BIOCHEMICAL PHARMACOLOGY 75 (2008) 1649–1658

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Insulin therapy restores impaired function and expression of P-glycoprotein in blood–brain barrier of experimental diabetes

Haiyan Liu [a], *Xiaodong Liu* [a,*], *Lee Jia* [b], *Yuchun Liu* [a], *Huiwen Yang* [a], *Guangji Wang* [a], *Lin Xie* [a]

[a] *Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China*
[b] *Toxicology and Pharmacology Branch, Developmental Therapeutics Program, National Cancer Institute/NIH, Bethesda, MD 20852, USA*

ARTICLE INFO

Article history:
Received 3 December 2007
Accepted 14 January 2008

Keywords:
Blood–brain barrier
Insulin
P-glycoprotein
Diabetes mellitus
mdr1a/mdr1b mRNA

ABSTRACT

We aimed to investigate effects of insulin on function and expression of P-glycoprotein (P-GP) in the blood–brain barrier of streptozotocin (STZ)-induced diabetic rats. Brain-to-plasma concentration ratio of vincristine (VCR) in rats was used as an indicator of in vivo function of P-GP. Western blot and quantitative real time-polymerase chain reaction were used to determine protein levels of P-GP and its *mdr1a/mdr1b* mRNA levels, respectively, in cerebral cortex of rats. In vitro effects of insulin on function and expression of P-GP in primarily cultured rat brain microvessel endothelial cells (rBMECs) were evaluated using rhodamine 123 (Rho123) uptakes and Western blot, respectively. The results showed that 3- and 5-week insulin treatment alleviated the impaired efflux function, expression and *mdr1a/mdr1b* mRNA levels of P-GP in cerebral cortex of diabetic rats. The 3- and 5-week insulin treatments also significantly enhanced P-GP levels and *mdr1a/mdr1b* mRNA levels in the cerebral cortex of normal rats. Addition of insulin to the insulin-deficient diabetic rat serum normalized the impaired function and expression of P-GP in rBMECs cultured in diabetic rat serum. When incubated with normal culture medium containing different levels of insulin, the rBMECs exhibited the enhanced P-GP levels and the reduced Rho123 uptake in a concentration-dependent manner. So we may conclude that appropriate level of insulin plays an important role in maintaining the normal function of BBB through regulating the function and expression of P-GP in the diabetic and normal rats.

1. Introduction

The impaired function of the endothelial barrier is an early feature of diabetic microangiopathy. In brain, the outcome (hemorrhagic or occlusive stroke, etc.) [1,2] of diabetes mellitus (DM) often correlates with changes in the function and integrity of blood–brain barrier (BBB) [3], which is constituted by the tight junction and brain microvessel

* *Corresponding author*. Tel.: +86 25 8327 1006; fax: +86 25 8530 6750.
E-mail address: xdliu@cpu.edu.cn (X. Liu).

Abbreviations: AGEs, advanced glycation end-products; BBB, blood–brain barrier; DM, diabetes mellitus; FBG, fasting blood glucose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hank's balance salt solution; MDR, multidrug resistance; P-GP, P-glycoprotein; Q-PCR, quantitative real time-polymerase chain reaction; rBMEC, rat brain microvessel endothelial cells; Rho123, rhodamine 123; STZ, streptozotocin; VCR, vincristine.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.01.004

endothelium [4]. Predominately located in the apical membranes of brain microvessel endothelium [5,6], kidney tubules, intestinal epithelium and cancer cells [7], P-glycoprotein (P-GP) is a member of the highly conserved superfamily of ATP-binding cassette transporter proteins. It is a 170-kDa glycoprotein encoded by *MDR1* and *MDR2* in humans and *mdr1a*, *mdr1b*, and *mdr2* in rodents although only *MDR1*, and *mdr1a*/*mdr1b* are able to confer the multidrug resistance (MDR) phenotype [8]. The polarized expression of P-GP is indicative of its role in controlling intestinal drug absorption [7,9] and is considered as a natural detoxification system in BBB and intestine [8]. Impairment of P-GP can result in dysfunction of central nervous system or lead to increased brain intoxication by drugs and toxins, resulting in neurotoxicity, or fundamentally altered pharmacological effects of the drugs on the central nervous system [10–13]. Kamei et al. reported that P-GP levels were significantly decreased in the whole cerebral cortex of diabetic mice [14]. Van Waarde et al. reported that hepatic gene and protein expression of *mdr*2 in the STZ-induced diabetic rats were changed [15]. These studies suggest that the function and expression of P-GP in BBB of the STZ-induced diabetic rats may be affected. Many components in the diabetic rat serum, including hormones and cell factors, become abnormal [16]. These abnormal factors may contribute to the impairment of the function and expression of P-GP in BBB of the diabetic rats. Insulin deficiency is a significant feature of DM and insulin treatment has been demonstrated to improve the DM symptoms. This suggests that insulin may be a potential factor regulating the function and expression of P-GP in BBB of the diabetic rats.

In our systematic investigation of the P-GP function and expression in BBB of STZ-induced diabetic rats, we found that there was no significant impairment on the integrity of BBB examined using Evans blue. Electron microscopy examination confirmed no significant damage occurred in the microvascular endothelium in the cerebral cortex of the STZ-induced diabetic rats. However, the protein level of P-GP in the cerebral cortex was significantly decreased in the diabetic rats [17]. As a result of the attenuated function and expression of P-GP in BBB of STZ-induced diabetic mice, phenobarbital distribution from blood into cerebral cortex was increased [13]. Since insulin is the most effective drug to treat DM, we herein tested the hypothesis that insulin treatment may restore impaired function and expression of P-GP in BBB of the STZ-induced diabetic rats, including changes in P-GP function and P-GP levels, and *mdr1a*/*mdr1b* mRNA levels in brain of rats. Although in vitro experimental models cannot resemble the complexity of a whole organism [18], their simplicity provides the ability to specifically manipulate and analyze single parameters. Therefore, in vitro effects of insulin on the P-GP function and expression were also examined in primarily cultured rat brain microvessel endothelial cells (rBMECs).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 180–220 g, were supplied by Center of Experimental Animals, China Pharmaceutical University. The rats were housed under the controlled environmental conditions (temperature, 23 ± 1 °C; humidity, $55 \pm 5\%$) with free access to standard laboratory diet and water. The studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

2.2. Diabetic induction and measurements of blood glucose and insulin in rats

The diabetic rats were induced by a single administration of STZ (i.p., 65 mg/kg). STZ (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in sodium citrate buffer (pH 4.5). The STZ-induced hyperglycemic state was considered as the type 1 DM model [14]. The age-matched control rats were injected with the vehicle. The fasting blood glucose (FBG) and insulin levels were measured using the reagent kit and ELISA kit (Jiancheng Biotech Co., Nanjing, China), respectively, on the 3rd day and thereafter as needed following injection of STZ or the vehicle. Rats with the FBG level higher than 11.1 mM [13] induced by STZ were considered diabetic.

2.3. Insulin administration to the diabetic and normal rats and sample schedules

Sixty rats were involved in the experiment and 20 rats were randomly chosen as age-matched normal control rats. The STZ-induced diabetic rats were randomly and equally divided into two groups: untreated diabetic rats and insulin-treated diabetic rats. Insulin treatment was initiated after confirmation of DM in the rats on the 3rd day. Insulin (40 U/ml), purchased from Wanbang Pharmaceutical Co. (Xuzhou, China), was administered to the rats at 10 U/kg/day (s.c., b.i.d.) for consecutive 3 and 5 weeks. The untreated diabetic rats and age-matched normal control rats only received saline. By the end of the 3-week treatment, 10 rats from each group were randomly chosen for measuring the P-GP function and P-GP levels as well as the *mdr1* mRNA levels following determination of the FBG and insulin levels. The rest continued to be treated for another 2 weeks, and were then subject to the same experiments as mentioned above.

In parallel to the treatment of the diabetic rats with insulin for 3 and 5 weeks, 40 normal rats divided into the insulin-treated and the untreated normal groups were subject to the same schedules for insulin administration and measurements of P-GP function, P-GP levels and *mdr1* mRNA levels.

2.4. Administration of vincristine (VCR) and determination of its levels in rat cerebral cortex and plasma

In order to measure the function of P-GP in the cerebral cortex of the rats, typical P-GP substrate VCR (Hangzhou Minsheng Pharmaceutical Co., Hangzhou, China) (3 mg/kg, i.v.) was administered to the experimental rats [17]. One hour after intravenous administration, the rats were sacrificed under light ether anesthesia, and whole cerebral cortex and blood samples were obtained. The concentrations of VCR in plasma and cerebral cortex were determined using the LC–MS method [17,19], the lowest limits of quantitation of VCR in cerebral cortex and plasma were 6.5 ng/g brain and 31.2 ng/ml, respectively.

Table 1 – Primer characteristics of *mdr1a*, *mdr1b* and *GAPDH*

Gene	GeneBank	Amplicon (bp)	Sequence forward	Sequence reverse
mdr1a	AF257746	221	5′-GCCCTGTTCTTGGACTGT-3′	5′-GGCCGTGATAGCTTTCTT-3′
mdr1b	AY082609	295	5′-GCCCATCCTGTTTGACTG-3′	5′-CGCTTCCTGGACGACCTT-3′
GAPDH	NM_002046	191	5′-AACGACCCCTTCATTGAC-3′	5′-TCCACGACATACTCAGCAC-3′

2.5. Q-PCR analysis of mdr1 mRNA

Q-PCR was used to measure *mdr1a*/*mdr1b* mRNA levels in rat cerebral cortex. The experimental rats were sacrificed under ether anesthesia and the brains were quickly removed. The cerebral cortex, weighing 50 mg, was homogenized under ice-cold condition. The Q-PCR procedure was conducted as previously described [12]. Briefly, 2 μg of total RNA from each original sample was converted into cDNA for each individual Q-PCR assay in a 38-cycle three-step PCR using the ABI Prism 7000 thermocycler. PCR primer sequences (Yingjun Biotech, Shanghai, China) were shown in Table 1. Amplification was performed in 20 μl reaction mixture: 2.0 μl of 10 × PCR buffer, 2.0 μl of 25 mM $MgCl_2$, 0.4 μl of 10 mM deoxyribonucleoside triphosphate (Shenneng Bocai Biotech Co., Shanghai, China), 250 nM of the appropriate forward and reverse primes (*mdr1* and *GAPDH*), and SYBR green I (Molecular Probes, OR, USA). For normalization of the gene levels, GAPDH was used to correct minor variations in the input RNA amount or inefficiencies of the reverse transcription. The results were calculated according to Applied-Biosystems [20].

2.6. Measurement of P-GP in rat cerebral cortex and rBMECs by Western blot

Western blot was used to measure P-GP levels in rat brain and the rBMECs. The experimental rats were sacrificed under ether anesthesia and the brains were quickly removed. The cerebral cortex weighing 300 mg was homogenized in ice-cold cell lysis buffer composed of 10 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM mercaptoethanol, 1% glycerol, and protease inhibitor cocktail (1 mM dithiothreitol and 2 mM phenylmethylsulfonylfluoride purchased from Sigma Chemical Co., St. Louis, MO, USA). The rBMECs, after various treatments (see the following sub-section), were rapidly pelleted by centrifugation and lysed using the ice-cold cell lysis buffer. An aliquot containing 25 μg of proteins was loaded onto each individual land on 8% SDS-polyacrylamide gel for electrophoresis separation and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked by a 60-min incubation of the nitrocellulose membranes in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and 5% of dried skim milk (blocking buffer). The membrane was incubated overnight at 4 °C with the primary monoclonal antibody C219 (Calbiochem-Novabiochem, Seattle, WA, USA) diluted 200-fold with PBS containing 0.1% Tween-20. After removal of the primary antibody, the membranes were washed with blocking buffer and incubated for 1 h at room temperature with goat anti-mice secondary antibody conjugated with the appropriate horse radish peroxidase (dilution: 1:5,000). The membranes were washed again three times in PBS containing 0.1% Tween-20. The P-GP bands were visualized using an enhanced chemiluminescence Western blotting analysis system. The relative expression was quantified densitometrically by using the software Quantity One (Bio-Rad Labs, Richmond, CA, USA) and calculated based on the density of the reference band β-actin (Boshide Biotech Co., Wuhan, China).

2.7. Culturing and treatments of rBMECs

Isolation and culture of the rBMECs were conducted according to methods previously described [21,22]. Briefly, the cells monolayer were preincubated with diabetic rat serum, control rat serum, or the same normal culture medium with increasing concentrations of insulin for additional 72 h before measurement of the uptake of rhodamine 123 (Rho123) by the cells and their P-GP expression using Western blot, respectively. The serum samples obtained from the 5-week STZ-induced diabetic rats and the age-matched rats showed the insulin levels at 39.0 ± 2.7 and 55.9 ± 3.1 mU/l ($n = 5$), respectively.

2.8. Rho123 uptake by rBMECs

Uptake experiments were performed according to previous method [17,25]. Briefly, the cultured rBMECs were preincubated at 37 °C in 1 ml Hanks' balanced salt solution (HBSS) for 30 min. Then the solution was replaced with HBSS (1 ml) containing Rho123 (0.1 μg/ml). After 120-min incubation, the uptake reaction was terminated by adding 1 ml of cold HBSS. The cells were washed with 1 ml of ice-cold HBSS for three times, 0.5 ml of purified water was added in each incubated well, frozen and melted repeatedly three times to break down cells. Amount of Rho123 in cells was measured using HPLC equipped with a fluorescence detector [23,24]. The lowest limit of quantitation of Rho123 in cells was 9.75 ng/mg protein. The protein content was measured by the method of Bradford [25] using BSA as the standard. Net uptake of Rho123 was expressed as the retained amount in cells (μg/mg protein).

2.9. Statistical analysis

Means are reported with their standard deviation (S.D.) and *n*. Two groups were compared by a *t* test. More than two groups were compared by ANOVA followed by a Newman–Keuls post hoc test.

3. Results

3.1. Effects of insulin treatment on diabetic rats and their P-GP function and expression and mdr1 mRNA in cerebral cortex

As expected, a single administration of STZ to the rats produced diabetic symptoms, including polyuria, polydipsia, polyphagia, loss of body weight, and significant increase in

Table 2 – Effects of insulin treatment on body weight, blood glucose and insulin of diabetic rats

3-Week treatment	Body weight (g)	Blood glucose (mM)	Insulin (mU/l)
Control	273 ± 13.6	6.1 ± 1.4	51.0 ± 3.2
Diabetic	212 ± 21.2**	19.5 ± 2.2**	41.1 ± 1.8**
Diabetic + insulin	225 ± 20.4##**	5.2 ± 1.5##	54.9 ± 6.9##
5-Week treatment	**Body weight (g)**	**Blood glucose (mM)**	**Insulin (mU/l)**
Control	319 ± 10.7	6.1 ± 1.4	55.9 ± 3.1
Diabetic	198 ± 16.3**	23.2 ± 2.7**	39.0 ± 2.7**
Diabetic + insulin	301 ± 10.2##**	4.7 ± 0.8#	59.5 ± 2.6##

The STZ-induced diabetic rats were treated with insulin (10 U/kg/day, s.c, b.i.d) for consecutive 3 or 5 weeks. The age-matched control rats and the untreated diabetic rats only received saline. The parameters were measured 8 h following injection of insulin. Each value represents the mean ± S.D. of five to six rats. **$P < 0.01$ vs. the control values; ##$P < 0.01$ vs. diabetic values by the ANOVA statistics.

blood glucose ($P < 0.01$) and decrease in the intrinsic insulin. Treatment of the diabetic rats with extrinsic insulin for consecutive 3 or 5 weeks restored the levels of blood glucose and insulin in the diabetic rats to those in the control rats (Table 2).

The brain-to-plasma concentration ratio of VCR in the 3-week and 5-week STZ-induced diabetic rats was significantly higher than that in the age-matched control rats, respectively (Table 3), indicating P-GP function impairment in rat cerebral cortex. At 1 h following i.v. administration of VCR, plasma concentration of VCR in diabetic rats significantly decreased. Whereas, the cerebral cortex concentrations of VCR significantly increased compared with those in the age-matched rats. Three-week and five-week treatment with insulin not only alleviated the diabetic syndrome as a whole, but also reversed the alterations in VCR concentrations in plasma and brain and decreased transport of VCR across the cerebral cortex in diabetic rats. As a result, the brain-to-plasma concentration ratio of VCR in the insulin-treated diabetic rats became close to that in the age-matched control rats. The results suggest that the function of P-GP in the cerebral cortex of the diabetic rats is impaired, and the 3-week treatment with insulin reverses the impairment.

In parallel, the Western blot was used to measure changes in P-GP expression in the cerebral cortex of the diabetic rats. The Western blot revealed a band of 170 kDa, corresponding to the P-GP [14]. The levels of P-GP in the cerebral cortex of the 3-week and 5-week diabetic rats was 73% and 75%, respectively, of that in the age-matched control rats, indicating a significant reduction in P-GP expression in the diabetic rats ($P < 0.01$; Fig. 1). Insulin treatment significantly increased P-GP expression in the diabetic rats, and brought P-GP levels in the 3-week and 5-week diabetic rats to 87% and 89%, respectively, of that in the age-matched control rats (Fig. 1).

In addition, Q-PCR was used to measure changes in *mdr1a*/*mdr1b* mRNA levels in the cerebral cortex of the same rats after diabetic induction by STZ and investigate the effect of insulin on the mRNA regulation. The *mdr1a*/*mdr1b* mRNA levels were regulated differently by the STZ-induced diabetes and insulin treatment. The *mdr1a* mRNA levels in the cerebral cortex of the 3-week and 5-week diabetic rats were significantly decreased to 41.1% and 59.2%, respectively, of that in the age-matched control rats. Insulin treatment for consecutive 3 and 5 weeks significantly restored the *mdr1a* mRNA to the levels closed to normal. On the other hand, 3 weeks after the diabetic induction the *mdr1b* mRNA level of the diabetic rats was significantly decreased to 6.8% of that of the age-matched control rats. Insulin treatment for 3 weeks significantly restored the *mdr1b* mRNA to the level closed to normal. However, the *mdr1b* mRNA level in the 5-week diabetic rats

Table 3 – Effects of 3-week and 5-week insulin treatment on VCR distribution in plasma and brain of the STZ-induced diabetic rats

3-Week treatment	Control	Diabetic	Diabetic + insulin
Plasma level (ng/ml)	741.9 ± 46.7	436.3 ± 114.5**	520.0 ± 76.5**
Brain level (ng/g brain)	15.0 ± 1.6	18.3 ± 2.6*	14.3 ± 3.0#
Brain-to-plasma concentration ratio (ml/g brain)	0.020 ± 0.001	0.041 ± 0.009**	0.028 ± 0.008#
5-Week treatment	**Control**	**Diabetic**	**Diabetic + insulin**
Plasma level (ng/ml)	604.8 ± 117.6	273.8 ± 47.4**	550.8 ± 50.8##
Brain level (ng/g brain)	17.7 ± 3.4	16.8 ± 1.3	14.9 ± 3.1
Brain-to-plasma concentration ratio (ml/g brain)	0.030 ± 0.005	0.062 ± 0.009**	0.027 ± 0.004##

The rats were treated with insulin (10 U/kg/day, s.c, b.i.d) for consecutive 3 weeks and 5 weeks. The age-matched control rats and untreated diabetic rats only received saline. The concentrations of VCR in plasma and cerebral cortex were measured at 1 h after injection of VCR (3 mg/kg, i.v.). Each value represents the mean ± S.D. of five to six rats. *$P < 0.05$, **$P < 0.01$ vs. the control values; #$P < 0.05$, ##$P < 0.01$ vs. the diabetic values using the ANOVA statistics.

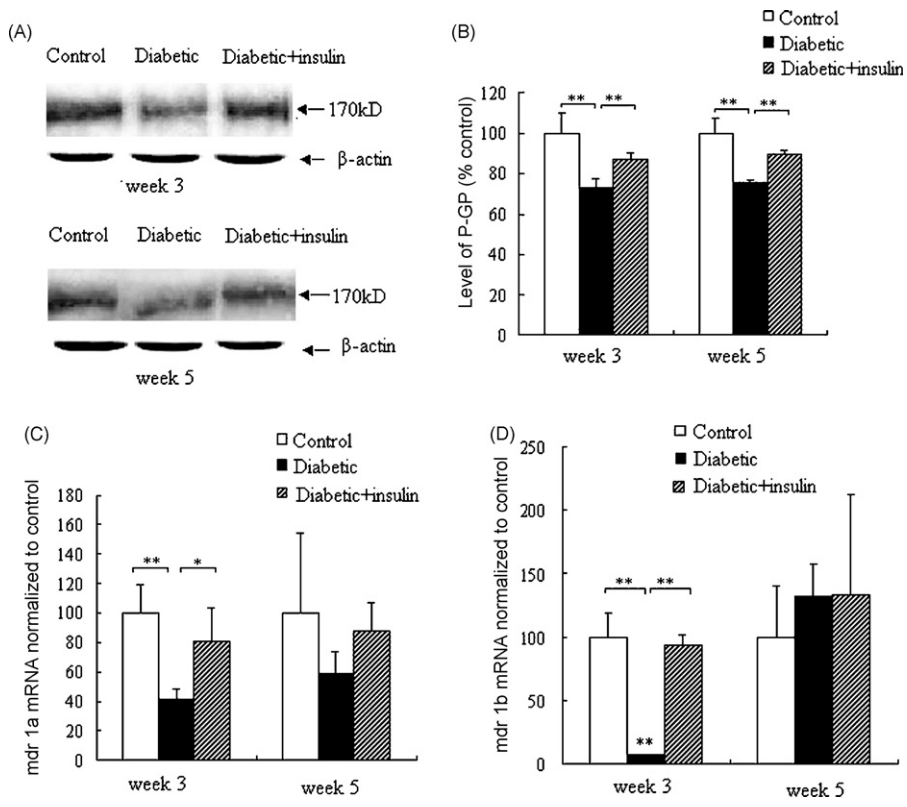

Fig. 1 – Effects of insulin treatment on P-GP levels and *mdr1a/mdr1b* mRNA levels in the cerebral cortex of the STZ-induced diabetic rats. The treated diabetic rats received insulin (10 U/kg/day, s.c., b.i.d.) for consecutive 3 or 5 weeks. Age-matched control rats and diabetic rats only received normal saline. Representative Western blot stains of P-GP (A), relative stain intensity for P-GP (B), *mdr1a* (C) and *mdr1b* mRNA (D) levels in the rat cerebral cortex of the treatment group are presented in comparison with those of the age-matched control rats. Each data represents mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ using ANOVA statistics.

increased, rather than decreased, to 132% of that in the age-matched control rats. The *mdr1b* mRNA level in the 5-week insulin-treated rats was similar to that in the untreated diabetic rats (Fig. 2). The Q-PCR results indicated that changes in the *mdr1a* were more correlated to the changes in P-GP function and expression induced by DM and treated with insulin in the same rats, while changes in the *mdr1b* by the end of the 5-week treatment were less correlative with the changes in P-GP function and expression under the same conditions.

3.2. *Effects of insulin on P-GP function and expression and mdr1 mRNA in cerebral cortex of normal rats*

One hour after a single i.v. administration of VCR to the normal rats given insulin for consecutive 3 or 5 weeks, plasma concentrations of VCR in the insulin-treated normal rats were significantly higher than those in the untreated normal rats. Whereas, cortex concentrations of VCR in the insulin-treated normal rats were significantly lower than those in the untreated normal rats (Table 4). As a result, the brain-to-plasma concentration ratio of VCR in the insulin-treated normal rats appeared to be significantly lower than that in the untreated normal rats, suggesting that long-term administration with insulin enhances the P-GP function.

The Western blot results further demonstrated that the P-GP expression in the cerebral cortex of normal rats given insulin for 3 or 5 weeks was significantly increased to about 260% and 200% of untreated normal rats, respectively ($P < 0.01$; Fig. 2). In addition, Q-PCR results showed that the *mdr1a/mdr1b* mRNA levels in the cerebral cortex were regulated differently by the insulin treatment: the *mdr1a* mRNA levels in the cerebral cortex of the normal rats treated with insulin for 3 and 5 weeks were induced to 120% and 130%, respectively, of those in the untreated normal rats. It was somewhat surprising that 3-week insulin treatment only induced *mdr1b* to an increase of 110%. However, 5-week insulin treatment resulted in an increase in *mdr1b* to 560% of the basal value of the untreated normal rats (Fig. 4). These results verified that insulin up-regulates the P-GP function and expression as well as *mdr1a/mdr1b* mRNA levels in the cortex of normal rats.

3.3. *Effects of insulin on P-GP function and expression in rBMECs*

When rBMECs were incubated with diabetic rat serum that was insulin-deficient (insulin level, 39.0 ± 2.7 mU/l), the uptake of Rho123 into the rBMECs was enhanced to 125% of that incubated with control rat serum (insulin level,

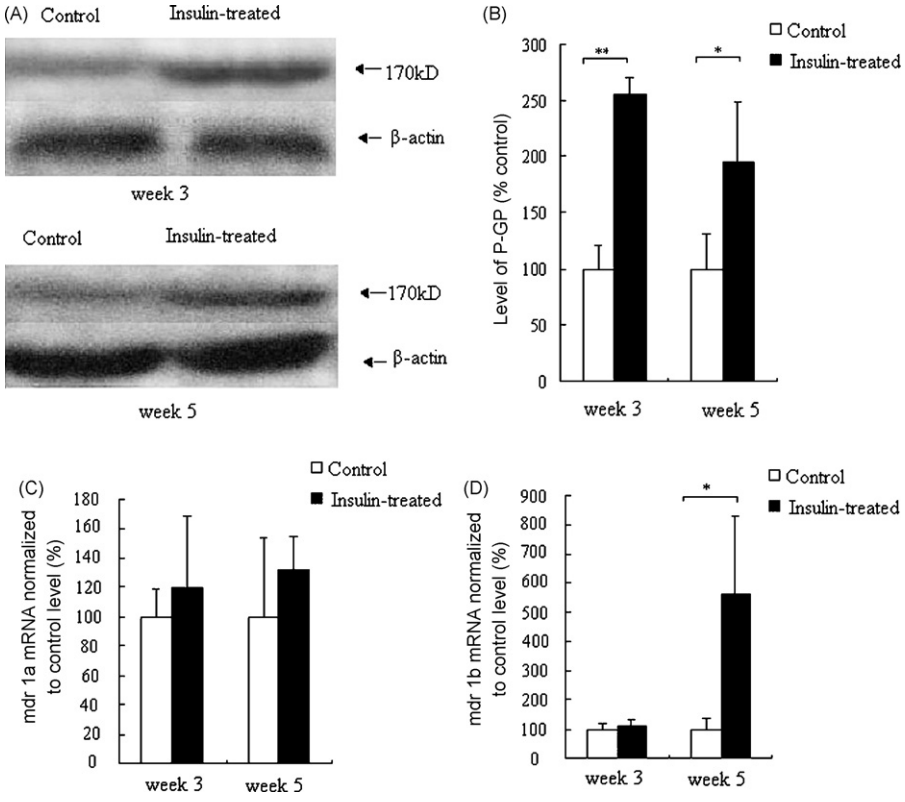

Fig. 2 – Effect of insulin treatment on P-GP levels and *mdr1a/mdr1b* mRNA level in cerebral cortex of normal rats. The insulin-treated rats received insulin (10 U/kg/day, s.c., b.i.d.) for consecutive 3 or 5 weeks. Untreated rats only received normal saline. Representative Western blot stains of P-GP (A), relative staining intensity for P-GP (B), *mdr1a* (C) and *mdr1b* mRNA (D) levels in the rat cerebral cortex of the treatment group in comparison with that of the untreated rats are presented. Each data represents mean ± S.D. ($n = 3$). *P < 0.05, **P < 0.01 using student's *t*-test.

55.9 ± 3.1 mU/l). The enhanced uptake was correlative with the decreased level of P-GP in the cells. The P-GP level in the cells incubated with diabetic rat serum was only 41.5% of that incubated with the control rat serum. Addition of 25 mU/l of insulin into the diabetic rat serum reversed the down-regulated function and level of P-GP in rBMECs (Fig. 3).

When the cells were incubated in the normal culture medium for 72 h with insulin added at 25, 50 or 100 mU/l, insulin reduced the Rho123 uptake by the cells and up-regulated P-GP levels in the rBMECs in a concentration-dependent manner (Fig. 4). These in vitro data are consistent with the in vivo observations, and they together conclude that insulin enhances activity of the transporter, P-GP.

4. Discussion

The present studies showed that the increased brain-to-plasma concentration ratio of VCR (Table 2) was correlated well to the decreased function and expression of P-GP and *mdr1a/mdr1b* mRNA levels (Fig. 1) in cerebral cortex of the STZ-induced diabetic rats. Treatment of the rats with insulin for 3 and 5 weeks significantly restored the brain-to-plasma

Table 4 – Effects of insulin on VCR distribution in plasma and brain of normal rats

	3-Week treatment		5-Week treatment	
	Untreated	Insulin-treated	Untreated	Insulin-treated
Plasma concentration (ng/ml)	719.6 ± 107.4	1115.6 ± 204.9**	736.4 ± 82.6	893.5 ± 97.0*
Brain concentration (ng/g brain)	28.7 ± 9.4	20.4 ± 4.7	30.2 ± 3.8	22.6 ± 5.6*
Brain-to-plasma concentration ratio (ml/g brain)	0.039 ± 0.01	0.019 ± 0.01**	0.041 ± 0.01	0.025 ± 0.01**

The insulin-treated rats were administered insulin (10 U/kg/day, s.c., b.i.d) for consecutive 3 or 5 weeks. Untreated rats only received saline. The concentrations of VCR in plasma and cerebral cortex were measured at 1 h after injection of VCR (3 mg/kg, i.v.). Each value represents the mean ± S.D. of five to six rats. *P < 0.05, **P < 0.01 vs. the untreated rats by the Student's *t*-test.

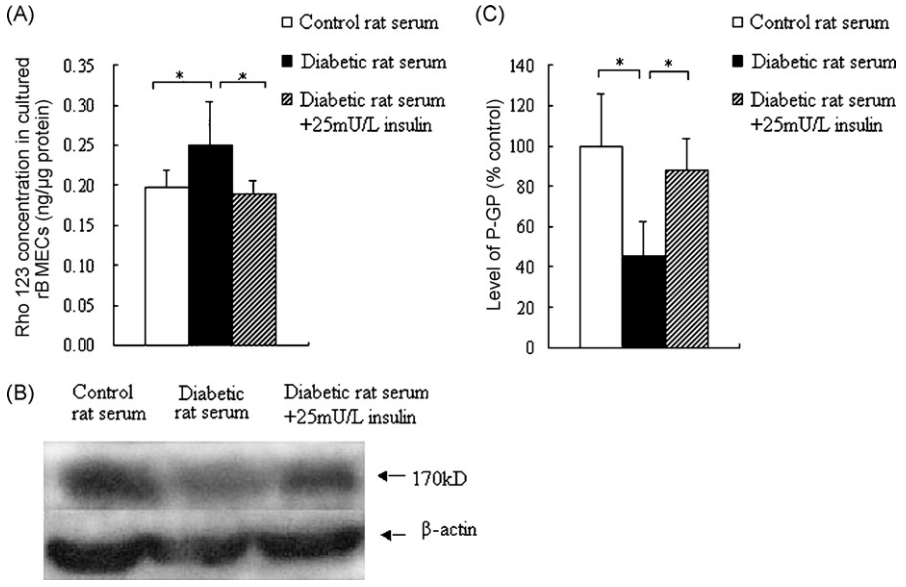

Fig. 3 – Effects of insulin on Rho123 uptake by rBMECs and P-GP levels in cells incubated with diabetic rat serum. The rBMECs were incubated for 72 h in control rat serum, diabetic rat serum and diabetic rat serum with insulin (25 mU/l) added, respectively. Uptake of Rho123 by rBMECs (A), representative Western blot stains of P-GP (B) and P-GP levels in the cells (C). Each data represents mean ± S.D. (n = 3 or 5). *$P < 0.05$ using ANOVA statistics.

concentration ratio of VCR and reversed the impaired P-GP expression and *mdr1a/mdr1b* mRNA levels in comparison with the untreated diabetic rats.

VCR is a typical substrate of P-GP, and is often used to evaluate the function of P-GP in vivo [26,27]. The brain-to-plasma concentration ratio of a drug is indicative of the degree of a drug's penetration across BBB. In this study, it was found that the brain-to-plasma concentration ratio of VCR in the STZ-induced diabetic rats was significantly higher than that in the age-matched control rats. It was noted that the concentration of VCR in the diabetic rat plasma was significantly lower than that in control rat plasma. This changed hepatic CYP450 level or changed renal clearance of VCR might be reasons that the concentration of VCR in plasma was changed in the STZ-induced diabetic rats and insulin-treated rats. But it was unclear and needed further investigation.

To our knowledge, this was the first report showing that in the diabetic rats, the *mdr1a/mdr1b* mRNA was affected differently. In the cerebral cortex of the 3-week diabetic rats, both *mdr1a/mdr1b* mRNA levels were significantly decreased.

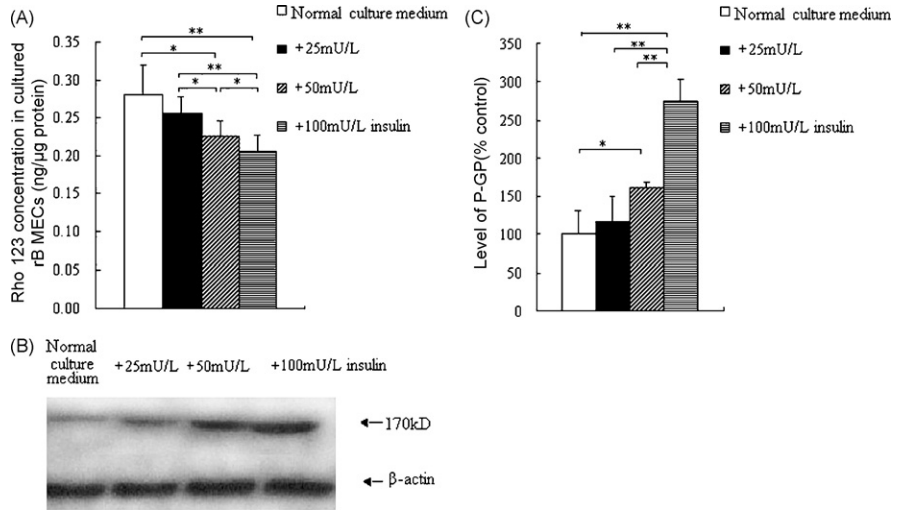

Fig. 4 – Effects of insulin on Rho123 uptake by rBMECs and P-GP levels in the cells. The rBMECs were incubated with increasing concentrations of insulin for 72 h. Uptake of Rho123 by rBMECs (A), representative Western blot stains of P-GP (B), and P-GP levels in the cells (C). Insulin inhibited the uptake and enhanced the function and expression of P-GP in the cells in a concentration-dependent manner. Each data represents mean ± S.D. (n = 3 or 5). *$P < 0.05$, **$P < 0.01$ using ANOVA statistics.

The degree of decrease in *mdr1b* mRNA was more significant than that in *mdr1a* mRNA (Fig. 1). Insulin treatment reversed the decreased levels of both *mdr1a*/*mdr1b* mRNA. However, 5 weeks after the diabetic induction, the *mdr1a* mRNA level in the cerebral cortex of the rats was still low compared with that in the age-matched control rats. Insulin treatment alleviated the low level of *mdr1a* mRNA. It was somewhat surprising that the *mdr1b* mRNA was overexpressed in the brain of the rats 5 weeks after the diabetic induction. The *mdr1b* mRNA level was 132% of that in the cerebral cortex of age-matched control rats. The mechanism of the change in either *mdr1* isoform was unknown although it was proposed that the alteration in the *mdr1b* might compensate for the change in the *mdr1a* [12]. In addition to their common action as a drug efflux transporter, individual *mdr1* isoform appeared to have its specific function: the *mdr1a* product had been proposed to regulate cell volume by influencing swelling activated chloride currents via a Protein kinase C sensitive phosphorylation site on P-GP [28], while the *mdr1b* was involved in apoptotic mechanisms [29] and cellular stress [30,31]. However, these points were still controversial. Some studies suggested that expression of the *mdr1* contribute to neuroprotection because a link was found between loss of proapoptotic protein p53 and expression of *mdr1a*/*mdr1b* [32,33]. The exact location of each *mdr1* isoform in the normal brain was not completely known although it was shown that *mdr1a* appeared to be preferentially expressed in the brain microvessel endothelium of BBB [34] and the *mdr1b* was mainly expressed in the cerebral cells (astrocytes or neurons) [35] and hippocampus [36]. In the STZ-induced diabetic rats, the *mdr1a* expression in the cerebral cortex was impaired, and the impairment was in line with the low P-GP levels (Fig. 1) and the high brain-to-plasma concentration ratio of VCR in the diabetic rats (Table 4). Although the expression of *mdr1b* gene was altered in the brain of the diabetic rats, the alteration was not correlated with the low P-GP levels and the high brain-to-plasma concentration ratio of VCR. Taken together, these results suggest that it is the impaired expression of the *mdr1a*, rather than the *mdr1b*, that cause the down-regulation of P-GP function and expression. It was reported that insulin-induced *mdr1b* expression by a NF-κB-mediated pathway in rat hepatoma cells [37]. Insulin might also affect the message levels of P-GP in vitro cultured rBMECs as well, and there might be effects on protein turnover and/or function as well, which needed further investigation.

It was also found that insulin administration for 3 and 5 weeks increased both P-GP and *mdr1a*/*mdr1b* mRNA levels not only in the cerebral cortex of the STZ-induced diabetic rats, but also in that of the normal rats (Fig. 2). In the parallel studies conducted in the same normal rats, the insulin administration decreased VCR levels in the cerebral cortex, resulting in a significant decrease in the brain-to-plasma concentration ratio of VCR compared with the control data ($P < 0.01$; Table 4). These results obtained from the direct measurement of P-GP function and VCR levels in the same rats suggest that insulin, by up-regulating the P-GP function, inhibits the penetration of VCR in blood across BBB into the brain.

The *in vitro* studies with rBMECs were used to further verify the effect of insulin on P-GP function and expression using the typical substrate Rho123 uptake and Western blot, respectively. The results showed that the function and expression of P-GP in the rBMECs were down-regulated by the diabetic rat serum that contained the low level of insulin (Fig. 3). Addition of insulin into the serum brought the Rho123 uptake and P-GP expression to the control levels, indicating the recovery of the P-GP function of the cells from insulin deficiency (Fig. 3). On the other hand, when the rBMECs were cultured in the normal culture medium, insulin decreased of Rho123 uptake by the cells and increased P-GP level in a concentration-dependent manner (Fig. 4), suggesting the up-regulation by insulin of the P-GP in the cells. The in vitro results verified the in vivo observations that long-term treatment with insulin for the diabetic rats and normal rats strengthened the function of P-GP in BBB.

Recent evidence indicates that insulin is transported through the blood–brain barrier and influences brain function via widely distributed insulin receptors on neurons [38]. Kondo reported that mice lacking insulin or insulin-like growth factor 1 receptors in vascular endothelial cells maintained normal blood–brain barrier, neither insulin nor IGF-1 signaling in vascular endothelial cells was required for development and maintenance of BBB [39]. So the changed permeability of P-GP substrates might not be influenced by insulin, but the changed function and expression of P-GP. Our observations posed a concern about the ''physiological'' regulatory roles of insulin on P-GP expression and function, and appropriate level of insulin might play an important role in maintaining the normal function of BBB through regulating the function and expression of P-GP in the diabetic and normal rats.

High levels of advanced glycation end-products (AGEs) [40] and glucose are significant features of DM. Although Thomas et al. reported that AGEs reduced organic cation transport in the kidney of the experimental DM [41], our treatment of the rBMECs incubated for 72 h with either high glucose (5.5 and 30 mM) or AGEs (6.5, 13, 38 and 76 AU/ml) did not show differences on uptake of Rho 123 by the cells (data not shown). These results indicated that neither high glucose nor high AGEs were a factor causing impairment of P-GP in rBMECs. In addition to insulin, other factors in diabetic rat serum may also impair the function and expression of P-GP in the rBMECs. These factors are produced by immunological and inflammatory reactions under the diabetic conditions and they are reported to down-regulate the P-GP function and expression as well [42,43].

In summary, the present studies revealed that the up-regulatory effects of insulin on the P-GP function and expression in both the in vivo and in vitro settings of the diabetic and normal models, and demonstrated the importance of appropriate levels of insulin in maintaining the normal function of BBB through regulation of P-GP, a natural detoxification system.

Acknowledgements

The work was supported by the National Natural Sciences Foundation of China (No. 30672499) and the Project of Innovation in Graduate Education, Jiangsu Province (No. 2006).

REFERENCES

[1] Hattori Y, Hattori S, Sato N, Kasai K. High-glucose-induced nuclear factor kB activation in vascular smooth muscle cells. Cardiovasc Res 2000;46:188–97.
[2] Cohen RA. Dysfunction of vascular endothelium in diabetes mellitus. Circulation 1993;87(Suppl. V):V67–76.
[3] Mooradian AD. Central nervous system complications of diabetes mellitus—a perspective from the blood–brain barrier. Brain Res Rev 1997;23:210–8.
[4] Jia L, Wong H. In vitro and in vivo assessment of cellular permeability and pharmacodynamics of S-nitrosylated captopril, a nitric oxide donor. Br J Pharmacol 2001;134:1697–704.
[5] Sun HY, Dai HQ, Shaik N, Elmquist WF. Drug efflux transporters in the CNS. Adv Drug Deliv Rev 2003;55:83–105.
[6] Kusuhara H, Sugiyama Y. Efflux transport systems for drugs at the blood–brain barrier and blood–cerebrospinal fluid barrier. Drug Discov Today 2001;6:150–6.
[7] Jia L, Schweizer J, Wang Y, Cerna C, Wong H, Revilla M. Effect of nitric oxide on cytotoxicity of Taxol: enhanced Taxol transcellular permeability. Biochem Pharmacol 2003;66:2193–9.
[8] Ambudkar SV, Dey S, Hrycyna CA, Ranmachandra M, Pastan I, Gottesman MM. Biochemical, cellular and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 1999;39:361–98.
[9] Jia L, Wong H, Wang Y, Garza M, Weitman SD. Carbendazim: disposition, cellular permeability, metabolite identification and pharmacokinetic comparison with its nanoparticle. J Pharm Sci 2003;92:161–72.
[10] Saunders NR, Habgood MD, Dziegielewska KM. Barrier mechanisms in the brain. I. Adult brain. Clin Exp Pharmacol Physiol 1999;26:11–29.
[11] Schinkel AH. P-glycoprotein, a gatekeeper in the blood–brain barrier. Adv Drug Deliv Rev 1999;36:179–94.
[12] Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, et al. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs. Cell 1994;77:491–502.
[13] Liu HY, Zhang DM, Xu X, Liu XD, Wang GJ, Xie L, et al. Attenuated function and expression of P-glycoprotein at blood–brain barrier and increased brain distribution of phenobarbital in streptozotocin-induced diabetic mice. Eur J Pharmacol 2007;561:226–32.
[14] Kamei J, Hirano S, Miyata S, Saito A, Onodera K. Effects of first- and second-generation histamine-H1-receptor antagonists on the pentobarbital-induced loss of the righting reflex in streptozotocin-induced diabetic mice. J Pharmacol Sci 2005;97:266–72.
[15] van Waarde WM, Verkade HJ, Wolters H, Havinga R, Baller J, Bloks V, et al. Differential effects of streptozotocin induced diabetes on expression of hepatic ABC-transporters in rats. Gastroenterology 2005;122:1842–2185.
[16] Girach A, Vignati L. Diabetic microvascular complications—can the presence of one predict the development of another? J Diabetes Complicat 2006;20:228–37.
[17] Liu HY, Xu X, Yang ZH, Deng YX, Liu XD. Impaired function and expression of P-glycoprotein in blood–brain barrier of streptozotocin-induced diabetic rats. Brain Res 2006;1123:245–52.
[18] Jia L, Liu XD. The conduct of drug metabolism studies considered good practice (II): in vitro experiments. Curr Drug Metab 2007;8:807–18.
[19] Guo P, Wang XM, Zhou F, Gallo JM. Determination of vincristine in mouse plasma and brain tissues by liquid chromatography–electrospray mass spectrometry. J Chromatogr B Biomed Sci Appl 2004;809:273–8.
[20] Applied-Biosystems. Relative quantification of gene expression user bulletin #2: ABI PRISM 7700 sequence detection system. 1997.
[21] Sun JJ, Xie L, Liu XD. Transport of carbamazepine and drug interactions at the blood–brain barrier. Acta Pharmacol Sin 2006;27:249–53.
[22] Zhang L, Liu XD, Xin L, Wang GJ. P-glycoprotein restricted transport of nimodipine across blood–brain barrier. Acta Pharmacol Sin 2003;24:903–6.
[23] Ando H, Nishio Y, Ito K, Nakao A, Wang L, Zhao YL, et al. Effect of endotoxin on P-glycoprotein mediated biliary and renal excretion of rhodamine-123 in rats. Antimicrob Agents Chemother 2001;45:3462–7.
[24] Zhang DM, Liu HY, Xie L, Liu XD. Effect of baicalin and berberine on transport of nimodipine on primary cultured rat brain microvascular endothelial cells. Acta Pharmacol Sin 2007;28:573–8.
[25] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[26] Perrière N, Demeuse P, Garcia E, Regina A, Debray M, Andreux JP, et al. Puromycin-based purification of rat brain capillary endothelial cell cultures. Effect on the expression of blood–brain barrier-specific properties. J Neurochem 2005;93:279–89.
[27] Watanabe T, Suzuki H, Sawada Y, Naito M, Tsuruo T, Inaba M, et al. Induction of hepatic P-glycoprotein enhances biliary excretion of vincristine in rats. J Hepatol 1995;231:440–8.
[28] Bond TD, Valverde MA, Higgins CF. Protein kinase C phosphorylation disengages human and mouse-1a Pglycoproteins from influencing the rate of activation of swelling activated chloride currents. J Physiol 1998;508(Pt. 2):333–40.
[29] Lecureur V, Thottassery JV, Sun D, Schuetz EG, Lahti J, Zambetti GP, et al. Mdr1b facilitates p53-mediated cell death and p53 is required for mdr1b upregulation in vivo. Oncogene 2001;20:303–13.
[30] Zhou G, Kuo MT. Wild-type p53-mediated induction of rat mdr1b expression by the anticancer drug daunorubicin. J Biol Chem 1998;273:15387–94.
[31] Ziemann C, Burkle A, Kahl GF, Hirsch-Ernst KI. Reactive oxygen species participate in mdr1b mRNA and P-glycoprotein overexpression in primary rat hepatocyte cultures. Carcinogenesis 1999;20:407–14.
[32] Bush JA, Li G. Regulation of the Mdr1 isoforms in a p53 deficient mouse model. Carcinogenesis 2002;23:1603–7.
[33] Marroni M, Agrawal ML, Kight K, Hallene KL, Hossain M, Cucullo L, et al. Relationship between expression of multiple drug resistance proteins and p53 tumor suppressor gene proteins in human brain astrocytes. Neuroscience 2003;121:605–17.
[34] Demeule M, Labelle M, Régina A, Berthelet F, Béliveau R. Isolation of endothelial cells from brain, lung, and kidney: expression of the multidrug resistance P-glycoprotein isoforms. Biochem Biophys Res Commun 2001;281:827–34.
[35] Ballerin P, Di IP, Ciccarelli R, Nargi E, D'Alimonte I, Traversa U, et al. Glial cells express multiple ATP binding cassette proteins, which are involved in ATP release. Neuroreport 2002;13:1789–92.
[36] Kwan P, Sills GJ, Butler E, Gant TW, Brodie MJ. Differential expression of multidrug resistance genes in naïve rat brain. Neurosci Lett 2003;339:33–6.
[37] Zhou G, Kuo MT. NF-κB-mediated induction of mdr1b expression by insulin in rat hepatoma cells. J Biol Chem 1997;272:15174–83.

[38] Kar S, Chabot JG, Quirion R. Quantitative autoradiographic localization of [^{125}I]insulin-like growth factor I, [^{125}I]insulin-like growth factor II, and [^{125}I]insulin receptor binding sites in developing and adult rat brain. J Comp Neurol 1993;15: 375–97.
[39] Kondo T, Hafezi-Moghadam A, Thomas K, Wagner DD, Kahn CR. Mice lacking insulin or insulin-like growth factor 1 receptors in vascular endothelial cells maintain normal blood–brain barrier. Biochem Biophys Res Commun 2004;317:315–22.
[40] Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovas Res 2004;63:582–92.
[41] Thomas MC, Tikellis C, Kantharidis P, Burns WC, Cooper ME, Forbes JM. The role of advanced glycation in reduced organic cation transport associated with experimental diabetes. J Pharmacol Exp Ther 2004;311:456–66.
[42] Tan K. Dyslipidaemia, inflammation and endothelial dysfunction in diabetes mellitus. Int Congr Ser 2004;1262:511–4.
[43] Goralski KB, Hartmann G, Piquette-Miller M, Renton KW. Down-regulation of mdr 1a expression in the brain and liver during CNS inflammation alters the in vivo disposition of digoxin. Br J Pharmacol 2003;139:35–48.