Alteration of nuclear transcription factors NF-κB and AP-1 with DNA binding activity in discrete brain structures of streptozotocin-induced diabetic rats with hyperglycemia

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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, DNA binding activities of nuclear factor (NF)-κB and activator protein-1 (AP-1) were determined in the brain from streptozotocin (STZ) -injected diabetic rats in order to explore particular proteins related with diabetes. Gel retardation electrophoresis using core consensus elements containing NF-κB or AP-1 probe revealed that STZ significantly potentiated NF-κB binding in the cerebral cortex and medulla-pons. Moreover, STZ markedly decreased NF-κB binding in the striatum and hypothalamus. However, no significant changes was detected in NF-κB binding in the hippocampus, midbrain and cerebellum. In contrast, STZ significantly potentiated AP-1 binding in the cerebellum and medulla-pons, whereas STZ markedly decreased AP-1 binding in the striatum, hypothalamus and midbrain. However, AP-1 binding was not markedly affected by the injection of STZ in the cerebral cortex and hippocampus. These results suggest that the STZ-injected diabetic rat shows differential alterations in DNA binding activities of transcription factors NF-κB and AP-1 in the brain.

Key words: Diabetes, Transcription factors, NF-κB, AP-1, Brain

Introduction

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 modulation of transcription by RNA polymerase II of those genes into mRNA. The transcription factor nuclear 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 transcription of inducible target genes, following formation of the dimeric protein complex NF-κB. In its inactive state, NF-κB is sequestered in the cytoplasm together with an inhibitor protein IκB1,2). In contrast, the transcription factor activator protein-1 (AP-1) is a homodimeric and/or heterodimeric protein complex of c-Jun and c-Fos protein families.

An 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. Complications of diabetes include neuropathy, retinopathy, nephropathy, cardiovascular disease in addition to periodontal disease. Periodontal disease are infections affecting the periodontium and resulting in the loss of tooth support. One of the prominent features of periodontal dis-
eases is structural and functional alterations in oral tissues. There is increasing evidence that certain nuclear transcription factors are rapidly and transiently expressed in response to alterations of hyperglycemia in diabetes. For example, hyperglycemia stimulates the production of superoxide anion and activation of NF-κB in endothelial cells, promotes leukocyte adhesion to endothelium through up-regulation of cell surface adhesion molecule expression, dependent on NF-κB activation\(^3\), and induces activation of NF-κB in vascular smooth muscle cells\(^4\). In addition, oxidative stress induces rapid and selective enhancement of NF-κB DNA binding activity in a variety of cells\(^5\,\!^6\). However, little attention has been paid to the DNA binding activities of particular transcription factors in the brain with diabetic diseases.

In this paper, therefore, DNA binding activities of NF-κB and AP-1 were determined in nuclear fractions of brain from streptozotocin (STZ)-injected diabetic rats in order to explore particular proteins related with mechanisms to neuropathy in diabetes.

**Materials and Methods**

1. **IDDM model**

The protocol employed here conforms with the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Osaka Dental University. All efforts were 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. Animals were injected with STZ (65 mg/kg, i.v.) or 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. Brain 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.

2. **Nuclear extracts**

Nuclear extracts were prepared by the method of Schreiber et al.\(^7\) with minor modifications\(^8\). In brief, 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 (GP), 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%, homogenates were centrifuged at 20,000 g for 5 min. Pellets were suspended in 10 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 10 mM NaF, 10 mM GP and the aforementioned protease inhibitors, followed by centrifugation at 20,000×g for 5 min. Supernatants thus obtained were stored at −80°C as nuclear extracts.

3. **Gel retardation electrophoresis**

An aliquot (10 μg protein) of nuclear extracts was incubated with 400 μmol of 5’-biotin-labeled NF-κB probe (5’-GATCGAGGGGACTTTCCCTAGC-3’) and AP-1 probe (5’-CTAGTGATGAGTCTAGCCGGATC-3’) (J BioS. Co.; Shizuoka, Japan) and 1 μg poly (dI-dC) in 20 μL of 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl\(_2\), 200 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 10% glycerol, 5 mM NaF, 5 mM GP, and 1 μg/mL protease inhibitors at 25°C for 30 min. The bound and free probes were separated by electrophoresis on a 6 % polyacrylamide gel in buffer (pH 8.5) containing 50 mM Tris, 0.38 M glycine and 2 mM EDTA at a constant voltage of 11 V/cm for 1.5 h in an ice bath, and subsequently blotted to a positively charged nylon membrane. The membrane was then incubated with streptavidin-alkaline phosphatase, and exposed to Kodak X-ray film. Densitometric determination was carried out with the aid of a 1D Image Analysis Software (Kodak, Rochester, NY) as described previously\(^9\).

4. **Statistical analysis**

Results were expressed as the mean ± SE. Statistical analysis was determined by the two-tailed Student’s t-test or one-way analysis of variance. Differences were considered to be significant when the P value was<0.05.
Result

1. Body weights and blood glucose levels

Control animals exhibited naturally increasing body weights, whereas, the rate of this increase was slower in STZ-injected diabetic animals as shown previously. STZ-injection significantly increased blood glucose levels in animals over a period of 24h after injection, and this persisted for 120 days as shown previously.

2. DNA binding of NF-κB

Figure 1 shows comparison of DNA binding activity of NF-κB in discrete brain regions of rats injected of vehicle or STZ. The administration of STZ potentiated NF-κB binding in CX (cerebral cortex) and MP (medulla-pons). In contrast, they exhibited a decreasing NF-κB binding in ST (striatum) and HT (hypothalamus). Other central structures did not display any marked alterations of NF-κB binding between control and STZ-injected rats.

Quantitative densitometric determination of these data derived from six separate animals revealed that STZ significantly induced the potentiation of NF-κB binding in CX and MP (Fig. 2). In contrast, STZ induced statistically marked decrease of NF-κB binding in ST and HT. However, no significant changes was detected in NF-κB binding in HC (hippocampus), MB (midbrain) and CL (cerebellum).

3. DNA binding of AP-1

Figure 3 shows comparison of DNA binding activity of AP-1 in discrete brain regions of rats injected of vehicle or STZ. The administration of STZ potentiated AP-1 binding in CL (cerebellum) and MP (medulla-pons). In contrast, STZ-injected rats resulted in an inhibition of AP-1 binding in ST (striatum), HT (hypothalamus) and MB (midbrain). Other central structures did not display any marked alterations of AP-1 binding between control and STZ-injected rats.

Quantitative densitometric determination of these data derived from six separate animals revealed that STZ significantly potentiated AP-1 binding in CL and MP (Fig. 4). In contrast, STZ statistically decreased AP-1 binding in ST, HT and MB. However, AP-1 binding was not markedly affected by the injection of STZ in CX.

Fig. 1 Distribution profiles of NF-κB binding in discrete brain regions of STZ-injected rats. Animals were decapitated after injection of STZ, followed by dissection of each region and preparation of nuclear extracts. Aliquots of nuclear extracts from each brain structure were incubated with NF-κB probe, followed by electrophoresis. Each lane corresponds to a sample from one animal.
**Fig. 2** Densitometric determination of effect of administration of STZ on NF-κB binding in discrete structures of rats' brain. Each value indicates the data from densitometric determinations for quantitative evaluation. The data are from six separate animals. *P<0.05, **P<0.01, when compared with vehicle-injected animals.

**Fig. 3** Distribution profiles of AP-1 binding in discrete brain regions of STZ-injected rats. Animals were decapitated after injection of STZ, followed by dissection of each region and preparation of nuclear extracts. Aliquots of nuclear extracts from each brain structure were incubated with AP-1 probe, followed by electrophoresis. Each lane corresponds to a sample from one animal.
Fig. 4 Densitometric determination of effect of administration of STZ on AP-1 binding in discrete structures of rats brain. Each value indicates the data from densitometric determinations for quantitative evaluation. The data are from six separate animals. *P<0.05, **P<0.01, when compared with vehicle-injected animals.

Discussion

This is the first attempt to determine possible involvement of DNA binding activities of transcription factors NF-κB and AP-1 with diabetes. The present study has clearly demonstrated that the DNA binding of transcription factors NF-κB and AP-1 are constitutively detected in a normal state and alters its DNA binding activities in brain structures in response to hyperglycemia following STZ-injection. About 120 days later that STZ-injection was performed, STZ was eliminated completely. It thus appears that hyperglycemia lasting for about 120 days was free from STZ-control. Recent studies in cultured sympathetic primary neurons found that high glucose levels induce apoptosis in neurons\(^1\). In addition, STZ-injected diabetic rats exhibit an induction of apoptosis in dorsal root ganglion neurons and Schwann cells\(^2\). Moreover, there have been several reports on diabetic mice indicating that high glucose can impair later neurite regeneration capacity at a normal glucose level\(^3\). 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. It is likely that polyol pathway hyperactivity may play an important role in pericyte apoptosis induced by high glucose\(^4\). In contrast, previous studies showed that high glucose prevents induction of apoptosis in cortex neurons by NMDA\(^5\). Therefore, the question of whether or not high glucose participates in apoptosis of cells in the central nervous system 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.

High glucose regulates TGF-β mRNA and protein, and also TGF-β receptor expression in endothelial cells\(^6\), 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 cell maintenance\(^7\). These previous findings, together with the data
obtained here, are consistent with the idea that after STZ-injection, de novo synthesis of several inducible target proteins could result in alteration of cellular functions such as apoptosis of cells and/or destruction of brain structures through transcriptional modulation by NF-κB or AP-1.

To our knowledge, this is the first direct demonstration of an alteration in DNA binding activities of NF-κB and AP-1 in the brain 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 vasodilation18). 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 cells30). Similarly, hyperglycemia induces activation of NF-κB in endothelial cells7) and in vascular smooth muscle cells5). Oxidative stress also induces rapid and selective enhancement of DNA binding activity of NF-κB in a variety of cells5,6). It was found that high glucose up-regulates cell proliferation in mesangial cells, depending on the protein kinase C and NF-κB pathway30). 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.

Conclusion

It thus concluded that hyperglycemia would lead to a variety of alterations of cellular functions associated with expression of various different target proteins through modulation of de novo synthesis at the level of gene transcription in a manner unique to the individual signals. This study is aimed at searching for a clue to elucidate DNA binding activities of particular transcription factors in diabetic animals and clearly shows that STZ-injected diabetic rats exhibit an alteration in DNA binding activities of NF-κB and AP-1 in the brain. It thus concluded that target proteins induced through activation of NF-κB or AP-1 may be involved in diabetes. NF-κB and AP-1 would play a critical role in the molecular mechanisms associated with consolidation of extracellular high glucose signals at the level of gene transcription into long-lasting and permanent alterations of cellular functions. Identification of target proteins is undoubtedly required for evaluation of molecular mechanisms underlying the diabetic diseases in future studies.

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References


ストレプトゾトシンによる糖尿病発症ラット脳内における核内転写因子 NF-κ B および AP-1 DNA 結合能の変動

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真核細胞ではほとんどの場合、タンパク質の de novo 生合成は核内転写制御因子による遺伝子転写レベルでの制御を受ける。今回われわれは、糖尿病発症に伴ない変動する転写制御因子を探索する目的で、ストレプトゾトシン (STZ) 投与による糖尿病発症モデルラットの脳を用いて、転写制御因子 NF-κ B と AP-1 の DNA 結合能について検討を行った。コア塩基配列を含むオリゴヌクレオチドプローブを用いたゲルシフトアッセイ法により解析したところ、NF-κ B の DNA 結合能は、大脳皮質および橋・延髄では増強が見られたが、線条体および視床下部では減少が認められた。しかしながら、海馬、中脳および小脳では NF-κ B 結合能に顕著な変化は観察されなかった。一方、AP-1 結合能は、小脳および橋・延髄では増強が観察されたが、線条体、視床下部および中脳では減少することが明らかとなった。しかしながら、大脳皮質と海馬では AP-1 結合能に著変は見られなかった。以上の結果より、STZ 投与糖尿病モデル動物脳内において、転写制御因子 NF-κ B と AP-1 の DNA 結合能が変動することが明らかとなった。

キーワード：糖尿病、転写因子、NF-κ B、AP-1、脳