

Advanced glycation end products cause increased CCN family and extracellular matrix gene expression in the diabetic rodent retina

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Abstract

Aims/hypothesis Referred to as CCN, the family of growth factors consisting of cystein-rich protein 61 (CYR61, also known as CCN1), connective tissue growth factor (CTGF, also known as CCN2), nephroblastoma overexpressed gene (NOV, also known as CCN3) and WNT1-inducible signaling pathway proteins 1, 2 and 3 (WISP1, -2 and -3; also known as CCN4, -5 and -6) affects cellular growth, differentiation, adhesion and locomotion in wound repair, fibrotic disorders, inflammation and angiogenesis. AGEs formed in the diabetic milieu affect the same processes, leading to diabetic complications including diabetic reti-

nopathy. We hypothesised that pathological effects of AGEs in the diabetic retina are a consequence of AGE-induced alterations in CCN family expression.

Materials and methods CCN gene expression levels were studied at the mRNA and protein level in retinas of control and diabetic rats using real-time quantitative PCR, western blotting and immunohistochemistry at 6 and 12 weeks of streptozotocin-induced diabetes in the presence or absence of aminoguanidine, an AGE inhibitor. In addition, C57BL/6 mice were repeatedly injected with exogenously formed AGE to establish whether AGE modulate retinal CCN growth factors in vivo.

Results After 6 weeks of diabetes, *Cyr61* expression levels were increased more than threefold. At 12 weeks of diabetes, *Ctgf* expression levels were increased twofold. Treatment with aminoguanidine inhibited *Cyr61* and *Ctgf* expression in diabetic rats, with reductions of 31 and 36%, respectively, compared with untreated animals. Western blotting showed a twofold increase in CTGF production, which was prevented by aminoguanidine treatment. In mice infused with exogenous AGE, *Cyr61* expression increased fourfold and *Ctgf* expression increased twofold in the retina.

Conclusions/interpretation CTGF and CYR61 are downstream effectors of AGE in the diabetic retina, implicating them as possible targets for future intervention strategies against the development of diabetic retinopathy.

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Diabetes mellitus · Diabetic retinopathy · Experimental ·
Extracellular matrix · Gene expression regulation

Abbreviations

CML	N ^ε -(carboxymethyl)lysine
CTGF	connective tissue growth factor
CYR61	cystein-rich protein 61
COL4A3	collagen type IV alpha 3
LAMB1	laminin beta 1
MSA	mouse serum albumin
NOV	nephroblastoma overexpressed gene
qPCR	quantitative PCR
TGFB	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase
VEGF	vascular endothelial growth factor
WISP	WNT1-inducible signalling pathway protein

Introduction

Diabetic retinopathy is a major complication of diabetes and a leading cause of blindness [1, 2]. Despite recent progress in understanding the pathogenesis of diabetic retinopathy, further research is warranted, as the disease remains neither preventable nor curable.

Diabetic retinopathy is preceded by an asymptomatic preclinical phase, in which a microangiopathy develops which is characterised by diffusely increased vascular permeability and capillary basement membrane thickening, resulting from excess accumulation of extracellular matrix components [3–5]. In later stages of preclinical diabetic retinopathy, endothelial cell and pericyte loss leads to vascular cell death and the development of acellular capillaries. Experimental prevention of basement membrane thickening has been shown to ameliorate these retinal vascular changes [6, 7]. Thus in galactose-fed rats, a model of diabetes, downregulation of fibronectin synthesis partly prevented retinal basement membrane thickening and also reduced pericyte and endothelial cell loss [6]. Combined downregulation of mRNA levels of the extracellular matrix components fibronectin, collagen type IV alpha 3 (*Col4a3*) and laminin beta 1 (*Lamb1*) not only prevented increases in their protein levels, but also reduced vascular leakage in the retina of rats with streptozotocin-induced diabetes [7]. These findings suggest that basement membrane thickening is not just an epiphenomenon of the diabetic state, but may be instrumental in the progression of sight-threatening diabetic retinopathy. Modulation of basement membrane thickening may therefore have a preventive effect on the development of diabetic retinopathy.

However, the mechanisms leading to diabetes-induced basement membrane thickening remain largely unknown. One of the postulated mechanisms is the formation of AGEs in the diabetic milieu. Inhibition of AGE formation by aminoguanidine has been shown to protect against

retinal capillary basement membrane thickening [8]. AGE have also been shown to induce extracellular matrix synthesis in the diabetic rat kidney. A similar induction of extracellular matrix synthesis was also shown to be mediated by connective tissue growth factor (CTGF, also known as CCN2) [9, 10], a member of the family of proteins referred to as CCN (for cystein-rich protein 61 [CYR61, also known as CCN1], CTGF and nephroblastoma overexpressed gene [NOV, also known as CCN3]). CTGF leads to accumulation of extracellular matrix by induction of collagen, fibronectin and laminin synthesis, as well as decreased proteolysis of extracellular matrix components as a result of increased production of tissue inhibitors of metalloproteases (TIMP) [9, 11–18]. Recently, we observed that *Ctgf*^{+/-} mice (lacking one functional allele for *Ctgf*) are protected from diabetes-induced basement membrane thickening of retinal and kidney glomerular capillaries ([19]; P. Roestenberg, F.A. Van Nieuwenhoven, R. Verheul et al., unpublished results).

We hypothesised in the present study that AGE-induced basement membrane thickening observed in the retina is at least partly mediated by CTGF. To establish the role of CTGF in the AGE-induced production of vascular basement membrane components and their mediators, we investigated the effects of aminoguanidine on the levels of CTGF, the other CCN family members (CYR61, NOV and WNT1-inducible signalling pathway protein 1, 2 and 3 [WISP1, -2, and -3, also known as CCN4, -5 and -6]) [20–22] and vascular basement membrane-related molecules in the retina of rats with streptozotocin-induced diabetes, as well as in the retina of mice infused with AGE.

Materials and methods

Animals All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments involving rats were reviewed and approved by the ethics committee for animal care and use of the Free University Medical Centre, Amsterdam, the Netherlands. All experiments involving mice were carried out in accordance with British Home Office regulations.

Streptozotocin-induced diabetic rat model Adult Wistar rats (Charles River, Maastricht, the Netherlands), weighing approximately 250 g, were randomly divided into three experimental groups: a control group (*n*=14), a diabetic group (*n*=16) and a diabetic group treated with aminoguanidine hydrogen carbonate (*n*=14; Fluka, Buchs, Switzerland). Diabetes was induced by a single i.p. injection of 60 mg/kg streptozotocin (Sigma, St Louis, MO, USA). Immediately prior to use, streptozotocin was dissolved in

cold 0.1 mol/l citrate buffer, pH 4.5. Control rats received a single i.p. injection of 0.1 mol/l citrate buffer only. Aminoguanidine was administered from day 1 at a dose of 1 g/l in drinking water. Serum glucose levels and body weight were monitored at the start and end of the experiment. Diabetes was verified by a serum glucose level >13.9 mmol/l. At 6 weeks, half of the rats were randomly selected from the three experimental groups and killed with a lethal dose of pentobarbital sodium (i.p.). At 12 weeks the remaining rats were killed. Eyes from each rat were rapidly enucleated, one being snap-frozen in liquid nitrogen and stored at -80°C , while the contralateral eye was fixed in 4% paraformaldehyde. Additionally, blood samples were collected and plasma levels of N^{ϵ} -(carboxymethyl)lysine (CML) were measured by stable-isotope dilution tandem mass spectrometry [23].

In vivo administration of exogenous AGE Female C57BL/6 mice (10–12 weeks old) were randomly assigned to two groups of equal size and injected i.p. with either native mouse serum albumin (MSA) or glycoaldehyde-modified MSA (10 mg/kg) daily for seven consecutive days. At 3–4 h after the final injections, mice were killed, eyes enucleated and retinas dissected freshly, before being snap-frozen in liquid nitrogen.

Preparation of AGE-modified albumin The glycoaldehyde-modified MSA preparation was made according to Nagai et al. [24]. Following dialysis against PBS, endotoxin was removed using an endotoxin-removing column (Pierce, Rockford, IL, USA). Glycoaldehyde-MSA and native MSA were passed three times through separate columns to ensure that all contaminating endotoxin was removed. Analysis of the CML content of glycoaldehyde-MSA and native MSA was performed using gas chromatography–mass spectrometry. The lysine content of the samples was analysed by cation exchange chromatography and the values for CML were corrected for lysine loss and expressed as mmol CML/mol lysine as previously reported [25].

RNA isolation and mRNA quantification Snap-frozen rat and mouse eyes were allowed to thaw in ice-cold RNAlater (Ambion, Austin, TX, USA). The anterior chambers of the eyes were removed and the retinas were carefully dissected. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The amount of total retinal RNA isolated was approximately 12 μg per retina (spectrophotometric measurements at 260 nm), with no significant differences between the experimental groups. The integrity of the RNA samples was verified using an automated electrophoresis system (Experion; Bio-Rad, Hercules, CA, USA). All samples had sharp ribosomal RNA bands with no sign of degradation.

A 2- μg aliquot of total RNA was treated with DNase I (amplification grade; Invitrogen) and reverse-transcribed into first strand cDNA with Superscript II and oligo(dT) 12–18 (Invitrogen).

Details of the primers are given in the Electronic supplementary material (ESM) (ESM Table 1). Specificity of the primers was confirmed by a nucleotide–nucleotide BLAST (<http://www.ncbi.nlm.nih.gov/blast/index.shtml>) search. The presence of a single PCR product was verified by both the presence of a single melting temperature peak and detection of a single band of the expected size on a 3% agarose gel.

Real-time quantitative PCR (qPCR) was performed in a sequence detection system (ABI Prism 5700; Applied Biosystems, Foster City, CA, USA). For each primer set a mastermix was prepared consisting of 1 \times SYBR Green PCR buffer (Eurogentec, Seraing, Belgium), 3 mmol/l MgCl_2 , 200 $\mu\text{mol/l}$ each of dATP, dGTP and dCTP, 400 $\mu\text{mol/l}$ dUTP, 0.5 U AmpliTaq Gold (Eurogentec) and 2 pmol primers. All cDNA samples were diluted 1:10 and amplified using the following PCR protocol: 10 min at 95°C , followed by 40 cycles of 15 s at 95°C and 60 s at 60°C and a melting program (60 – 95°C). Relative gene expression was calculated using the equation: $R = E^{-C_t}$, where E is the mean efficiency of all samples for the gene being evaluated and C_t is the cycle threshold for the gene as determined during real-time PCR. All qPCR experiments were performed at least twice.

Real-time qPCR data from the mouse experiments were normalised using 18S rRNA, which was determined to be stably expressed in all experimental groups. For the rat experiments, no suitable housekeeping genes that were not regulated by the diabetic background could be found. Therefore, the rat data were normalised using the relative starting amounts of cDNA, which was determined using a novel technique recently developed in our laboratory (J. M. Hughes, I. Klaassen, W. Kamphuis, C. J. F. Van Noorden and R. O. Schlingemann, unpublished results). In brief, reverse transcription reactions were carried out in duplicate with one set of reactions containing the normal dNTP mix and the parallel set of reactions containing a dNTP mix with α - ^{32}P -labelled dCTP. From each sample 4 μl of the α - ^{32}P -labelled dCTP-incorporated cDNA were pipetted on to separate nitrocellulose filters, which were allowed to air-dry. After washing with 0.1 mol/l phosphate buffer, radioactivity of the filters was measured using a scintillation counter (Beckman Coulter, Fullerton, CA, USA).

Western blotting Protein was isolated from paraformaldehyde-fixed retinal tissue as described by Shi et al. [26]. In brief, retinas were dissected from the 4% paraformaldehyde-fixed rat eyes and pooled in 1.5 ml Eppendorf vials in antigen-retrieval buffer (20 mmol/l Tris, 2% SDS, pH 7). The pooled samples were then dissociated using a pestle

and incubated at 100°C for 20 min followed by 2 h at 60°C. Supernatant fractions were collected after centrifugation at 4°C for 15 min at 10,000g. Protein concentrations were determined with a bicinchoninic acid protein assay kit (Perbio, Etten-Leur, the Netherlands) and adjusted to 2.5 µg/µl. For SDS-PAGE and western blots, proteins were separated using 13% mini gels under reducing conditions. Following gel electrophoresis, proteins were transferred to a nitrocellulose filter (Whatman Schleicher & Schuell, Brentford, Middlesex, UK) using a semi-dry transfer cell (Bio-Rad). At the end of the transfer, the filter was blocked in blocking buffer (1% non-fat skimmed milk powder, 1% BSA, 1 mmol/l NaN₃ in Tris-buffered saline and 0.05% Tween) overnight at 4°C while being gently rocked. The filter was incubated in blocking solution for 2 h at room temperature, with the relevant antibodies as defined below. Following three washes in TBS/0.05% Tween-20, the blots were incubated in blocking solution with horseradish peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse antibodies for 1 h at room temp. After extensive washing, blots were developed using a chemiluminescent kit (Super-Signal West Pico; Perbio). Filters were exposed to X-ray film (Kodak-Biomax, Herts, UK).

Primary antibodies (ESM Table 2) were diluted with 0.3% skimmed milk powder in TBS/Tween and horseradish peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse (Perbio) was diluted 1:20,000. Intensity of bands was quantified by densitometry using AlphaEase software (AlphaInnotech, San Leandro, CA, USA).

Immunohistochemistry Cryostat sections (10-µm thick) were stained using an indirect immunoperoxidase procedure as previously described [27]. Primary antibodies are listed in ESM Table 2. Primary antibody was omitted for negative controls. Indirect immunoperoxidase staining was performed using histostaining reagents (Poversion; ImmunoVision, Daly City, CA, USA) for all sections except those incubated with the TIMP1 antibody. The TIMP1 sections were indirectly stained using horseradish peroxidase-labelled rabbit-anti-goat antibody (P0160; Dako, Glostrup, Denmark).

Statistics CML data were log₁₀ transformed to obtain a normal distribution. Significant differences ($p < 0.05$) in glucose and CML plasma levels, and in gene expression levels among groups were calculated with single ANOVA. The Bonferroni post hoc test was used to perform pairwise comparisons of groups.

Results

Glucose and CML levels in control and diabetic rats Induction of diabetes and the degree of hyperglycaemia in

streptozotocin-treated rats was established by serum glucose levels (Fig. 1a). Streptozotocin treatment resulted in a three- to fourfold increase in serum glucose concentration after 6 and 12 weeks irrespective of aminoguanidine treatment.

Plasma levels of CML were measured to determine the efficacy of aminoguanidine treatment. CML plasma levels were elevated by twofold at 6 and 12 weeks after streptozotocin-treatment (Fig. 1b). Aminoguanidine treatment had no effect on CML levels at 6 weeks, but at 12 weeks the CML levels were decreased by approximately 25%.

CCN family gene expression in control and diabetic rats After 6 weeks of diabetes, *Cyr61* mRNA levels in the diabetic retina were increased by threefold against control retina. Treatment with aminoguanidine reduced *Cyr61* expression to levels that were not significantly different from control levels (Fig. 2). CYR61 protein was mainly localised in the ganglion cell layer (Fig. 3). No differences in staining patterns were found between experimental groups.

Ctgf mRNA levels were elevated by twofold at 12 weeks of streptozotocin-induced diabetes. Aminoguanidine treat-

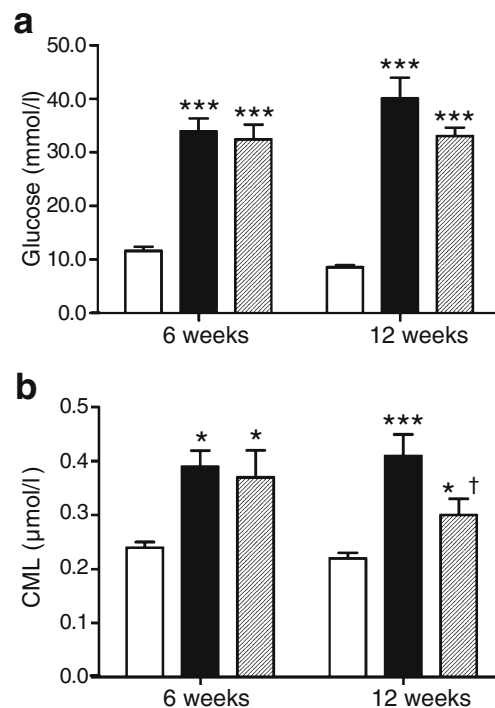


Fig. 1 Plasma glucose (a) and CML (b) levels in control and diabetic rats at 6 and 12 weeks of streptozotocin-induced diabetes. *White bars*, control rats; *black bars*, diabetic rats; *cross-hatched bars*, diabetic rats treated with aminoguanidine. * $p < 0.05$ and *** $p < 0.001$ for difference between experimental and control group. The aminoguanidine group was only significantly different from the groups with diabetes at 12 weeks († $p < 0.05$). The error bars show the standard deviation for each group

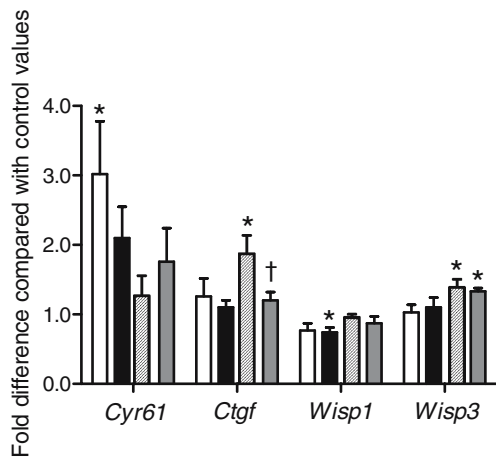


Fig. 2 Gene expression of CCN family members. Fold change, compared with control values, in retinal mRNA levels of CCN family members in streptozotocin-induced diabetic rats at 6 (white bars) and 12 weeks (cross-hatched bars) after streptozotocin-induction and in aminoguanidine-treated, streptozotocin-induced diabetic rats at 6 (black bars) and 12 weeks (grey bars) of diabetes. * $p < 0.05$ for difference between experimental group and control group; † $p < 0.05$ for difference between aminoguanidine-treated diabetic group and diabetes-only group

ment almost completely prevented this increase (Fig. 2). Western blotting showed a 1.8-fold increase in CTGF protein levels in the retina of diabetic rats at 12 weeks, whereas aminoguanidine treatment also prevented this effect (Fig. 4). CTGF immunostaining was mainly found in the ganglion cell layer and was more diffuse throughout the outer plexiform layer, inner nuclear layer and inner plexiform layer (Fig. 3). Differences in staining between experimental groups were not observed.

Wisp1 and *Wisp3* mRNA levels were low in retinas of all groups of rats. The levels never differed more than 1.4-fold between experimental and control groups. Due to small standard deviations, significant differences were found (Fig. 2), but it is doubtful whether these small differences

Fig. 3 Immunohistochemical staining patterns of **a** CYR61, **b** CTGF and **c** TIMP1 in control rat retinas. Intense staining of CYR61 and CTGF was present in large cell bodies of the ganglion cell layer (GCL) and weak staining in the inner plexiform layer (IPL). Intense uniform immunostaining of TIMP1 was found in the GCL and weak staining in the IPL. *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer, *RCL* rod and cones layer, *RPE* retinal pigment epithelium. Magnification: $\times 150$

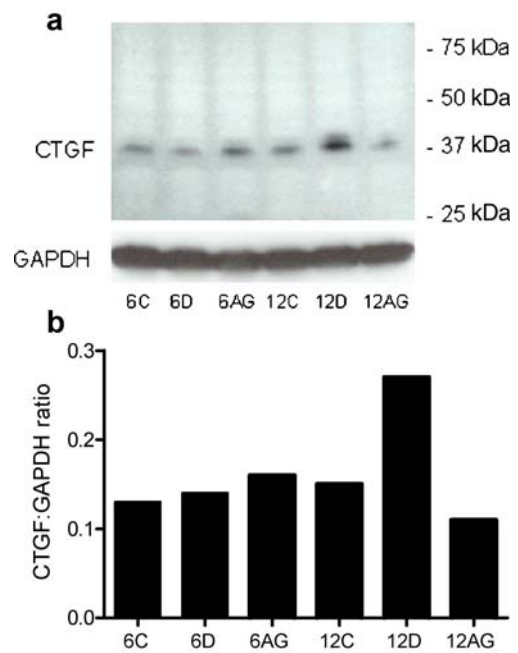
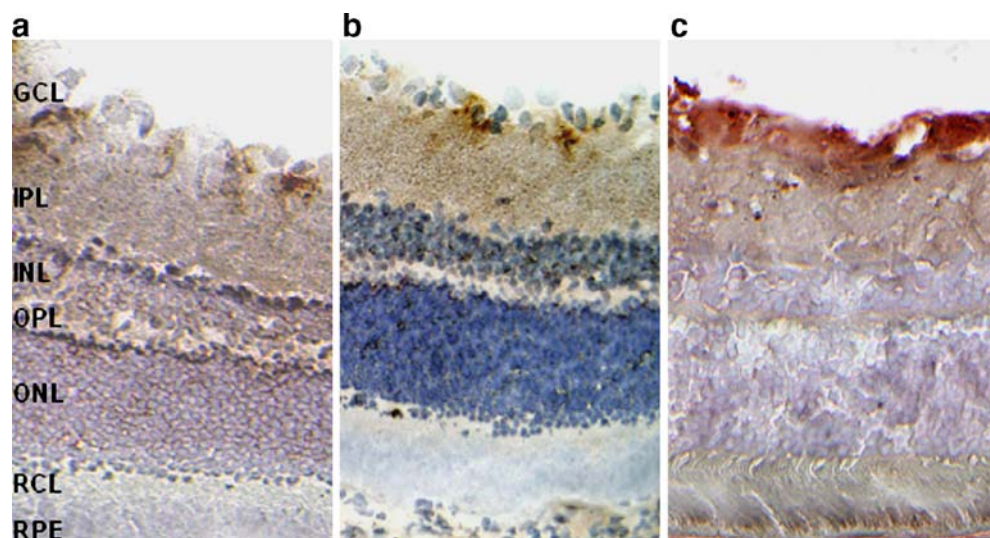


Fig. 4 CTGF protein levels in retina of control and diabetic rats. **a** Western blots of CTGF and GAPDH as loading control. Samples were pooled for each group. A prominent band of CTGF protein is present in the 12-week diabetic group (12D; $n=8$), whereas protein bands were similar in all other groups (control rats at 6 [6C; $n=6$] and 12 [12C; $n=8$] weeks, diabetic rats at 6 [6D; $n=8$] weeks and diabetic rats treated with aminoguanidine at 6 [6AG; $n=7$] and 12 [12AG; $n=7$] weeks). **b** The blots were quantified by densitometry and expressed as a ratio of CTGF:GAPDH

are biologically meaningful. *Nov* and *Wisp2* mRNA expression levels were too low to be detected in all groups of rats.

Expression of transforming growth factor beta 1 and 2 in control and diabetic rats To determine whether: (1) transforming growth factor beta (TGFB), an upstream regulator of CTGF production, is induced by diabetes; and (2) this

induction is prevented by aminoguanidine in rat retina, we examined the mRNA levels of its two most common isoforms, *Tgfb1* and *Tgfb2*. *Tgfb1* mRNA expression was decreased by approximately 30% in retina of rats with diabetes for 6 weeks (Fig. 5). Whether this difference is biologically relevant remains to be determined. In all other experimental groups, *Tgfb1* and *Tgfb2* mRNA levels were similar to control levels (Fig. 5). Immunohistochemical analysis of TGF β 1 revealed a vascular pattern of staining in all experimental groups (Fig. 6).

Expression of extracellular matrix molecules in control and diabetic rats CTGF and CYR61 are known modifiers of the extracellular matrix. Therefore, we investigated expression patterns of various extracellular matrix components. *Col4a3* mRNA levels in the rat retina were elevated by threefold after 6 weeks of diabetes. Aminoguanidine treatment inhibited this induction of *Col4a3* mRNA levels by 30% (Fig. 7). *Lamb1* mRNA levels showed a 1.5-fold increase in 12-week diabetic rats, which was virtually unaffected by aminoguanidine treatment. Fibronectin mRNA levels were not affected by streptozotocin-induced diabetes. *Timp2* mRNA levels were not affected either, but *Timp1* mRNA levels were elevated by 2.5-fold in retina of 12-week diabetic rats, this increase being completely prevented by aminoguanidine treatment (Fig. 7). Laminin and fibronectin were localised immunohistochemically in microvessels in rat retina (Fig. 6). This staining pattern was similar in all groups. TIMP1 immunostaining was restricted to the ganglion cell layer in all groups of rats (Fig. 3).

CCN family mRNA expression in control and AGE-treated mice AGE-treated MSA infusion of mice induced an increased retinal expression of *Cyr61* and *Ctgf* mRNA by 3.7-fold and twofold, respectively, compared with control

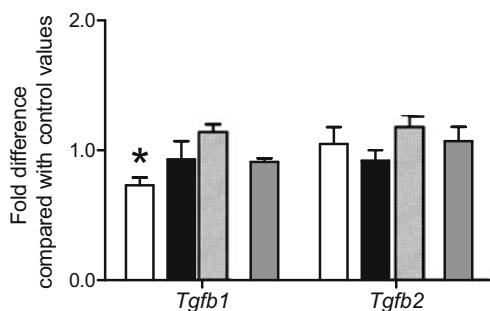


Fig. 5 Fold change of *Tgfb1* and *Tgfb2* expression in diabetic rats at 6 (white bars) and 12 weeks (cross-hatched bars) after streptozotocin-induction and in aminoguanidine-treated diabetic rats at 6 (black bars) and 12 weeks (grey bars) after streptozotocin-induction. *Tgfb1* was significantly decreased in the diabetic rats after 6 weeks (* $p < 0.05$ vs control). This difference was not observed in the aminoguanidine-treated group at 6 weeks. *Tgfb2* expression was not significantly altered at either time point

mice (Fig. 8). *Wisp1* and *Wisp3* mRNA expression was not affected by AGE-treated MSA infusion (data not shown).

Summary of results In the retina of rats with streptozotocin-induced diabetes, mRNA levels of the CCN family members *Cyr61* and *Ctgf* were increased threefold at 6 weeks and twofold at 12 weeks, respectively, whereas expression of all other CCN family members was not notably affected. CTGF protein levels in retina were also elevated twofold at 12 weeks of diabetes. In the aminoguanidine-treated diabetic rats these increases were partly counteracted by treatment with this AGE inhibitor. In line with these findings, treatment of mice with exogenous AGE induced elevated retina mRNA levels of *Cyr61* and *Ctgf*, but not of the other CCN family members. In parallel, mRNA levels of some extracellular matrix components were also increased in the retina of diabetic rats, an effect also prevented by aminoguanidine treatment.

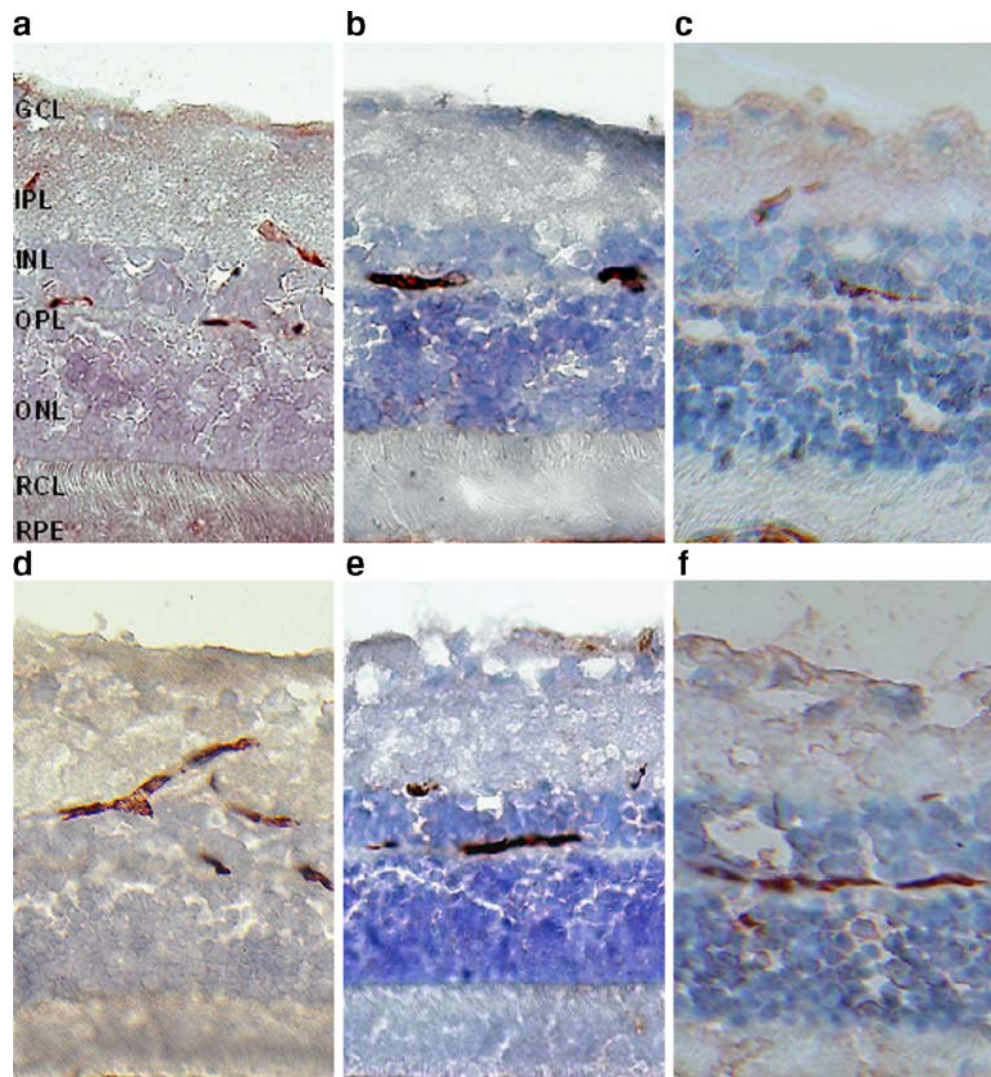
Discussion

We present here a comprehensive expression analysis of the CCN family of fibrosis-inducing cytokines in the retina of rats with streptozotocin-induced diabetes. Messenger RNA and protein levels of CYR61 and CTGF, both known to be capable of modulating the extracellular matrix, were increased in diabetic rats, whilst the AGE inhibitor aminoguanidine attenuated these effects of diabetes. We also found that exogenously administered AGEs are capable of inducing *Cyr61* and *Ctgf* expression in the adult mouse retina in vivo. Taken together, these data present evidence that AGEs are both necessary and sufficient to cause increased levels of CYR61 and CTGF in the diabetic retina.

Expression of CTGF at the mRNA or protein level has previously been demonstrated in vivo in normal and diabetic rat [28] and human retina [29], as well as in cultured retinal microvascular cells [30] and astrocytes [31]. Our data on CTGF are in agreement with a previously reported twofold increase in *Ctgf* expression in the diabetic rat retina [28]. In our study, immunostaining with a polyclonal anti-CTGF antibody showed staining in the ganglion cell layer [28] and diffusely throughout the larger part of the inner rat retina. In contrast, in a previous study of the human diabetic retina employing a monoclonal anti-CTGF antibody, CTGF was detected in microglia and pericytes in the microvasculature of the inner retina [29]. These varying patterns of CTGF protein distribution may be species-related or due to differences in specificity of the antibodies used.

Gene expression analysis was also performed on the other five known members of the CCN family. We found

Fig. 6 Immunohistochemical staining patterns of TGF β 1 (a, d), laminin (b, e) and fibronectin (c, f) in retina of control rats (a–c) and rats 12 weeks after streptozotocin-induced diabetes (STZ) (d–f). Immunostaining of TGF β 1, laminin and fibronectin was confined to the retinal microvasculature and did not notably differ between control and streptozotocin sections. Magnification: $\times 150$



Cyr61 expression in the normal adult rat retina and upregulation in the diabetic retina. In addition to CTGF, this suggests a possible role for CYR61 in the development of diabetes-related retinal sequelae. The lack of detectable *Nov* and *Wisp2* expression argues against a role for these two proteins in normal or diabetic retina. This is in agreement with a previous study, which demonstrated that *Wisp2* mRNA was not present in tissues of the adult rat [32]. Expression of *Wisp1*, which is much less studied but known to suppress cancer cell growth in vivo [33], was slightly decreased in diabetic retina, whereas *Wisp3*, a cell growth suppressor and inhibitor of angiogenesis [34], was increased. The significance of these findings remains unclear, as the functions of the CCN family members are complex. Still, such opposing actions may be reflective of a regulatory balance or may indicate that some CCN family members are redundant, although this is currently not considered to be the case in other tissues [35].

The role of CTGF in diabetic renal pathology has been clearly established. CTGF is responsible for mesangial

expansion [36] and increased extracellular matrix deposition [37, 38] as observed in early stages of diabetic nephropathy. Therefore, CTGF and/or CYR61 may have a similar role in diabetic retina and be responsible for the thickening of microvascular basement membranes observed in early stages of diabetic retinopathy.

We also therefore examined expression patterns of the genes encoding several extracellular matrix-related molecules. Expression of the basement membrane component *Col4a3* was found to be increased concomitantly with *Cyr61* expression after 6 weeks of diabetes, an increase significantly reduced in aminoguanidine-treated diabetic rats. At 12 weeks of diabetes, *Timp1* and *Lamb1* expression demonstrated a concomitant increase with *Ctgf*, which was significantly attenuated in the aminoguanidine-treated group (not statistically significant for *Lamb1*). These findings suggest a causal role of AGEs in the diabetes-induced production of CTGF, CYR61, COL4A3 and TIMP1 in the retina. Whether increased *Lamb1* expression is also mediated by AGEs remains to be determined.

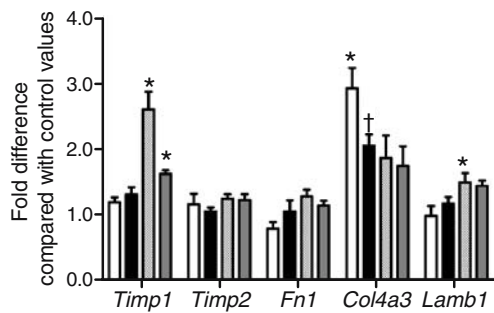


Fig. 7 Gene expression of extracellular matrix components. Fold change of extracellular matrix gene expression as indicated in diabetic rats at 6 (white bars) and 12 weeks (cross-hatched bars) after streptozotocin-induction and in aminoguanidine-treated diabetic rats at 6 (black bars) and 12 weeks (grey bars) after streptozotocin-induction. * $p < 0.05$ for difference between experimental and control group; † $p < 0.05$ for difference between aminoguanidine-treated diabetic group and diabetes-only group

However, this possibility is supported by a previous study showing that protein and mRNA levels of the ribosomal protein SA (previously known as laminin receptor 1 [67 kD, ribosomal protein SA]) are upregulated by AGEs in cultured retinal microvascular endothelial cells [39].

AGE CML plasma levels, used in our study as a marker of AGE formation in the rat diabetes model, were not altered at 6 weeks in diabetic rats, making an effect of AGEs at this time point questionable. However, it should be noted that CML is merely one of many types of AGE known to be generated under hyperglycaemic conditions [40] and that aminoguanidine has been shown to decrease serum AGE levels in diabetic rats as early as 6 weeks after diabetes induction [41].

In previous studies in the diabetic rat kidney, *Ctgf* and fibronectin gene expression both increased after 32 weeks of streptozotocin-induced diabetes [10]. These changes

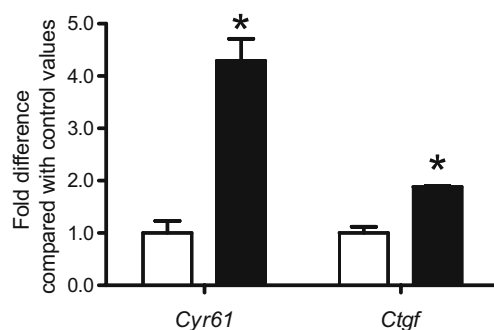


Fig. 8 Relative mRNA levels of *Cyr61* and *Ctgf* in retinas of control (white bars) and AGE-treated (black bars) mice, depicted as fold change in comparison with control mice. * $p < 0.05$ for effect of AGE treatment on *Cyr61* and *Ctgf* mRNA levels

were prevented by aminoguanidine treatment. As AGE accumulation in the diabetic kidney was prevented by aminoguanidine, those authors surmised that the anti-fibrotic effects of aminoguanidine could be at least partially mediated by a decrease in CTGF expression [10]. Our study may have been too short to observe an increase of fibronectin in the diabetic retina, but otherwise our results are in line with these findings in kidney. Additional studies will be necessary to further elucidate the ability of CYR61 and CTGF to directly modulate these extracellular matrix molecules in retinal vascular cells.

TGFB, considered to be the most important fibrotic factor, has been shown to upregulate CTGF in many cell types in vitro [42–44] and in vivo [45, 46]. Although our findings indicate that TGFB production is not increased in the diabetic retina, a role for TGFB in the observed upregulation of CTGF cannot be ruled out, as the regulation of TGFB bioavailability is complicated and not solely dependent on the level of TGFB production [47].

We have recently demonstrated that vascular endothelial growth factor (VEGF) increases expression of *Ctgf* and *Cyr61* in the rat retina in vivo as well as in retinal vascular endothelial cells in vitro (E. J. Kuiper, J. M. Hughes, I. M. C. Vogels et al., unpublished results). As AGEs are known inducers of VEGF in retinal cells [48, 49], it is possible that the increases in CTGF and CYR61 observed in our animal models are the result of AGE-induced VEGF.

A major finding of our study was the ability of aminoguanidine to attenuate the increase in *Ctgf* expression observed in the diabetic rat retina. Tikellis et al. [28] have reported that perindopril, an ACE inhibitor, prevents increased *Ctgf* expression in diabetic rat retina [28]. This suggests that both interventions may affect a common molecular mechanism leading to the upregulation of CTGF. As ACE inhibition has been shown to prevent AGE accumulation in diabetic tissues [50], it is feasible that inhibition of AGE formation could be a common molecular mechanism allowing both ACE inhibitors and aminoguanidine to inhibit the increase of retinal CTGF.

In summary, this study provides the first evidence that, in addition to CTGF, the CCN family molecules CYR61, WISP1 and WISP3 play possible roles in the development of early stages of experimental diabetic retinopathy. At the very least, these results warrant further study into the functional aspects of these molecules in the eye, and of how these aspects pertain to the development of diabetic retinopathy. Additionally, we demonstrate for the first time that AGEs directly upregulate both CTGF and CYR61 levels in the retina in vivo and that aminoguanidine inhibits these diabetes-induced increases. This provides the first evidence that CTGF and CYR61 are downstream effectors of AGEs in the diabetic retina and implicates them as possible targets for future intervention strategies.

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Duality of interest None of the authors have or have in the previous 12 months had a relevant duality of interest with a company whose products or services are directly related to the subject matter of this paper.

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