Increased Glucose Uptake and Metabolism in Mesangial Cells Overexpressing Glucose Transporter 1 Increases Interleukin-6 and Vascular Endothelial Growth Factor Production: Role of AP-1 and HIF-1 α

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Key Words

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Abstract

Previous results indicate that enhanced glucose transporter (GLUT)1 expression mediates the deleterious effects of metabolic and hemodynamic perturbations leading to diabetic kidney disease. First screening for altered gene expression in GLUT1 overexpressing cells (GT1) by Affymetrix microarray analysis revealed upregulation of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) expression, which was verified by RT-PCR. Subsequently, IL-6 and VEGF protein production was more than 3-fold increased in the GT1 cells. This upregulation was independent from each other. Studies on the underlying transcriptional mechanisms by gelshift assays and siRNA approach implicated activation of AP-1 in the increased expression of both, IL-6 and VEGF. We

found also increased nuclear protein levels of hypoxia-inducible factor (HIF)-1 α and enhanced DNA binding activity to a hypoxia responsible element located in the VEGF promoter. Knock-down of HIF-1 α reduced the VEGF expression to 50% with an additive effect of AP-1 gene silencing down to 24%. The IL-6 expression was not affected by reducing HIF-1 α . In conclusion our results link increased GLUT1 levels leading to excess glucose metabolism under normoglycemic conditions and altered gene expression of pathogenetic factors involved in diabetic kidney disease.

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Introduction

The ubiquitous glucose transporter (GLUT)1 is the most important glucose transporter in kidney [1] and mesangial cells express predominantly GLUT1 [2].

GLUT1 is a high-affinity ($K_m = 0.5\text{--}3.7\text{ mM}$), low capacity transporter operating close to its maximal transport velocity under physiological glucose concentrations [2–6]. Thus, increases in extracellular glucose concentrations cause not per se enhanced glucose uptake and subsequent metabolism, but are limited by the expression level of GLUT1 in mesangial cells [7].

Following this line, there is circumstantial evidence that elevated levels of GLUT1 are an important mediator in the development of diabetic kidney disease. Both, elevated blood glucose levels and increased blood pressure lead to GLUT1 expression thereby increasing glucose uptake and metabolism [8, 9]. Several characteristic markers of the (pre)diabetic milieu have been recognized to increase GLUT1 expression in glomerular mesangial cells. These include: Hyperglycemia [5, 9], TGF- β 1 [10], IGF-1 [5], hypoxia [11], and noteworthy, angiotensin II and mechanical stress [8, 12].

The functional importance of enhanced GLUT1 expression has been demonstrated in rat mesangial cells overexpressing GLUT1 (GT1) cultured in physiological glucose concentrations. The increases in glucose uptake and metabolism in GT1 cells resulted in increased proliferation and enhanced accumulation of extracellular matrix proteins [6]. The authors conclude that presence of high glucose concentrations is not necessarily required for the development of diabetic kidney disease. Since stable overexpression of antisense GLUT1 in mesangial cells (GT1AS) blocks D-glucose-induced GLUT1 expression and subsequently increased glucose metabolism, increased proliferation and enhanced matrix production [13] the GLUT1 gene transcription appears to be regulated in a positive feedback mechanism.

Our studies on the underlying molecular pathways activated by GLUT1 overexpression revealed clear differences to the deleterious changes induced by ambient high glucose concentrations: we found no increase in oxidative stress and no activation of the p38 MAPK or the extracellular signal-related kinases 1 and 2 MAP kinase pathway and subsequently no increase in TGF- β 1 expression in GLUT1 overexpressing mesangial cells [14]. Thus it appears that the cellular events activated by hyperglycemia or by elevated GLUT1 expression in normoglycemic conditions including both increased glucose uptake and metabolism are not identical.

To get more insight in the molecular mechanisms activated by GLUT1 expression we performed gene expression analysis using Affymetrix microarrays. Based on the data obtained we studied the upregulated gene expression of the cytokines IL-6 and VEGF and investi-

gated the underlying transcriptional mechanisms.

Material and Methods

Cell culture

Preparation of rat mesangial cells stably transfected with the human GLUT1 transporter gene (GT1) or with bacterial β -galactosidase as control cells were described previously [6, 13]. Cells were cultured in RPMI 1640 containing 8 mM glucose corresponding to normal i.e. physiological glucose levels in rats, 2% Ultrosor, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Materials

Oligonucleotides were synthesized by invitrogen (Karlsruhe, Germany). Cell culture media, supplements, Ultrosor and fetal calf serum (FCS) were from Gibco (Eggenstein, Germany). VEGF and IL-6 were from R&D Systems, Wiesbaden, Germany, CoCl₂ was from Sigma-Aldrich (Schnellendorf, Germany). Rabbit polyclonal HIF-1 α -antibodies were from Novus (NB 100-134; Acris Antibodies, Hiddenhausen, Germany).

Oligonucleotide array analysis

Total RNA was isolated using the Rneasy Kit (Qiagen, Hilden, Germany) including a DNase digestion step. The same amount of RNA from three independent cell culture experiments was pooled. Double-stranded DNA was synthesized from total RNA with the Superscript Choice system (Invitrogen, Karlsruhe, Germany) using HPLC-purified T7-(dT)24 primers (Geneset, Paris, France). The cDNA was resuspended in RNase free water and used as a template for in vitro transcription in the presence of biotinylated UTP and CTP to generate labeled cRNA using the BioArray high yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). The labeled cRNA was fragmented in fragmentation buffer (40 mM TRIS acetate (pH 8.1), 10 mM potassium acetate, 30 mM magnesium acetate) and hybridized to the microarrays according to the protocol of the manufacturer. The arrays were rat U34A arrays (Affymetrix, Santa Clara, CA) containing approximately 7000 genes and 1000 ESTs. The microarrays were then washed and stained using the Genechip fluidics station according to the manufacturer's instructions. Data were collected by scanning and analyzed with Microarray Suite 5.0 analysis software (Affymetrix).

RT-PCR and Real-time quantitative PCR analysis

Reverse transcription of total RNA (1 μ g) was performed in a volume of 20 μ l using random hexamers and Avian Myeloblastosis Virus reverse transcriptase with the First strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany) as described [15]. Aliquots (2 μ l) of the reverse transcription reactions were then submitted in triplicate to online quantitative PCR with the Light Cycler system (Roche, Mannheim, Germany) with SYBR® green using the FastStart DNA-MasterSYBR Green I (Roche, Mannheim, Germany). The following primer pairs were used: rat IL-6 sense: CCG GAG AGG AGA CTT CAC AG, antisense: ACA GTG CAT CAT CGC TGT TC, product of

Table 1. Sequences of oligonucleotide probes for EMSAs, ¹according to rat IL-6 promoter sequence, ²according to rat VEGF promoter sequence

Factor	position	Sequence
AP-1	-279/-255 ¹	TCAAGTGCTGAGTCACTTTTAAAGA
CRE	-168/-138 ¹	TTGGATGCTAAATGACGTC ACATTGTGCAAT
NF-IL6	-159/-129 ¹	AATGACGTCACATTGTGCAATCTTAATAAG
NFκB	consensus	GTTAGTTGAGGGGACTTTCCCAGGC
HIF-1α	-83/-61 ²	CCTGTGGAAGCCCACGTATGCACTGT

Table 2. Increased gene expression in GT1 cells. The gene expression profiles of control and GT1 cells cultured for 24 h were analyzed and assessed as described under Methods. The numerical intensity values shown are the “average differences” calculated by Affymetrix software and represents the averaged differences of the hybridization intensities to the specific oligonucleotides minus the hybridisation intensities of the corresponding mismatched oligonucleotides. The tabulated genes were arranged according to their increasing induction in GT1 cells compared to control cells cultured. An independent duplicate microarray experiment was performed with the GT1 cells obtaining similar results. ¹indicates that the expression of the gene by the Affymetrix software was classified as absent.

GenBank TM accession no.	gene description	control cells	GT1 cells	x-fold change
M26744_at	interleukin-6	4.0 ¹	161	28.8
X67948_at	aquaporin 1	132	1823	13.9
M58040_at	transferrin receptor	30	144	4.7
U46118_at	cytochrome P450	45	247	4.1
X17053cds_s_at	small inducible gene JE	328	1491	3.9
U97146_at	lysophospholipase	49	183	3.8
U38379_at	gamma-glutamyl hydrolase	49	267	3.5
S68135_s_at	glucose transporter 1	109	347	3.3
X56551cds_at	fibroblast growth factor-7	42	140	3.3
rc_AI070295_at	Gadd 45a	28	106	3.3
rc_AI175959_at	v-Jun oncogene homolog	105	318	3.1
U03491_g_at	TGF beta-3	657	1541	3.0
rc_AA946108_at	laminin-5 alpha 3 chain	14 ¹	44	2.8
rc_AI180442_at	Farnesyl diphosphate-synthase	114	596	2.8
D63834_at	monocarboxylate transporter-1	86	303	2.7
U23146cds_s_at	gravin	139	414	2.6
rc_AA899253_at	myristoylated alanine-rich PKC substrate	36	135	2.6
D85189_at	fatty acid-CoA ligase	106	247	2.5
AF013144_at	MAP kinase phosphatase	312	670	2.3
K01932_f_at	glutathione-S-transferase (α)	67	140	2.0
X55286_g_at	HMG-CoA reductase	57	117	2.0

161 bp; rat JunD sense: AGC ATG CTG AAG AAA GAC GC; antisense: ATG ATC AGC CTT TCC AGC TC, product of 181 bp; rat VEGF sense: CAA TGA TGA AGC CCT GGA GT, antisense: TTT CTT GCG CTT TCG TTT TT, product of 210 bp. The PCR was performed in a volume of 20 µl: 2 µl FastStart DNA-MasterSYBR Green I, MgCl₂ 4 mM, and primers according

to a primer concentration of 1 µM. The instrument settings were: After denaturing at 95°C for 10 minutes, cycling was performed by denaturing at 95°C for 15 s, annealing at 68°C for 5 s, elongation for 6 s for rat IL-6; the number of cycles was 45; annealing at 67°C for 5 s, elongation for 8 s for rat JunD; the number of cycles was 40; annealing at 62°C for 10 s, elongation

Table 3. Decreased gene expression in GT1 cells. The gene expression profiles of control and GT1 cells cultured for 24 h were analyzed and assessed as described under Methods. For details see also legend to Table 2.

GenBank™ Accession no.	gene description	control cells	GT1 cells	x-fold change
M96601_at	taurine transporter	200	2.8 ¹	0.02
rc_A1230247_s_at	selenoprotein P	137	4.4 ¹	0.03
X62660mRNA_at	glutathione transferase subunit 8	155	5.1 ¹	0.04
rc_A1172247_at	xanthin dehydrogenase	63	2.3 ¹	0.04
AB009999_g_at	CDP-diacylglycerol synthase	50	8.7 ¹	0.16
D30647_at	acyl-CoA dehydrogenase	42	7.5 ¹	0.18
U09401_s_at	tenascin	104	12 ¹	0.18
M64711_at	endothelin 1	68	12 ¹	0.22
U07181_at	lactate dehydrogenase B	1400	272	0.22
U26663_s_at	prostaglandin F receptor	80	26 ¹	0.23
D78359_at	sushi-repeat-containing protein	585	147 ¹	0.28
AF016296_at	neuropilin	124	34 ¹	0.30
M92340_at	interleukin-6 signal transducer/ gp130	372	129	0.30
M65149_at	C/EBP delta	246	84	0.34
S81497_I_at	cholesterol esterase	2020	769	0.38
M80367_at	guanylate binding protein 2	135	42	0.39
U69272_at	interleukin-15	215	47	0.40
U38376_s_at	phospholipase A2, group IVA	135	70	0.41
rc_A1171844_at	F1-ATPase epsilon subunit	1010	493	0.45
L48209_s_at	cytochrome c oxidase	2121	963	0.47
rc_A1102505_g_at	cytochrome c oxidase subunit VIIIa	4621	2230	0.48

for 9 s for rat VEGF; the number of cycles was 40. Quantification was performed by online monitoring for identification of the exact time point at which the logarithmic linear phase was distinguishable from the background. Serially diluted samples obtained by PCR with the above mentioned primers from wild type rat mesangial cells, purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany) were used as external standards in each run. The cycle numbers of the logarithmic linear phase were plotted against the logarithm of the concentration of the template DNA and the concentration of cDNA in the different samples was calculated with the Light Cycler software (version 5.32).

Enzyme-linked immunosorbent assay for IL-6 and VEGF

Supernatants of GT1 and control cells were collected at the indicated time points and IL-6 or VEGF protein concentrations were measured by the Quantikine Immunoassay (R&D Systems, Minneapolis, MN).

Western blotting

Cytosolic or nuclear extracts from GT1 and control cells were obtained and Western blotting was performed as described recently [15].

Determination of DNA concentrations

DNA in total extracts was measured by fluorometry with bisbenzimidazol [16].

Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded oligonucleotides (Table 1) were radioactively end labeled and incubated with up to 25 µg nuclear proteins in 20 µl of 7 mM HEPES-KOH (pH 7.9), 100 mM KCl, 3.6 mM MgCl₂ and 10% glycerol on ice for 20 minutes [17]. For HIF gel-shift assays, the incubation was performed in 50 mM KCl, 1 mM MgCl, 1 mM EDTA, 10 mM TRIS and 5 mM DTT (pH 7.7). As an unspecific competitor 0.05 mg/ml poly[d(I-C)] was added. The samples were run on a 5% non-denaturing

Fig. 1. IL-6 expression and production is elevated in GLUT1 overexpressing cells. (A) mRNA expression levels of IL-6 of control (con) and GT1 cells grown for 24 h were determined by quantitative PCR analysis. The mRNA level of IL-6 measured in control cells was set as 100%. The data are presented as the mean $\pm$ SD ($n = 3$). (B) IL-6 protein concentration in cell culture supernatants of control cells and GT1 cells grown for 24 h or 48 h. The data are presented as means $\pm$ SD ($n = 3$).

polyacrylamide gel in a buffer containing 25 mM Tris-HCl (pH 8.0), 190 mM glycine, and 1 mM EDTA. Gels were dried and analyzed by autoradiography. Specificity of binding was ascertained by competition with excess of unlabeled consensus oligonucleotides or by supershift.

Small interfering RNA (siRNA)

siRNA versus IL-6 and junD were designed, synthesized and annealed at Dharmacon Research, Lafayette, CO. For siRNA targeting the rat IL-6 mRNA (GenBank accession no. NM_12589), the sequence AAC AGC GAT GAT GCA CTG TCA and for siRNA targeting rat JunD mRNA (GenBank accession no. NM_138875) the sequence AAC CGC ATC GCC GCT TCC AAA was used. For silencing HIF-1 α , the sequence GCA GTT ACT CAT GCA TAT A (Genbank accession number NM_024359) was used. The corresponding RNA oligonucleotides were synthesized at MWG-Biotech (Ebersberg, Germany). Transfection was performed with Oligofectamin (Invitrogen, Karlsruhe, Germany) with 0.2 μ M siRNA according to the instructions of the manufacturer, cells were harvested after 48 h. An unrelated siRNA targeting firefly luciferase was used as control in all experiments.

Statistical analysis

Results presented were derived from at least three independent experiments ($n = 3$). Means $\pm$ SD were calculated and groups of data were compared using Student's t test. Statistical significance was set at $p < 0.05$.

Results

Effects of GLUT1 overexpression on gene expression profiles

As previously shown, GLUT1 overexpression resulted in 5-fold increased glucose uptake and subsequently elevated glucose metabolism determined as lactate production [6, 18]. We applied a DNA microarray approach to screen for short term effects of GLUT1 overexpression on mesangial gene expression. By using Affymetrix Genechips the gene expression profiles of control and GT1 cells grown for 24 h in normo-physiological glucose concentrations were determined. An overview of the predominantly GLUT1-regulated genes is presented in the table 2 and 3. Among the induced genes, the expression of the inflammatory cytokine interleukin-6 (IL-6) was upregulated to the highest extend (28-fold). When we analyzed the effect of high glucose (30 mM) on gene expression in control cells by Affymetrix Genechips a 3.4-fold increase of IL-6 mRNA was found (data not shown). Screening for altered expression of other cytokines we found also an increase in vascular endothelial growth factor (VEGF) expression (1.7-fold). Thus increased glucose metabolism led to substantial

Fig. 2. VEGF expression and production is elevated in GLUT1 overexpressing cells independent of IL-6. (A) mRNA expression levels of VEGF of control (con) and GT1 cells grown for 24 h was determined by quantitative PCR analysis. The mRNA level of VEGF measured in control cells was set as 100%. The data are presented as the mean $\pm$ SD (n = 7). (B) VEGF protein concentration in cell culture supernatants of control cells and GT1 cells grown for 48 h or 72 h (b.d. below detection limit of the ELISA). Bars show means $\pm$ SD (n = 6). (C) VEGF protein concentration in cell culture supernatants of control cells grown for 72 h stimulated with 20 ng/ml IL-6 at time points 0, 16 and 40 and 64 h or of unstimulated cells (-). Bars show means $\pm$ SD (n = 3). (D) Effect of IL-6 knock-down on VEGF production. GT1 cells were treated with IL-6 siRNA or an unrelated siRNA targeting firefly luciferase (-) for 72 hours. Bars show means $\pm$ SD for n=3 experiments.

expression of IL-6 and to enhanced expression of VEGF, two cytokines associated with macro- and diabetic microvascular disease including diabetic nephropathy [19-21] and the development of type 2 diabetes mellitus [22].

GLUT1 overexpression enhances IL-6 expression and production in mesangial cells

The induction of IL-6 expression was confirmed by RT-PCR and ELISA analysis. The quantification of IL-6 mRNA levels using real-time PCR revealed an 11.3 ± 2 -fold increase in GT1 cells compared to control cells (Fig. 1A). IL-6 protein production was also elevated in the GT1 cells. In the supernatant of control cells 20.4 ± 5 pg/ml IL-6 protein was detected after 24 h compared to 64.0 ± 6.6 pg/ml of GT1 cells (Fig. 1B). After 48 h, control cells produced 27.1 ± 5.1 pg/ml IL-6 protein and GT1

cells produced 91.3 ± 10.9 pg/ml. A similar induction of IL-6 protein synthesis by the GT1 cells was obtained when the data were corrected for the DNA content of the cells (data not shown). These results show that the increase in IL-6 mRNA expression in the GT1 cells leads to subsequent elevated production and secretion of IL-6 protein.

GLUT1 overexpression enhances VEGF expression and production in mesangial cells

Next we studied VEGF expression and production by RT-PCR and ELISA. We found an 2-fold increase in VEGF mRNA expression in GT1 cells compared to control cells (Fig. 2A) and a pronounced effect on VEGF protein production: in the supernatant of control cells VEGF protein was below detection level after 48 h and

Fig. 3. Transcription factors involved in increased IL6 expression in GT1 cells. Electrophoretic mobility shift assays with nuclear extracts obtained from control cells (con) or GT1 cells using binding sites for AP-1 (A), CRE and NF-IL6 (B) and NFκB (C) as shown in Table 1. 5x, 15x cold probe excess: addition of x-fold molar excess of cold AP-1 binding site (A); + TNFα stimulation of GT1 cells with 1nM TNFα for 15 min (C). Specific binding is marked by brackets, free labeled DNA probes are not shown. (D) Effect of JunD knock-down on IL-6 mRNA expression. GT1 cells were treated with JunD siRNA or an unrelated siRNA (firefly luciferase) (-) for 48 hours. The IL-6 mRNA was measured by quantitative RT-PCR from 1 μg total RNA. IL-6 expression of GT1 cells treated with firefly luciferase was set as 100%. Bars show means ± SD (n = 14).

at 9.6 ± 4.9 pg/ml after 72 h (Fig. 2B) while GLUT1 overexpression led to markedly increased synthesis of VEGF protein (36.8 ± 20.6 pg/ml after 48 h, 37.7 ± 12.8 pg/ml after 72 h).

Because previous studies have shown that IL-6 may induce VEGF via IL-6 responsive elements located in the VEGF promoter and the IL-6 production in the GT1 cells precedes the VEGF production by approximately 24 h, we reasoned that the overproduced IL-6 may stimulate VEGF gene expression [23]. However, stimulation of control cells with 20 ng/ml IL-6 had no effect on VEGF protein levels (Fig. 2C) and using IL-6 siRNA, which reduced the increased IL-6 expression to approximately 20 % (data not shown), we found no change in VEGF production, both results indicating that the enhanced VEGF expression in GT1 cells is not mediated by the increased IL-6 concentrations (Fig. 2D).

Transcriptional mechanisms for increased IL-6 production

We then investigated which transcriptional mechanisms are involved in the pronounced upregulation of IL-6 in the GT1 cells. In previous studies several activating regulatory elements in the rat IL-6 promoter have been identified together with the involved transcription factors CRE, NF-IL6, NFκB and AP-1 [24-26]. We tested the binding activity of nuclear extracts of control cells and GT1 cells to these potential regulatory DNA sequences using EMSA. A representative result for the potential regulatory DNA sequences AP-1 (-279/-255), CRE (-168/-138) and NF-IL6 (-159/-129) is shown in Figure 3A and B, respectively. We observed a clearly enhanced DNA binding activity to the AP-1 site. The specificity of the binding was verified by addition of increasing amounts of competing cold probe (Fig. 3A).

Fig. 4. Transcription factors involved in increased VEGF expression in GT1 cells. (A) Western blotting of nuclear extracts obtained from unstimulated (-) or CoCl_2 -treated (+; 0,2 mM for 4 h) control and GT1 cells. Arrow indicates the 116 kD band detected by the anti-HIF-1 α antibodies. (B,C) Electrophoretic mobility shift assays with nuclear extracts obtained from control and GT1 cells using the HRE (-83/-61) site of the rat VEGF promoter (Table 1). + 10, 50 x cold: addition of 10- or 50-fold molar excess of cold HRE (B), + anti-HIF-1 α , + anti-NF κ B: addition of the corresponding antibodies (C). Specific binding to the HRE is shown by brackets, arrows indicate super-shifted bands, free labeled DNA probe is not shown. (D,E,F) Effect of HIF-1 α and JunD knock-down on mesangial VEGF mRNA expression. GT1 cells were treated with HIF-1 α siRNA (D), JunD siRNA (E) or both (F) for 48 hours. The VEGF mRNA was measured by quantitative RT-PCR from 1 μ g total RNA. VEGF expression of GT1 cells treated with control siRNA targeting firefly luciferase (-) was set as 100%. Bars show means $\pm$ SD (n = 6).

In contrast, no difference was found for binding to CRE and NF-IL6 sequences, although we observed protein binding to these promoter sites (Fig. 3B). We detected no specific binding of the nuclear extracts to a consensus NF κ B binding site although stimulation of GT1 cells with TNF α , a known strong activator of NF κ B, resulted in specific binding to the NF κ B consensus sequence (Fig. 3C). These results suggest an involvement of AP-1, but not of NF κ B, NF-IL6 or CRE regulatory elements in the increased IL-6 expression in GT1 cells.

Since we have previously shown that JunD is markedly increased in nuclear extracts of GT1 cells and that Jun proteins are involved in the increased formation of an AP-1 complex in GT1 cells [14] we studied the role of this protein in IL-6 overexpression. Using an siRNA approach JunD expression was reduced to approximately 40% (not shown) which in turn resulted in significantly decreased IL-6 expression of 76% (Fig 3D). Together, the data show that the increased glucose metabolism in GT1 cells leads to enhanced activation of AP-1 and

subsequent elevated IL-6 expression.

Transcriptional mechanisms for increased VEGF production

The most prominent transcription factor involved in transcriptional activation of the VEGF gene is hypoxia-inducible factor (HIF)-1 α . Using Western blotting we observed increased nuclear HIF-1 α protein levels in GT1 cells compared to control cells (Fig. 4A). Treatment of the cells with CoCl₂ to induce hypoxia served as positive control. Next we studied DNA binding to the hypoxia responsible element (HRE) -83/-61 located in the VEGF promoter by EMSA. We found a markedly enhanced binding of nuclear extracts obtained from GT1 cells to this HRE (Fig. 4B). The specificity of the binding is confirmed by competition with excess cold HRE (Fig. 4B) and HIF-1 α was identified in the DNA binding complex by supershifted bands after addition of anti-HIF-1 α antibodies (Fig. 4C). Addition of anti-NF κ B antibodies served as negative control. Thus enhanced glucose uptake and metabolism activates HIF-1 α .

To demonstrate the functional relevance of HIF-1 α in the elevated VEGF expression in the GT1 cells, we silenced HIF-1 α expression using siRNA oligonucleotides. The knock-down of HIF-1 α resulted in clearly reduced VEGF mRNA expression (Fig. 4D). Since the VEGF promoter contains also AP-1 sites we studied also the effect of reducing the activated AP-1 complex in the GT1 cells by JunD siRNA. Knock-down of JunD reduced VEGF expression to 56% compared to control transfected cells (Fig. 4E), and silencing of both, JunD and HIF-1 α had an additive effect ($24 \pm 8\%$ VEGF expression compared to control transfected cells; Fig. 4F). Thus, increased VEGF expression in the GT1 cells is mediated by activation of AP-1 and HIF-1 α . In contrast, knock-down of HIF-1 α had no effect on the enhanced IL-6 expression in the GT1 cells (data not shown).

Discussion

The first major finding of this study is that increased glucose uptake and metabolism in mesangial cells led to induced expression and secretion of the two cytokines IL-6 and VEGF. The upregulation of the cytokines in GLUT1 overexpressing cells was found (a) by using the Affymetrix chip, (b) by quantitative real-time PCR, and (c) at the protein level by immunological determination of the IL-6 and VEGF protein accumulation in the cell culture supernatant.

The enhanced expression of IL-6 in human diabetic nephropathy has been demonstrated by high resolution *in situ* hybridization [19]. Most cells in the area of mesangial proliferation were strongly stained for IL-6 mRNA while only few cells were positive in advanced glomerular lesions. Thus, IL-6 mRNA is expressed by glomerular resident cells and interstitial cells and its expression is associated with mesangial proliferation in diabetic nephropathy. Of note, GT1 cells exhibit higher proliferation rates compared to control cells [13] which was prevented by knock-down of IL-6 (C. Weigert, unpublished data). Thus IL-6 could act as growth factor on mesangial cells [27]. Furthermore, an increased urinary excretion of IL-6 in diabetic patients has been reported [20]. These data indicate that IL-6 is primarily expressed in glomeruli in early stages of diabetic nephropathy and that urinary IL-6 levels may be a good indicator for the progression of the disease.

The expression of VEGF in diabetic kidney has been studied in more detail. Glomerular VEGF expression was highest in type 1 and 2 diabetic patients with mildest sclerotic changes [28, 29] while patients with diabetic nephropathy show fewer glomerular VEGF mRNA positive cells than controls [28, 30]. Similar results were obtained with experimental animal models for type 1 and type 2 diabetes [21]. Urinary VEGF excretion was also elevated in patients with early diabetic nephropathy (Haller H: personal communication). In sum, the data indicate that induction of IL-6 and VEGF expression is an early event in the development of diabetic nephropathy.

The second major finding of this study is the activation of the transcription factor HIF-1 α by the enhanced glucose uptake and metabolism. We found increased nuclear protein levels of HIF-1 α in the GT1 cells and markedly elevated DNA binding activity to the HRE located in the VEGF promoter. Subsequently, HIF-1 α accounts for approximately 50% of the VEGF expression in the GT1 cells as demonstrated by siRNA silencing. Hyperglycemia has been shown to induce pathophysiological changes and cellular disturbances similar to hypoxia. Since this occurred under normoxic conditions, this phenomenon has been termed "hyperglycemic pseudohypoxia" [31]. However, the activation of HIF-1 α and HIF-dependent gene activation could also be blunted by hyperglycemia [32, 33]. In the present study using the cell model of GLUT1 overexpressing mesangial cells we could clearly discriminate the effects of high extracellular glucose concentrations and increased glucose metabolism. We could demonstrate that increased glucose uptake and

metabolism in the absence of elevated extracellular glucose concentrations are effective and sufficient to activate HIF-1 α and subsequently VEGF expression. In a recent paper it has been shown that lactate and pyruvate, the endproducts of glycolysis, could be responsible for the accumulation of HIF-1 α protein by inhibition of HIF-1 α degradation independently of hypoxia [34]. We hypothesized that this could also be the underlying mechanism of HIF activation in the GT1 cells, since these cells exhibit 2-3 times higher lactate production compared to control cells [18]. Thus increased glycolytic flux per se is sufficient to activate HIF-1 α -dependent gene expression.

Activation of the transcription factor complex AP-1 is involved in the upregulation of both, VEGF and IL-6 expression. In a previous study we demonstrated enhanced nuclear protein levels of Jun proteins in the GT1 cells and subsequently increased DNA binding activity to a consensus AP-1 binding site [14]. Here we showed specific and increased binding to the AP-1 motif -279/-255 of the rat IL-6 promoter [24, 35]. Binding to the AP-1 sites located in the VEGF promoter [36] has not been tested in the present study. The causal involvement of activated AP-1 in increased expression of IL-6 and VEGF was proven by the knock-down of the AP-1 component JunD, which reduced the mRNA expression of VEGF and lowered the expression of IL-6 to a minor extend. The activation of AP-1 has been implicated in the development of diabetic nephropathy in several studies [17, 37, 38]. Transfection with AP-1 decoy oligonucleotides strongly inhibited high glucose- and angiotensin II-induced cell proliferation and expression of matrix genes in cultured mesangial cells. Administration of these decoy oligonucleotides into diabetic rat kidney virtually abolished TGF- β 1 and plasminogen activator inhibitor-1 expression [37]. Collectively, the data indicate that AP-1 activation is crucial for mesangial proliferation and matrix production in response to high glucose and angiotensin II.

Since the knock-down of both, AP-1 and HIF-1 α , prevented the VEGF expression almost completely, we suggest that these are the major important factors for the upregulation of VEGF in the GT1 cells. The mechanism of the increased IL-6 expression remains less clear. Among all the known regulatory elements of the rat IL-6 promoter tested we observed enhanced DNA binding activity only to one AP-1 site. Particularly the activation of NF κ B, which is an important factor for IL-6 gene activation [39], was not detectable in the GT1 cells. In contrast to VEGF expression, the knock-down of HIF-1 α had no effect on IL-6 expression. Since reduction of

JunD using siRNA had only a minor, even though significant effect on IL-6 expression further yet unknown transcription factors must be responsible for the upregulated IL-6 expression in the GT1 cells.

Several previous studies revealed essential biochemical pathways involved in the pathogenesis of diabetic nephropathy. Among them, activation of PKC isoforms and enhanced flux through the sorbitol pathway are shared pathomechanisms of hyperglycemia and increased GLUT1 expression [14, 40]. In GT1 cells activated PKC- α and - β lead to increased DNA binding of AP-1 subsequently enhancing transcription of genes of ECM proteins as shown in our previous study [14] and presumably genes like IL-6 and VEGF. Noteworthy a recent report demonstrates that PKC- α deficient diabetic mice show no increase in glomerular VEGF protein expression and no elevation of urinary albumin excretion [41]. In the present study we could demonstrate a novel molecular mechanism for the altered gene expression possible involved in the development of diabetic nephropathy: the enhanced glucose uptake and glycolysis is responsible for activation of HIF-1 α -dependent gene activation.

In conclusion, increased GLUT1 expression in mesangial cells represents a key event in the development of diabetic nephropathy. The present data provide a molecular link of the enhanced glucose uptake and known pathomechanisms of diabetic late-complications, since the subsequently elevated glucose metabolism activates gene expression of cytokines as IL-6 and VEGF.

Abbreviations

AP-1 (activated protein 1); CRE (cAMP responsive element); ECM (extracellular matrix); EMSA (electrophoretic mobility shift assay); GLUT1 (glucose transporter 1); GT1 (GLUT1 overexpressing cells); IL-6 (interleukin-6); HIF (hypoxia inducible factor); NF κ B (nuclear factor κ B); PKC (protein kinase C); siRNA (small interfering RNA); TGF- β (transforming growth factor β); VEGF (vascular endothelial growth factor).

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