.7×10^{-3}	0.8×10^{-3}	1.1×10^{-3}	1.5×10^{-3}

*a–b*Means within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: BME-UV cells were exposed to cytokine TNF-α for 24- (TNF-24h); 72- (TNF-72h) and 120-hr (TNF-120H) prior to performing the transport study.

[2]SE: standard error.