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**High glucose induction of DNA binding activity of the transcription factor NF κ B
in patients with diabetic nephropathy**

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SUMMARY

The aim of this study was to investigate whether high glucose induces aldose reductase (AKR1B1) expression through NFκB, which may contribute to the pathogenesis of diabetic nephropathy. 34 Caucasoid patients with type 1 diabetes were recruited; 20 nephropaths and 14 long-term uncomplicated subjects. Peripheral blood mononuclear cells (PBMCs) were cultured under normal or high glucose (25 mmol/l of D-glucose) with or without an aldose reductase inhibitor (ARI). High glucose increased NFκB binding activities in the PBMCs from nephropaths compared to the uncomplicated subjects (1.77 ± 0.22 vs. 1.16 ± 0.04 , $p=0.02$). ARI induced a substantially greater decrease of NFκB binding activities in the nephropaths compared to the uncomplicated subjects (0.58 ± 0.06 vs. 0.79 ± 0.06 , $p=0.032$). AKR1B1 protein levels in the nephropaths were increased under high glucose conditions and decreased in the presence of an ARI, whilst the silencing of the NFκB p65 gene in-vitro reduced the transcriptional activities of AKR1B1 in luciferase assays. These results show that NFκB induces AKR1B1 expression under high glucose conditions, and the pattern of expression differs between nephropaths and the uncomplicated subjects.

INTRODUCTION

A large number of patients with type 1 and type 2 diabetes mellitus will develop one or more chronic microvascular complications during the course of their diabetes. The precise mechanisms that initiate and promote diabetic microvascular complications are still being elucidated. It is well established that high glucose stimulates increased flux through the polyol pathway, and this is linked to abnormalities such as osmotic and oxidative stresses that have been cited as promoters of diabetic microvascular disease [1-3].

Aldose reductase (AKR1B1) is the first and rate-limiting enzyme of the polyol pathway and its expression is tightly regulated by intracellular osmolality at the transcriptional level [4]. This is mediated through the osmotic response elements (OREs) located in the 5' flanking sequences of the AKR1B1 gene [5]. There are three OREs, (OREA, OREB and OREC), that act as specific binding sites for the transcription factor, nuclear factor of activated T cells 5 (NFAT5) or the TonE binding protein (TonEBP) [5]. Our previous study has demonstrated that high glucose significantly increased the DNA-binding activity of NFAT5 to the OREs, in particular to the OREC [6]. Furthermore, this increase was significantly higher in patients with type 1 diabetes and nephropathy compared with those patients without microvascular complications. These results indicate that AKR1B1 is up-regulated by the transcriptional factor NFAT5 under high glucose conditions.

Nuclear factor kappa B (NF κ B) is a redox-sensitive transcriptional factor which shares the binding site with OREC in the promoter region of AKR1B1 gene with NFAT5 [7]. NF κ B is a widely expressed and is an inducible transcriptional factor with an inactive cytoplasmic and active nuclear DNA-binding form. Canonically, upon stimulation, NF κ B is activated through a rapid phosphorylation of I κ B α , which is dissociated from NF κ B and

is rapidly translocated to the nucleus where it binds to a κ B site and activates its target genes. High glucose-increased flux through the polyol pathway induces redox change and oxidative stress, which activates NF κ B. As a result, increased NF κ B activity may induce the expression of AKR1B1 by binding to the κ B motif in the promoter region of AKR1B1 gene under high glucose conditions. At the same time, NF κ B binds to other target genes to increase transcription of these genes, such as macrophage chemotactic protein (MCP-1) and metallo-matrix-proteinases (MMPs) which are involved in the accumulation of extracellular matrix and contributes to the development of diabetic complications [8-10]. Increased activity of NF κ B in patients with type 1 as well as type 2 diabetes with microvascular complications have been observed by several groups [11-14]. Ramana et al. [15] demonstrated that inhibition of AKR1B1 prevented hyperglycemia-induced NF κ B activation in vascular smooth muscle cells. They observed that aldose reductase inhibitors (ARI) inhibited the translocation of NF κ B and decreased NF κ B DNA binding activities.

The aims of the present study were to determine whether the AKR1B1 gene expression is regulated by NF κ B under high glucose conditions, and whether this expression is different between those patients with or without nephropathy. Finally, we investigated the effects of ARI on the NF κ B binding, and the effects of the silencing of the NF κ B p65 subunit on the expression of AKR1B1 gene in vitro.

MATERIALS AND METHODS

Subjects

The following Caucasoid subjects were included in this study: 34 patients with type 1 diabetes. All patients with type 1 as defined by The Expert Committee On The Diagnosis And Classification Of Diabetes Mellitus [16] had attended the Diabetes Clinic,

Derriford Hospital, Plymouth. The study was approved by the Local Research Ethical Committee and informed consent was obtained from all subjects. The criteria for diabetic microvascular complications have been published previously [17].

Uncomplicated: (Uncompls: n=14) have been diagnosed with type 1 for at least 20 years but remain free of retinopathy (fewer than five dots or blots per fundus), proteinuria (urine Albustix negative on at least three consecutive occasions over 12 months) and neuropathy (overt neuropathy was defined if there was any clinical evidence of peripheral or autonomic neuropathy).

Diabetic nephropathy: (Nephropaths: n=20) have had type 1 for at least 8 years with persistent proteinuria (urine Albustix positive on at least three consecutive occasions over 12 months or three consecutive total urinary protein excretion rates $>0.5\text{g}/24\text{h}$) in the absence of hematuria or infection on midstream urine samples. Diabetic nephropathy was always associated with retinopathy. Retinopathy was defined as more than five dots or blots per eye; hard or soft exudates, new vessels, or fluorescein angiographic evidence of maculopathy or previous laser treatment for pre-proliferative or proliferative retinopathy; and maculopathy or vitreous haemorrhage. Fundoscopy was performed by both a diabetologist and an ophthalmologist.

Cell isolation and cultures

Peripheral venous blood samples (20ml) were collected into 5% EDTA Vacutainers (Becton Dickinson, UK). The PBMC were separated by using Histopaque (Sigma, Dorset, UK) and grown in RPMI 1640 supplemented with D-glucose at a 5.5 mmol/l of concentration, 10% calf serum and 2 mmol/l L-glutamine, 100units/ml penicillin G sodium and 100mg/ml streptomycin sulphate with PHA-P at 5 $\mu\text{g}/\text{ml}$ of concentration in a 37⁰C incubator with a controlled, humidified atmosphere of 95% air/5% CO₂. These cells were divided into three groups from each individual in 200ml-flasks.

Group 1 (NG): cells were cultured in the above medium. Group 2 (HG): 19.5mmol/l extra D-glucose was added into above-mentioned medium. Group 3 (ARI+HG): sorbinil (10 μ mol/l) which is an ARI was added into above-mentioned medium and 3 hours later 19.5mmol/l extra D-glucose was added to the culture medium [15]. All cells were incubated for 5 days. At the end of the incubation time, cells were harvested and nuclear and cytoplasmic proteins were extracted from PBMC as below. A portion of the PBMCs in some samples were cultured with mannitol for 5 days (a final concentration at 25mM) as positive controls. HEK 293 were cultured in EMEM medium supplemented with D-glucose at a 5.5 mmol/l of concentration, 10% calf serum and 2 mmol/l L-glutamine, 100units/ml penicillin G sodium and 100mg/ml streptomycin sulphate for 5 days at NG, HG and ARI+HG conditions.

Extraction of nuclear protein and cytoplasmic proteins

Cells were collected and re-suspended in 100 μ l of buffer A (10 μ mol/l, HEPES, pH 7.9, 1.5 mmol/l $MgCl_2$, 0.5 mmol/l dithiothreitol (DTT), 0.2% NP-40, 100 mmol/l 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 18.4 mg/ml sodium orthovanadate, 42 mg/ml sodium fluoride and 2.2 mg/ml aprotinin) and held on ice 15 minutes. The resulting cell lysate was then centrifuged at 13000rpm for 10 minutes. The supernatant containing cytoplasmic proteins was transferred into a fresh tube for Western blotting and the nuclear pellets were re-suspended in 50 μ l of buffer C (20 mmol/l HEPES pH 7.9, 25% glycerol, 0.42 mol/l NaCl, 1.5 mmol/l, $MgCl_2$, 0.5 mmol/l DTT, 0.2 mmol/l EDTA, 100 mmol/l AEBSF, 18.4 mg/ml sodium orthovanadate, 42 mg/ml sodium fluoride, 2.2 mg/ml aprotinin), and incubated on ice for 10 minutes. After centrifugation at 13000 rpm for 10 minutes the supernatant containing the nuclear protein was transferred into a fresh tube and stored at $-70^{\circ}C$ until use. The concentrations of both nuclear and cytoplasmic proteins

were determined using a Coomassie® Plus Protein Assay kit (Peribo Science Ltd., Chest, UK).

Electrophoretic mobility shift assay (EMSA)

The NF κ B probe with consensus sequence to κ B motif AAATGGGAAATCACCGGC [7] (Fig. 1A) of AKR1B1 gene was labelled with [α - 32 P] deoxy-ATP by T4 polynucleotide kinase (Amersham Pharmacia Biotech, Buckinghamshire, UK). The labelled probe along with a gel binding buffer was incubated with 25 μ g of nuclear proteins at room temperature for 20 minutes. The binding mixtures were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel at 100V for 3 to 4 hours. The gel was exposed to X-Omax photographic paper. The specificity of the DNA binding protein for the putative binding sites was established by specific competitors and antibodies against human NF κ B p65 (Insight Biotechnology Ltd, Wembley, UK), respectively.

Western blotting

Briefly, a total 50 μ g of cytoplasmic proteins were loaded onto a 7.5 or 10% precast SDS-PAGE (BIO-RAD Laboratory Ltd, Hempstead, UK), electrophoresed for 2-3 hours at 100 volts, transferred to nitrocellular membrane (Amersham, USA) overnight. Next, the membrane was blocked with 5% non-fat milk and 0.05% Tween 20 -PBS for one hour at room temperature. Immunoblotting was performed with primary rabbit antibodies against human AKR1B1 and I κ B- α (Abcam Ltd, Cambridge, UK) as well as with primary mouse antibodies against NF κ B p65, phosphorylated I κ B- α (Insight Biotechnology Ltd, Wembley, UK) in appropriate dilutions, respectively. Secondary antibodies against rabbit or mouse IgG of horse-radish peroxidase-conjugated were used in a 1:10,000 dilution (Sigma, UK). A chemiluminescence kit (Pierce, UK) and Kodak X-Omat film (Amersham, UK) were used to detect the amount of proteins. Meantime, in order to have

an equal amount of proteins in all wells, β -actin levels were measured by using a mouse antibody against human β -actin in 1:10,000 dilution (Sigma, UK) and a secondary antibody against mouse IgG of horse-radish peroxidase-conjugated in 1:10,000 dilution (Sigma, UK).

DNA binding activities of NF κ B and protein levels of AKR1B1, NF κ B p65, phosphorylated I κ B- α and I κ B- α were analysed and quantified by using a phosphoimager (BIO-RAD, UK) with multi-analyst software. All results were expressed as means of fold increases due to high glucose treatment, calculated by dividing the amount of density in high glucose-treated cells by the amount of density in untreated cells or as means of fold decreases due to ARI treatment, calculated by dividing the amount of density in high glucose with ARI treated cells by the amount of density in high glucose-treated cells.

SiRNA assay

In order to investigate effects of NF κ B on the transcription of AKR1B1 gene under high glucose conditions, we silenced the NF κ B gene by RNA interference. Three siRNA duplex sequences were pre-designed from Ambion (Ambion Limited, UK), targeting to exon 7, 8 and 9 of the NF κ B p65 gene, respectively. A negative control siRNA was purchased from Ambion as well.

Transfection of siRNA was carried out by using siFECT siRNA transfection reagent (Promega, Southampton, UK) and transfection of plasmids was performed by using Tfx-20 reagent (Promega, Southampton UK). The day before transfection, HEK 293 cells (which express NF κ B p65 identified by RT-PCR, data not shown) were seeded at 30% to 50% confluence in 96- well plates and each transfection was performed in duplicates. siRNA at a concentration range from 10 nmol/l to 1000 nmol/l was tested and a 500 nmol/l of siRNA gave a detectable silencing effect. Among three siRNAs, the one targeting exon 9 gave the highest silencing. Therefore, siRNA targeting at exon 9 and

negative control at a 500 nmol/l were used in our study. For analysing the effect of silencing of NFκB p65 on the transcription of AKR1B1 gene, recombinants pGL3 reporter plasmids at 98 ng which contain the promoter region with OREC/κB motif and haplotypes Z-2/C-106 or Z/C-106 of the AKR1B1 gene [6, 18] and firefly luciferase gene downstream were co-transfected with pRL-TK control plasmids which contain the Renilla reniformis luciferase gene into the cells at 2 ng after 24 hours of siRNA transfection. Next day, cells were divided into four groups: (a) cells were maintained in original media; (b) cells were switched to HG (a final concentration at 25 mmol/l of D-glucose) and (c) cells were switched to ARI (sorbiniol at 10 μmol/l); (d) cells were switched to HG and ARI conditions (ARI was added 3 hours earlier before D-glucose was added). All cells were cultured for another 30 hours. At the end of culture, cells were lysed using 20 μl of lysis buffer (Promega, Southampton, UK). AKR1B1 transcription activity was measured using a Dual-luciferase reporter assay system (Promega, Southampton, UK) in a MLX luminometer (Dynex Technologies Inc., USA). For each transfection, the relative activity was defined as firefly luciferase activity normalised by the activity of the internal control (Renilla luciferase) as described previously [6, 18].

Statistical Analysis

All data were expressed as the means \pm standard error of the means (SE). Student t-test was used to compare differences between two groups and ANOVA was used to compare differences between more than two groups. A *p* value of less than 0.05 was considered to be significant.

RESULTS

Clinical characteristics of patients with type 1 diabetes with or without nephropathy are shown in Table 1. There were no differences in age, gender, age at onset of diabetes, duration of diabetes, Hb1Ac and glucose levels between two groups.

High glucose significantly increased NFκB binding activities to the κB motif site in PBMCs

We observed that two binding bands were formed in the gel shift with NFκB probe, which were consistent with Ramana et al. study [15]. In the presence of cold (unlabelled) NFκB probe, the band was abolished (Figure 1B). The super shift analysis of NFκB showed that following the incubation with antibodies against human NFκB p65, the binding complex with the probe to κB motif were shifted upwards as indicated in Figure 1B.

The DNA-binding activities of NFκB to the κB motif in PBMCs exposed to either normal glucose, high glucose or, ARI with high glucose conditions, from a nephropath and an uncomplicated subject are shown in Figure 1C. Following the incubation with high glucose there was a significantly increased DNA binding activities of NFκB to κB motif in PBMCs in both the nephropaths and uncomplicated subjects. These increases were particularly significant in the nephropaths compared with uncomplicated group (1.77 ± 0.22 vs. 1.16 ± 0.04 , $p=0.02$) (Table 2). ARI treatment significantly decreased the DNA-binding activities of NFκB to κB motif under high glucose conditions in both the nephropaths by 67% (1.77 ± 0.22 vs. 0.58 ± 0.06 , $p=0.0001$) and uncomplicated by 32% (1.16 ± 0.04 vs. 0.79 ± 0.06 , $p=0.0002$) groups, respectively (Table 2). The ARI-linked decrease in NFκB binding activity was significantly greater in the nephropaths compared to the uncomplicated (0.58 ± 0.06 vs. 0.79 ± 0.06 , $p=0.032$) (Table 2).

High glucose significantly increased protein level of the AKR1B1

Western blots were used to measure the AKR1B1, NFκB p65, IκB-α and phosphorylated IκB-α protein levels (Figure 2). In the presence of high glucose there was a significant increase in AKR1B1 protein in PBMCs from the nephropaths compared to the uncomplicated (1.26 ± 0.08 vs. 0.93 ± 0.07 , $p=0.02$). This is consistent with our earlier study of elevated AKR1B1 mRNA and proteins by high glucose in the nephropaths [6, 19]. In the presence of the ARI, the elevation of AKR1B1 protein by high glucose was significantly suppressed in PBMCs from the nephropaths (1.26 ± 0.08 vs. 0.76 ± 0.07 , $p=0.01$), but not from uncomplicated subjects (0.93 ± 0.07 vs. 0.81 ± 0.06 , $p=0.24$) (Table 3). High glucose slightly increased the expression of NFκB p65 and phosphorylated IκB-α and IκB, respectively, in PBMC from the nephropaths, but those increases were not significantly different compared to PBMC from the uncomplicated subjects. In the presence of ARI, those protein levels were decreased by various degrees from 10 to 20% compared with that under high glucose conditions from the nephropaths, respectively. Again, these changes were not statistically significant. All results are summarised in Table 3.

Silencing of NFκB reduces AKR1B1 gene transcription

These results were consistent with our previous studies showing [6,18] that the recombinants with the Z-2/C-106 AKR1B1 haplotype had the highest transcriptional activity among all haplotypes. The Z/C-106 AKR1B1 haplotype had the second highest transcriptional activity in transfected HEK 293 cells in luciferase assays. High glucose significantly increased AKR1B1 transcription by 1.2 fold in the HEK293 cells transfected with both Z-2/C-106 and Z/C-106 AKR1B1 haplotypes, respectively (Figure 3A and B). In the presence of the siRNA targeting NFκB p65, transcriptional activities of AKR1B1 in the Z-2/C-106 recombinants were reduced by 50%, 56%, 60% and 50% under NG, HG,

ARI and ARI with HG, respectively (Figure 3A). Similarly, transcriptional activities of AKR1B1 with Z/C-106 recombinants were decreased by 50%, 57%, 56% and 37% under NG, HG, ARI and ARI with HG, respectively (Figure 3B). The silencing of NF κ B p65 gene was confirmed by Western blotting by using an antibody against human NF κ B p65 (Figure 3C). However, the ARI did not decrease transcriptional activities in those cells transfected with either Z-2/C-106 or Z/C-106 AKR1B1 haplotypes without siRNA transfection. There were no significant changes in transcriptional activities in transfected cells with the control siRNA compared to cells without siRNA between either normal glucose, high glucose or, high glucose and ARI for both haplotypes (data not shown).

DISCUSSION

Chronic hyperglycaemia is a major initiator of microvascular complications in diabetes. Accelerated flux through the polyol pathway may be a key to the development of diabetic nephropathy and other complications [3]. There is strong evidence that those patients with diabetic nephropathy have increased expression and activity of AKR1B1. We previously demonstrated that the increased expression of AKR1B1 gene is accompanied by elevated AKR1B1 protein level, is ORE-dependent, and regulated by NFAT5 under high glucose conditions [6]. High glucose induced NFAT5 binding activities to the OREs has been also observed in the myo-inositol oxygenase (MIOX) gene [20, 21], which is closely linked to the polyol pathway and has been suggested to play an important role in the development of diabetic nephropathy [22].

NF κ B is activated by a large variety of stimuli including cytokines, viruses, oxidative stress and genotoxic drugs. There is growing evidence that high glucose as well as diabetes can activate NF κ B expression [14, 23-25]. Previous studies have shown that

NF κ B-DNA binding activity was increased in response to elevated glucose in cultured porcine vascular smooth muscle cells [23] and PBMCs from patients with type 1 diabetes [14] and the activity correlated with blood glucose levels. In present study, we demonstrated that the binding activity of NF κ B to the κ B motif of the AKR1B1 gene was significantly increased under high glucose conditions in nephropaths. In contrast, there was no increase in NF κ B-DNA binding activity in patients without microvascular complications. The exact mechanisms have still to be elucidated and explored, but genetic factors or an imbalance of redox probably make important contributions. Our previous report [26] indicated there was a defect of anti-oxidant gene expression in patients with nephropathy. Since high glucose causes excess free radical generation [27, 28], this implies that the increase in NF κ B binding activity may be due to a generalized response to oxidative stress. This is supported by our previous report that PBMCs of nephropaths have decreased expression of cytoplasmic anti-oxidant genes in response to excess glucose, whilst those from patients with no complications have a marked increase in expression of these genes [26]. Since NF κ B activation is redox dependent [29, 30], and increased metabolic flux through AKR1B1 is strongly linked to a change in redox potential within the cell [31], it is possible that NF κ B could provide positive feedback to regulate the level of AKR1B1 expression through κ B motif. This notion is supported by our results that ARI decreased the DNA binding activities of NF κ B in both the nephropaths and uncomplicated subjects. It is also supported by Ramana et al. study [15] that ablation of AKR1B1 by siRNA prevented high glucose induced NF κ B activation in vitro. Furthermore, silencing of NF κ B p65 significantly decreased the transcriptional activity of AKR1B1 in vitro in this study, which provided solid evidence that high glucose regulates the expression of AKR1B1 gene through the transcriptional factor NF κ B. The susceptible haplotype (Z-2/C-106) to the diabetic nephropathy showed the highest transcriptional activity and the

common haplotype (Z/C-106) in population showed the second higher transcriptional activity in luciferase assays, which are consistent with our previous results [6, 18]. The different haplotypes may change the binding activity of NF κ B p65 to the κ B motif in the promoter region of the AKR1B1 gene as we have seen in nephropaths. Taken together, reduced luciferase activities by silencing of both NFAT5 [6] and NF κ B p65 expression in HEK 293 cells transfected with those recombinants supports the notion that interaction between NFAT5 and NF κ B is required for transcription of AKR1B1 gene although we believe that there are more accessory proteins involved in the binding complex because our supershift analysis could not completely abolish the bands by using antibodies against NF κ B and NFAT5 (data not shown).

We have demonstrated that increases in NF κ B binding activities in response to high glucose were prevented by treatment with an ARI in PBMCs, particularly in the nephropaths. These findings together with those reported in cell lines and other species [15, 27, 32-34] strongly suggest that increased expression and activity of AKR1B1 is intricately linked with the activation of the pro-inflammatory transcription factor NF κ B. The precise mechanism underlying these observations has still to be determined. Recently it has been shown that inhibition of aldose reductase either by ARIs or SiRNA can abolish many of the pro-inflammatory effects of NF κ B in animal models of systemic inflammation. The inhibition of aldose reductase can modify a number of NF κ B related molecular and cellular pathways within the cell including mitogenic signalling and the accumulation of products of lipid peroxidation [35]. A recent study from the same group showed that high glucose induced NF κ B activation could be prevented by ARI through inhibition of TNF- α synthesis and secretion in vascular smooth muscle cell [36]. All those results indicate that ARI could be modulating the response by decreasing the action of AKR1B1 through its action as a pro-inflammatory mediator [32].

The absence of any significant detectable changes in protein levels of cytoplasmic NF κ B, I κ B- α and P-I κ B- α in the patient groups may be due to a number of reasons including higher DNA binding affinity of NF κ B in the diabetic nephropaths compared with diabetic controls. This might be influenced by genetic variation in the promoter region [18]. In addition, previous reports suggest that the reservoir of individual nuclear proteins is far greater than the total amount of NF κ B protein actually required for binding to the DNA binding motifs [37]. Finally, it is likely that access to these motifs differs between patients with or without nephropathy [18, 38]. Consequently, in our study there may be reduced requirement for NF κ B to translocate from the cytoplasm to the nucleus.

We cannot fully explain patterns of phosphorylated I κ B- α and I κ B- α protein levels under various conditions in both groups. Theoretically, an increase in phosphorylated I κ B- should be accompanied by a decrease in total I κ B due to phosphorylation. A recent review [39] suggested that there are three major NF κ B activating pathways responding to various stimuli: the canonical, the alternative and atypical pathways. Oxidative stress may lead to the atypical pathway with a weak and slow NF κ B signal (with peak activities reached after 2-4 hours) [40] and long-term high glucose may generate oxidative and genotoxic stresses, which may partly induce NF κ B activities in an IKK-independent way. Therefore, the absence of significant decreases in I κ B- α expression in PBMCs may reflect this fact, cells exposed to chronic high glucose. Our observation is consistent with previous reports showing sustained NF κ B activation in the absence of significant decreases in I κ B- α expression in PBMCs exposed to chronic high glucose [14].

In conclusion, the exposure of human PBMCs to high glucose in vitro caused increased binding of NF κ B to the κ B motif. These increases were significantly higher in patients with the nephropathy compared to the uncomplicated subjects and were accompanied with an increase in protein level of AKR1B1. ARI decreased the binding

activity of NF κ B to the κ B motif in PBMCs and the effect was greater in PBMC from nephropaths compared to the uncomplicated subjects. Silencing of NF κ B p65 decreased AKR1B1 transcription in transfected cells, which indicates that the polyol pathway is partly up-regulated through NF κ B under high glucose conditions.

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ACCEPTED MANUSCRIPT

LEGENDS

Figure 1. A: A sequence scheme of the promoter region of AKR1B1 gene. (AC)_n is the dinucleotide repeats located at -2100 and (C/T) is a single nucleotide polymorphism located at -106 of the gene. OREC is underlined and κ B motif is aligned above the OREC.

B: DNA binding activities of NF κ B to κ B motif in HEK 293 cells, which were cultured under normal conditions. Reactions were incubated with 25 μ g of nuclear proteins and labelled NF κ B probe without competitor (lane 1: no protein) and lane 2, or with 50 times competitors corresponding to unlabeled probes OREC (lane 3), NF κ B (Lane 4), a mutated probe which is 5-bp mutation of NF κ B probe (lane 5) and AP1 (lane 6). Lane 7 is a supershift assay with a poly-rabbit antibody against human NF κ B p65. **C:** Representative image of DNA binding of NF κ B in nephropath and uncomplicated subjects. The PBMCs were incubated under normal (NG), high glucose (HG: D-glucose at 25 mmol/l) or aldose reductase inhibitor (ARI: sorbinil at 10 μ mol/l) with HG conditions, respectively.

Figure 2. Western blotting results with antibodies against human AKR1B1, NF κ B p65, phosphorylated I κ B, I κ B and β -actin, respectively. Peripheral blood mononuclear cells (PBMCs) from the nephropaths and uncomplicated subjects were cultured in normal (NG), high glucose (HG: D-glucose at 25 mmol/l) or ARI (sorbinil at 10 μ mol/l) with HG conditions, respectively.

Figure 3. Data are means+SE (5 experiments were performed). Results are relative luciferase activity normalised by the activity of the internal control. We defined the transcriptional activity of Z-2/C-106 haplotype under normal conditions (NG) as 100%. All other values were calculated against it. Duplicated wells were performed for each transfection. Transfected cells were cultured under NG, HG (D-glucose at 25 mmol/l), ARI (sorbinil at 10 μ mol/l) and ARI with HG conditions, respectively (see methods). **A:** Cells were co-transfected with plasmids (promoter of AKR1B1 Z-2/C-106 haplotype) and

siRNA targeting to NFκB p65 exon 9. **B:** Cells were co-transfected with plasmids (promoter of AKR1B1 Z/C-106 haplotype) and siRNA targeting to NFκB p65 exon 9. *, #, § and ¶ indicate significant differences of the transcriptional activity between cells transfected with and without siRNA under NG, HG, ARI and ARI+HG conditions for both **A** and **B** ($p < 0.005$), respectively. // indicates that HG significantly increased AKR1B1 transcriptional activity in cells transfected with either Z-2/C-106 or Z/C-106 haplotype, respectively ($p < 0.05$). **C.** Representative Western blotting results ($n=4$) with antibody against NFκB p65 and β-actin in transfected cells. Lane 1: cells were co-transfected with negative control siRNA and plasmids (Z-2/C-106); Lane 2 (NG) and 4 (HG): cells were co-transfected with siRNA targeting NFκB p65 exon 9 and plasmids (Z-2/C-106); Lane 3 (NG) and 5 (HG): cells were transfected with plasmids (Z-2/C-106), but without siRNA.

Table 1. Clinical characteristics of patients with *type 1* diabetes

	Nephropaths (n=20)	Uncomplicated (n=14)
Male:Female	6:14	4:10
Age (years)	49.6±13.9 (30-82)	48.1±14.9 (19-74)
Age at onset of diabetes (years)	12.3±8.8 (1-35)	15.2±9.4) (2-38)
Duration of diabetes (years)	34.9±8.1 (21-49)	34.3±9.4) (20-53)
Plasma Glucose (mmol/L)	9.8±0.8	10.1±1.5
HbA1c (%)	8.2±0.3	8.3±0.3

Data are means±SE (ranges) in years. Levels of plasma and Hemoglobin A1c (HbA1c) are expressed as means±SE in mmol/l and %, respectively.

Uncomplicated: patients have been diagnosed with *type 1* for at least 20 years but remain free of retinopathy (fewer than five dots or blots per fundus), proteinuria (urine Albustix negative on at least three consecutive occasions over 12 months) and neuropathy (overt neuropathy was defined if there was any clinical evidence of peripheral or autonomic neuropathy).

Diabetic nephropaths: patients have had *type 1* for at least 8 years with persistent proteinuria (urine Albustix positive on at least three consecutive occasions over 12 months or three consecutive total urinary protein excretion rates >0.5g/24h) in the absence of hematuria or infection on midstream urine samples.

**Table 2. DNA binding activities of NFκB to κB motif in PBMCs
under HG and ARI+HG conditions**

	NFκB	
	HG/NG	ARI+HG/HG
Nephropaths (n=20)	1.77±0.22* ^ϕ	0.58±0.06 [§]
Uncomplicated (n=14)	1.16±0.04 [#]	0.79±0.06

Data are expressed as means of fold increases±SE under high glucose conditions compared to normal conditions (HG/NG) or under the aldose reductase inhibitor plus high glucose conditions compared to high glucose conditions without the aldose reductase inhibitor (ARI+HG/HG) in peripheral blood mononuclear cells (PBMCs). The density value was defined as 1 under normal conditions (NG: D-glucose at a final concentration of 5.5 mmol/l). High glucose conditions (HG) are D-glucose at a final concentration of 25 mmol/l and aldose reductase inhibitor conditions (ARI) are sorbinil at a final concentration of 10 μmol/l.

* versus the uncomplicated under HG conditions (p=0.02);

ϕ versus the nephropaths under ARI+HG conditions (p=0.0001);

versus the uncomplicated under ARI+HG conditions (p=0.0002);

§ versus the uncomplicated under ARI+HG conditions (p=0.032).

Table 3. Protein levels of AKR1B1, NFκB and phosphorylated IκB-α and IκB-α in PBMCs under HG and ARI with HG

Proteins	Stress	Nephropaths	Uncomplicated
AKR1B1	HG/NG	1.26±0.08*, # (n=8)	0.93±0.07 (n=4)
	ARI+HG/HG	0.76±0.07 (n=8)	0.81±0.06 (n=4)
NFκB (p65)	HG/NG	1.15±0.17 (n=5)	1.06±0.11 (n=3)
	ARI+HG/HG	0.75±0.12 (n=5)	0.87±0.07 (n=3)
P-IκB-α	HG/NG	1.08±0.15 (n=6)	1.05±0.19 (n=4)
	ARI+HG/HG	1.08±0.12 (n=6)	0.92±0.18 (n=4)
IκB-α	HG/NG	1.18±0.16 (n=6)	1.15±0.08 (n=5)
	ARI+HG/HG	0.88±0.07 (n=6)	0.96±0.05 (n=5)

Data are expressed as means of fold increases ±SE under HG (D-glucose at 25 mmol/l) to normal conditions (HG/NG) or under ARI (sorbinil at 10 μmol/l) with HG conditions to HG conditions (ARI+HG/HG) in peripheral blood mononuclear cells (PBMCs). Sample sizes are showed within parentheses.

* versus the uncomplicated under HG conditions (p=0.02)

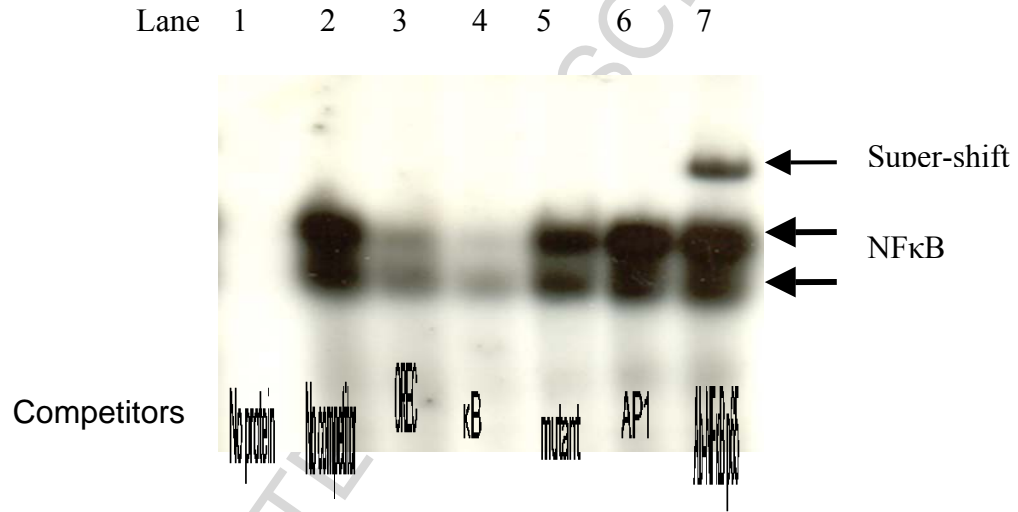
versus the nephropaths under ARI+HG conditions (p=0.01).

A

GGGANNTCAC (κ B motif)
 -2280(AC)_n....CCAAA TGGAAAATCAC CGGCATGGAGTTTA....(C/T).....+10
 (OREC)

The promoter region of the AKR1B1 gene

B



C

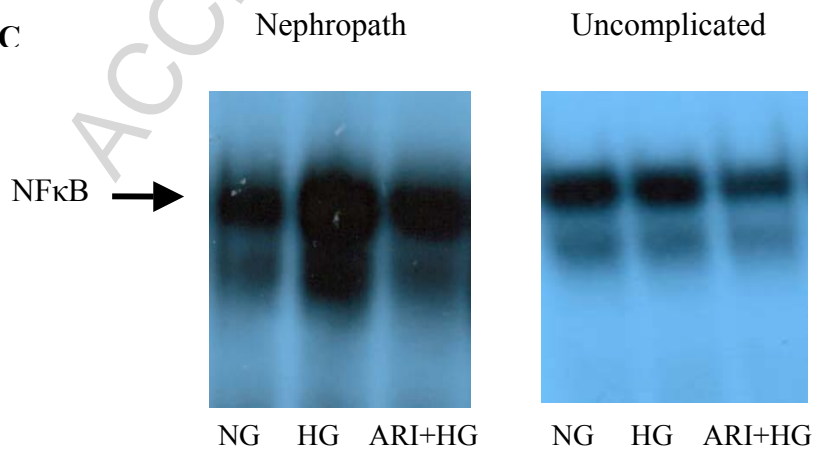


Figure 1

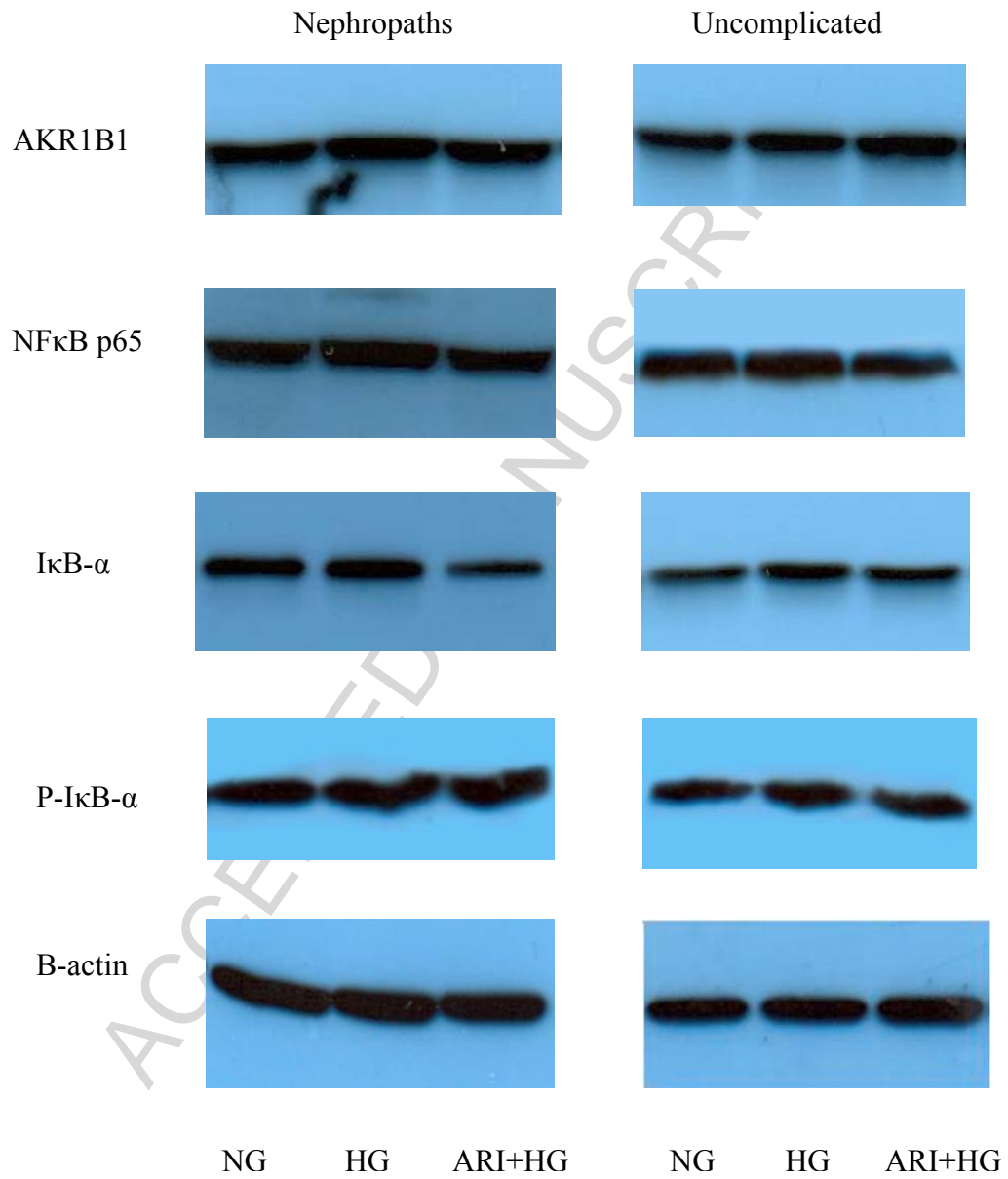


Figure 2

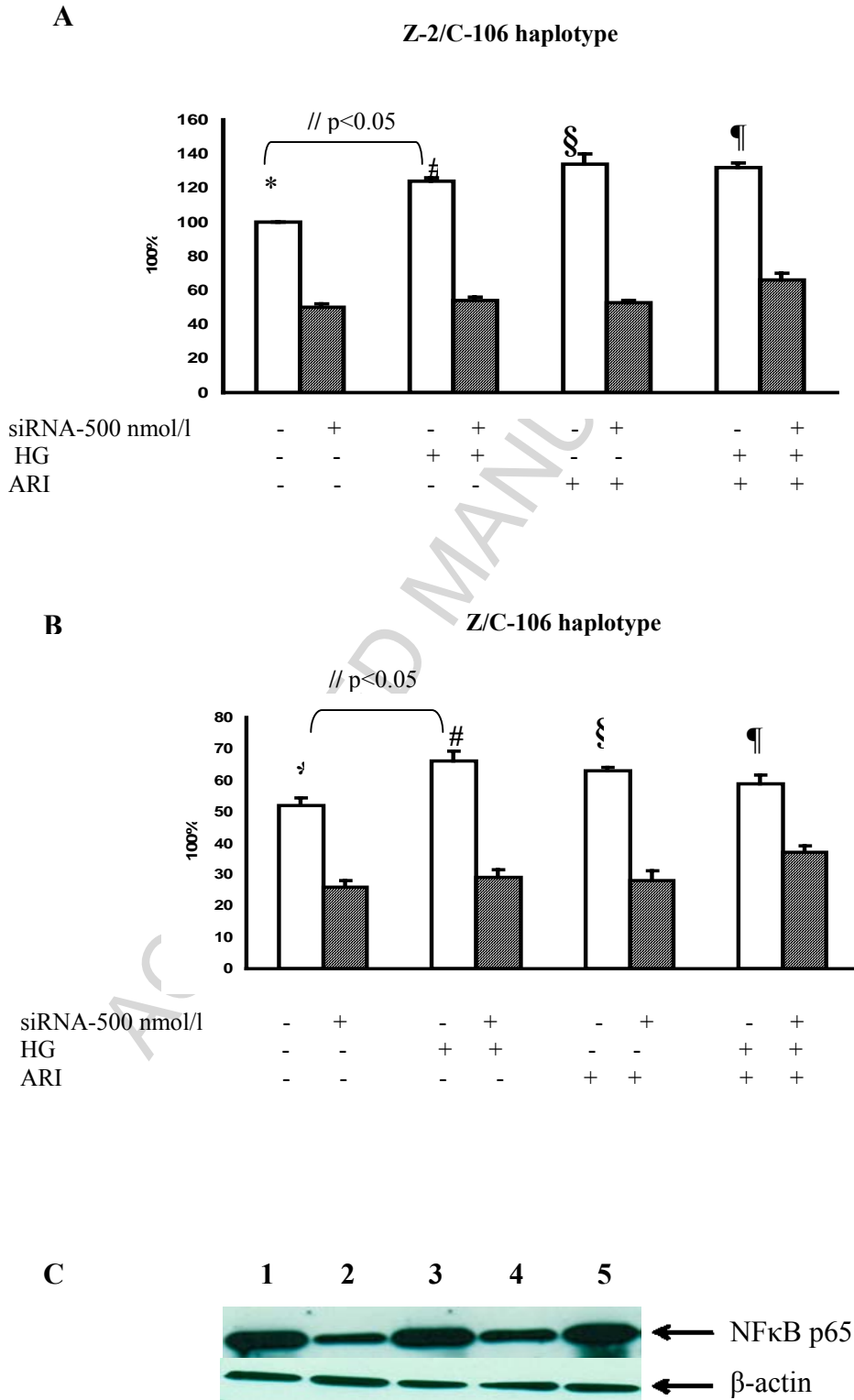


Figure 3