Expression and Nuclear Localization of p65 in Brain and Retina of Streptozotocin-induced Diabetic Rats with Hyperglycemia


*Department of Pediatric Dentistry, Osaka Dental University
**Department of Pharmacology, Osaka Dental University
***High Technology Research Center, Osaka Dental University
****8-1 Kuzuhahanazono-cho, Hirakata, Osaka 573-1121, Japan

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Abstract: De novo protein synthesis in eukaryotes is mainly controlled at the level of gene transcription by transcription factors in the nucleus. In this communication, the expression and nuclear localization of the Rel/NF-κB family member p65 in nuclear and cytosolic fractions of the brain and retina from streptozotocin (STZ)-injected diabetic rats were determined in order to evaluate the possibility that the transportation of Rel/NF-κB family members from the cytoplasm to the nucleus could play a role in diabetes. Immunoblotting assays with a specific antibody revealed a much higher expression of immunoreactive p65 in nuclear fractions than in cytosolic fractions of the cerebral cortex, hippocampus, midbrain and cerebellum after diabetes induction by STZ administration. In contrast, STZ administration suppressed the p65 nuclear localization detected in the striatum, hypothalamus, medulla-pons and retina. These results suggest that STZ-injected diabetic rats show differential alterations in the nuclear localization of p65 protein in the brain and retina.

Introduction

The association between diabetes and periodontitis is a widely accepted phenomenon that has been shown in numerous studies. Most studies indicate a higher incidence of periodontitis in diabetes compared to healthy controls. The complications of diabetes include neuropathy, retinopathy, nephropathy, and cardiovascular disease in addition to periodontal dis-
Diabetic retinopathy is the major cause of visual loss in the working population. Periodontal diseases are infections affecting the periodontium and resulting in the loss of tooth support. One of the prominent features of these diseases is structural and functional alterations in the tissue. There is increasing evidence that certain nuclear transcription factors are rapidly and transiently expressed in response to hyperglycemia alterations in diabetes. For example, hyperglycemia stimulates the production of superoxide anions and the activation of nuclear factor (NF)-κB in endothelial cells, promotes leukocyte adhesion to the endothelium through the up-regulation of cell surface adhesion molecule expression, dependent on NF-κB activation, and induces NF-κB activation in vascular smooth muscle cells. In addition, oxidative stress induces the rapid and selective enhancement of NF-κB DNA binding activity in a variety of cells.

In eukaryotic cells, de novo protein synthesis is mainly controlled at the level of gene transcription. Transcription factors bind with high affinity to particular nucleotide sequences at promoter or enhancer regions on double stranded DNA in the nucleus, followed by transcriptional modulation by RNA polymerase II of those genes into mRNA. The transcription factor NF-κB is a homodimeric and/or heterodimeric protein complex of the Rel/NF-κB protein families. These proteins, which consist of p50, p52, p65, Rel B and C-Rel, are able to modulate the transcription of inducible target genes, following the formation of the dimeric protein complex NF-κB. In its inactive state, NF-κB is sequestered in the cytoplasm together with an inhibitor protein IκB.

These proteins must be transported from the cytoplasm to the nucleus in order to function specifically as transcription factors after de novo synthesis at the ribosome. Moreover, it has been proposed that transcription factor translocation to the nucleus and DNA binding activities are modulated by mutual protein modification and protein-protein interactions. Recent studies have demonstrated that these nuclear transcription factors can translocate to the nucleus because they themselves have nuclear localization signals. However, little attention has been paid to the nuclear localization of particular transcription factors in diabetic diseases. In this study, therefore, the expression and nuclear localization for p65 were determined in nuclear fractions and cytosolic fractions of the brain and retina (RT) from STZ-injected diabetic rats in order to establish whether the nuclear translocation of p65 protein could alter in diabetes.

**Materials and methods**

1. **Materials**
A rabbit polyclonal antibody against p65 was provided by Santa Cruz Biotechnology (Santa Cruz, CA). An anti-rabbit IgG antibody was supplied by DAKO A/S (Glostrup, Denmark) and ECL detection reagents from Amersham Life Science (Buckinghamshire, U.K.). The other chemicals used were all of the highest purity commercially available.

2. **IDDM model**
The protocol employed here conforms with the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for the Ethical Use of Experimental Animals at Osaka Dental University. Every effort was made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to in vivo techniques. Adult male Wistar rats weighing 200—250 g at the beginning of the experiment were housed in metallic breeding cages in a room with a light-dark cycle of 12 h/12 h and humidity of 55±2% at 25°C, with free access to food and water for at least seven days before use. The animals were injected with streptozotocin (STZ) (65 mg/kg, i.v.) or a vehicle (50 mM citrate buffer). Hyperglycemia was defined as non-fasting blood glucose>300 mg/dl at 24 h after the injection. STZ-induced diabetes resulted in visual loss 120 days following administration. The brain and RT were quickly removed within 3 min after decapitation and immersed in ice-cold homogenizing buffer at 2°C for at least 5 min, followed by dissection of the cerebral cortex (CX), hippocampus (HC), striatum (ST), hypothalamus (HT), midbrain (MB), cerebellum (CL) and medulla-pons (MP) as required.
3. Fractionation

Each region was homogenized in 50 volumes of 10 mM HEPES-NaOH buffer (pH 7.9) containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 10 mM NaF, 10 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate and 1 μg/ml of various protease inhibitors [(p-amidinophenyl)methanesulfonyl fluoride, benzamidine, leupeptin and antipain] using a Dounce homogenizer with a B-type pestle at 2°C unless indicated otherwise. Following the addition of 10% Nonidet P-40 to make a final concentration of 0.6%, the homogenates were centrifuged at 20,000 g for 5 min. The supernatants thus obtained were stored at −80°C as cytosolic fractions enriched in cytoplasmic constituents and plasma membrane lysates. The pellets were suspended in 5 volumes of 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and the aforementioned protease inhibitors. The lysates thus obtained were stored at −80°C as nuclear fractions.

4. Immunoblotting assay

The nuclear and cytosolic fractions were mixed at a ratio of 4:1 v/v with 10 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecylsulfate, 0.01% bromphenol blue and 5% mercaptoethanol, followed by boiling at 100°C for 10 min, respectively. Aliquots (50 μg protein for nuclear fractions or 25 μg protein for cytosolic fractions) were electrophoresed on a 10% polyacrylamide gel at a constant current of 17 mA/plate for 2 h at room temperature and subsequent blotting to a PVDF membrane previously treated with 100% methanol. After blocking with 5% skim milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20 (TBST), the PVDF membrane was reacted with a rabbit polyclonal antibody against p65, diluted 500 fold with TBST containing 1% skim milk, followed by reaction with a peroxidase-conjugated anti-rabbit IgG antibody. Finally, the proteins reactive with the anti-p65 antibody were visualized with the aid of ECL™ detection reagents.

Results

1. Body weights and blood glucose levels

The control animals injected with the vehicle exhibited naturally increasing body weights, whereas, the rate of this increase was slower in STD-injected diabetic animals (Fig. 1A). STZ-injection significantly increased the blood glucose levels in the animals over a period of 24 h after injection, and this persisted for 120 days (Fig. 1B).

2. Immunoreactivity of p65 protein

Figure 2 shows typical autoradiograms obtained from the nuclear fractions of each region in the animals after the injection of STZ or the vehicle. Immunoreactivity with the anti-p65 antibody was detected at 65,000 mol. wt in nuclear fractions and cytosolic fractions of all tissues examined. STZ-injected
Fig. 2 Effects of STZ-injection on nuclear immunoreactivities to an antibody against p65 in murine brain and retina. Animals were decapitated after STZ injection, followed by dissection of each region and preparation of nuclear fractions. Aliquots of individual nuclear fractions were subjected to SDS-PAGE, followed by immunoblotting assay using an antibody against p65. Typical autoradiograms from six independent animals are presented.

Diabetic animals showed a slight increase in immunoreactivity detected at the p65 position in CX, HC and CL. In contrast, they exhibited decreasing p65 immunoreactivity in ST, MP and RT. In HT and MB from STZ-injected animals, however, no marked alteration in immunoreactivity was seen with an antibody against p65 protein.

Figure 3 shows typical autoradiograms obtained from the cytosolic fractions of each region in the animals after the injection of STZ or the vehicle. STZ-injected diabetic animals showed a much more marked decrease of p65 immunoreactivity in CX. However, no difference was evident in p65 immunoreactivity between the cytosolic fractions obtained from the other regions of animals injected with STZ or the vehicle.

3. Quantitative analysis

Quantitative densitometric determination of the above data revealed that p65 immunoreactivity in the nuclear fractions of CX, HC and CL from STZ-injected animals was increased compared with control rats injected with the vehicle alone (Fig. 4, left panel). On the other hand, STZ-injected diabetic animals exhibited a marked decrease of p65 immunoreactivity in the nuclear fractions of ST, MP and RT. In the nuclear fractions of HT and MB from STZ-injected animals, however, no marked immunoreactivity alteration was seen with this antibody.

In contrast, p65 immunoreactivity in the cytosolic fractions of HT and MP was increased compared with the control rats injected with the vehicle alone, while immunoreactive p65 in the cytosolic fractions of CX was decreased (Fig. 4, right panel). However, STZ-injected diabetic animals did not show significant alterations in p65 immunoreactivity in the cytosolic fractions of HC, ST, MB, CL and RT.

4. Nuclear localization of p65 protein

Nuclear localization of p65 was determined in discrete structures. CX and HC exhibited the most immunoreactive p65 localized to nuclear fractions.
Fig. 3 Effects of STZ-injection on cytosolic immunoreactivities to an antibody against p65 in murine brain and retina. Animals were decapitated after STZ injection, followed by dissection of each region and preparation of cytosol fractions. Aliquots of individual cytosolic fractions were subjected to SDS-PAGE, followed by immunoblotting assay using an antibody against p65. Typical autoradiograms from six independent animals are presented.

Fig. 4 Densitometric determination of the effects of STZ-injection on nuclear and cytosolic immunoreactivities to an antibody against p65 in murine brain and retina. Data from six independent animals are shown. * p<0.05, ** p<0.01, when compared with vehicle-injected animals.
Fig. 5 Nuclear localization of p65 protein in the brain and retina following STZ-injection. Each value indicates the data from densitometric determinations of p65 in cytosolic fractions over nuclear fractions. * p<0.05, when compared with vehicle-injected animals.

following STZ-injection. In addition, progressively increasing levels of p65 immunoreactivity in the nuclear fractions were found in MB and CL with STZ-injected diabetic animals (Fig. 5). In ST, HT, MP and RT from STZ-injected diabetic animals, on the other hand, immunoreactive p65 was highly expressed in the cytosolic fractions. However, HT and MB alterations were not significant.

Discussion

This is the first attempt to determine the possible involvement of p65 nuclear localization with diabetes. This study clearly demonstrated that the transcription factor p65 is constitutively expressed in a normal state and alters its nuclear localization in brain and retinal structures in response to hyperglycemia following STZ-injection. Recent studies in cultured sympathetic primary neurons found that high glucose levels induce apoptosis in neurons\(^{11}\). In addition, STZ-injected diabetic rats exhibit apoptosis induction in dorsal root ganglion neurons and Schwann cells\(^{12}\). However, previous studies showed that high glucose prevents the induction of apoptosis in cortex neurons by NMDA\(^{13}\). Therefore, the question of whether or not high glucose participates in the apoptosis of neurons remains to be addressed before drawing any conclusions. Alternatively, the possibility that a direct response to high glucose by neurons may be involved in their vulnerability to high glucose cannot be ruled out.

There have been several reports on the retinal structures of diabetic mice indicating that high glucose can impair later neurite regeneration capacity at a normal glucose level\(^{14}\). Moreover, high glucose induces apoptosis in retinal microvascular pericytes, the loss of which contributes to diabetic retinopathy. This apoptosis of pericytes caused by high glucose is prevented by aldose reductase inhibitors. Therefore, it is likely that polyl pathway hyperactivity may play an important role in pericyte apoptosis induced by high glucose\(^{15}\). Furthermore, high glucose regulates TGF-β mRNA and protein, and also TGF-β receptor expression in retinal endothelial cells\(^{16}\), suggesting that potential angiogenic factors may influence diabetic disease progression. In addition, it was found that hyperglycemia potentiates the uptake of taurine that is essential for retinal maintenance\(^{17}\). These previous findings, together with the data obtained here, are consistent with the idea that after STZ-injection, the *de novo* synthesis of several inducible target proteins could result in the alteration of cellular functions such as retinal cell apoptosis and/or the destruction of retinal structures through transcriptional modulation by p65-containing NF-κB. In this study, the nuclear localization of p65 protein was found to be decreased in retinal structures from STZ-injected diabetic animals with visual loss. Therefore, it is conceivable that proteins induced in retinal structures through the activation of NF-κB may be essential for retinal maintenance.

In general, short amino acid sequences, often referred to as nuclear localization signals, are required for the translocation of a protein from the cytoplasm into the nucleus. In fact, Rel/NF-κB family members have putative bipartite nuclear targeting signals in their molecules. The protein phosphatase calcineurin is essential for facilitating the nuclear import of particular transcription factors through the formation of a protein-protein complex. These previous findings
all raise the possibility that the individual family members may first be imported into the nucleus through their nuclear localization signals, followed by dimerization to generate NF-κB in the nucleus. Several independent lines of evidence are available in the literature supporting the concept of the rapid export of proteins from the nucleus through nuclear export signals. In this investigation, STZ-injected diabetic animals showed the nuclear localization of p65 immunoreactivity in CX, HC, MB and CL, but the cytoplasmic localization in ST, HT, MP and RT. Indeed, it seems likely that the balance between the import and export of p65 protein may differ in each distinct brain region in the STZ-induced diabetes model.

To our knowledge, this is the first direct demonstration of an alteration in the nuclear localization of p65 in the brain and retina of STZ-injected diabetic rats. STZ-injected animals are shown to exhibit hyperglycemia, intravascular oxidant production, DNA strand breakage, poly (ADP-ribose) polymerase activation, NF-κB activation, and a selective loss of endothelium-dependent vasodilation. There have been several reports on the relationship between diabetes and NF-κB indicating that reactive oxygen intermediates mediate the activation of NF-κB and thus play an important part in the high glucose-mediated induction of apoptosis in human endothelial cells. Similarly, hyperglycemia induces the activation of NF-κB in endothelial cells and vascular smooth muscle cells. Oxidative stress also induces the rapid and selective enhancement of the DNA binding activity of NF-κB in a variety of cells. It was found that high glucose up-regulates cell proliferation in mesangial cells, depending on the protein kinase C and NF-κB pathway. Indeed, high glucose activated NF-κB in a variety of cells. The question of whether alterations leading to the activation of NF-κB in response to high glucose are beneficial or toxic to these cells remains to be established.

As outlined here, in any event, hyperglycemia leads to a variety of alterations of cellular functions associated with the expression of various different target proteins through the modulation of de novo synthesis at the level of gene transcription in a manner unique to the individual signals. This study aimed to document the nuclear localization of particular transcription factor family members and clearly shows that STZ-injected diabetic rats exhibit an alteration in p65 nuclear localization in the brain and retinal structures. It is likely that target proteins induced through NF-κB activation may be involved in diabetes. NF-κB plays a critical role in the molecular mechanisms associated with the consolidation of extracellular high glucose signals at the level of gene transcription into long-lasting and permanent alterations of cellular functions. The identification of each family member is undoubtedly required to evaluate the molecular mechanisms underlying the import and export of nuclear proteins with DNA binding activities in future studies.

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References