

THE ROLE OF ADVANCED GLYCATION IN REDUCED ORGANIC CATION TRANSPORT ASSOCIATED WITH EXPERIMENTAL DIABETES

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Running title:

AGEs and organic cation clearance in diabetes

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Paper statistics:

Number of text pages = 30

Number of tables = 2

Number of figures = 7

Number of references = 39

Word count:

Abstract = 240

Introduction = 399

Discussion = 1337

Non-Standard abbreviations used in the paper:

Advanced glycation end products (AGEs), Carboxymethyllysine (CML),

Effective renal plasma flow (ERPF), Glomerular filtration rate (GFR),

Low molecular weight (LMW), N-methylnicotinamide (NMN),

Organic cation transporters (OCTs), Renin-angiotensin system (RAS)

Abstract

Tubular dysfunction is an important early manifestation of diabetic nephropathy. Reduced renal expression of organic cation transporters (OCTs) potentially contributes to impaired cation clearance in diabetes. This study examines the role of advanced glycation end-products (AGEs) in mediating these changes. Experimental diabetes was induced with streptozotocin (55mg/kg). Rats were randomly treated with the AGE inhibitor, aminoguanidine, for 32-weeks. In a second protocol, diabetic rats were followed with and without low dose insulin therapy (2U/day) for 4-weeks. Expression of OCTs was determined by real time RT-PCR and western blotting. As a marker of cation transport, the fractional clearance of endogenous N-methyl-nicotinamide (NMN) was determined by HPLC. Both short- and long-term diabetes was associated with reduced gene and protein expression of the three renal OCT isotypes. This was associated with a reduction in the fractional clearance of NMN compared to control animals by over 50%. These changes correlated with the accumulation of renal and plasma AGEs. Treatment with the AGE inhibitor, aminoguanidine, restored the expression of OCT-2 and OCT-3 in diabetic animals and normalized renal NMN clearance. NMN clearance was also improved in diabetic animals receiving low-dose insulin, correlating with a reduction in AGEs and improvement in effective renal plasma flow. These studies demonstrate an early impairment of expression of OCTs and cation clearance associated with diabetes. These changes correlate with the accumulation of AGEs and may be partly attenuated by an AGE-inhibitor, implicating a role for AGEs in organic cation transport.

Introduction

Prolonged exposure to hyperglycemia and carbonyl stress in diabetes results in the formation and accumulation of advanced glycation end products (AGEs). These chemically heterogeneous compounds have a wide range of chemical, cellular and tissue effects implicated in the development and progression of diabetic nephropathy [Forbes *et al.*, 2003]. The accumulation of AGEs, both in tissue and serum, correlates with the severity of renal complications in diabetes [Makita *et al.*, 1991; Suzuki *et al.*, 1999]. However, AGEs may be considered downstream mediators of renal injury in diabetes. This is illustrated by studies showing that inhibition of advanced glycation is able to attenuate renal injury, without influencing glycemic control [Soulis-Liparota *et al.*, 1991]. In addition, administration of exogenous AGEs to non-diabetic animals is able to generate changes in renal structure and function, similar to those seen in diabetic nephropathy in the absence of hyperglycemia [Vlassara *et al.*, 1994].

Proximal tubular cells represent the principal site of uptake of filtered AGEs [Asano *et al.*, 2002], and may therefore be uniquely vulnerable to AGE-mediated injury. Tubular dysfunction can be identified as one of the earliest manifestations of diabetic nephropathy, preceding the development of hyperfiltration or glomerular lesions [Thomson *et al.*, 2001]. For example, the apical uptake of gentamicin into proximal tubular cells is reduced after as little as four days of experimental diabetes [Vaamonde *et al.*, 1984]. In addition, basolateral tubular transport is also disrupted in diabetes. In experimental diabetes, a significant reduction in tubular cation clearance can be observed, associated with a down-regulation of the expression of tubular organic cation transporters (OCTs) on the basolateral surface of the proximal tubule [Thomas *et al.*, 2003; Grover *et al.*, 2004]. OCTs are important for the renal homeostasis of a number of

physiologically important endogenous cations including monoamine neurotransmitters, agmatine and prostaglandins. OCTs are also necessary for the renal clearance of a broad range of exogenous substrates including toxins, xenobiotics and commonly used drugs (eg. metformin, β -blockers).

The mechanism of reduced OCT expression in diabetes remains to be established. However, the fact that blockade of the renin angiotensin system was able to correct this, in the presence of ongoing hyperglycemia, suggests organic cation transport in diabetic animals may be influenced by some common downstream mediator of both hyperglycemia and the renin angiotensin system. We hypothesize that AGEs represent one such mediator of tubular dysfunction in diabetes. This study examines the role of AGEs in organic cation clearance associated with experimental diabetes. In addition, we demonstrate the protective effects of aminoguanidine, an inhibitor of advanced glycation, on transporter expression and cation clearance in experimental diabetes.

Materials and Methods

Animals

Male Sprague–Dawley rats (Biological Resource Laboratory, Heidelberg, Australia), aged between 8 and 9 weeks and weighing between 200 and 250g, were used in this study. Experimental diabetes was induced by intravenous injection of the β -cell toxin, streptozotocin (Sigma Chemical Co, St. Louis, MO, USA) at a dose of 55 mg/kg, following an overnight fast. Animals with a plasma glucose concentration in excess of 15 mmol/L one week after the induction of diabetes were included in the study as diabetic. Sham injected control animals (sodium citrate buffer pH 4.5) were followed concurrently. The protocols for animal experimentation and the handling of animals were in accordance with the principles established

by the Animal Welfare Committee of the Austin and Repatriation Medical Centre. Throughout the study, animals were given free access to water and an identical diet (Barrastoc GR2 rat and mouse breeder ration, Clayton, VIC, Australia).

Protocol A: 4-week diabetes model

Experimental diabetes was induced, as described above, and animals were followed for 4 weeks. A 4-week time point was selected specifically to examine the effect of diabetes on organic cation clearance prior to the onset of proteinuria, which usually takes place after at least 8 weeks of diabetes in this model. Rats with diabetes were randomized to receive either a daily dose of 2 units of Ultralente insulin (Ultratard HM, Novo Industries, Bagsvaerd, Denmark) to prevent ketonuria (n=10) or no insulin therapy (n=10).

Protocol B: 32-week diabetes model

After baseline measurements of physiological and biochemical parameters, 20 diabetic and 20 control animals were followed for 32-weeks to examine for changes in the context of overt nephropathy. Diabetic and control animals were further randomized to receive aminoguanidine (Fluka Chemica, Buchs, Switzerland) in drinking water at a dose of 1 g/L for diabetic rats and 4 g/L for control rats for 32 weeks (n=10 each group). Aminoguanidine, an inhibitor of advanced glycation, has previously been shown to attenuate the development and progression of renal disease in experimental diabetes [Soulis-Liparota *et al.*, 1991]. In addition, diabetic animals were given 2 units per day of Ultralente insulin (Ultratard HM, Novo Industries, Bagsvaerd, Denmark) to maintain body weight and prevent ketoacidosis, without significantly lowering blood glucose levels. Complete insulin deficiency was not ethically appropriate, because of adverse effects on animals' health and high animal mortality. Nonetheless, this model is closer

to what may be achieved clinically in patients with type 1 diabetes, who all receive systemic insulin.

Measurement of physiological and biochemical parameters

The following parameters were serially measured: body weight; blood glucose, measured using a glucometer (Accutrend, Boehringer Mannheim G.m.b.H. Biochemical, Mannheim, Germany); systolic blood pressure, measured by tail-cuff plethysmography in conscious, warmed rats [Buñag, 1973]; glycated hemoglobin, measured by high performance liquid chromatography (HPLC) (CLC330 Analyzer; Primus, Kansas City, MO, USA) [Cefalu *et al.*, 1984]. Urine was collected from animals that were placed for 24 hours in individual metabolic cages (Iffa Credo, L'Arbresle, France) for measurement of albumin excretion rate (AER) by radioimmunoassay [Soulis-Liparota *et al.*, 1995]. Glomerular filtration rate (GFR) was measured by a single-injection isotopic technique [^{99}Tc -DTPA (^{99}Tc -diethylenetriaminepenta-acetic acid)] developed in our laboratory [Cooper *et al.*, 1988]. At completion of the study, rats were sacrificed by decapitation. The kidneys were rapidly dissected out and bisected, with one half snap frozen in liquid nitrogen and stored at -80°C prior to use and the other half embedded in paraffin wax.

Real time reverse transcription-polymerase chain reaction (RT-PCR)

The upper pole of the right kidney, containing only cortical tissue was homogenised (Polytron, Kinematica AG, Lucerne, Switzerland) and total RNA isolated according to the Trizol protocol (Gibco BRL, Grand Island, NY, USA). RNA samples were treated with RNase-free DNase (Gibco BRL). RNA quality was assessed by formaldehyde gel electrophoresis to ensure that all samples used were of equivalent quality based on the 18s:28s ratio. Gene expression of mRNA for OCT-1, OCT-2 and OCT-3 was assessed with quantitative real-time RT-PCR using the

TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer Inc, PE Biosystems, Foster City, CA, USA) as previously described [Thomas *et al.*, 2003]. Briefly, 3µg of RNA were used to synthesize cDNA with Superscript First Strand synthesis system (Gibco BRL, Grand Island, NY, USA). Genomic DNA contamination was removed by subjecting sample RNA to DNase treatment (Ambion, Austin, TX, USA). Samples were further subjected to PCR amplification, without a reverse transcriptase step, to confirm the absence of genomic DNA. To control for variation in the amount of DNA available for PCR in the different samples, gene expression of OCTs was normalized simultaneously (by multiplexing) in relation to the expression of the housekeeping gene 18S ribosomal RNA (rRNA) (18S rRNA TaqMan Control Reagent kit; ABI Prism 7700, Perkin-Elmer Inc, Foster City, CA, USA), as an endogenous control. Primers and TaqMan probe for OCTs and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700, Perkin-Elmer Inc, Foster City, CA, USA). The forward primer for OCT-1 was 5'-[TGGTGTTTAGGCTGATGGAA]-3' and the reverse primer for OCT-1 was 5'-[GCCCAAACCCCAAACAAA]-3'. The probe specific to OCT-1 was FAM-5'-TTTGGCAAGCCCTGCCCTCA-3'-TAMRA; where FAM = 6-carboxyfluorescein, and TAMRA (quencher) = 6-carboxy-tetramethylrhodamine. The forward primer for OCT-2 was 5'-[GCGAGCATGGCTGGGTATAC]-3' and the reverse primer for OCT-2 was 5'-[CAGGTAAACTCAGTGACAATGG]-3'. The probe specific to OCT-2 was FAM-5'-ACACTCCCGGCTCC-3'-TAMRA. The forward primer for OCT-3 was 5'-[TTGCACCGAATTTTTCTGTGTT]-3' and the reverse primer for OCT-3 was 5'-[CAGGCCCCCTTTCCAAAC]-3'. The probe specific to OCT-3 was FAM-5' – ATCTTCCGCTTCCTGC-3'-TAMRA. PCR amplification was done in a 25 µL volume, including 1x TaqMan buffer A, 200 µmol/L dATP, 200 µmol/L dCTP, 200 µmol/L dGTP, 400

μmol/L dUTP, 100 nmol/L fluorogenic probe, 200 nmol/L each of forward and reverse primers, 0.5 U uracil-N-glycosylase, 1.25 U DNA polymerase (PE Biosystems, Foster City, CA, USA), and 100 ng sample cDNA. The amplification was performed with the following time course: 50°C, 2 minutes and 10 minutes at 95°C; followed by 40 cycles of 94°C, 20 seconds, 60°C, 1 minute. Each sample was tested in triplicate. Results were expressed as proportional to relevant control animals.

In situ hybridization

The site-specific expression of OCT-1 and OCT-2 mRNA was determined by radioactive *in situ* hybridization using methods carried out as previously described [Thomas *et al.*, 2003]. Because of the low level of expression of mRNA, *in situ* hybridization for OCT-3 was not performed. In brief, cDNAs coding for OCTs were prepared from rat cDNA and shortened by alkaline hydrolysis to an average length of 250 bases. Purified probes were then cloned into pGemT easy vector (Promega, Madison, WI, USA) and linearized with Sal-1. Anti-sense riboprobes for OCTs were then generated using T7 RNA polymerase. Background hybridization was controlled by the inclusion of a sense riboprobe or treatment with RNase prior to hybridization. Four micron sections were hybridized with riboprobes and then exposed to BioMax MR film for 3-5 days.

Western Blot and immunohistochemistry

Forty μg of protein extracted from kidney samples was separated on a 12% SDS-PAGE gel and transferred to PVDF membrane. OCT-1, OCT-2 and OCT-3 were detected using polyclonal antibodies (Alpha Diagnostics International, San Antonio, TX, USA) incubated overnight at 4°C at a dilution of 1:1000. Bound antibody was detected on X-ray film by enhanced

chemiluminescence with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2000) and Supersignal® West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA).

Renal expression of OCT-1, OCT-2 and OCT-3 were detected using commercial polyclonal antibodies (Alpha Diagnostics International, San Antonio, TX, USA) at a dilution of 1:500, 1:250 and 1:250 respectively. For each of these antibodies, antigen retrieval prior to incubation with the primary antibody was performed using 0.4% pepsin (Sigma Chemical Co., St Louis, MO, USA) in 0.01N hydrochloric acid for 5 minutes at 37°C. Negative control slides were included without addition of primary antibody. Vector biotinylated goat anti-rabbit immunoglobulin was used as the secondary antibody, followed by Vector ABC (Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was identified by reaction with 3,3'-diaminobenzidine tetrahydrochloride tablets (Sigma Chemical Co., St Louis, MO, USA). Sections were counterstained with hematoxylin, dehydrated and mounted with DePeX (BDH, Melbourne, Australia).

Measurement of N-methylnicotinamide clearance

N-methylnicotinamide (NMN) is an endogenous cation that is cleared by the kidney *via* OCT-dependent pathways [Urakami *et al.*, 1998]. Urine and serum concentrations of NMN were measured HPLC, as previously described [Thomas *et al.*, 2003]. Renal NMN clearance was estimated from the daily excretion of NMN divided by the plasma NMN concentration.

Estimation of renal plasma flow

Effective renal plasma flow (ERPF) was estimated from the clearance of endogenous hippuric acid clearance in conscious animals, as previously described [Igarashi *et al.*, 1987]. Serum samples were taken in the morning, at the time of constant low hippurate excretion. Urine samples were collected in metabolic cages, as detailed above, and analysed in a 1:50 dilution.

Hippuric acid was measured in serum and urine samples using HPLC. Briefly, 300 μ l of acetonitrile containing internal standard, isopropanoic acid, was added dropwise to 100 μ l of serum or 1:50 diluted urine. The sample was then centrifuged to remove precipitated protein and the supernatant dried under nitrogen. Subsequently, the sample was reconstituted in a mobile phase consisting of 10% acetonitrile, 1% trinitrofurane and 0.5% phosphoric acid. The sample was then injected into a 250 x 3mm, 5 micron phenyl-hexyl column (Phenomenex, CA, USA) with a mobile phase at 1 ml/minute. The hippurate peak was detected at 10.2 minutes, using an on-line UV detector set at 249 nm. As serum and urine were run sequentially, ERPF was calculated as the urinary volume multiplied by the area under the urine hippurate curve divided by that of plasma hippurate and expressed as ml per minute per m² of body surface area.

Quantification and Localization of AGEs

Plasma AGEs: Incomplete degradation of AGE-modified proteins results in the production of low molecular-weight (LMW) AGEs that accumulate in the serum in diabetes. These LMW-AGEs parallel the level of tissue AGE-modification in diabetes [Forbes *et al.*, 2003, Suzuki *et al.*, 2001]. In addition, LMW-AGEs may be directly pathogenic to the proximal tubule, as they are both freely filtered at the glomerulus and actively reabsorbed in the proximal nephron. LMW-AGE levels present in plasma samples were assayed using on-line spectrofluorometric detection in a flow system, as previously described by our group [Forbes *et al.*, 2003]. Briefly, 20 μ l of serum was deproteinated with 480 μ l of trichloroacetic acid and mixed with 100 μ l of chloroform. The resulting aqueous phase was injected directly into a carrier stream (deionized water) in which total AGE-fluorescence was measured (370/440nm, Waters 470 fluorescence spectrophotometer; Waters, Milford, Massachusetts, USA). Injection volume was corrected by the measurement of UV absorption at 280nm (A₂₈₀), using a Hewlett Packard, L3500

spectrophotometer detector placed in series. Samples were run in triplicate. LMW-AGE content was expressed as arbitrary units defined by the ratio of the area under the fluorescence curve divided by the area under the A_{280} curve. Results were normalized to the values for hydrolysed AGE-modified bovine serum albumin prepared using standard methods.

Tissue AGE content: AGE-fluorescence (370/440nm) was determined in homogenized renal cortical samples after reduction with freshly prepared NaBH_4 (1 M NaBH_4 in 0.1 M NaOH overnight at 4°C) followed by enzymatic hydrolysis into peptide fragments using proteinase K. The resulting low molecular weight peptides were then analysed using the same flow injection assay described above. Results were expressed after being normalized for the protein content of the supernatant, which was determined prior to acid hydrolysis using the BCA assay (BioRad, USA).

Immunohistochemical localization: Localization of renal AGE content was determined by immunohistochemistry, as previously described by our group [Soulis *et al.*, 1997]. Briefly, formalin fixed paraffin sections of kidney were de-waxed and hydrated. Following incubation with 0.3% hydrogen peroxide for 20 minutes, sections were incubated with protein blocking agent (Lipshaw, PA, USA) for 20 minutes and then incubated with a polyclonal AGE antibody that recognizes the non-fluorescent AGE, carboxymethyllysine (CML), as its primary epitope [Reddy *et al.*, 1995]. Tissue sections were stained consecutively with biotinylated IgG for 10 minutes and avidin-biotin horseradish peroxidase complex for 15 minutes (Vectastain ABC ELITE kit, Vector Laboratories Inc., Burlingame, CA, USA) before a substrate solution of 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St Louis, MI, USA) was added. Sections were counterstained in Harris' hematoxylin and mounted in dePex (BDH, Merck, Poole, UK).

Statistical analysis

Data are expressed as mean \pm the standard error of the mean with the exception of AER which is shown as the geometric mean $\times/_{\pm}$ a tolerance factor. Normally distributed variables in different groups were compared by ANOVA. Comparisons between group means were performed by Fisher's least significant difference method. A p value of <0.05 was considered statistically significant.

Results

Animals

Physiological parameters for the *4-week diabetes study* are shown in **table 1**. Animals with experimental diabetes not treated with insulin (DO₄) had increased GHb levels and daily food and water intake compared to diabetic animals receiving a low dose of insulin (DI₄) and control animals (C₄) after 4-weeks of study. DO₄ animals were also smaller than DI₄ ($p<0.01$). GFR was significantly increased in animals with diabetes with or without insulin treatment (*vs* control $p<0.01$). AER was not elevated at this time-point in any group.

Physiological parameters for the *32-week diabetes study* animals are shown in **table 2**. After 32-weeks of study, animals with experimental diabetes (DI₃₂) had increased GHb levels and daily food and water intake compared to control animals (C₃₂). Neither GHb nor feeding behaviour was influenced in animals treated with aminoguanidine (DIAG₃₂). The albumin excretion rate (AER) was increased in DI₃₂ but attenuated in DIAG₃₂, consistent with the previously documented renoprotective effect of aminoguanidine in this model (4,5). GFR was increased in animals with diabetes. However, aminoguanidine had no significant effect on hyperfiltration ($p=0.52$).

Estimation of AGE-exposure

Circulating levels of LMW-AGEs were higher in animals with diabetes with significant differences detectable at all time points. DO₄ animals had higher levels of LMW-AGEs than DI₄ animals receiving insulin. Treatment with the AGE-inhibitor, aminoguanidine, attenuated this rise in levels of LMW-AGEs in diabetic animals, without altering glycemic control. Renal AGE-fluorescence was significantly increased in diabetic animals, both at 4- and 32-weeks, although the absolute level of this AGE-fluorescence was greater at the latter time-point (**tables 1 & 2**). Treatment with aminoguanidine and insulin both reduced renal AGE-fluorescence compared to untreated animals. Total renal AGE-fluorescence was correlated with level of LMW-AGEs in control ($R^2 = 0.43$, $p < 0.01$) and diabetic animals ($R^2 = 0.56$ $p < 0.01$).

Immunostaining for renal AGEs was most pronounced in distal renal tubules and the macular densa (**figures 1 & 2**). This pattern of intense distal staining for CML was largely unchanged between control and diabetic animals and was not significantly modified by any intervention. By contrast, there was a significant increase in proximal tubular staining for AGEs in diabetic animals at both early and late time-points, compared to respective controls. This tubular staining was significantly reduced in animals treated with aminoguanidine (**figure 2**) and treatment with low dose insulin reduced immunostaining for AGEs when compared to DO₄ animals (**figure 1**).

Expression of OCT mRNA

The major cation transporter expressed in the rat kidney was OCT-2, constituting over 75% of the total expression of mRNA for renal OCTs in both control and diabetic animals. OCT-1 was the next most common transporter expressed, constituting ~23% of all renal OCT mRNA. The gene expression of OCT-3 in the rat kidney was more than 500 times less than OCT-2 and

OCT-1, suggesting it does not play an important role in cation transport in the rodent kidney. This distribution (ratio) of OCT mRNA expression was not significantly different between control and diabetic rats or between early and late time-points. In addition, this pattern was not significantly modified with prolonged duration of diabetes or by treatment with insulin in the short-term model.

The quantitative gene expression for each of the OCTs, as measured using real time RT-PCR, was significantly lower in animals with diabetes (**Figure 3**). At both 4-weeks and 32-weeks, the reduction in gene expression was of a similar magnitude for each of the OCTs, despite the absence of proteinuria at the 4-week time-point. Treatment with low-dose insulin significantly increased the expression of OCT-2 and OCT-3 compared to untreated animals with diabetes ($p < 0.05$), albeit still lower than control animals. However, the expression of OCT-1 remained unchanged as measured by real time RT-PCR (**Figure 3a**). Treatment with aminoguanidine significantly increased OCT-2 and OCT-3 mRNA expression in animals with diabetes, reaching levels not significantly different from control rats. However, the expression of OCT-1 was equivalent to that of untreated animals with diabetes (**Figure 3a**).

Notably, the gene expression of the OCTs were correlated with one another in both control and diabetic animals, with the single exception of OCT-3 and OCT-1 in diabetic animals (**Figure 4**). This close correlation in renal expression is consistent with the co-segregation of the OCTs and the close physical linkage of these three organic cation transporter genes in the rat genome [Eraly et al., 2003].

In all renal tissues, OCT-1 mRNA was predominantly expressed in the *pars convoluta* (S1) and cortical *pars recta* (S2) of the proximal tubule as demonstrated by *in situ* hybridization (**Figure 5**). Expression of OCT-2 mRNA was highest in the medullary *pars recta* (S3). Although the

level of expression was reduced for OCT-1 and OCT-2, the localization of gene expression in diabetic kidneys was not significantly different to that seen in control animals. However, the reduced expression of OCT-1 in diabetic animals was most marked in the superficial cortical zones. Neither aminoguanidine nor insulin had any significant effect on the tissue distribution of OCT-1 or OCT-2 gene expression as assessed by *in situ* hybridization although aminoguanidine increased the overall level of OCT-2 expression (**Figure 5**).

OCT Protein expression

Proximal tubular immunostaining was specifically basal and lateral for all the OCTs. OCT-1 protein was expressed predominantly in the cortical S1 and S2 tubules and OCT-2 in the juxtamedullary S3 tubule with no staining in the renal medulla (**Figures 1 & 2**). As previously reported, OCT-3 was present in both distal and proximal tubules in the rat kidney [Wu *et al.*, 2000]. The proximal tubular staining was limited to the cortical S1 and S2 tubule, and expressed in a similar pattern to OCT-1.

Correlating with the changes seen in gene expression, the protein expression of the OCTs was diminished in diabetic kidneys both after 4 and 32 weeks, as shown quantitatively on Western blot (**Figure 6**) and by immunohistochemistry (**Figures 1 & 2**). Although the level of protein expression was reduced, the localization of OCT-1 and OCT-2 expression in diabetic kidneys was not significantly different to that seen in control animals and was similar at both early and late time points. However, immunostaining for OCT-3 was reduced only in proximal tubules, with no clear difference between control and diabetic animals on distal tubular staining.

As for mRNA, protein expression of OCT-2 and OCT-3 in diabetic animals was increased following treatment with aminoguanidine (**Figure 6a**). However, treatment with low-dose insulin had no detectable effect on the protein expression of OCTs (**Figure 6b**).

N-methylnicotinamide clearance

There was a significant decline in the fractional clearance of NMN in animals with diabetes over the 32-week study period (**Figure 7a**). This reduction was apparent after 8-weeks of diabetes, prior to the onset of proteinuria, and persisted throughout the study period. After 32-weeks, tubular excretion of NMN was reduced by over 60% in diabetic compared to control animals (DI_{32} : 43 ± 6 ml/min; C_{32} 150 ± 12 ml/min, $p < 0.01$). These changes in the fractional clearance of NMN were independent of hyperfiltration associated with diabetes. However, the observed decline in cation clearance as a result of advanced diabetes was attenuated by correcting for the decline in renal blood flow as estimated by the clearance of endogenous hippurate. When expressed as a ratio of NMN clearance to that of hippurate, organic cation clearance appears to be uniformly reduced at all time-points in animals with diabetes. This is consistent with the uniform reduction in OCT mRNA and protein expression observed at both the early and late time-points (**Figure 7b**).

In the four-week study, only in untreated animals with diabetes was there a significant decline in the fractional clearance of NMN (DO_4 , 2.3 ± 0.2 ; C_4 , 3.5 ± 0.2 , $p < 0.01$). Organic cation clearance in diabetic animals treated with low dose insulin was not significantly different from control animals (DI_4 3.2 ± 0.3 ; C_4 3.5 ± 0.2 , $p = \text{NS}$). Treatment with aminoguanidine also restored cation clearance to control levels ($DIAG_{32}$ 139 ± 12 ml/min), despite having no effect on hyperfiltration.

Correlations with OCT expression

In the 32-week model, the level of expression of each of the OCTs was correlated with albuminuria in both diabetic ($R^2=0.33$, $p<0.01$) and control animals ($R^2=0.60$, $p<0.01$). On the other hand, there was no clear relationship between 'normoalbuminuria' and OCT expression in the 4-week study, reflecting the absence of pathological albuminuria at this time-point. However, the concentration of serum LMW-AGEs was correlated with OCT expression in animals with and without diabetes (e.g LMW-AGE vs OCT-2, diabetes $R^2=0.32$, $p<0.05$ control $R^2=0.76$, $p<0.01$).

Discussion

The renal clearance of organic cations is significantly reduced following the induction of experimental diabetes. As has been recently demonstrated by our group [Thomas *et al.*, 2003] and others [Grover *et al.*, 2004] this decline is temporally associated with reduced gene and protein expression of the organic cation transporters in the diabetic proximal tubule. Although OCT expression is reduced in proportion to tubular injury in advanced diabetes [Thomas *et al.*, 2003], a significant reduction in OCT expression was detectable after 4-weeks of diabetes, well before the development of albuminuria and significant renal damage. This suggests that the reduction in OCT expression observed in these studies is not simply an epiphenomenon or marker of renal injury. This finding is further supported by findings recently published in JPET, showing a reduction in OCT-1 and 2 protein after as little as 14 days of experimental diabetes [Grover *et al.*, 2004].

The fact that the expression of all the renal OCTs was reduced in the diabetic kidney is also significant. Knockout models of OCT-1 or OCT-2 alone demonstrated little effect on renal cation clearance, possibly because of receptor redundancy [Jonker *et al.*, 2003; Jonker *et al.*,

2001]. On the other hand, a double knockout of both genes results in complete loss of renal cation transport [Jonker *et al.*, 2003]. In this study, restoration of OCT-2 following treatment with aminoguanidine was sufficient to restore NMN clearance to within control parameters, without significant effects on OCT-1 expression.

Although OCT-3 is expressed at a low level in the rat kidney and has limited affinity for NMN [Okuda *et al.*, 2000] a reduction in its expression in diabetic animals may still be significant. OCT-3 is the major cation transporter in the human kidney [Hayer-Zillgen *et al.*, 2002]. In addition, selective blockade of OCT-3 in rats results in increased plasma catecholamines and metanephrines [Eisenhofer *et al.*, 1996]. However, OCT-3 knockout mice appear to show no evidence of a monoamine imbalance [Zwart *et al.*, 2001], possibly because of redundancy conferred through expression and/or activity of other cation transporters. It is conceivable that a reduction in OCT-1 and OCT-2 expression, on top of changes in OCT-3, could contribute to chronically elevated sympathetic tone or catecholamine sensitivity associated with diabetes and aging.

The mechanism by which OCT expression is reduced in experimental diabetes is unclear. It is likely that the changes are not specific to OCTs as other tubular transport pathways may also be disrupted in early diabetes [Vaamonde *et al.*, 1994], suggesting that these changes are a symptom of a more general tubular dysfunction. Renoprotective therapy with inhibitors of advanced glycation and blockade of the renin-angiotensin system [Thomas *et al.*, 2003] are both able to normalize the expression of OCTs in this diabetic model, despite having no effect on hyperglycemia, hyperfiltration or polyuria. This suggests that a common downstream mediator of both hyperglycemia and the renin angiotensin system, such as PKC or cGMP, may influence tubular transport in diabetic animals. Certainly, tubular PKC is activated in early diabetic

nephropathy and this effect is attenuated by both ACE inhibition and aminoguanidine [Osicka *et al.*, 2000]. In addition, AGEs may also represent a common mediator of tubular injury. Indeed, Grover *et al* recently suggested that AGEs may contribute to changes in OCT proteins [Grover *et al.*, 2004]. Of all the cells in the kidney, proximal tubular cells are unique in their ability to assimilate filtered AGEs and are therefore most susceptible to AGE-mediated damage in diabetes. Exposure of tubular cells to AGEs results in a variety of functional changes relevant to cation transport, including a reduction in trans-epithelial polarity and loss of the epithelial phenotype [Forbes *et al.*, 2003]. AGEs are also capable of independently activating a range of tubular growth and differentiation factors, including known mediators of organic cation transport such as PKC. In this study, serum LMW-AGEs levels were correlated with the renal expression of OCTs both at early and late time-points in diabetic animals. In addition, aminoguanidine, which inhibits the formation of AGEs, restored the expression of OCT-2 and OCT-3 in diabetic animals. Similarly ramipril, which reduces circulating and tubular levels of AGEs through a different mechanism [Forbes *et al.*, 2002] proved equally beneficial [Thomas *et al.*, 2003]. It is also conceivable that RAS and advanced glycation pathogenic pathways may act in a synergistic fashion, and combination therapy may offer better results than either treatment alone [Davis *et al.*, 2004]. In this study expression of OCT-1 was not significantly altered by aminoguanidine, although expression of OCT-2 and OCT-3 was fully restored. However, in our previous study, ramipril restored OCT-1 as well as OCT-2 in diabetic animals but had no significant effect on OCT-3 (*author, unpublished results*). It seems likely that a combination of hemodynamic and metabolic approaches may offer the best chance of restoring normal tubular function in diabetes [Gilbert *et al.*, 1999].

Dynamic changes in tubular blood flow are an early event in diabetic nephropathy and NMN clearance has been considered a valid measure of tubular blood flow [Musfield *et al.*, 2001].

The declining blood flow in animals with advanced diabetes and renal injury also contributes to the reduced NMN clearance observed in our study. Similarly, the lower renal plasma flow in untreated animals with diabetes compared to those treated with low-dose insulin (as originally described by Brenner and colleagues [Hostetter *et al.*, 1981]) suggests that effective NMN clearance may be even lower in untreated animals. However, even after correcting for changes in renal plasma flow, organic cation clearance is consistently reduced in animals with diabetes, paralleling the uniform reduction in OCT gene and protein expression observed at both early and late time-points.

Studies by our group have previously demonstrated that treatment of diabetic animals with aminoguanidine is able to restore a variety of aberrant tubular functions [Osicka *et al.*, 2000]. With the demonstration of a reduced expression of OCTs after 4 -weeks of diabetes, this effect is clearly more than just renoprotection. Although aminoguanidine was used in this study as an inhibitor of advanced glycation, aminoguanidine also inhibits the activity of inducible nitric oxide synthase (iNOS), which may be activated in early diabetes [Ziyadeh *et al.*, 1995]. However, other inhibitors of iNOS (L-NAME and methylguanidine) are not renoprotective in diabetes [Soulis *et al.*, 1997], while chemically unrelated inhibitors of glycation, such as pyridoxamine, confer some degree of renal protection in diabetes [Degenhardt *et al.*, 2002]. Furthermore, recent cell culture studies have suggested that nitric oxide has no effect on the activity of organic cation transporter pathways [Song *et al.*, 2002]. Aminoguanidine also has the added ability to inhibit semicarbazide-sensitive amine oxidase (SSAO) [Yu and Zuo, 1999], which has, as its major substrate, a variety of cationic polyamines. SSAO is increased in diabetic animal models from as early as nine days [Hayes and Clarke, 1990]. However, regulatory effects on SSAO activity on OCT expression, though plausible, remain to be established.

It is also likely that insulin influences OCT expression, as animals not treated with insulin for 4 weeks have a greater reduction in fractional NMN clearance. This was largely explained by the effect of insulin on renal blood flow and GFR [Hostetter *et al.*, 1981, Allen *et al.*, 1990], although the renal expression of the OCTs was also marginally increased by low dose insulin supplementation, without correcting hyperglycemia. Lack of insulin is certainly an important driving force for many of the pathogenic changes seen in experimental diabetes. However, that these changes may be partly reversed by therapies that do not alter insulin levels (ie AGE inhibition or ACE inhibition), insulin deficiency alone cannot explain the changes. We speculate that insulin deficiency, activation of the RAS and hyperglycemia must share common downstream mediators, one of which may be AGEs. Notably, small doses of insulin were sufficient to reduce the circulating level of LMW-AGEs. It is possible to speculate that some of the beneficial effects of insulin may be mediated *via* a reduction in AGE levels. Equally, as many AGEs carry a charge at physiological pH, augmentation of clearance pathways, such as the renal OCTs, may directly act to reduce circulating LMW-AGEs.

In summary, reduced gene and protein expression of OCT-1, OCT-2 and OCT-3 has been demonstrated in experimental diabetes, associated with the reduced clearance of the endogenous cation NMN. This change may be detected after as little as 4-weeks of diabetes. The reduction may be further compounded by a reduced renal plasma flow associated with advanced diabetes and complete insulin deficiency. Organic cation clearance and the expression of OCTs was restored following treatment with aminoguanidine, suggesting a possible role for AGEs in the disruption of tubular organic cation transport associated with diabetes.

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Footnotes

Dr Merlin Thomas is supported by a National Health and Medical Research Council (NHMRC) biomedical scholarship. Dr Josephine Forbes is supported by a Juvenile Diabetes Research Foundation (JDRF) post-doctoral fellowship. Wendy Burns is supported by an Australian Kidney Foundation (AKF) postgraduate scholarship.

The Danielle Alberti Memorial Centre for Diabetes Complications at the Baker Medical Research Institute is funded by a program grant from the JDRF.

Figure legends:

Figure 1: Immunolocalization of OCT-1, OCT-2, OCT-3 and AGEs in 4-week control animals (left panel), experimental diabetes (middle panel), diabetes treated with insulin (right panel).

Figure 2: Immunolocalization of OCT-1, OCT-2, OCT-3 and AGEs in control animals (left panel), experimental diabetes (middle panel), diabetes treated with aminoguanidine (right panel)

Figure 4: Renal expression of OCTs mRNA in the 4-week model (a) the 32-week model (b).

Figure 3: Correlation in the renal expression of mRNA for each of the OCTs in 32-week control (triangle) and diabetic animals (square).

Figure 5: Localisation of OCT gene expression of mRNA in the 32-week model.

Figure 6: Renal expression of OCT protein in the 4-week model (a) in the 32-week model (b).

Figure 7: Renal clearance of N-methyl-nicotinamide corrected for GFR (a) and ERPF (b) in control animals (open square), diabetes (black circle) and diabetes treated with aminoguanidine (grey triangle).

Table 1.

Biochemical parameters in the short-term (4-week) experimental model

Mean \pm SEM, * *vs* control, $p < 0.05$; # *vs* diabetes $p < 0.05$.

4-week study	Control	Diabetes	Diabetes +I
Body weight (g)	446 \pm 8	278 \pm 7*	330 \pm 9*#
Renal weight (g/m ²)	56 \pm 1	65 \pm 2*	85 \pm 2*#
Plasma glucose (mmol/L)	6.2 \pm 0.1	28.8 \pm 0.6*	25.9 \pm 0.8*
Glycated Hb (%)	3.7 \pm 0.1	13.8 \pm 0.3*	11.1 \pm 0.4*#
Systolic BP (mmHg)	122 \pm 4	142 \pm 8*	146 \pm 10*
AER (mg/day)	0.4 $\times/_{+}$ 1.2	1.2 $\times/_{+}$ 1.1	0.4 $\times/_{+}$ 1.2
GFR (ml/min/m ²)	60 \pm 3	78 \pm 4*	102 \pm 5*#
ERPF (ml/min/m ²)	287 \pm 11	193 \pm 15*	386 \pm 22*#
LMW-AGEs (AU/ml)	6.1 \pm 0.2	12.9 \pm 2.0*	8.6 \pm 0.4*#
Renal AGE fluorescence	1.0 \pm 0.1	1.9 \pm 0.1*	1.5 \pm 0.1*#

Table 2.

Biochemical parameters in the long-term-term (32-week) experimental model

Mean \pm SEM, * *vs* control, $p < 0.05$; # *vs* diabetes $p < 0.05$.

32-week study	Control	Diabetes	Diabetes +AG
Body weight (g)	710 \pm 24	538 \pm 17*	458 \pm 15* [#]
Renal weight (g/m ²)	52 \pm 1	88 \pm 3*	83 \pm 4*
Plasma glucose (mmol/L)	6.3 \pm 0.2	28.2 \pm 1.5*	28.0 \pm 1.5*
Glycated Hb (%)	4.2 \pm 0.2	15.3 \pm 0.8*	14.0 \pm 0.6*
AER (mg/day)	4.2 $\times/_{\pm}$ 1.3	52 $\times/_{\pm}$ 1.3*	4.2 $\times/_{\pm}$ 1.3 [#]
GFR (ml/min/m ²)	122 \pm 4	227 \pm 9*	226 \pm 16*
ERPF (ml/min/m ²)	410 \pm 25	576 \pm 18*	560 \pm 12*
LMW-AGEs (AU/ml)	14.8 \pm 1.0	23.6 \pm 1.9	15.9 \pm 0.8* [#]
Renal AGE fluorescence	2.0 \pm 0.2	4.6 \pm 0.9*	2.2 \pm 0.3* [#]

Figure 1.

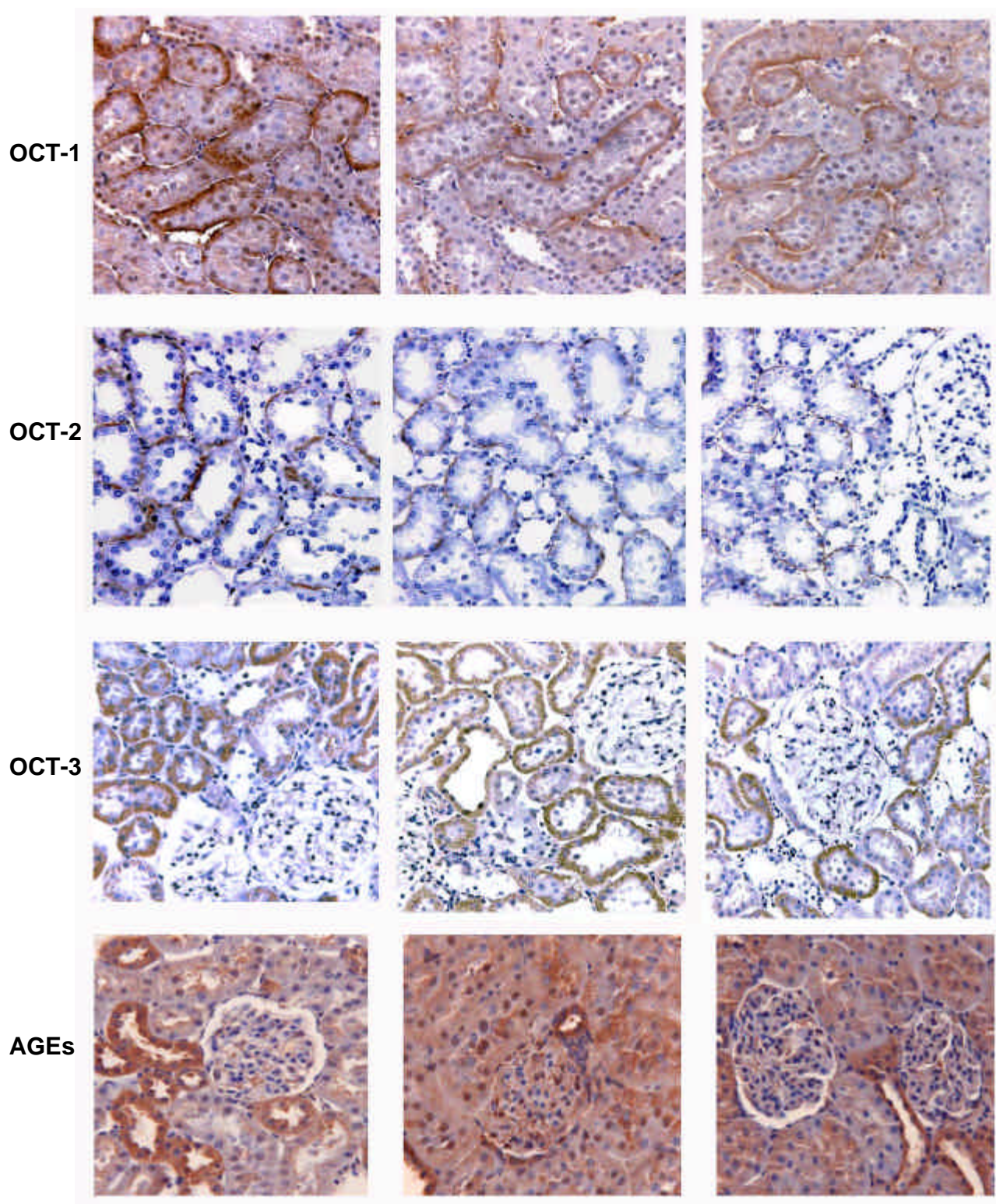


Figure 2

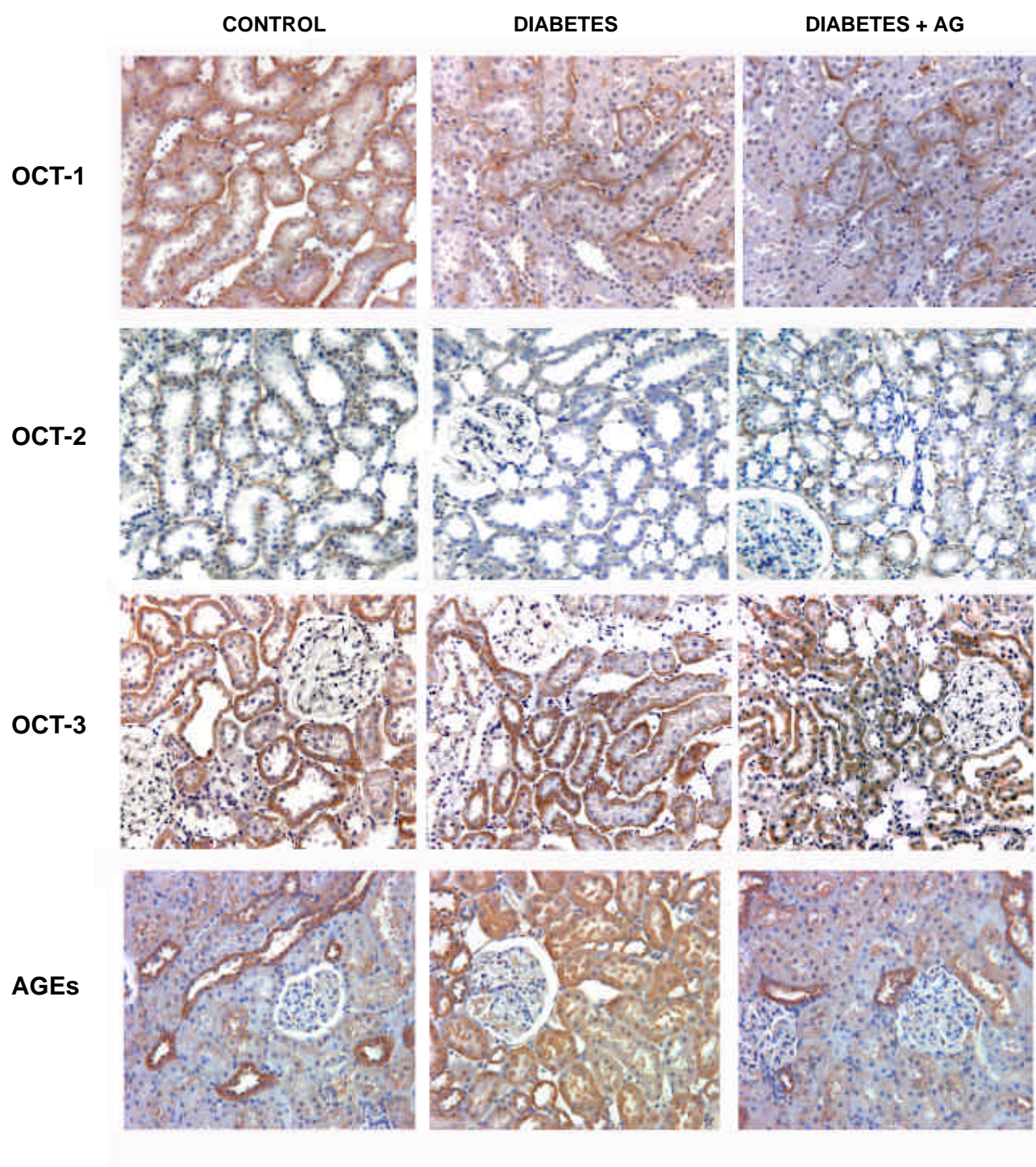


Figure 3.

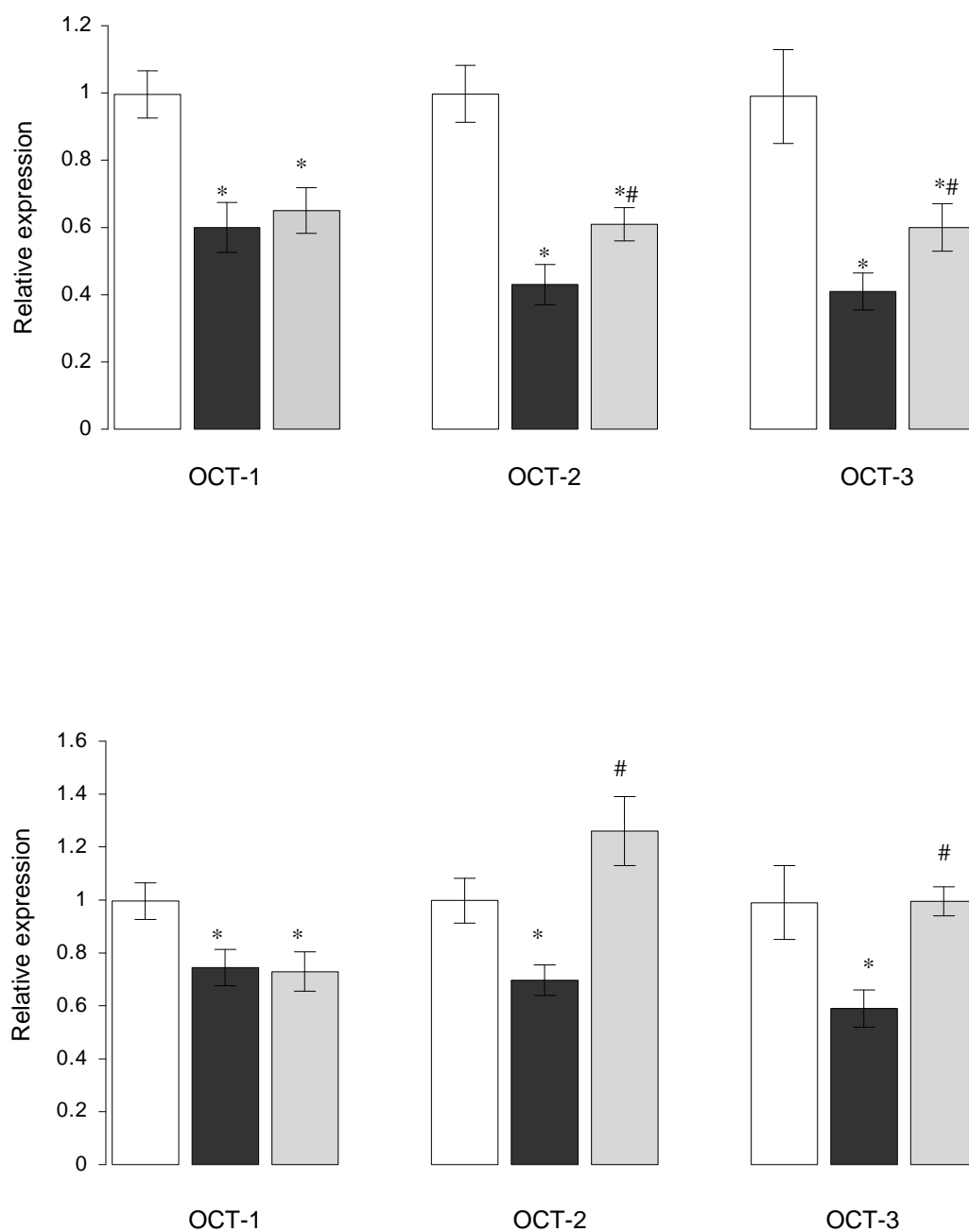


Figure 4.

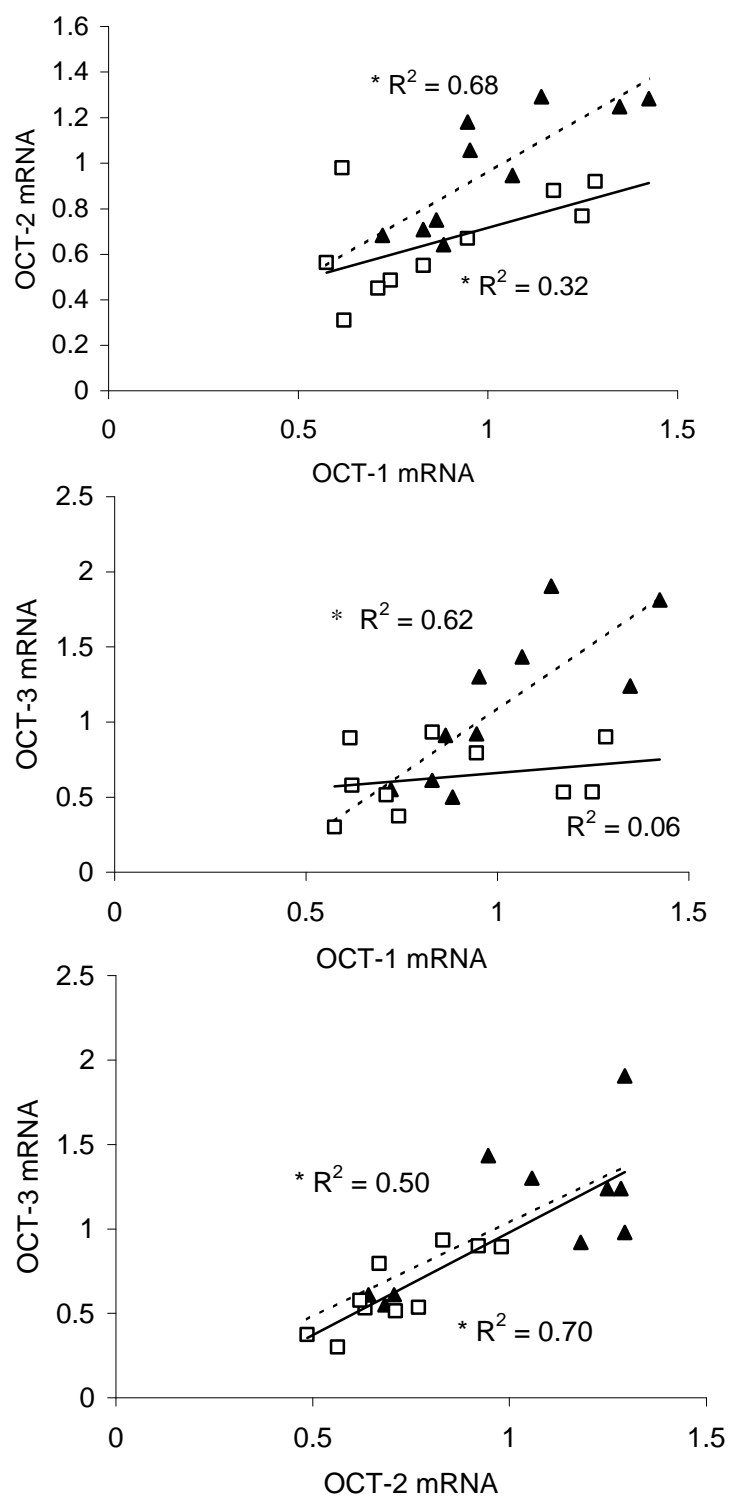


Figure 5.

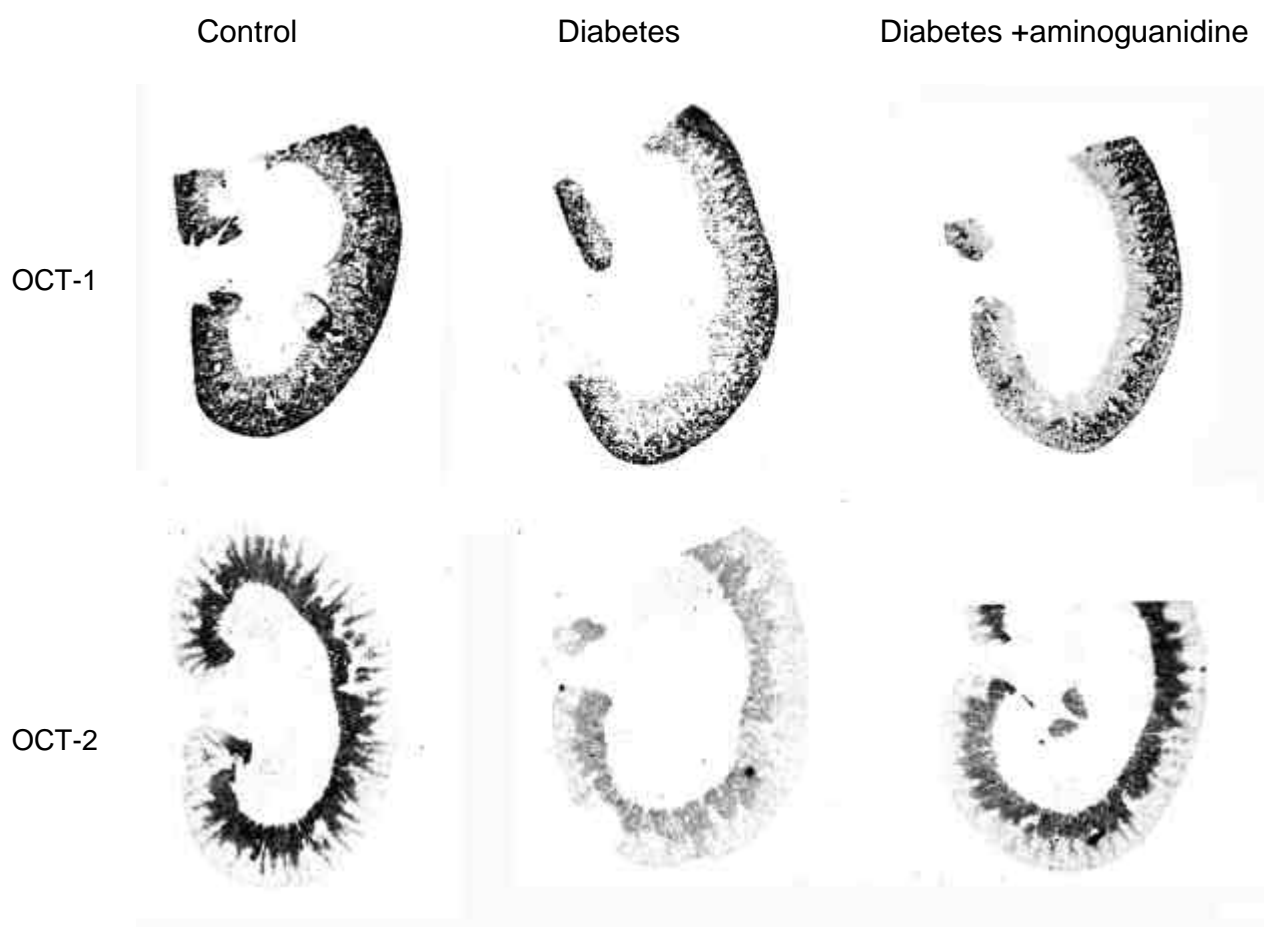
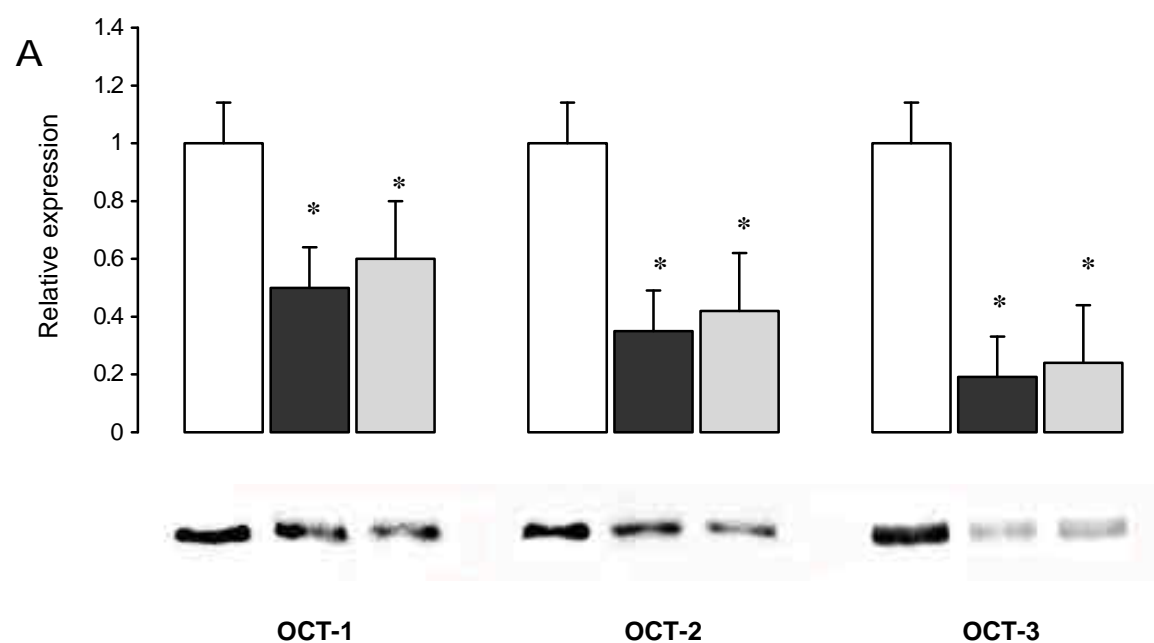


Figure 6.



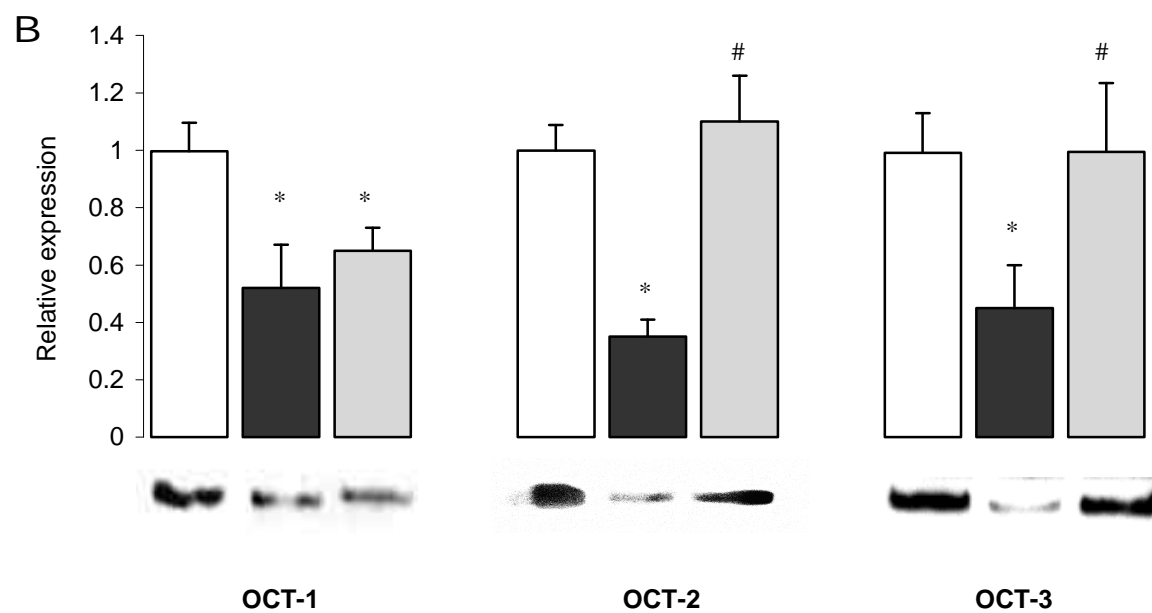


Figure 7.

