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Influence of tumor necrosis factor-α on the expression and function of P-glycoprotein in an immortalised rat brain capillary endothelial cell line, GPNT

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Abstract

Drug cerebral pharmacokinetics may be altered in the case of inflammatory diseases. This may be due to a modification of drug transport through the blood–brain barrier, in particular through drug interaction with the membrane efflux transporter, P-glycoprotein. The objective of this study was to investigate the influence of the inflammatory cytokine, tumor necrosis factor (TNF)-α, on the functionality and expression of P-glycoprotein, and on mdr1a and mdr1b mRNA expression in immortalised rat brain endothelial cells, GPNT. Cells were treated with TNF-α for 4 days. Levels of mdr1a and mdr1b mRNAs were quantitated using real-time RT-PCR analysis and expression of P-glycoprotein was analyzed by Western blot. The functionality of P-glycoprotein was studied by following the accumulation of [^{3}H]vinblastine in the cells without and with a pre-treatment with a P-glycoprotein inhibitor, GF120918. TNF-α increased the levels of mdr1a and mdr1b mRNAs while no effect was observed on protein expression. TNF-α increased [^{3}H]vinblastine accumulation indicating a time and concentration-dependent decrease of P-glycoprotein activity. This effect was eliminated when the cells were pre-treated with GF120918. Our observation of a decrease in P-glycoprotein activity could suggest that in the case of inflammatory diseases, brain delivery of P-glycoprotein-dependent drugs can be enhanced.

Keywords: TNF-α; P-glycoprotein; Brain capillary endothelial cell; GPNT; Blood–brain barrier; Multidrug resistance

1. Introduction

P-gp is an efflux plasma membrane protein overexpressed in tumor cells and acting as a pump, effluxing anticancer drugs from the cells. It confers to these cells a multidrug resistance. It is also expressed in non-malignant tissues such as lung, intestine, kidney, epithelia, testis and brain capillary endothelium [1–3]. P-gp is encoded by a gene family comprising two *mdr* genes (*MDR1* and *MDR2*) in humans and three *mdr* genes (*mdr1a*, *mdr1b* and *mdr2*) in rodents [4], respectively, hABCB1 and hABCB2 in humans and rAbcb1a, rAbcb1b and rAbcb2 in rodents according to the new ''nomenclature.'' However, only the expression of human MDR1 and rodent mdr1a and mdr1b appears to selectively confer multidrug resistance. In the endothelium of rat brain capillary, mdr1a is exclusively expressed and the P-gp mdr1a isoform contributes solely to the efflux of drugs from the brain back into the blood circulation. Experiments with knock-out mice deficient in mdr1a have shown the role of mdr1a and P-glycoprotein in the brain uptake of many drugs with potential targets in the central nervous system [5,6].

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Abbreviations: BBB, blood–brain barrier; P-gp, P-glycoprotein; TNF-α, tumor necrosis factor α; mdr, multidrug resistance.

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Central nervous system (CNS) inflammatory pathologies such as multiple sclerosis, bacterial and fungal meningitis, Alzheimer's disease and AIDS dementia complex may modify the integrity of the BBB. Inflammatory cytokines (TNF-α; interleukin-1β, IL1β; and interleukin-6, IL6) are predominantly secreted into the CNS after injury or inflammation by macrophages, microglial cells, astrocytes and capillary endothelial cells [7–9].

Contradictory studies have reported that inflammatory cytokines could modify the expression and the functionality of P-gp. Cellular accumulation of doxorubicin was increased by TNF-α in Caco-2 cells suggesting a decrease in P-gp functionality [10], while rhodamine 123 accumulation was decreased by TNF-α in primary rat hepatocyte culture indicating an increased functionality of P-gp [11].

At the BBB level, Mandi *et al*. [12] observed that TNF-α (10^3 U/mL) did not influence the expression of P-gp and seemed to decrease its functionality on human BB19 brain capillary endothelial cells. However, no statistics were applied to these data and the expression of mdr1a and mdr1b mRNAs was not investigated.

The objective of our study was to investigate the modification of the expression and functionality of P-gp by different concentrations of TNF-α in an immortalised rat brain capillary endothelial cell line, GPNT. Cells were treated with TNF-α at 0.1, 1, and 10 ng/mL for 2–96 hr. We have studied the TNF-α effect on the expression of mdr1a and mdr1b mRNA and P-gp protein, and we have measured the intracellular accumulation of [^{3}H]vinblastine with or without the P-gp inhibitor, GF120918.

2. Materials and methods

2.1. Materials

Media and foetal calf serum (FCS) of USA origin were obtained from Life Technologies. [^{3}H]vinblastine sulfate (8.1 Ci/mmol) was purchased from Amersham Pharmacia Biotech. The P-gp inhibitor GF120918 (GF120918) was provided by Glaxo SmithKline. GF120918 was dissolved in dimethylsulfoxide (DMSO) with a final DMSO concentration in the cell incubation media of 0.01% (v/v). Rat recombinant TNF-α was purchased from R&D Systems. The chemiluminescence detection kit (ECL) was purchased from Pierce and Hybond-N+ sheets were purchased from Amersham. All other reagents were purchased from Life Technologies or Sigma.

2.2. GPNT rat brain endothelial cell line

The GPNT cell line was obtained from a previously characterised rat brain endothelial cell line, GP8 [13]. GP8 cells were re-transfected with the plasmid pcDNA3-RSV containing the puromycin resistance gene. After repeated limiting dilution cloning of the parent line, a single clone, designated GPNT (from GP8 and the French Company Neurotech SA), was selected according to morphological criteria and retention of endothelial cell markers. GPNT cells were repeatedly treated with 5 μg/mL puromycin.

GPNT cells were plated on collagen I-coated plates and grown in culture medium A: α-MEM/Ham's F-10 (1:1) containing 10% heat-inactivated foetal calf serum, 0.5 ng/mL basic fibroblast growth factor, 5 μg/mL transferrin, 5 μg/mL insulin, 5 ng/mL selenium and 5 μg/mL puromycin, in humidified 5% CO_2/95% air at 37°. The expression and activity of P-gp is greatly increased by the repeated treatment of cells with puromycin [14].

2.3. Treatment of GPNT cells with TNF-α

TNF-α was solubilised in α-MEM/F-10 (1:1) with 0.1% of BSA (dilution of this solution was made in culture medium B at final concentrations of 0.1, 1, and 10 ng/mL). Culture medium B is culture medium A without the serum and completed with 1% BSA and 550 nM hydrocortisone. Control and TNF-α treated confluent GPNT cells were cultured in parallel in culture medium B for the following time periods: 2, 6, 14, 24, 48, 72, and 96 hr. Culture medium B was replaced every 24 hr.

2.4. MTT assay for cell viability

The cytotoxic effect of TNF-α on GPNT cells was studied using a MTT-based colorimetric assay. Cells were treated with TNF-α at 0.1 or 10 ng/mL for 48 and 96 hr. After treatment of cells with TNF-α, medium was removed, wells were replenished with fresh medium and 100 μL aliquot of MTT (500 ng/mL) were added to each well. After addition of a lysate buffer and plate mixing to ensure complete solubility, optical density was read at 570 nm. The cytotoxicity was calculated as the percent reduction in absorbance relative to controls.

2.5. Real-time reverse transcription polymerase chain reaction

10^5 GPNT cells were grown to confluence in 60 cm^2 Petri dishes in culture medium A and then treated for 2–96 hr with TNF-α in culture medium B. Total RNAs were extracted using Trizol® and the Chomczynski method [15]. Of the total RNA isolated, 1.25 μg was used for RT with enhanced avian reverse transcriptase (Sigma) according to the manufacturer's instructions. Of the cDNA product, 5 μL were added to 45 μL containing platinum quantitative PCR super Mix-UDG [60 U/mL, Platinum Taq DNA polymerase, 40 mM Tris–HCl (pH 8.4), 100 mM KCl, 6 mM $MgCl_2$, 400 μM dGTP, 400 μM dATP, 400 μM dCTP, 800 μM dUTP, 40 U/mL UDG], each primer at 300 nM, and iCycler PCR Reaction Mix SYBR Green I (Bio Rad). The primers used and PCR conditions are summarized in Table 1. After amplification, a melting

Table 1
RT-PCR and primers used for the determination of *mdr1a* and *mdr1b* genes

Genes	Primer sequence (5′ to 3′)
Rat *mdr1a*	5′-atcaactcgcaaaagcatcc-3′ (FP)
	5′-aattcaacttcaggatccgc-3′ (RP)
Rat *mdr1b*	5′-cactggtgcctctgagttga-3′ (FP)
	5′-gcacatcttcatccacatcct-3′ (RP)
Rat β-actin	5′-gcccagagcaagagaggtat-3′ (FP)
	5′-ggccatctcttgctcgaagt-3 (RP)

FP: forward primer; RP: reverse primer.

curve was obtained by heating at 55° and fluorescence data were collected at 0.5°/s.

2.6. Quantification of real-time PCR for mdr1a and mdr1b

Quantitative analysis of the iCycler iQ™ data was performed employing iCycler analysis software as previously described [16]. The data analysis is divided into two phases: a specificity control for the amplification reaction using the melting curve program of the iCycler iQ™ software, followed by use of the quantification program. The SYBR Green I signal of each sample is plotted against the number of cycles. The iCycler iQ™ analysis software is used to remove background by setting a noise band. This fluorescence threshold is used to determine cycle numbers that correlate inversely with the log of the initial template concentration. As described previously [16] we used the expression of β-actin to normalize the expression data of *mdr1a* and *mdr1b* genes. β-Actin was used as an active and endogenous reference to correct for differences in the amount of total RNA added to a reaction and to compensate for different levels of inhibition during reverse transcription of RNA and during PCR.

2.7. Semi-quantitative determination of P-glycoprotein expression by Western blot

10^5 GPNT cells were grown to confluence in 60 cm^2 Petri dishes in culture medium A and then treated for 24–96 hr with TNF-α in culture medium B. Total proteins were extracted. The culture medium was removed and cells were washed twice with PBS 1× without calcium and magnesium. Cells were then kept frozen at −20°.

Lysate buffer (200 μL) with 1% octyl-glucoside containing protease inhibitor in PBS 1× was added to frozen cells. The surface of the Petri dish was scratched and the protein solution was transferred to a polypropylene tube. Samples were kept for 30 min at +4°, shaken and then centrifuged at 12,000 *g* for 10 min at +4°. The supernatant was kept frozen at −20°.

Sixteen microliter of the mixture (SDS 8%, glycerol 40%, bromophenol blue 0.005%, 2-mercaptoethanol 10%) was added to 20 μg of each protein sample solubilised in 40 μL of lysate buffer. 20 μg of protein were separated onto a 7.5% SDS–polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to a PDVF hybond-P membrane. The membrane was washed twice, then blocked in Tris–buffer saline (TBS) containing 0.05% Tween 20 (TBST) and finally blocked in TBST containing 5% skim dried milk for 1 hr at room temperature. The primary monoclonal antibody, C219 (DAKO), was diluted in TBST–5% skim dried milk (1 μg/mL) and incubated overnight at +4°. After five washes in TBST buffer, a secondary polyclonal antibody [goat anti-mouse IgG peroxidase conjugate (DAKO)] was diluted 400-fold in TBST–5% skim dried milk and incubated at room temperature for 1 hr. The membrane was washed twice for 5 min and three times for 10 min in TBST. P-gp protein in crude membranes was detected by chemiluminescence using a supersignal chemiluminescence substrate (Pierce). The integrity of the protein gel loading and transfer was checked with Ponceau red staining before immunostaining.

2.8. Drug accumulation studies

GPNT cells were grown to confluence in 24-multiwell plates in culture medium A and then treated for 2–96 hr with TNF-α in culture medium B. Cellular uptake of radiolabelled [^{3}H]vinblastine was measured as previously described [17]. On the day of experiment, cells were at first starved of puromycin and bFGF by incubation for 2 hr in a culture medium C, which is culture medium B without either of these molecules. Cells were then washed three times with phosphate buffered saline (PBS) 1× at 37° and preincubated for 30 min at 37° in a shaking waterbath with culture medium C, with or without the P-gp inhibitor GF120918 (1 μM). [^{3}H]vinblastine, at a final concentration of 10 nM, was then added to the medium in the 24-multiwell plates and left for 60 min, preliminary experiments having shown that vinblastine uptake was at equilibrium after 50 min of contact (data not shown). The plates were shaken during both preincubation and incubation periods to reduce the effect of the aqueous boundary layer on drug accumulation. The reaction was stopped by removing the medium rapidly by three rapid washes with ice-cold PBS 1× to eliminate the extracellular drug and lysed with 500 μL 0.1 M NaOH. The amount of radiolabelled drug retained in the cells was counted in Pico Fluor by β-scintillation counting (Packard 1990 TR). An aliquot of cell lysate was used in parallel to determine cellular protein concentration following the method described by Lowry *et al*. [18]. Intracellular vinblastine concentrations were expressed in nmol/g protein.

2.9. Statistical analysis

The results of [^{3}H]vinblastine accumulation and the effect of different concentrations of TNF-α were analyzed by the ANOVA test and the multiple-comparison Fisher test. In all cases, a value of $P < 0.05$ was considered significant.

3. Results

3.1. Cell viability after TNF-α treatment

After treatment with 0.1 and 10 ng/mL TNF-α for 48 and 96 hr, cell viability was not modified (within 10% of control).

3.2. RT-PCR analysis

To establish *mdr1a* and *mdr1b* gene expression production by GPNT cell line in response to TNF-α treatment, amplification of cDNA was assessed. To quantify the relative abundance of the mdr1a and mdr1b transcripts, we performed real-time PCR using specific primers (Table 1). We obtained the slopes of the standard curves (threshold cycle vs. log[amplicon]) that gave values of 3 with both primers, validating our determinations. In addition, the adequacy of the fluorescent measurements with SYBR Green I was confirmed by the finding with agarose gel electrophoresis (data not shown) of a single product of the appropriate size (244 and 224 for mdr1a and mdr1b, respectively). Moreover, real-time PCR showed that the mdr1a-mRNA precede mdr1b-mRNA expression. mdr1a was 11-fold higher in treated GPNT cells than in the control 6 hr following treatment (Fig. 1A, B and G), whereas the expression of the control β-actin gene did not differ between untreated and TNF-α-treated GPNT cells (data not shown). This expression decreases significantly at 24 hr (Fig. 1C and G). mdr1b was 3- and 21-fold higher at 6 and 24 hr, respectively, in treated GPNT cells than in the untreated GPNT cells (Fig. 1E, F and G), whereas the expression of the control β-actin gene did not differ between untreated and TNF-α-treated GPNT cells (data not shown).

3.3. Immunoblot analysis

In the immunoblot experiment using an anti-P-gp monoclonal antibody C219 (Fig. 2), the protein with a molecular size of 150–160 kDa corresponds to P-gp in GPNT cells [14]. TNF-α has no influence on the level of P-gp expression whatever the concentration of cytokine or the duration of treatment. Parallel expression of β-actin protein was not changed by the treatment (data not shown).

3.4. Functional analysis

The influence of TNF-α on the accumulation of [^{3}H]vinblastine, without pre-treatment of the cells with the P-gp inhibitor GF120918, is represented in Fig. 3a. The influence of TNF-α concentrations was better represented through calculation of the ratio between accumulation of the substrate after TNF-α treatment, and the control without TNF-α (Fig. 3b). It showed that TNF-α increased the accumulation of [^{3}H]vinblastine in GPNT cells, suggesting that the efflux was decreased by TNF-α. After 14 hr of contact, the decrease in efflux was dependent on the TNF-α concentration added to the medium ($P < 0.05$) and was maximal after 3 days of contact.

The involvement of P-gp in this phenomenon was suggested by the performance of the same experiment, treating the cells with the P-gp inhibitor GF120918 (1 μM). It showed that the influence of TNF-α on the accumulation of [^{3}H]vinblastine disappeared whatever the concentration of TNF-α added to the medium and whatever the duration of contact between the cells and the cytokine (Fig. 4a and b). Moreover, by pre-treating the cells with GF120918, vinblastine intracellular concentrations increased by a 10-fold factor (2–5 nmol/g protein) when compared to concentrations without GF120918 (0.2–0.5 nmol/g protein). DMSO (0.01% in the culture medium), used to dissolve the P-gp inhibitor GF120918, did not change the accumulation of [^{3}H]vinblastine in GPNT cells (data not shown).

4. Discussion

TNF-α induced an increase in mdr1a and mdr1b mRNA expression, whereas no change in the protein level was observed. Moreover, treatments with every tested TNF-α concentration resulted in a P-gp activity decrease, which could not be correlated with any change in P-glycoprotein expression.

4.1. Duration and TNF-α concentrations in TNF-α treatment

The treatment of GPNT cells with TNF at 0.1 and 10 ng/mL up to 96 hr did not modify cell viability. The effect on P-glycoprotein activity is dependent on TNF-α concentration and on treatment duration, with a maximal effect after 3 days.

The influence of TNF-α was investigated over a contact period of 2–96 hr with the cytokine. In order to avoid interference with the cytokines present in fetal bovine serum, this serum was eliminated from the cell medium. For this reason, 550 nM of hydrocortisone were added to the medium to increase survival of cells cultured without serum [19]. Concentrations of TNF-α (0.1, 1, 10 ng/mL) tested in our study were selected in accordance with the concentrations used in other studies and were similar to the concentrations reported in brain tissue affected by inflammatory diseases [20].

4.2. mdr1a and mdr1b by RT-PCR analysis

Our results on mdr1b mRNA regulation are similar to those obtained with rodents in other laboratories. On the contrary, the increase in mdr1a expression that we observed differs from other studies. Expression of mdr1b mRNA was significantly increased in mouse liver 24 hr

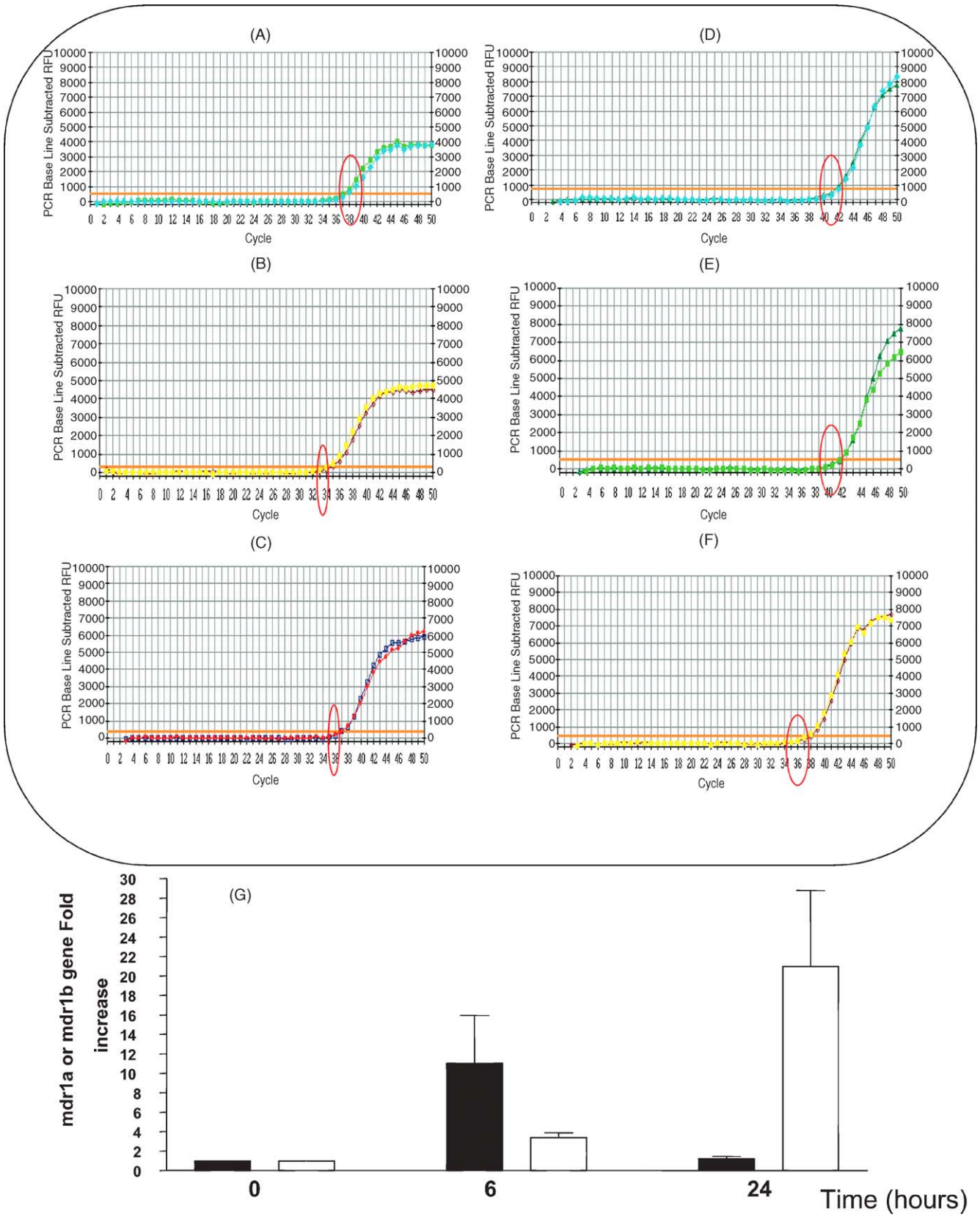

Fig. 1. Real time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mdr1a and mdr1b messenger RNA expression in immortalised rat brain capillary endothelial GPNT cells without and with pre-treatment with TNF-α (10 ng/mL), where A, B, and C represent *mdr1a* gene expression after 0, 6 and 24 hr of contact with TNF-α and D, E, and F represent *mdr1b* gene expression after 0, 6, and 24 hr of contact with TNF-α. G represents the quantification of *mdr1a* (black histogram) and *mdr1b* (open histogram).

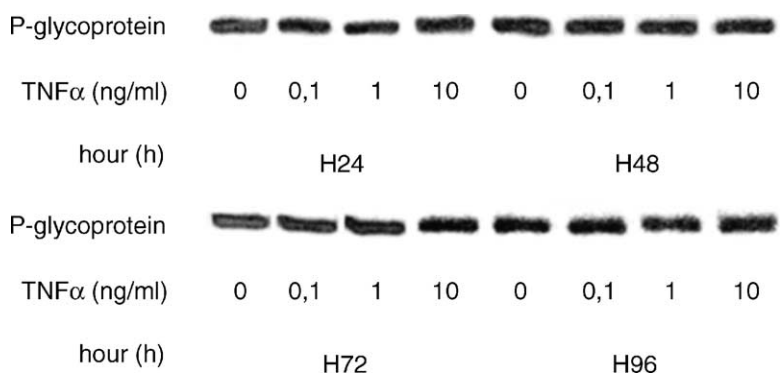

Fig. 2. Western blot analysis of P-glycoprotein expression in immortalised rat brain capillary endothelial GPNT cells without pre-treatment with TNF-α (0 ng/mL) or after pre-treatment with different concentrations of TNF-α (0.1, 1, and 10 ng/mL) over periods of 24, 48, 72 or 96 hr.

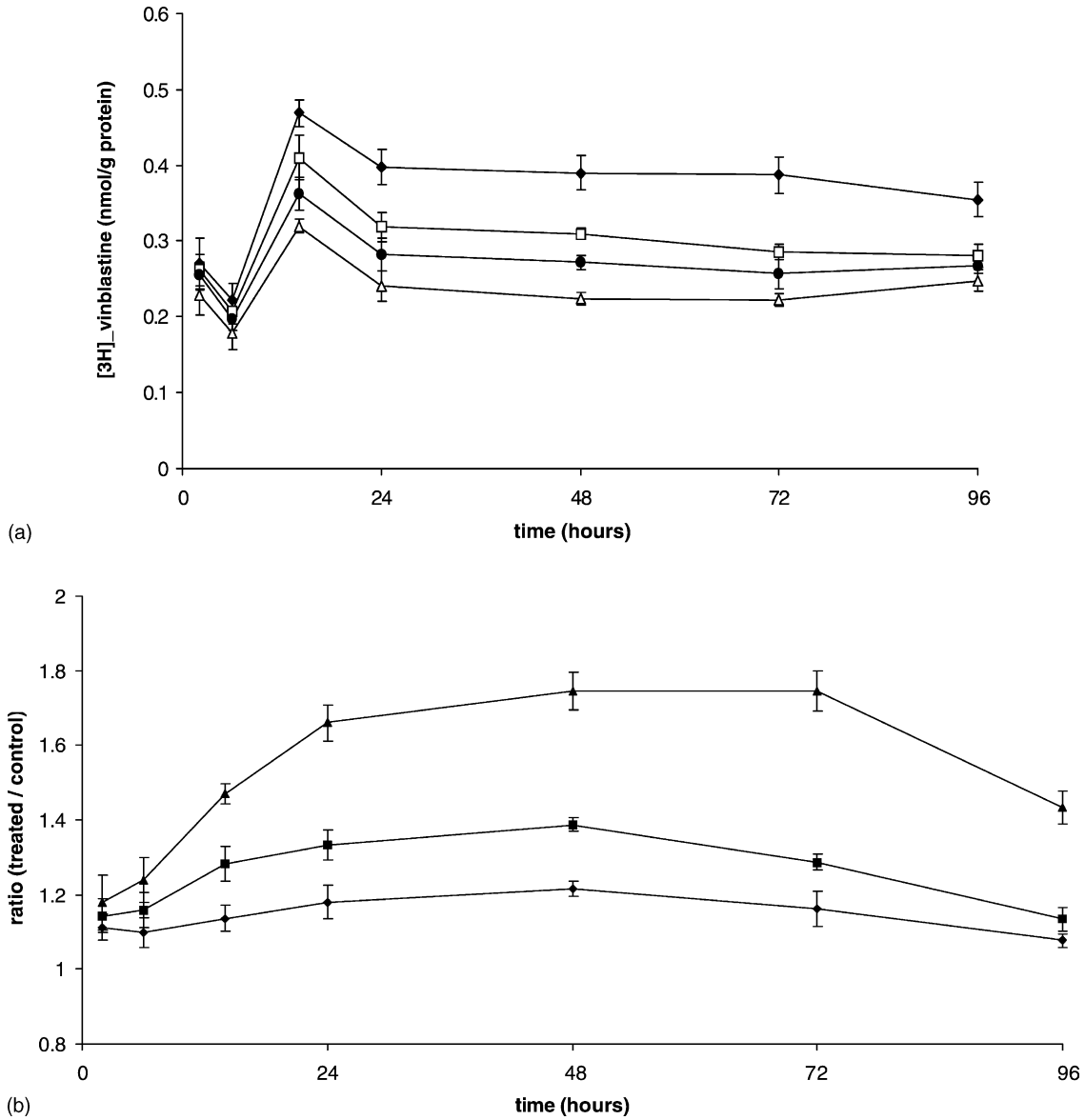

Fig. 3. Influence of TNF-α at different concentrations (0.1, 1, 10 ng/mL) on the functionality of P-glycoprotein in an immortalised rat brain capillary endothelial cell line, GPNT. (a) Accumulation of [3H]vinblastine, where (◆) 10 ng/mL, (□) 1 ng/mL, (●) 0.1 ng/mL, (△) 0 ng/mL (data are mean ± SD, N = 6). (b) Ratio of accumulation of [3H]vinblastine obtained in wells treated with TNF-α divided by the mean accumulation in control wells without TNF-α, where (▲) 10 ng/mL, (■) 1 ng/mL, (◆) 0.1 ng/mL. Data are mean ± SD, N = 6.

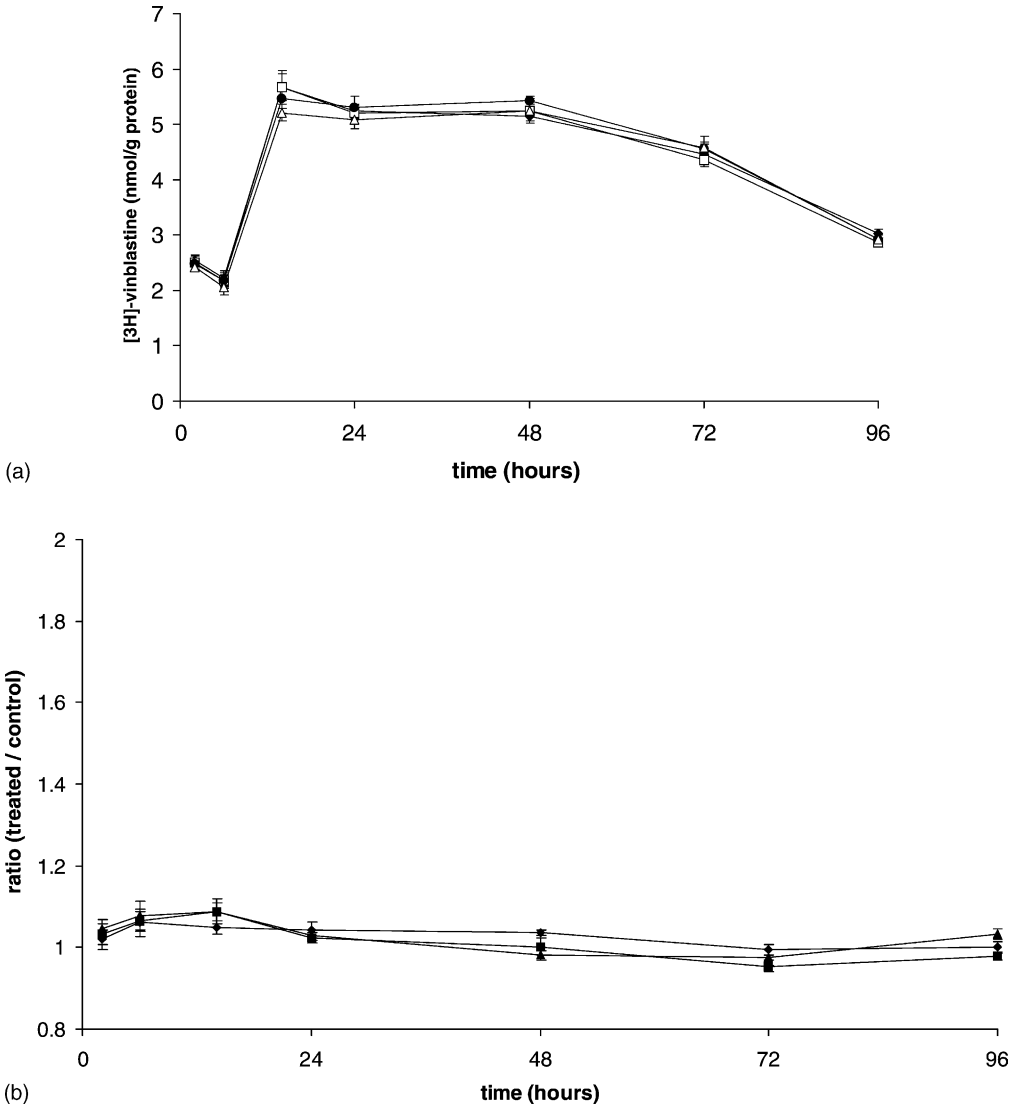

Fig. 4. Influence of TNF-α at different concentrations (0.1, 1, 10 ng/mL) on the functionnality of P-glycoprotein in an immortalised rat brain capillary endothelial cell line, GPNT treated with a P-glycoprotein inhibitor GF120918 (1 μM). (a) Accumulation of [3H]vinblastine, where (◆) 10 ng/mL, (□) 1 ng/mL, (●) 0.1 ng/mL, (△) 0 ng/mL (data are mean ± SD, N = 6). (b) Ratio of accumulation of [3H]vinblastine obtained in wells treated with TNF-α divided by the mean accumulation in control wells without TNF-α, where (◆) 10 ng/mL, (■) 1 ng/mL, (▲) 0.1 ng/mL. Data are mean ± SD, N = 6.

after intraperitoneal administration of TNF-α, whereas mdr1a mRNA expression remained unchanged [21]. In primary rat hepatocyte cultures, mdr1b mRNA was the major mdr mRNA types induced by TNF-α [11]. After intraperitoneal injection of bacterial endotoxin to rats, mdr1b mRNA was greatly increased throughout the liver or in isolated hepatocytes, whereas mdr1a and mdr2 mRNA did not significantly change [22]. After intraperitoneal injection of *Klebsiella pneumoniae* endotoxin to rats, the expression of mdr1b mRNA in the liver was increased, whereas the expression of mdr1a mRNA decreased 6 hr after endotoxin injection and returned to control levels after 24 hr. In this last study, these changes could be associated with increased plasma TNF-α levels [23]. All these results are in agreement with the observation that cytotoxic agents, heat shock, irradiation, DNA-damaging agents, cytokines, protein synthesis inhibitors and reactive oxygen species could induce stress and increase the expression of *mdr1b* gene, which is thus considered as a "stress-responsive" gene [24].

4.3. Functionality analysis

The increase of intracellular vinblastine concentrations after TNF-α treatment could be explained by a cytotoxic effect of TNF-α resulting in increase of membrane fluidity, an increase in drug diffusion or by an inhibition of drug efflux from the cells. The MTT-based colorimetric assay demonstrated that a cytotoxic effect of TNF-α did not occur when cells were treated up to 96 hr. An eventual effect of TNF-α on BBB permeability in our experiments can be ruled out as there is no difference in intracellular vinblastine concentrations between control cells and TNF-treated cells when the cells are pre-treated with the efflux

inhibitor, GF120918, indicating that the effect of TNF-α is on efflux from the cells and not on entry into the cells.

Several transporters involved in drug efflux have been described such as the multidrug resistance MDR1/P-glycoprotein, the multidrug resistance-associated proteins (MRPs), the breast cancer resistance protein ABCG2/MXR/BCRP (BCRP), . . . , P-glycoprotein and MRPs have been characterised in BBB and recent report suggested that BCRP could also be expressed in BBB [25]. GF120918 has been described as P-gp and BCRP inhibitor [26] but did not show any effect on MRP [27]. The P-gp substrate, vinblastine, was not extruded from transfected cells overexpressing BCRP indicating that vinblastine is not a substrate of this protein [28]. As GF120918 is an inhibitor of both P-gp and BCRP and as vinblastine is a substrate of P-gp but not of BCRP, the effect of GF120918 on vinblastine efflux is specific to P-glycoprotein. In our experiments, TNF-α induced a decrease in vinblastine efflux in the immortalised brain endothelial cells, GPNT and this effect was eliminated when cells were pre-treated with GF120918, indicating that this phenomenon is therefore specific to the P-glycoprotein efflux.

The 10-fold factor observed between vinblastine intracellular concentrations after inhibition of P-gp functionality by GF120918 and those after inhibition of P-gp functionality by TNF-α shows that the effects of TNF-α is rather small in proportion.

Several laboratories have observed a decrease in P-gp activity after treatment with TNF-α, as was observed in our study. Doxorubicin accumulation was increased in human colon carcinoma cells HCT15 and HCT116 α, with a maximal effect after 2 days of treatment [10]. Rhodamine-123 uptake was enhanced in U373MG human glioblastoma cells in which the human TNF-α gene was transduced [29]. Moreover, after intraperitoneal injection of *K. pneumoniae* endotoxin to rats, P-glycoprotein-mediated biliary and renal excretion of rhodamine-123 was decreased in correlation with increased plasma TNF-α level [23].

4.4. Relation between mdr mRNA level, P-gp expression and P-gp activity after TNF-α treatment

Contradictory results have also been obtained in different laboratories studying TNF-α effects on mdr mRNA level and P-gp expression and activity. Hartman *et al*. [21] did not observe any change in the immunodetectable P-glycoprotein level in mouse hepatocytes 24 hr after intraperitoneal administration of TNF-α, whereas the mdr1b mRNA expression was significantly increased. However, a time-dependent reduction in P-gp expression, in agreement with the data on MDR1 mRNA level, was observed in human colon carcinoma HTC15 and HCT16 cells exposed to TNF-α [10]. TNF-α-induced P-gp overexpression was correlated with MDR1 overexpression in human myeloid leukemia TF-1 cells [30] and with mdr1b overexpression in primary rat hepatocyte cultures [11].

At the BBB level, the decrease of P-gp functionality without modification of the protein expression was also observed by Mandi *et al*. [12] on human BB19 cerebral capillary endothelial cells. They observed that TNF-α at the concentration of 10^3 U/mL did not influence the P-gp expression investigated by immunofluorescence analysis and seemed to moderately increase the uptake of rhodamine measured by flow cytometry. Moreover, change in P-gp activity without any corresponding change in P-gp expression in GPNT cells has already been described by Regina *et al*. [14], who demonstrated an increased P-gp activity without any increase in P-gp expression after treatments with dexamethasone.

The increase in mdr1a and mdr1b mRNAs may be explained by transcriptional activation or by processes involving mRNA stabilisation. In the first case, the increase in mRNA should be followed by an increase in the level of the corresponding protein. In the second hypothesis, TNF-α could modify the half life of mdr1a and mdr1b mRNAs with no direct consequence on the level of P-gp protein transcription. A similar modification of mRNA half-life has already been described for β-actin [31]. This second hypothesis could explain the apparent contradiction between the increase of mdr1a and mdr1b mRNAs and the unchanged level of P-gp observed in our experiments.

Regarding P-gp functionality, the treatment of GPNT cells with TNF-α resulted in a decrease of P-gp activity with an unchanged level of protein. This decrease of functionality with a similar level of protein may be explained either by an interference of TNF-α with P-gp cellular distribution [32] or by a decrease of phosphorylation of the protein resulting in a decrease of its efflux properties. The effect of TNF-α on protein localisation inside the membrane or phosphorylation could be direct and/or through synthesis of some regulator protein.

5. Conclusion

All these apparent discrepancies in the regulation of mdr mRNA expression, protein expression, and P-gp activity indicate that different results may be obtained depending on the species, the type of cell model, the procedures of culture used and the treatment protocols. Indeed, the numerous mechanisms involved in these processes (such as transcriptional and translational regulations, mRNA stability, phosphorylation state of the protein and changes of it physico-chemical properties) could interact differently according to the experiment characteristics [24].

In so far as the cell model achieved with the GPNT cells enable us to draw conclusions on the properties of *in situ* rat or human blood–brain barrier endothelial cells, our observation of a decrease in P-glycoprotein activity could suggest that in the case of neurological inflammatory diseases such as meningitis and AIDS dementia, brain delivery of P-glycoprotein-dependent drugs could be modulated.

These changes in drug cerebral pharmacokinetics in the case of inflammatory diseases may be used advantageously to increase cerebral uptake of some P-glycoprotein dependent drugs in the treatment of cerebral pathologies.

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References

[1] Fojo AT, Ueda K, Slamon D, Poplack D, Gottesman MM, Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA 1987;84:265–9.
[2] Thiebaut F, Tsuoro T, Hamada H, Gottesman MM, Pastan I, Willingham M. Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: evidence for localization in brain capillaries and cross reactivity of one antibody with a muscle protein. J Histochem Cytochem 1989;37:159–64.
[3] Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. J Histochem Cytochem 1990;38:1277–87.
[4] Ng WF, Sarangi F, Zastawny RK, Veinot-Drebot L, Ling V. Identification of members of the P-glycoprotein multigene family. Mol Cell Biol 1989;9:1224–32.
[5] Schinkel AH, Wagenaar E, van Deemter L, Mol CA, Borst P. Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. J Clin Invest 1995;96:1698–705.
[6] Schinkel AH, Wagenaar E, Mol CA, van Deemter L. P-glycoprotein in the blood–brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Clin Invest 1996;97: 2517–24.
[7] De Vries H, Blom-Rosmalen M, Oosten M, de Boer A, van Berkel T, Breimer D, Kuiper J. The influence of cytokines on the integrity of the blood–brain barrier *in vitro*. J Neuroimmunol 1996;64:37–43.
[8] De Vries H, Kuiper J, de Boer A, van Berkel T, Breimer D. The blood–brain barrier in neuroinflammatory diseases. Pharmacol Rev 1997;49:143–55.
[9] Descamps L, Cecchelli R, Torpier G. Effect of tumor necrosis factor on receptor-mediated endocytosis and barrier functions of bovine brain capillary endothelial cell monolayers. J Neuroimmunol 1997;74: 173–84.
[10] Stein U, Walther W, Shoemaker R. Modulation of mdr1 expression by cytokines in human colon carcinoma cells: an approach of multidrug resistance. Br J Cancer 1996;74:1384–91.
[11] Hirsch-Ernst K, Ziemann C, Foth H, Kozian D, Schmitz-Salue C, Kahl G. Induction of mdr1b mRNA and P-glycoprotein expression by tumor necrosis factor alpha in primary rat hepatocyte cultures. J Cell Physiol 1998;176:506–15.
[12] Mandi Y, Ocsovszki I, Szabo D, Nagy Z, Nelson J, Molnar J. Nitric oxide production and MDR expression by human brain endothelial cells. Anticancer Res 1998;18:3049–52.
[13] Greenwood J, Pryce G, Devine L, Male DK, Dos Santos WLC, Calder VL, Adamson P. SV40 large T immortalised cell lines of the rat blood–brain and blood–retinal barriers retain their phenotypic and immunological characteristics. J Neuroimmunol 1996;71:51–63.
[14] Regina A, Romero IA, Greenwood J, Adamson P, Bourre JM, Couraud PO, Roux F. Dexamethasone regulation of P-glycoprotein activity in an immortalised rat brain endothelial cell line, GPNT. J Neurochem 1999;73:1954–63.
[15] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.
[16] Didier N, Banks WA, Créminon C, Dereuddre-Bosquet N, Mabondzo A. HIV-1-induced production of endothelin-1 in an *in vitro* model of the human blood–brain barrier. Neuroreport 2002;13:1179–83.
[17] El Hafny B, Cano N, Piciotti M, Regina A, Scherrmann JM, Roux F. Role of P-glycoprotein in colchicine and vinblastine cellular kinetics in an immortalised rat brain microvessel endothelial cell line. Biochem Pharmacol 1997;53:1735–42.
[18] Lowry O, Rosenbrough N, Farr A, Randall R. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
[19] Hoheisel D, Nitz T, Franke H, Wegener J, Hakvoort A, Tilling T, Galla HJ. Hydrocortisone reinforces the blood–brain barrier properties in a serum free cell culture system. Biochem Biophys Res Commun 1998;244:312–6.
[20] Mark KS, Miller DW. Increased permeability of primary cultured brain microvessel endothelial cell monolayers following TNF-α exposure. Life Sci 1999;64:1941–53.
[21] Hartmann G, Kim H, Piquette-Miller M. Regulation of the hepatic multidrug resistance gene expression by endotoxin and inflammatory cytokines in mice. Int Immunopharmacol 2001;1:189–99.
[22] Vos TA, Hooiveld GJEJ, Koning H, Childs S, Meijer DKF, Moshage H, Jansen PLM, Müller M. Up-regulation of the multidrug resistance genes, *mrp1a* and *mdr1b*, and down-regulation of the organic anion transporter, mrp2, and the bilt salt transporter, spgp, in endotoxemic rat liver. Hepatology 1998;28:1637–44.
[23] Ando H, Nishio Y, Ito K, Nakao A, Wang L, Zhao YL, Kitaichi K, Takagi K, Hasegawa T. Effect of endotoxin on P-glycoprotein-mediated biliary and renal excretion of rhodamine-123 in rats. Antimicrob Agents Chemother 2001;45:3462–7.
[24] Sukhai M, Piquette-Miller M. Regulation of the multidrug resistance genes by stress signal. J Pharm Pharm Sci 2000;3:268–80.
[25] Eisenblatter T, Galla HJ. A new multidrug resistance protein at the blood-brain barrier. Biochem Biophys Res Commun 2002;293:1273–8.
[26] Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg ACLM, Schinkel AH, van de Vijver MJ, Scheper RJ, Schellens JHM. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res 2001;61:3458–64.
[27] Evers R, Kool M, Smith AJ, van Deemter L, de Haas M, Borst P. Inhibitory effect of the reversal agents V-104, GF120918 and pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. Br J Cancer 2000;83:366–74.
[28] Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, Miyake K, Resau JH, Bates SE. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci 2000;113:2011–21.
[29] Walther W, Stein U, Pfeil D. Gene transfer of human TNF alpha into glioblastoma cells permits modulation of mdr1 expression and potentiation of chemosensitivity. Int J Cancer 1995;9:832–9.
[30] Bailly JD, Pourquier P, Jaffrezou JP, Duchayne E, Casar G, Bordier C, Laurent G. Effect of 5637-conditionned medium and recombinent cytokines on P-glycoprotein expression in a human GM-CSF-dependent leukemia myeloid cell line. Leukemia 1995;9:1718–25.
[31] Kohno K, Hamanaka R, Abe T, Nomura Y, Morimoto A, Izumi H, Shimizu K, Ono M, Kuwano M. Morphological change and destabilization of beta-actin mRNA by tumor necrosis factor in human microvascular endothelial cells. Exp Cell Res 1993;208:498–503.
[32] Puddu P, Fais S, Luciani F, Gherardi G, Dupuis ML, Romagnoli G, Ramoni C, Cianfriglia M, Gessani S. Interferon-gamma up-regulates expression and activity of P-glycoprotein in human peripheral blood monocyte-derived macrophages. Lab Invest 1999;79:1299–309.