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Diabetes-induced alterations in tissue collagen and carboxymethyllysine in rat kidneys: Association with increased collagen-degrading proteinases and amelioration by Cu(II)-selective chelation

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ABSTRACT

Advanced glycation end-products (AGEs) comprise a group of non-enzymatic post-translational modifications of proteins and are elevated in diabetic tissues. AGE-modification impairs the digestibility of collagen *in vitro* but little is known about its relation to collagen-degrading proteinases *in vivo*. N^{ε}-carboxymethyllysine (CML) is a stable AGE that forms on lysyl side-chains in the presence of glucose, probably *via* a transition metal-catalysed mechanism.

Here, rats with streptozotocin-induced diabetes and non-diabetic controls were treated for 8 weeks with placebo or the Cu(II)-selective chelator, triethylenetetramine (TETA), commencing 8 weeks after disease induction. Actions of diabetes and drug treatment were measured on collagen and collagen-degrading proteinases in kidney tissue.

The digestibility and CML content of collagen, and corresponding levels of mRNAs and collagen, were related to changes in collagen-degrading-proteinases. Collagen-degrading proteinases, cathepsin L (CTSL) and matrix metalloproteinase-2 (MMP-2) were increased in diabetic rats. CTSL-levels correlated strongly and positively with increased collagen-CML levels and inversely with decreased collagen digestibility in diabetes. The collagen-rich mesangium displayed a strong increase of CTSL in diabetes. TETA treatment normalised kidney collagen content and partially normalised levels of CML and CTSL.

These data provide evidence for an adaptive proteinase response in diabetic kidneys, affected by excessive collagen-CML formation and decreased collagen digestibility. The normalisation of collagen and partial normalisation of CML- and CTSL-levels by TETA treatment supports the involvement of Cu(II) in CML formation and altered collagen metabolism in diabetic kidneys. Cu(II)-chelation by TETA may represent a treatment option to rectify collagen metabolism in diabetes independent of alterations in blood glucose levels.

1. Introduction

CML is a major AGE that is said to form *via* the 'glycoxidation pathway' (also known as 'autoxidative glycation'), which involves the reaction of glucose with proteins, catalysed by transition metals such as copper [1,2]. The AGE formation is enhanced in diabetes [3] where it has been linked to decreased collagen digestibility [4,5] and altered proteolytic processing of albumin *in vitro* [6].

The capacity of the proteolytic system may eventually become exhausted by excessive AGE formation and AGE accumulation in lysosomes has been demonstrated *in vitro* [7]. Consequently, proteinases involved in the degradation of collagen could be affected by altered AGE content of substrate proteins in diabetes, but data concerning *in vivo* collagen-AGE formation that pertain to roles of collagen-degrading proteinases are lacking. Distinguishing between substrates modified with AGEs *in vitro* compared with *in vivo* is recognised as an important question, and has generated discussion regarding the interaction of AGE-modified proteins with the receptor for AGEs (RAGE) [8].

MMP-2 and certain cathepsins are implicated in soft-tissue collagen turnover [9,10]. Previous reports regarding collagen degradation in diabetic kidneys are contradictory. Increased proteolytic activity towards

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glomerular basement-membrane collagen has been reported in glomerular homogenates from rats with streptozotocin (STZ)-induced diabetes after 4 and 10 weeks' hyperglycaemia [11]. This finding contrasts with data obtained using artificial substrates for collagen-degrading cathepsins as well as gelatine zymography for metalloproteinases, whereby decreased activity was reported in glomeruli of STZ-diabetic rats with up to 5-weeks' diabetes [12]. Activity of CTSL was reportedly decreased in kidneys of STZ-diabetic rats 10 and 30 days after disease induction [13] while activity was decreased and unchanged after 10 days and 6-months in proximal-tubular cells, respectively [14].

The aim of this study was to investigate the relation between modifications of collagen and collagen-degrading proteinases in healthy and STZ-diabetic rats. The Cu(II) chelator TETA, was previously shown to prevent albuminuria and heart failure in diabetic rats [15,16], and was here investigated as an interventional treatment against AGE formation and linked effects on collagen homeostasis.

2. Materials and methods

2.1. Animal studies

All protocols were approved by the University of Auckland Animal Ethics Committee and complied with the 'Principles of Laboratory Animal Care' (NIH 1985): this report is consistent with current guidelines for the proper reporting of animal experiments [17]. Male Wistar rats 8 weeks of age with body-weights of 300 g (±30 g) (mean ± SEM) were injected once *via* the tail vein with a solution of saline vehicle or STZ (S0130, Lot No. 119K1591, Sigma-Aldrich, USA; 55.0 mg/kg body-weight) in saline solution. Rats were fed chow (Teklad Diet No. 2018; Harlan Laboratories, USA) and water *ad libitum*. Thereafter, body-weights of animals were measured weekly and non-fasting blood-glucose levels fortnightly. Treatment of rats with placebo (water) or TETA dihydrochloride (~17 mg/day; T5033, Sigma-Aldrich, USA) was started 8 weeks after injection in one-half of the STZ- and saline-injected animals, and was administered *via* the drinking water as previously described [15]. Kidneys were perfused and collected at the end of experimental week 16, following 8-weeks' drug treatment. RNAlater (AM7021, Life Technologies, USA) was added to those samples destined for RNA analysis and samples were stored at −80 °C. Light microscopy of haematoxylin-and-eosin-stained renal-cortical sections was performed, and showed that rats had not developed renal tumours, consistent with a lack of renal STZ toxicity.

2.2. Gene expression analysis

RNA was extracted from renal cortical tissue using spin columns (QIAGEN, Germany). Complementary DNA synthesised using the Transcriptor cDNA kit with an oligo-dT primer (Roche, Germany) and subsequently analysed using the Light Cycler 480 system and SYBR green MasterMix (both Roche). Target gene levels were normalised to three reference genes, which were stably and similarly expressed in all four groups of animals. All primers (Table 1) were designed to be intron-spanning.

Table 1
Primer sequences for RT qPCR.

List of primers		
Gene abbreviation	Forward primer sequence	Reverse primer sequence
Col1a1	ACATGTTCAGCTTTGTGGACC	TTAGGGACCCTTAGGCCATT
Col1a2	AGAAAATGGCATCGTTGGTC	ACCAGGGAAGCCAGTCATAC
Col3a1	TATTTTGGCACAGCAGTCCA	CAGAGGACAGATCCCGAGTC
Col4a1	GTCCTCACTGTGGATTGGCT	CGATGAATGGGGCACTTCTA
Col4a4	CAGATGGACCGAGTCCTACG	TCTTGCTGCTCCCATATTCA
Col4a5	GATCTCCAGGTGACCAAGGA	CCTGAAATGCCAGTTCCAAT
Col6a1	GCTGAGCAAGGACGAGCTAG	CACGTGTTCTTGCATCTGGT
Col6a2	GACTTTGGTCTGAAAGGAGCA	GGGTTCTCCCTCAGGTCCT
Col6a3	TACCGAGCATCTGAGGAGCT	GAACTCCACTTGCATCAGCC
Ctsl	AAGCCCTCATGAAGCCTGTAG	CCTTGCTGCTACAGTTGGGTT
Lox	CTGACTTCTTACCAAGCCGC	GCAGGTCGTAGTGGCTGAAT
Mmp2	CTTTGATGGCATTGCTCAGA	GTCACGTGGTGTCACTGTCC
Rpl13a	ACAAGAAAAAGCGGATGGTG	TTCCGGTAATGGATCTTTGC
Tbp	AGAACAATCCAGACTAGCAGCA	GGGAACTTCACATCACAGCTC
U2af	CCATTGCCCTCTTGAACATT	CCTCCCCGTACTTCTCTTCC

2.3. Western blotting

Protein levels of lysyl oxidase (LOX) (sc-32410; Santa Cruz, USA) and CTSL (ab6314; Abcam, UK) in kidney-cortex lysates were detected by Western blotting as described [16], and normalised to corresponding beta-actin values (sc-47778; Santa Cruz).

2.4. Gelatinase activity assay

Tissue samples were lysed (10 mM $CaCl_2$, 0.25% v/v Triton-X100) and run on gelatine-containing polyacrylamide gels (EC 61752, Life Technologies, USA). These were equilibrated at RT for 30 min in renaturation buffer (LC2670, Life Technologies), and then incubated in developing buffer (LC2671, Life Technologies) for 30 min at RT. Buffer was then refreshed, and gels incubated for 72 h at 37 °C. Gels were stained with Coomassie blue [18]. EDTA (10 mM) was added as a negative control to the first incubations on a separate control gel to confirm that the activity was metalloproteinase-dependent.

2.5. CTSL activity assay

CTSL activity was assayed using a peptide substrate, Ac-HRYR-ACC (Cat # 219497; Merck, Germany), coupled to the probe, ACC (7-amino-4-carbamoylmethylcoumarin), which fluoresces following cleavage. The assay was performed as described by Choe et al. [19] with minor modifications. In brief, renal cortex was lysed (30 mM Tris–HCl, 0.1% Brij-35 v/v, 1 mM DTT) and mixtures centrifuged (15 min, 16,100 *g*, 4 °C). Supernatants were collected on ice, and 10 μL (~30 μg) of each was added in triplicate to a black 96-well plate. The activity-assay buffer (100 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, 0.01% Brij-35 (v/v), DTT 10 mM, pH 5.5 with substrate/DMSO to 5 μM) was pre-warmed to 30 °C, and added alongside a standard curve. Fluorescence was measured (excitation, 380 nm; emission, 460 nm; cut-off, 435 nm) after 10 min incubation at 30 °C. Activity was normalised for protein concentration as determined by BCA assay. TETA (25 μM) and/or $CuCl_2$ (25 μM) were added for relative CTSL activity assay, and comparisons made to wells containing the lysate without either of these compounds (100% activity).

2.6. Collagen extraction

Collagen was extracted from kidney tissues after removal of the renal pelvis and major calyces, and purified according to a published protocol [20].

2.7. Quantification of collagen levels

Collagen was quantitated by measurement of hydroxyproline levels in acid hydrolysates (6 M HCl at 105 °C for 16 h) of the kidney or collagen extracts according to the procedure of Bergman and Loxley [21].

2.8. Determination of collagen pepsin digestibility

Collagen extracts were digested with 1 mg/ml pepsin/0.5 M acetic acid at a concentration of 1 mg/ml for 24 h at 4 °C. Digests were then centrifuged (16,100 *g*, 4 °C, 30 min). Hydroxyproline levels in supernatants and pellets were measured, and the digestibility presented as the percentage of total collagen.