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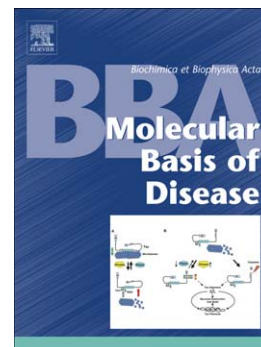
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Increase in P-Glycoprotein Accompanied by Activation of Protein Kinase C α and NF- κ B p65 in the Livers of Rats with Streptozotocin-induced Diabetes

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§ Abbreviations: ABC, ATP-binding cassette; MDR, multidrug resistance;

P-gp, P-glycoprotein; STZ, streptozotocin; MRP, multidrug resistance-

associated protein; OAT, organic anion transporter; OCT, organic cation

transporter; PKC, protein kinase C; NF- κ B, nuclear factor κ B;

I κ B, inhibitory κ B; PCR, polymerase chain reaction.

Abstract

It is known that protein kinase C (PKC) signal transduction is enhanced in a diabetic state, and that PKC activator phorbol esters increase the gene expression of MDR1 in human tumor cells. To clarify the expression of the liver transporters under diabetic conditions and the roles of PKC α and the transcription factor NF- κ B, we investigated the expression levels of Mdr1a, Mdr1b, Mdr2, Mrp2, Bcrp, Bsep, Oct1, Oat2, and Oat3 transporters, PKC α , I κ B, and NF- κ B in the liver of rats with STZ-induced hyperglycemia. A selective increase in the gene expression of Mdr1b was detected by RT-PCR. Western blotting with C219 antibody revealed an increase in P-glycoprotein. Although the mRNA level of PKC α was not affected, translocation of PKC α to the microsomal fraction was detected. NF- κ B p65, I κ B α and I κ B β mRNA levels were increased as was the level of nuclear NF- κ B p65. From these findings, it was suggested that STZ-induced hyperglycemia caused the upregulation of mdr1b P-gp expression through the activation of PKC and NF- κ B.

Key words. Mdr1b, PKC α , NF- κ B p65, I κ B, diabetes, rat, liver, streptozotocin

Running title; Upregulation of P-glycoprotein in STZ-induced diabetes

Introduction

Hepatocytes express various transporter proteins that take up and export xenobiotics and bile components. These transporters include the solute carrier family proteins organic cation transporter 1 (Oat1), organic anion transporter (Oat) 2, and Oat3, and ATP-binding cassette (ABC) transporters, Mdr1a (Abcb1a), Mdr1b (Abcb1b), Mdr2 (Abcb4), Mrp2 (Abcc2), Bcrp (Abcg2), and Bsep (Abcb11). Mdr1a and Mdr1b are homologues of human MDR1 P-glycoprotein (P-gp) which transports xenobiotics such as doxorubicin and cyclosporin A [1]. The Mdr2 P-gp, a homologue of MDR3 in humans, transports phosphatidylcholine into bile. Mrp2 is a transporter of organic anions such as bilirubin glucuronides. Bcrp is known as a transporter of methotrexate, and Bsep is an export pump of bile acids.

Several reports have indicated that the expression of transporters was affected by various chemical substances and pathophysiological conditions [2-5]. In rats with streptozotocin (STZ)-induced diabetes, van Waarde et al. [6] showed that the Mdr2 gene expression and the protein level were increased in the liver and that the secretion of biliary phospholipids was enhanced. Human MDR1 gene expression is well known to be enhanced by various stimulants including antitumor drugs [7] and protein kinase C activator phorbol esters [8, 9]. As for two rat Mdr1 genes, it has been reported that the Mdr1a gene lacked any response to STZ while the Mdr1b gene showed a massive

increase in expression in both the liver and kidney after STZ treatment [10]. The different responses of these two Mdr1 genes have also been seen in LPS-induced endotoxemic rat liver [11] and in doxorubicin-treated rat astrocytes [12], in which only the Mdr1b gene was upregulated. Thus, the Mdr1b gene prefers to respond to external stimulations as in the case of the human MDR1 gene, although whether the P-gp level is affected under diabetic conditions has remained unclear.

It is widely recognized that the PKC signal transduction system is enhanced in the diabetic state [13]. PKC expression is persistently upregulated and this is recognized as an initial event leading to insulin resistance, cardiac disease, and nephropathy in diabetes [14]. It is also indicated that the activation of PKC is involved in the hyperglycemia-induced sustained activation of the transcription factor NF- κ B [15, 16]. PKC consists of a family of at least eleven isoforms, which are categorized into the classic (α , β 1, β 2, γ), the novel (δ , ϵ , η , θ , μ), and the typical (ζ , ι) [17, 18]. Among these isoforms, PKC α activated in kidney cells, is indicated to mediate NF- κ B's activation [19].

In the present study, to clarify the changes in the expression of the liver transporters under diabetic conditions and the role of PKC isoforms, we investigated the levels of various transporters and PKC α in the liver of rats with STZ-induced hyperglycemia. The results indicate that the increased expression of Mdr1b P-gp is

associated with the translocation of the PKC α isoenzyme to membrane. Together with evidence for the activation of NF- κ B, we added a discussion about the system contributing to the up-regulation of Mdr1b P-gp in the diabetic rat liver.

Materials and Methods

Animals.

Male Wistar rats (9 weeks old, 280-300 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and housed in a controlled environment. The rats were kept on a commercial laboratory chow (CRF-1; Charles River Laboratories Japan, Inc., Yokohama, Japan) and had free access to tap water. The STZ-induced diabetes was established with a single intravenous injection of STZ (50 mg /Kg body weight, Sigma) into the tail. The control rats were injected with the vehicle (sodium citrate buffer, pH4.5). On the 7th day after the injection, the increased urinary excretion of glucose was checked with a commercial urine dipstick (Wako Pure Chemicals, Japan) and the next day the rats were killed after starvation for 24 hours. Blood was collected from the aorta abdominalis under anesthesia with an intraperitoneal injection of pentobarbital (40mg/Kg) and the liver was excised after perfusion with a 0.9% NaCl solution from the portal vein. Small pieces of the liver were immersed into Trizol (Invitrogen Japan K.K.)

for the isolation of total RNA, and other parts of the tissue were frozen on dry ice and stored at -80°C until further use. All experiments were performed according to the institutional guidelines for the care and use of laboratory animals in research.

Gene expression studies.

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. Then, cDNA was prepared by incubation of 0.5-1.0 μg of the RNA with random primers (12.5 ng, Invitrogen), RNase inhibitor (RNaseOUT, 20 units, Invitrogen), 0.5 mM deoxynucleotides (dNTPs, Promega, WI), and 100 units of RNA reverse transcriptase (ReverTra Ace, TOYOBO, Japan) in 20 μL of reaction buffer according to the ReverTra Ace data sheet. Following inactivation of the enzyme by incubation at 99°C for 5 min, polymerase chain reaction (PCR) was carried out with 0.625 units of G-Taq Taq DNA polymerase (Hokkaido System Science Co., Japan) in 25 μL of PCR solution (1 μL of cDNA solution, 0.4 μM primers, 0.2 mM dNTPs, and 2.5 μL of 10x PCR buffer) in a Thermal Cycler PxE (Thermo Fisher Scientific, MA). PCR primers (Table 1) were synthesized by Hokkaido System Science. The PCR products (10 μL) were electrophoresed on a 1.5% agarose gel and visualized with ultraviolet light after immersion in an ethidium bromide solution (1 $\mu\text{g}/\text{mL}$) for 15 min. Images were taken with a digital camera C-3030 zoom (Olympus, Japan) equipped with a BPB-60 filter (Fujifilm Japan). Densitometric analysis was performed using Scion Image for

Windows supplied by the Scion Corporation.

Western blot analysis of PKC α and P-gp.

Small pieces of the liver were homogenized with a Potter-Elvehjem homogenizer in 5 volumes of Buffer A containing 10 mM Tris-HCl (pH7.5), 0.1 mM phenylmethylsulfonylfluoride (Sigma), and 1mM dithiothreitol (Sigma). The homogenates were centrifuged at 800 x g for 10min at 4°C and then the supernatant was centrifuged at 15,000 x g for 30 min at 4°C. The 15,000 x g supernatant was further centrifuged at 100,000 x g for 30 min at 4°C to obtain a cytosolic fraction (supernatant) and a microsomal fraction (pellet). The pellet was re-suspended in a small volume of Buffer A. These fractions were used to detect PKC isoforms. The 15,000 x g pellet was solubilized in Buffer A with 1% Triton X100 and centrifuged at 15,000 x g for 30 min at 4°C. The resultant supernatant (Fraction PM) was used for the detection of P-glycoprotein.

The protein concentration was determined by using a DC protein assay kit (Bio-Rad Laboratories, Inc.).

Detection of PKC α : The cytosolic and microsomal fractions were used for the detection of PKC α . Each fraction (20-100 μ g protein) was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel), and the separated proteins were transferred onto an Immobilon-P transfer membrane (Nihon Millipore, Japan).

After blocking with skim milk, the membrane was treated with an isoform-specific antibody for PKC α (Santa Cruz Biotechnology, Inc., CA) followed by horseradish-labeled goat anti-mouse IgG₁ antibody (Santa Cruz Biotechnology, Inc.), and the specific immunoreactive band was detected using an ECL-detection kit (Amersham Japan). Densitometric analysis was performed using Scion Image for Windows.

Detection of P-gp : As was done for the detection of PKC isoforms, Fraction PM was isolated by SDS-PAGE (8% gel) and then P-gp was detected with the C219 anti-MDR1 P-gp mouse monoclonal antibody (Centocor, Inc., Malvern, PA) as a primary antibody and horseradish-labeled goat anti-mouse IgG_{2a} antibody (Santa Cruz Biotechnology, Inc.) as a secondary antibody. The level of P-gp was detected and evaluated as described above.

Western blot analysis of NF- κ B p65

Cytoplasmic and nuclear fractions were prepared according to the report by Kiemer et al.(2000). Each fraction (75-100 μ g protein) was isolated by SDS-PAGE (10% gel), and then NF- κ B p65 was detected with anti-NF- κ B p65 antibody (Cell Signaling Technology, Inc., MA) as a primary antibody and horseradish-labeled goat anti-rabbit IgG antibody (Cell Signaling Technology, Inc.) as a secondary antibody. The level of NF- κ B p65 was detected and evaluated as described above.

Statistical analysis. Data are shown as means \pm S.D. for five animals. Statistical

analyses were performed using Student's t-test.

Results

Phenotype of the control and STZ-induced diabetic rats

On the 8th day after the injection of STZ or vehicle, diabetic rats had a significantly lower body weight than the control rats (Table 2). As the liver weight was not affected by the injection of STZ, the liver weight/body weight ratio was significantly increased. The average fast blood glucose level of STZ-treated rats was 310mg/dL compared to 128mg/dL in control rats. Then, the STZ-treated rats were used as an animal model of diabetes.

Effects on hepatic drug transporters.

As shown in Figure 1(A, B), the semi-quantitative PCR-based analysis of nine transporters revealed the upregulation (2-fold) of Mdr1b mRNA expression in the liver of the diabetic rats. By contrast, Mdr1a was not affected by the STZ-treatment as shown previously [10]. To determine whether the increase in Mdr1b mRNA affects the P-gp level, we conducted a Western blot analysis. As shown in Figure 2, the expression of P-gp was significantly upregulated (2-fold) in the diabetic rat liver. These results indicate that the increase in Mdr1b mRNA level caused by STZ-treatment is large enough to raise the liver concentration of P-gp. Although the mRNA levels of Mdr2, Mrp2, Bcrp, Bsep, Oct1, Oat2, and Oat3 were investigated as shown in Figure 1(A), no detectable

changes were observed. These results indicate that Mdr1b is most sensitive to hyperglycemic conditions among these apical and basolateral transporters in the liver.

Effects on PKC expression.

PKC is a stimulating factor of P-gp expression [8, 9] and is activated in hyperglycemic conditions [13, 14, 20]. Moreover, PKC α and PKC β 2 isoforms have been indicated to be increased in STZ-induced diabetes [21]. However, there are conflicting reports that PKC α levels were lower in the hepatocytes of diabetic rats than normal rats [22] and that the approximately 80kDa PKC α was not increased in the cytosolic or membrane fraction in the diabetic rats while the 90kDa protein was [23]. Then, we investigated the mRNA and protein levels of PKC α as shown in Figure 3. The results showed that PKC α mRNA levels were not affected in the STZ-induced diabetic model, while the Western blot analysis revealed that the 80kDa PKC α protein in the microsomal fraction was significantly increased while in the cytosolic fraction, the PKC α protein level was not affected. These results indicated that the production of PKC α protein was not induced and most of the protein persistently translocated to the membrane in STZ-induced diabetes.

Effects on I κ B and NF- κ B expression.

NF- κ B, a major transcription factor of the MDR1 gene [24, 25], has been indicated to be persistently activated in diabetic state, and the activation of NF- κ B is suggested to be

associated with the de novo synthesis of NF- κ B p65 in the presence of newly synthesized I κ B α and I κ B β . Furthermore, it has been indicated that NF- κ B was activated by PKC α [26]. Then we investigated the mRNA levels of NF- κ B p65, I κ B α , and I κ B β in STZ-treated rats compared with the control rats. As shown in Figure 4, the NF- κ B p65 mRNA level was increased by 50% and I κ B α and I κ B β mRNA levels were increased 50% and 100% respectively in the STZ-exposed rat liver. Next, the nuclear translocation of NF- κ B p65 was investigated by Western blotting. As shown in Figure 5, we detected increased nuclear translocation of p65 in STZ-treated rats and a slight increase in cytoplasmic p65 levels.

Discussion

In the present study, we demonstrated an increase in P-gp among nine hepatic canalicular and basolateral drug transporter genes investigated, and the translocation of PKC α to the membrane and of NF- κ B p65 to the nucleus, in the liver of rats with STZ-induced diabetes. Although P-gp is encoded by the Mdr1a and Mdr1b genes in rats, only the Mdr1b gene expression was upregulated and contributed to the increase in P-gp.

As for the level of P-gp under diabetic conditions, Tramonti et al. [27] reported that, while renal tubular the Mdr1a and Mdr1b genes were upregulated in their expression, P-

gp levels were not affected in diabetic rats. Contrary to this report, Liu et al. [28] showed that P-gp levels in the brain were lower in diabetic rodents than control animals. Thus the present study clearly showed that the expression of liver P-gp responded to the diabetic state in a different manner. Considering that P-gp regulates the absorption, distribution, and disposition of a large number of medicines and that hepatic P-gp confers vectorial flow of various lipophilic compounds into the canalicular space, the increase in P-gp probably leads to the accelerated excretion of medicines or xenobiotics into bile in the diabetic state.

In the present study, concurrently with the upregulation of P-gp expression in the liver, the level of PKC in the membrane fraction increased which generally means the activation of the enzyme. PKC-related upregulation of MDR1 gene expression has been observed in multidrug-resistant human tumor cells [8]. Generally, such resistant cells express high levels of the PKC α isoform, and it has been shown that PKC α contributed to the overexpression of P-gp [29, 30]. Independently of P-gp research, isoform-specific increases in PKC have been indicated by several studies in the rats with STZ-induced diabetes. In renal and myocardial tissues, selective upregulation of the expression of the PKC α isoform has been shown [31], and in hepatocytes, increased levels of PKC α and - β 2 isoforms and the translocation of PKC ϵ have been noted [21]. Considering these findings, the present study is the first to suggest an association of PKC α with the

upregulation of *mdr1b* gene expression and P-gp level in an animal model of diabetes.

Van Waarde et al. [6] reported that STZ-induced advanced diabetes with high levels of serum bile acids induced *mdr2* gene expression in the liver. However, we found no change of *mdr2* gene expression in the present study. As the expression of the human MDR3 gene is downregulated by PKC β in vitro [32], and that of both the MDR3 and *mdr2* genes can be upregulated by bile acids through the farnesoid X receptor [33 34], we may have to consider the influence of these factors and duration of disease on the expression of *mdr2* in models of diabetes that use rats.

As for the persistent upregulation of PKC expression in diabetes, it was indicated that it was generally accompanied by an increased diacylglycerol (DAG) level and was caused by hyperglycemia [14]. On the other hand, it is reported that hyperglycemia increased mitochondrial reactive oxygen species (ROS) production and also increased ROS-mediated PKC activation [35]. As it has been shown that PKC induced ROS production [36], PKC activation and ROS production may mutually contribute to the sustained activation of PKC in the diabetic state. Based on these reports, although further investigation is needed, DAG and ROS production contribute to the induction of P-gp expression and the increase in the level of PKC in the membrane.

In the present study, we also indicated the activation of the transcription factor NF- κ B by showing upregulation of the transcription and nuclear translocation of NF- κ B

p65 accompanied by increased I κ B transcription. It has been shown that phorbol ester, an activator of PKC, induced the degradation of I κ B and appearance of active NF- κ B (p65) [37]. Furthermore, it has been shown that PKC induced the activation of NF- κ B [26] and that NF- κ B is one of the major transcription factors of the human MDR1 gene [24, 38] and rat *mdr1b* gene [39]. Then, the activation of NF- κ B in STZ-treated rats seems to contribute to the transduction of PKC activity to upregulate the expression of P-gp.

I κ B consists of I κ B α and I κ B β subunits and forms a complex with NF- κ B (a heterodimer of p50 and p65) in the cytosol and plays a major role in the regulation of NF- κ B activity. Degradation of I κ B α after phosphorylation by IKK is followed by the translocation of NF- κ B p65 and also by the expression of I κ B mRNA [40, 41]. In calorie non-restricted aged rats, increases in the mRNA and protein levels of I κ B α were found consistent with the upregulated nuclear translocation of NF- κ B p65 and in correlation with age-related oxidative status [42, 43]. These findings imply that in chronic pathophysiological conditions both the nuclear translocation of NF- κ B and the expression of I κ B α were persistently upregulated. On the other hand, I κ B β binds to NF- κ B in place of I κ B α and the complex translocates to the nucleus and it has been suggested that this translocation contributed to the sustained activation of NF- κ B [44, 45]. Considering the nuclear translocation of NF- κ B p65 accompanied by increased

I κ B β transcription in the present STZ-generated model of diabetes, hyperglycemia seems to cause persistent activation of NF- κ B and to induce overexpression of hepatic P-gp.

In conclusion, we found a marked increase in the level of mdr1b P-gp in the liver at a relatively early stage of STZ-induced diabetes. Thus we indicated that the distribution of xenobiotics is affected through the upregulation of P-gp expression in the diabetic state. Concomitantly with this upregulation, we also found activation of the PKC and NF- κ B transcription system. From these findings, it is strongly suggested that STZ-induced hyperglycemia caused the upregulation of mdr1b P-gp expression through the activation of PKC and NF- κ B.

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Table 1. Primer sets for PCR

	Forward (5'-3')	Reverse (5'-3')	Annealing temperature	Product size	Ref.
Mdr1a	GGGCCACATGATCAAGACGG	AGCGTCATTGGCAAGCCTGG	65	447	2
Mdr1b	ACAGAAACAGAGGATCGC	AGAGGCACCAGTGTCCT	55	352	15
Mdr2	ACAGAAACAGAGGATCGCCA	ATGCGTGCTTTCCAGCCA	55	386	15
Bsep	TGCTTATGGGAGGCGTAT	GGGCTGACAGCAAGAATC	55	565	16
Mrp2	GGCTGAGTGCTTGGAC	CTTCTGACGTATCCTCAC	55	789	17
Bcrp	CAATGGGATCATGAAACCTG	GAGGCTGGTGAATGGAGAA	60	536	18
Oct1	GATCTTTATCCCGCATGAGC	TTCTGGGAATCCTCCAAGTG	60	478	19
Oat2	CGCTCAGAATTCCTCTCCAC	ACATCCAGCCACTCCAACTC	60	311	20
Oat3	TGAGAAGTGTCTCCGCTTCG	CTGTAGCCAGCGCCACTGAG	65	508	21
PKC α	GGAACTCAGGCAGAAGTTCG	CAGTTCCTCTGTCCCTTCC	58	196	Original
IkBa	GACGAGGATTACGAGCAGAT	CCTGGTAGGTTACTCTGTTG	60	634	22
IkBb	CCCAAGAGATGCCTCAGATA	GCCTTATCTCTGTTGTCAC	65	520	22
NF κ B	AAGATCAATGGCTACACGGG	CCTCAATGTCTTCTTTCTGC	60	493	23
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTGGCTGTA	58	452	24

Table 2. Comparison of body weight, liver weight, and plasma glucose level.

Group	Body weight (g)	Liver weight (g)	Ratio of liver to body weight (%)	Plasma glucose (mg/dl)
Control	309 ± 4.2	7.7 ± 0.4	2.5 ± 0.1	128 ± 11
STZ	265 ± 15 ^{**}	8.1 ± 0.6	3.0 ± 0.1 ^{**}	310 ± 93 ^{**}

** $p < 0.01$ vs. control

Figure legends

Figure 1. The expression levels of hepatic transporter genes in rats with STZ-induced diabetes. The expression of transporter genes was investigated by semi-quantitative RT-PCR methods using optimal concentrations of total RNA and primer sets shown in Table 1. The photograph (A) is typical of each group. Column graph data are given relative to the level of GAPDH and expressed as means \pm S.D. for five animals. *P<0.05, compared with the control.

Figure 2. The increase of hepatic P-gp levels in rats with STZ-induced diabetes. A liver membrane fraction was subjected to a Western blot analysis of P-gp as described in Materials and Methods. Column graph data are means \pm S.D. for five animals. *P<0.05, compared with the control. The photograph (A) is typical of each group.

Figure 3. Expression and translocation of PKC α in the liver of rats with STZ-induced diabetes. (A) The PKC α mRNA was examined by semi-quantitative RT-PCR using optimal concentrations of total RNA and primer sets shown in Table 1. (B) Western blot analysis of PKC α . The microsomal and cytosolic fractions of the liver were prepared as described in Materials and Methods. The photograph is typical of each group. Column graph data are means \pm S.D. for five animals. *P<0.05, compared with the control.

Figure 4. Upregulation of the expression of I κ B genes in the liver of rats with STZ-induced diabetes. The expression of I κ B α and I κ B β was examined by semi-quantitative RT-PCR using optimal concentrations of total RNA and primer sets shown in Table 1. The photograph (A) is typical of each group. Column graph data are given relative to the level of GAPDH and expressed as means \pm S.D. for five animals. *P<0.05, **P<0.01, compared with the control.

Figure 5. Upregulation of the expression of the NF- κ B p65 gene in the liver of rats with STZ-induced diabetes. The expression of the gene was examined by semi-quantitative RT-PCR using optimal concentrations of total RNA and primer sets shown in Table 1. The photograph (A) is typical of each group. Column graph data are given relative to the level of GAPDH and expressed as means \pm S.D. for five animals. **P<0.01, compared with the control.

Figure 6. Increase of nuclear NF- κ B p65 in the liver of rats with STZ-induced diabetes. A liver nuclear fraction was subjected to a Western blot analysis of NF- κ B p65 as described in Materials and Methods. Column graph data are means \pm S.D. for five animals. *P<0.05, compared with the control. The photograph (insert) is typical of each

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Kameyama N, Upregulation of P-glycoprotein in STZ-induced diabetes

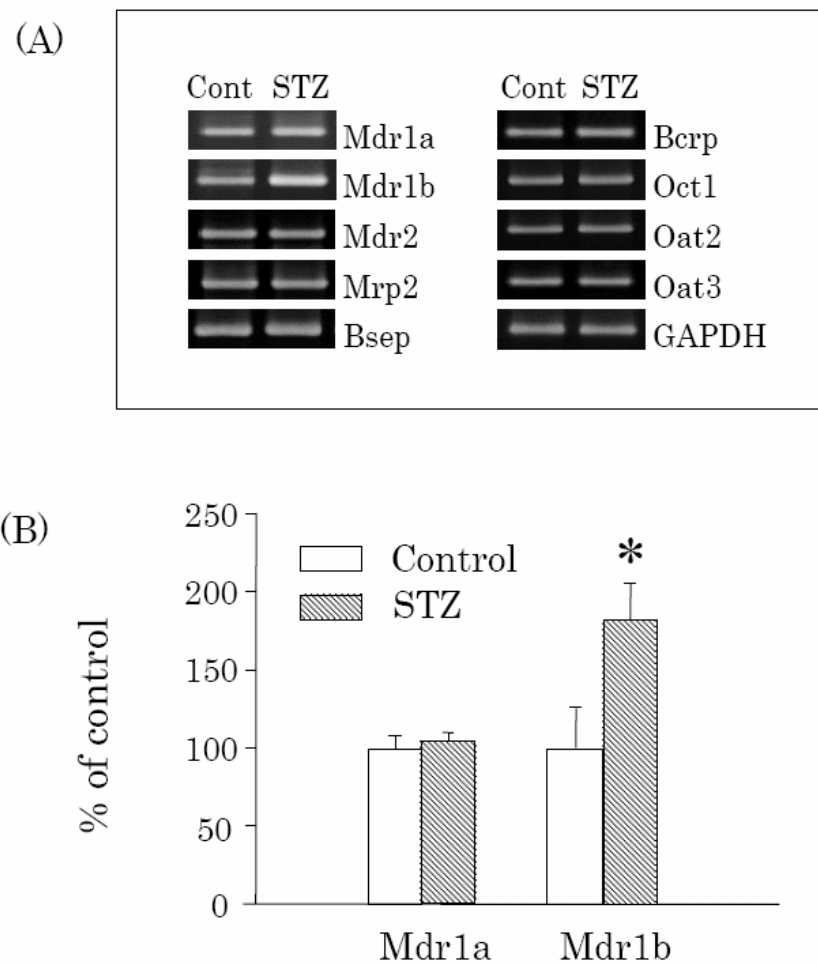


Figure 1

Kameyama N, Upregulation of P-glycoprotein in STZ-induced diabetes

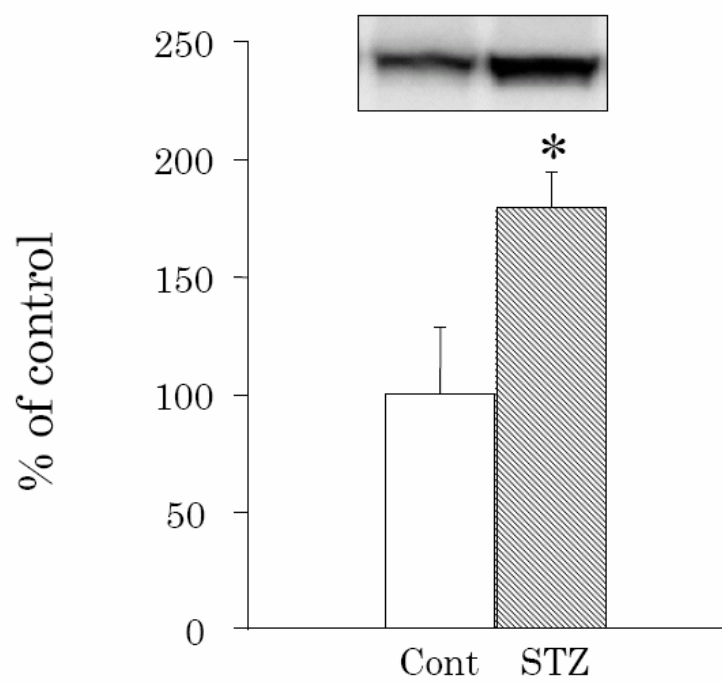


Figure 2

Kameyama N, Upregulation of P-glycoprotein in STZ-induced diabetes

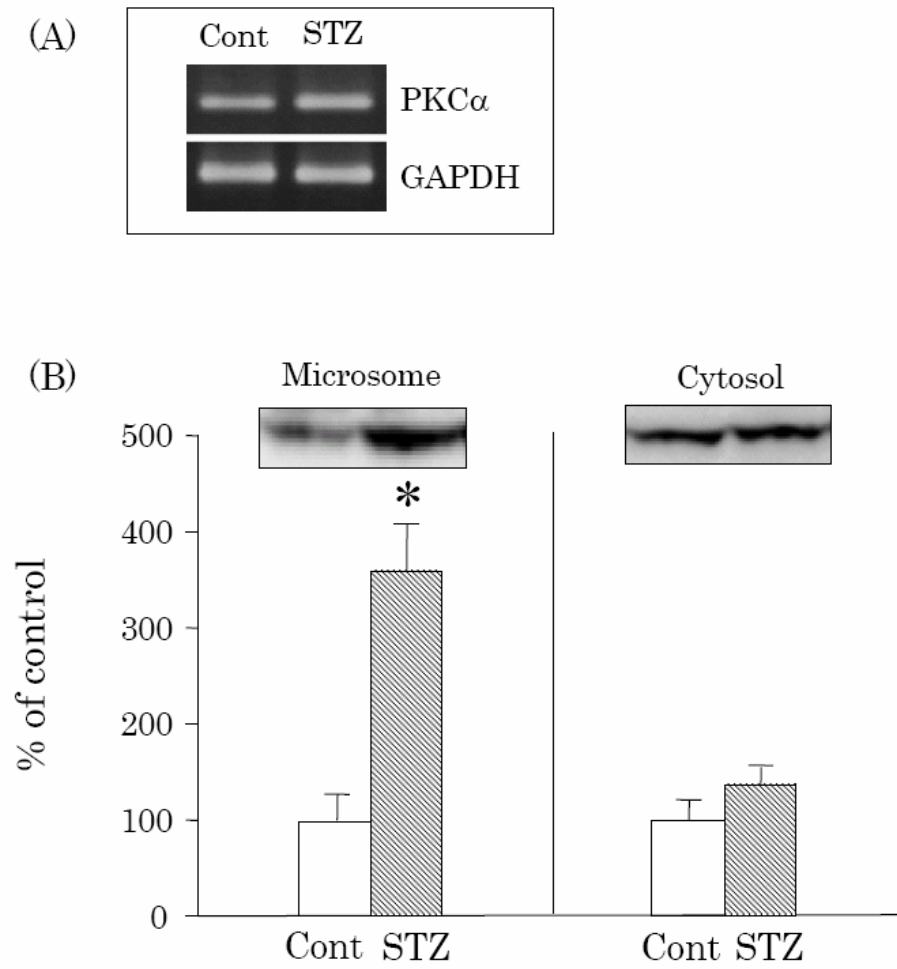


Figure 3

Kameyama N, Upregulation of P-glycoprotein in STZ-induced diabetes

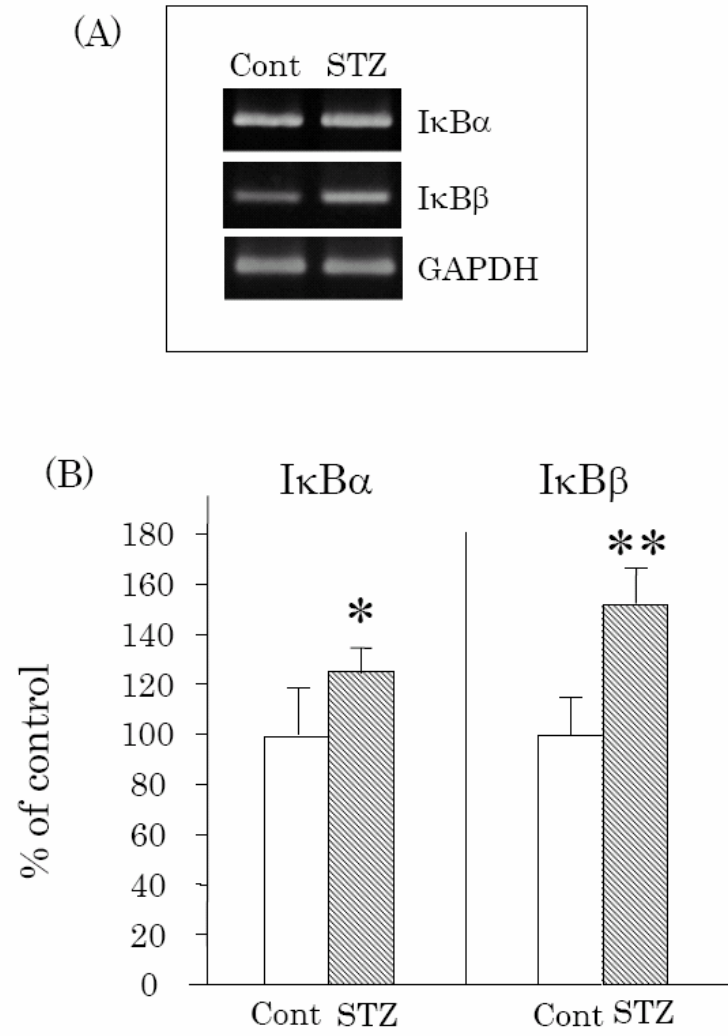


Figure 4

Kameyama N, Upregulation of P-glycoprotein in STZ-induced diabetes

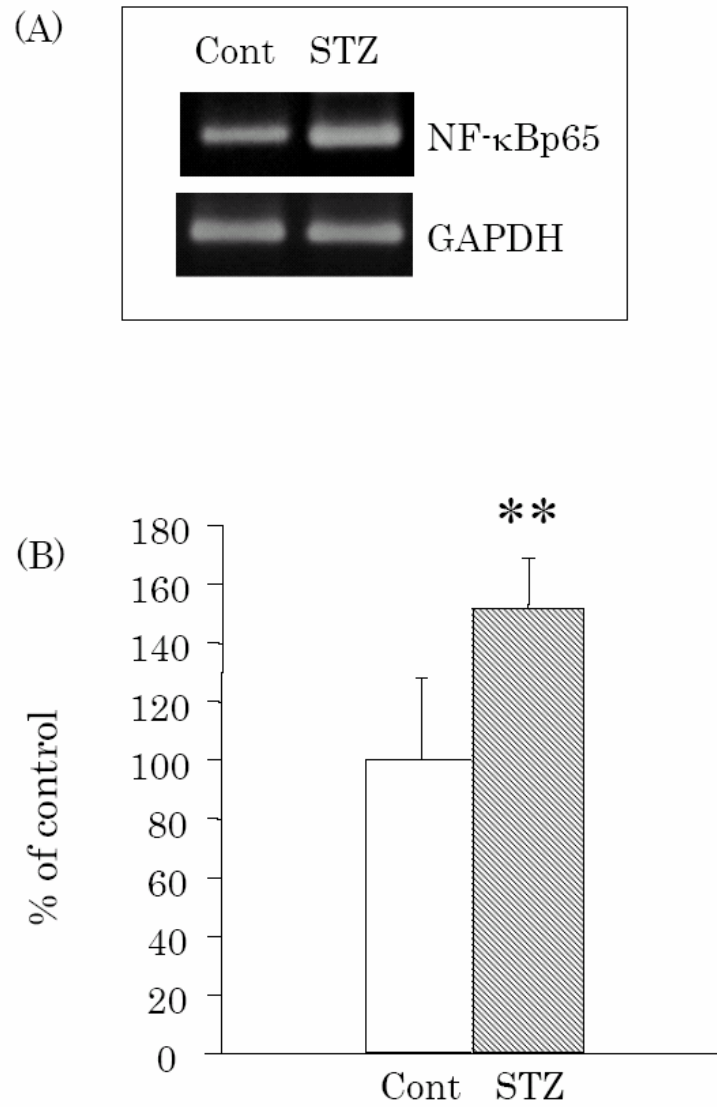


Figure 5

Kameyama N, Upregulation of P-glycoprotein in STZ-induced diabetes

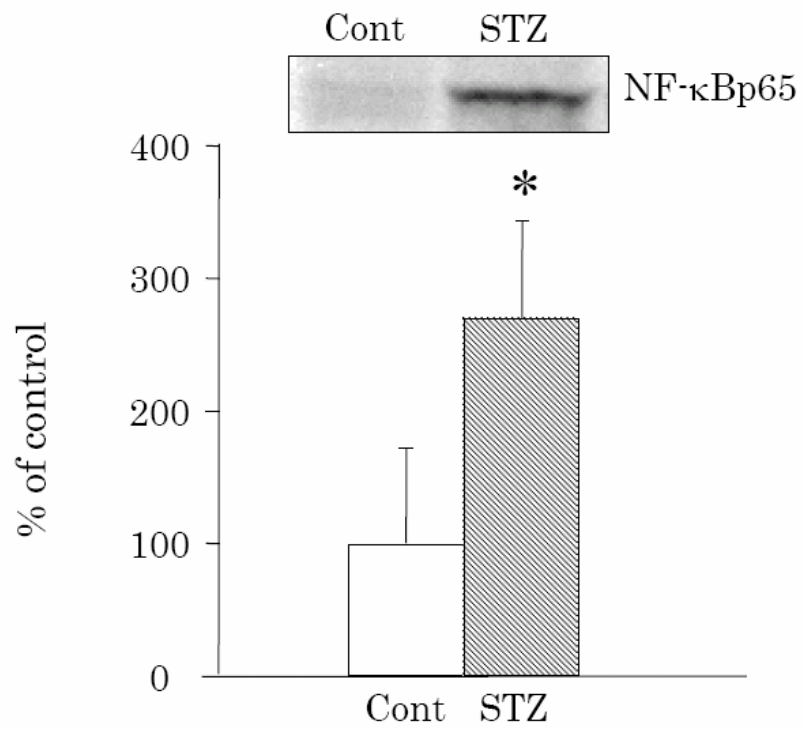


Figure 6