THE ROLE OF RENIN-ANGIOTENSIN SYSTEM INHIBITORS IN DIABETES-INDUCED MULTIORGAN DAMAGE

Ph.D. thesis

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1. Introduction

The prevalence of diabetes mellitus (DM) is increasing at an alarming rate; consequently, the risk of developing diabetic complications is becoming a concern for an increasing number of individuals. Diabetic kidney disease (DKD) is one of the most common and detrimental microvascular complications of DM, associated with cardiovascular disease (CVD) and comorbid depression.

Significant progress has been made in treating DM-associated multiorgan complications over the past decade. However, the treatment strategies currently available remain inadequate for disease prevention. Pharmacological modulation of key regulatory pathways such as fibrosis or inflammation, may present a promising approach in DM pathophysiology.

International guidelines identify renin-angiotensin-aldosterone system inhibitors (RAASi) as the gold-standard therapy in diabetic patients with micro- and macrovascular complications. Various studies demonstrate that different RAASi reduce functional and structural multiorgan damage in DM. However, it remains uncertain whether their protective properties are linked to or confined to their blood pressure-regulating effects, and the precise molecular pathways involved have yet to be thoroughly investigated.

Furthermore, although the aldosterone escape phenomenon has been reported in DKD patients treated with angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor 1 blocker (ARB), aldosterone antagonists are still primarily used only as diuretics to manage hypertension. Thus, further evidence is needed to facilitate their monotherapeutic use.

The activity of the hexosamine biosynthesis pathway is increased in DM, leading to protein O-linked N-acetylglucosamine modification (O-GlcNAcylation), a central contributor of glucose toxicity. During the process, O-GlcNAc transferase (OGT) adds a single O-GlcNAc moiety to serine and/or threonine residues of various proteins, while O-GlcNAcase (OGA) removes the modification. The two enzymes maintain the rate of glycoslyation and deglycosylation in a dynamic equilibrium, finely tuning multiple cellular and metabolic processes in a glucose-dependent manner. Hyperglycemia-increased glucose flux

activates O-GlcNAcylation, which promotes insulin resistance and disrupts the function and metabolic balance of vascular, neuronal, and renal cells.

These pathways, combined with the increased activation of the RAAS, stimulate the production of proinflammatory and profibrotic factors such as nuclear factor kappa B (NFκB), transforming growth factor β (TGFβ), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF).

All the above processes lead to the progression of DKD and the development of kidney fibrosis.

Previously, glomerulosclerosis was regarded as the primary site of fibrosis in DKD; however, recent studies provide increasing evidence that hyperglycaemia-induced tubulointerstitial lesions also play a significant causative role. Hyperglycaemia elevates the tubular glucose load, resulting in tubular hyperplasia and hypertrophy due to early functional changes, such as primary proximal tubular hyperreabsorption. Enhanced DNA synthesis encourages the transition to senescence, during which local pro-inflammatory cytokine release, growth factor production, and extracellular matrix (ECM) components establish the foundation for tubulointerstitial fibrosis.

Fibroblasts are the primary effector cells of fibrosis, and their activation is marked by the production of alpha-smooth muscle actin (αSMA). Upon activation, they serve as the primary collagen-producing cells and contribute to the accumulation of ECM. This increases ECM components, such as type I and type III collagen and fibronectin.

The impaired turnover (formation and degradation) of ECM components leads to the secretion of specific pro-collagen terminal fragments and protein neo-epitopes into the urine, which have recently been identified as novel, early non-invasive urinary biomarkers of renal fibrosis. Two examples of these are neo-epitopes of matrix metalloproteinase (MMP)-9-mediated degradation of type III collagen (C3M) and the alpha 3 chain of type IV collagen, known as tumstatin (TUM). Numerous recent studies have confirmed the association of urinary C3M with renal fibrosis in various rat models. In T1DM, these markers have yet to be tested, and the effect of RAASi treatment on their excretion has not been investigated either.

Renal and cardiac fibrosis exhibit focal characteristics; the precise localisation of the injury is often unpredictable, particularly when dealing with small samples obtained from rats. Due to the focal nature of fibrotic organ injuries, conventional sample preparation methods can scarcely yield consistent false-positive or false-negative results. In contrast, lyophilised tissue can be easily pulverised and a portion of a homogenous powder used for biochemical research investigations is more representative of the entire sample than segmented parts taken from frozen tissues.

Cardiovascular (CV) complications in diabetic patients significantly contribute to the rising rates of morbidity and mortality. Prolonged hyperglycaemia leads to accelerated arterial stiffening. Diabetic cardiomyopathy (DCM), which occurs independent of hypertension or coronary heart disease, is associated with about 30% mortality. Furthermore, non-diabetes-specific macroangiopathic complications such as atherosclerosis are more prevalent in type 2 DM (T2DM). At the same time, CVD is also common in T1DM, particularly in the presence of albuminuria, due to the microangiopathy of small intramural coronary arteries. Additionally, T1DM patients experience a further loss of life years due to CV events.

The development of DCM is independent of hypertension or coronary heart disease. The underlying pathological factors are complex and include metabolic disturbances, systemic and cardiac inflammation, oxidative stress, hypoxia, and overactivation of the RAAS. Increased angiotensin II (Ang II) and aldosterone activity promote cardiomyocyte loss, cell hypertrophy, inflammation, and extensive myocardial fibrosis, leading to cardiac remodeling and atherosclerosis.

DKD and CVD have a unique bidirectional relationship. DKD is related to cardiac overload, inducing cardiac hypertrophy and fibrosis, which aggravate the worsening of kidney function. On the other hand, CVD, like pre-existing chronic heart failure (HF), also increases the risk of chronic kidney disease (CKD) in diabetic patients (cardiorenal syndrome).

Accelerated arterial stiffening due to prolonged hyperglycemia and metabolic changes further increases the elevated risk of CVD in diabetic patients. Carotid–femoral pulse wave velocity (PWV) is the gold standard non-invasive method for measuring central arterial stiffness,

with a 1 m/s increment corresponding to a 15% rise in vascular events and mortality risk. Intima–media thickness (IMT) is another crucial, non-invasive surrogate marker for vascular damage and atherosclerosis. It is recommended as a routine examination for identifying macrovascular risk in diabetic patients.

Current treatment strategies are insufficient to prevent the progression of CVD in DM. Although sodium–glucose cotransporter-2 inhibitors are recommended to reduce the risk of adverse CV events for T2DM patients with CKD, CVD, or HF, they are unsuitable for treating T1DM patients due to the increased risk of ketoacidosis. RAAS serves as another primary pharmacological target in CV medicine. Nonetheless, RAASi are used exclusively when hypertension and albuminuria are present or for diabetic patients with macrovascular complications alongside hypertension, HF, or DKD. RAASi are not advised for diabetic normotensive patients without pre-existing CVD, and clinical studies involving low-dose RAASi in T1DM-related CV complications are lacking.

DM is often linked to depression, resulting in a reduced quality of life and poorer long-term prognosis. The incidence of cognitive decline and depression is two to three times higher among diabetic patients. However, most cases remain underdiagnosed. Conversely, depression doubles the risk of developing DM. Despite the apparent bidirectional relationship, the pathophysiology connecting the two diseases remains unclear.

Mounting evidence underscores the role of inflammation in the pathophysiology of both DM and depression, which could indicate a potential common mechanism of the two disorders. Neuroinflammation and glial activation induce various pathological changes that contribute to the onset of metabolic and neuropsychiatric diseases. Inflammatory processes reduce brain-derived neurotrophic factor (BDNF) production, which may impair synaptic plasticity and neuronal survival.

BDNF is highly expressed in the central nervous system. It is synthesised as a pre-pro peptide, then converted to its precursor form (proBDNF) and finally proteolytically cleaved to mature BDNF (mBDNF). It is now widely accepted that diminished BDNF signaling is associated with psychiatric disorders. mBDNF

mediates neuroplasticity processes such as neuronal survival, neurogenesis, and synaptic activity. Patients with depression have lower serum BDNF levels. In parallel, BDNF is decreased in hippocampal samples of patients suffering from depression.

According to recent data, the RAAS has also been implicated in the pathomechanism of depression. Several clinical observations suggest a connection between RAASi and reduced depressive symptoms, although the underlying mechanisms remain unclear. Furthermore, case-control and cohort studies demonstrate that patients taking RAASi have reduced risk of mood disorders.

It has been established that RAAS functions beyond a mere circulating hormonal system. Components of the classic RAAS are present in the brain, kidneys, and heart, where they regulate: i) blood pressure, cerebral and renal circulation, central sympathetic activity, and behaviour; ii) vascular resistance, filtration, epithelial cell hypertrophy, mesangial cell contraction and ECM expansion; iii) cardiac inflammation, myocardial fibrosis, and hypertrophy. In DM, both systemic and local RAAS are excessively activated, with an elevated level of Ang II.

2. Objectives

The studies presented evaluate changes in specific target organs and examine the protective effect of RAASi treatment against complications induced by T1DM and their underlying mechanisms.

Our primary goal was to investigate the underlying mechanisms of the pleiotropic effect of monotherapeutic nondepressor-dose RAASi in protecting against T1DM-induced renal, cardiac, and cerebral complications, focusing on fibrosis and inflammation.

To fulfil these aims, we have established the following specific objectives:

1. To investigate *in vitro* how RAASi influence glucose-induced and profibrotic changes in human proximal tubular cells and rat kidney fibroblast cells
2. To assess the renoprotective and antifibrotic effect of RAASi in a rat model of T1DM

3. To assess the impact of lyophilisation on reproducibility of fibrotic tissue sample processing
4. To analyse the beneficial effects of RAASi on diabetic vascular impairment, cardiac tissue damage, fibrosis, hypoxic and inflammatory damage in T1DM rat hearts
5. To assess the protective potential of RAASi against T1DM-induced neuroinflammation and central BDNF pathway in rat hippocampi

3. Methods

3.1. *In vitro* experiments

3.1.1. Cell culture and experimental design

Human kidney 2 (HK-2) proximal tubular epithelial cells were maintained under standard (5.5 mM) or high glucose (HG; 35 mM) conditions for 24 or 48 hours. To distinguish between the direct glucotoxic and osmotic effects of HG, HK-2 cells were cultured in isosmotic control mannitol (5.5 mM glucose + 29.5 mM mannitol) media. The HG groups were treated with 10 μM ramipril, 10 μM losartan, or 10 μM eplerenone. Drug doses were adopted from the literature.

Normal rat kidney fibroblast (NRK-49F) cells were cultured in DMEM containing 25 mM D-glucose. NRK-49F cells were treated with PDGF (10 ng/mL) or CTGF (10 ng/mL) and various RAASi, as previously described.

Control cells were treated with vehicle (dimethyl sulfoxide) alone (n = 6 wells/group). RNA and protein were isolated with the same buffers as for tissue homogenates.

3.1.2. Immunocytochemistry

For immunocytochemical examination, NRK-49F cells were cultured in tissue culture chambers. Following repeated washes, cells were fixed in formalin, washed again, and permeabilised with Triton X-100. After blocking with 5 % bovine serum albumin (BSA), the samples were incubated with specific anti-PDGFR-β primary antibody diluted 1:100. After repeated washes, the slides were incubated with specific goat anti-rabbit Alexa fluor 568 secondary antibody diluted 1:100. F-actin was

immunostained by incubation with phalloidin-TRITC diluted 1:300. The samples were washed and nuclei were counterstained with Hoechst 33342 diluted 1:1000. After drying, the sections were fixed with Vectashield Mounting Medium. Coverslipped slides were analysed using a Zeiss LSM 510 Meta Image Examiner confocal laser scanning microscope with 63× or 100× magnification objectives or, for phalloidin-stained slides, with an Olympus IX81 fluorescence microscope at 100× magnification.

3.2. *In vivo* experiments

All animal procedures and handling were approved by the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at Semmelweis University, Budapest, Hungary (PEI/001/380-4/2013). All experiments were conducted according to international ethical principles and regulations. Before the *in vivo* experiments, a power analysis determined the minimum number of animals.

3.2.1. Animals and experimental design

Eight-week-old male Wistar rats (*Rattus norvegicus*) were purchased from "Toxi-Coop" Toxicological Research Centre and housed in groups of three in plastic cages under 12-hour dark/light cycle at a constant temperature and humidity with *ad libitum* access to standard rodent chow and drinking water.

T1DM was chemically induced by administering a single intraperitoneal injection of 65 mg/bwkg of streptozotocin (STZ), dissolved in citrate buffer. Blood glucose levels were measured three times from the tail vein using a Dcont IDEAL device after an overnight fast. Rats were considered diabetic and enrolled in the study if their peripheral blood glucose value exceeded 15 mmol/L 72 hours after the STZ injection and remained elevated.

Five weeks after the onset of DM, rats were randomised into four groups to ensure equal average blood glucose values (n = 7 animals/group) and treated daily for 2 weeks *per os* by oral gavage with: (i) isotonic saline as vehicle; or (ii) ACEi ramipril (10 μg/bwkg/day); or (iii) ARB losartan (20 mg/bwkg/day); or (iv) mineralocorticoid receptor antagonist eplerenone (50 mg/bwkg/day). The RAASi drug doses were determined based on our previous experiments in accordance with the literature, in which effective

blockade of RAAS was achieved without alterations in systemic blood pressure.

Healthy, non-diabetic animals matched for age and body weight (Control; n = 7 animals/group) received an equivalent volume of citrate buffer without STZ once, alongside the same amount of salineadministered daily by oral gavage daily, concurrently with the diabetic animals throughout the 2-week treatment period.

Diabetic rats did not receive insulin treatment. Consequently, their physical condition gradually deteriorated alongside the metabolic changes. The overall well-being of the animals was monitored daily, and those whose pain signals elevated significantly were anaesthetised. After the experimental protocol, rats were placed in metabolic cages to collect urine for 24 hours. Based on protein excretion rates, this experimental model indicates that the animals show signs of chronic renal damage after 5 weeks of untreated DM.

Lastly, at 7 weeks into the experimental period, rats were anaesthetized using a mixture of ketamine and xylazine administered intraperitoneally and were sacrificed by terminal blood drawn from the abdominal aorta. Blood, urine, kidney, heart, aorta, and hippocampal samples were collected, immediately snap-frozen, or stored in formalin for further investigation.

3.2.2. Measurement of arterial blood pressure and pulse wave velocity

After two weeks of treatment, PWV and blood pressure measurements were taken under isoflurane anaesthesia on a heating pad maintained at 37 °C. The CODA tail cuff standard monitor system employs clinically validated proprietary volume pressure recording technology to non-invasively measure the tail vein's systolic and diastolic blood pressure. Mean arterial pressure (MAP) was calculated from these values. Non-invasive PWV registration was performed using PulsePenLab applanation tonometry device while recording electrocardiogram. Pulse waves were detected simultaneously over the carotid and femoral arteries on the same side. The time difference between the two waves was divided by 80 % of the direct carotid-femoral surface distance, measured with a high-precision digital calliper.

3.2.3. Metabolic and renal function parameters

Blood and urine samples were centrifuged for 6 minutes at 3600 rpm. Serum metabolic parameters (glucose, fructosamine, total cholesterol) and renal parameters (blood urea nitrogen, serum creatinine, and creatinine from urine collected for 24 hours in metabolic cage) were determined photometrically using commercially available kits on a Hitachi 912 chemistry analyser. Creatinine clearance was calculated by the formula: (urine creatinine x urine volume / serum creatinine) / urine collection time minutes / bodyweight x 100.

3.2.4. Enzyme-linked immunosorbent assay (ELISA)

Collected rat blood samples were centrifuged at 3600 rpm for 6 minutes, after which the separated serum samples were diluted to 1:10. Serum Cardiac Troponin I and Klotho levels were measured using commercially available rat-specific sandwich ELISA kits, following the manufacturer's protocols. Concentrations were calculated based on absorbance measurement at 450 nm, with wavelength correction at 650 nm, using SPECTROstar Nano microplate reader.

3.2.5. Histology

Kidney, left ventricular muscle, and aorta sections were dissected, fixed in formalin, dehydrated in graded series of alcohols, and embedded in paraffin. 5 μm thick sections were cut and deparaffinised in xylene, rehydrated and mounted on coated glass microscope slides. Images were taken with AxioImager A1 light microscope on specific stained sections, and histological examination was performed under ×200 magnification using Panoramic Viewer software version 1.15.2. Analysis was performed in a double-blinded fashion with computer-assisted morphometry using ImageJ software version 1.5.

Picrosirius red staining was performed on the kidney cortex and left ventricular muscle to evaluate fibrosis. Ten areas from each kidney cortex and heart left ventricular muscle cross-section were randomly selected, and the specifically stained fibrotic area was measured. The number of pixels containing stained fibrotic tissue was divided by the total number of pixels in the area to obtain the percentage of fibrosis and collagen deposition.

For IMT measurement, aorta sections were immersed in 1 % orcein at 60 ºC for 30 min, followed by differentiation in acid-alcohol for 10 seconds to remove the dye excess, and then washed with distilled water. Orcein-stained elastic fibres made IMT measurement possible on cross sections of each aorta and the mean value of ten measurements was calculated.

Masson's trichrome staining was performed to evaluate the media layer's fibrosis. The specifically stained interstitial areas were measured within the intima-media layer on each aorta cross-section. The number of pixels containing blue-stained fibrotic tissue was divided by the number of other pixels in the area to obtain the ratio of medial fibrosis.

3.2.6. Lyophilisation

Collected kidney and heart tissue samples were stored at -80 °C in 2 ml Eppendorf tubes, optimally arranged to maximise surface exposure for lyophilisation. Freeze-drying was performed using ScanVac CoolSafe Touch Superior device. The tubes remained open through the entire process. After 1 hour of pre-freezing at -40 °C, pressure was lowered to 0.07 hPa. Primary drying involved six 2-hour steps at 0.22 hPa with gradually increasing temperatures up to 30 °C, while secondary drying was conducted at 0.1 hPa at 40 °C for 3 hours. Dried tissue products were manually crushed using 20-gauge needles and pulverised with 5 mm stainless steel balls in the TissueLyser LT. Powdered dry tissue samples were stored at 4 °C until further processing and measurements.

3.3. Measurement of extracellular matrix formation and degradation biomarkers

C3M, PRO-C3 (measuring type III collagen formation) and TUM biomarkers were assessed in rat urine samples. FBN-C (measuring C-terminal of fibronectin turnover) and PRO-C4 (measuring type IV collagen formation) were evaluated in the supernatant of HK-2 and NRK-49F cells. Nordic Bioscience developed both competitive ELISA kits. To standardise for urine output, urinary biomarker levels were divided by urinary creatinine levels measured with the QuantiChrom™ Creatinine kit. The assays were performed at Nordic Bioscience Laboratories following previously described protocols. Briefly, streptavidin-precoated 96-well ELISA plates were incubated with biotinylated peptide. The plates were washed five times with washing

buffer and then incubated with the standard peptide or sample along with HRP-conjugated monoclonal antibody for 20 hours at 4 °C (C3M, PRO-C3, FBN-C) or 1 hour at 20 °C (TUM, PRO-C4). Following repeated washing steps, incubation with 3,3′,5,5-tetramethylbenzidine occurred in the dark. The reaction was halted by adding sulfuric acid, and density values were measured with an ELISA reader at 450 nm, using 650 nm as a reference.

3.4. Quantitative real-time reverse transcript polymerase chain reaction

Total RNA was extracted from HK-2 and NRK-49F cells and rat kidney, heart, and hippocampal samples using Total RNA isolation Mini Kit. The quality and quantity of the isolated RNA were measured with NanoDrop ND-1000 spectrophotometer. RNA was reverse-transcribed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR. Specific mRNA expressions of interest were determined in duplicates from cDNA samples obtained by qPCR using SYBR Green I Master enzyme mix and particular primers, with sequences designed based on nucleotide sequences from the National Centre for Biotechnology Information's nucleotide database. Results were analysed using LightCycler 480 software version 1.5.0. The mRNA expressions of interest were normalized to the mRNA expression of the 18S ribosomal RNA housekeeping gene from the same samples as a reference transcript.

3.5. Western blot analysis

Total protein was extracted from homogenised HK-2 and NRK-49F cells and rat kidney, heart, and hippocampal samples at 4°C using lysis buffer with the TissueLyser LT homogeniser. To measure O-GlcNAc, 40 μM O-(2-acetamido-2-deoxy-d-glucopyranosylidene)-amino-N-phenyl-carbamate was added to lysates to inhibit OGA activity. Lysates were centrifuged at 13000 rpm at 4 °C for 10 minutes. The protein concentration of the supernatant samples was measured using a detergent-compatible Bradford dye-binding method protein assay kit in 96-well microplates at 650 nm with a SPECTROstar Nano microplate reader. Protein samples were mixed in a ratio of 1:3 with Laemmli buffer and heated at 95 °C for 5 minutes for denaturation.

Solubilised sample lysates containing the appropriate amounts of protein per lane were loaded onto Mini-PROTEAN TGX polyacrylamid precast gels, electrophoretically separated, and transferred to nitrocellulose membranes using the high-efficiency semi-dry Trans-Blot Turbo Transfer System. Ponceau S staining was conducted to verify protein transfer and evaluate the total loaded protein. Membranes were blocked in 5 % w/v non-fat dried milk or BSA in Tris-buffered saline for 1 hour at room temperature and immunoblotted with specific primary antibodies at 4 °C overnight. After repeated washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence was detected using Luminata Forte substrate and immunoreactive bands of interest were quantified densitometrically using Versadoc Quantity One Analysis software. After background subtraction, the integrated optical density was adjusted based on Ponceau S staining to correct for variations in total protein loading. Blots were also normalised to an internal control to compare bands across separate membranes.

3.6. Statistical analysis

Analyses were performed using GraphPad Prism software version 7.0. The minimum required number of items per group was determined with power analysis before the experiments to reach an appropriate statistical strength. Kolmogorov-Smirnov normality test was performed to test if the values were from a Gaussian distribution.

A linear regression fit was applied to dataset pairs to test correlation.

Data were analysed using two-tailed paired t-tests for all parametric comparisons to compare two groups. In the case of nonparametric data, the Wilcoxon test on ranks was employed. To assess the effectiveness of homogenisation, variances within each group were compared using Levene's test, conducted in Microsoft Office Excel, where single-factor ANOVA was applied to the absolute differences of values from the mean.

For comparing more groups, data were analysed by one-way ANOVA, followed by Bonferroni's multiple-comparison *post hoc* test for all parametric comparisons, or in the case of nonparametric data, Kruskal-Wallis ANOVA on ranks, followed by Dunn correction. Significance was set *a priori* at $P < 0.05$, corrected for multiple comparisons.

4. Results

4.1. *In vitro* experiments

4.1.1. RAASi prevent glucose-induced changes in HK-2 cells

To distinguish the *per se* effect of hyperglycaemia and hyperosmolarity on proximal tubules, HK-2 cells were cultured under standard, HG or mannitol-containing isosmotic conditions. Following hyperglycaemia, profibrotic changes were observed, as mRNA expressions of TGFβ1, PDGFB and CTGF increased in HG-treated HK-2 cells. In contrast, while modelling hyperosmolarity without hyperglycaemia, mannitol treatment did not alter TGFβ1 and PDGFB levels but enhanced CTGF mRNA expression.

Concerning the contribution of growth factors produced by proximal tubular cells under hyperglycaemic conditions to fibrosis, we aimed to investigate the potential protective effect of RAASi on profibrotic changes in HK-2 cells. In HG-treated cells, RAASi significantly reduced both PDGFB and CTGF expression to the level of controls; however, no effect of RAASi was noted on TGFβ1 expression.

We measured secreted collagen formation and degradation by-products from supernatants as novel non-invasive biomarkers. Hyperglycaemia reduced the level of FBN-C (a biomarker of decreased fibronectin turnover), which was restored to control levels by RAASi treatments. The amount of internal epitope of the 7S domain of type IV collagen (PRO-C4) secreted into the cells' supernatant was identical across all groups, suggesting no changes in type IV collagen formation in HK-2 cells.

Time-dependent effects of hyperglycaemia-induced protein O-GlcNAcylation and expression of OGT and OGA enzymes were also investigated in HK-2 cells. Protein O-GlcNAcylation level was elevated after 24 and 48 hours of HG treatment, compared to control cells cultured in standard glucose. OGT was increased after 24 hours of HG treatment, but compared to the 24 hours' value, it decreased after 48 hours. OGA changed oppositely, decreased below the level of control after 24 hours, but increased after 48 hours.

RAASi did not affect hyperglycaemia-induced protein O-GlcNAcylation or the OGT enzyme level after 24 hours of HG

treatment in HK-2 cells, whereas OGA was increased by RAASi treatments.

4.1.2. RAASi ameliorate profibrotic changes in NRK-49F cells

Since profibrotic factors produced by proximal tubular epithelial cells can act directly on renal fibroblasts, we also evaluated the effect of growth factor treatment on NRK-49F cells. As we could not detect significant changes in TGFB1 expression due to RAASi treatment in HK-2 cells, we further focused our analyses on PDGFB and CTGF treatment.

Firstly, using fluorescent immunocytochemistry, we proved that NRK-49F cells express PDGFR-β, which is, as discussed above, essential for the action of both PDGF and CTGF.

The effects of various RAASi on fibroblast proliferation was assessed by methyl-thiazolyl-tetrazolium assay. PDGF and CTGF treatment induced cell proliferation, but it was not abated by any RAASi.

Additionally, more specific markers, such as proliferating cell nuclear antigen (PCNA) and Ki67, were measured to evaluate the antiproliferative effects of RAASi. These factors are among the most established components of cell proliferation. PCNA plays a role in DNA synthesis and repair, and it is one of the regulators of the cell cycle. Ki67 can only be detected in the nuclei of proliferating cells. In our study, there was a tendency for increased expression of proliferation markers following both growth factor treatments; however, this elevation reached significance only in the case of PDGF-treated NRK-49F cells. RAASi decreased PCNA expression, but not Ki67.

Next, we investigated morphological changes/cytoskeleton rearrangements of NRK-49F cells caused by PDGF or CTGF treatment using phalloidin staining, which is a highly selective bicyclic peptide used for staining actin filaments (also known as F-actin).

Control cells were slightly elongated and exhibited a diffuse network of thin actin filaments. Changes in fibroblast cytoskeleton morphology, particularly the reorganisation of stress fibres along a longitudinal axis parallel to the formation of F-actin bundles, were noted following both treatments. However, the elongation of cells and F-actin bundles was more pronounced after PDGF treatment. Additionally, PDGF and

CTGF induced actin-clump formation, especially at the edges of fibroblasts.

Lastly, we measured αSMA content and PRO-C4 secretion of NRK-49F cells after treatment with growth factors and RAASi. PDGF and CTGF treatments increased αSMA protein levels, and all RAASi drugs hindered these changes by diminishing αSMA production to the level of controls. Type IV collagen formation did not change due to growth factor treatment or RAASi.

4.2. *In vivo* experiments

Non-depressor doses were adopted from previous studies in line with literary data to investigate the *per se* effect of monotherapeutic RAASi on multiorgan complications of DM.

Seven weeks after the induction of T1DM, diabetic rats had impaired weight gain, elevated serum glucose, fructosamine and total cholesterol levels, demonstrating the development of DM. MAP remained unaltered in all groups, confirming that the examined effects of RAASi are independent of their antihypertensive properties. RAASi did not affect body weight or metabolic parameters, either.

4.2.1. RAASi improve renal function and kidney fibrosis

DM-induced renal impairment presented in higher blood urea nitrogen levels and lower creatinine clearance. These functional parameters of DKD were ameliorated by RAASi treatment. Deteriorated renal function (lower creatinine clearance) was associated with more severe DM (higher blood sugar levels).

Besides functional decline, massive collagen accumulation was observed in untreated diabetic rats, as key histological feature of renal fibrosis. All of the RAASi minimised fibrosis as reflected by fewer Sirius Red-positive areas.

As novel, non-invasive biomarkers of kidney fibrosis, urinary markers of ECM remodelling showed increased collagen IV turnover (TUM) in diabetic animals as well as elevated rates of collagen III formation (PRO-C3) and degradation (C3M). Eplerenone treatment decreased the urinary level of C3M.

4.2.2. Lyophilisation improves reproducibility in fibrotic tissues

During our experiments, we faced the problem that fibrotic tissue accumulation is most often inhomogeneous in the samples; significant differences can be seen between certain kidney and heart regions. Depending on the tissue region used for protein extraction, this may cause higher scatter and more outlier data points within groups and between different isolations.

Therefore, we decided to introduce lyophilisation as an additional method to our tissue preparation process. To test whether the problem of focality can be resolved, protein and RNA were extracted 4 times from lyophilized, pulverized homogenates and from four parts of the same frozen samples. Both mRNA and protein levels of αSMA showed lower variances in the lyophilized, homogenized samples than within the conventionally processed group from frozen samples.

4.2.3. RAASi ameliorate diabetic vascular impairment

To evaluate changes in arterial stiffness, PWV was measured in diabetic rats as the earliest detectable manifestation of pathological vascular remodelling. PWV values correlated with the animals' blood pressure and body weight. There was no significant difference in MAP values between the groups, so PWV was adjusted for body weight. Elevated weight-corrected PWV reflected the increased arterial stiffness in DM.

Aortic histological changes were evaluated with Orcein and Masson's trichrome staining. IMT was measured on Orcein-stained aortic sections, where diabetic rats displayed intimal thickening, irregularities, and diffused elastic membranes. The increased thickness in diabetic animals returned to the control value following treatment with ramipril and eplerenone. Aortic media layer thickness correlated with serum total cholesterol levels. While assessing the accumulation of fibrotic ECM components, Masson's trichrome staining revealed a disorganized arrangement of elastic lamina. The increased blue-stained medial connective tissue ratio in diabetic aortas was reduced by eplerenone.

4.2.4. RAASi minimize cardiac tissue damage and fibrosis

As regular molecular markers of cardiac tissue damage, serum troponin I levels were elevated in diabetic rats, indicating myocardial injury. The mRNA expression of B-type natriuretic peptide (BNP), specific to

chronic left ventricular expansion, was also increased in the hearts of diabetic rats. All RAASi treatments decreased troponin I and BNP levels to normal control values. Klotho, a newly recognised protective marker that regulates fibrosis, oxidative stress, and inflammation, exhibited reduced serum levels in diabetic rats. This adverse event was offset by eplerenone treatment. Along the same pathway, related to kidney damage, the counter-regulatory fibroblast growth factor 23 protein was elevated in the heart muscles of diabetic animals, and all RAASi treatments mitigated this response.

Left ventricular fibrosis was assessed on Sirius Red-stained sections. Fibrotic connective tissue was increased in diabetic hearts, while all RAASi decreased the amount of collagen components to control levels. As another specific marker of cardiac fibrosis, αSMA protein was elevated in diabetic rat hearts, while ramipril and eplerenone treatment decreased it. Since DCM-associated growth factors induce fibrotic differentiation, we also measured the expression of certain profibrotic factors. Increased heart CTGF and PDGF mRNA expressions were observed in DM, which were reduced by all RAASi treatments.

4.2.5. RAASi decrease cardiac hypoxic and inflammatory injury

While exploring the inflammatory aspects of cardiac tissue damage, we detected higher levels of interleukin (IL-6) cytokine mRNA expression and NFκB protein in diabetic heart muscles, which were reduced by losartan treatment. In contrast, tumour necrosis factor alpha (TNFα) cytokine expression was lower in DM, while ramipril and losartan treatment compensated for this change.

On the other pathway, hypoxic marker vascular endothelial growth factor protein level was elevated in DM and decreased by RAASi. In line with TNFα changes, the mRNA expression of its suppressor heat shock protein 72 was doubled in DM and decreased by all RAASi.

4.2.6. RAASi mitigate diabetes-induced inflammation in the hippocampi

Since inflammatory processes are linked to depression and DM, we also investigated inflammation in the brain by measuring pro-inflammatory cytokine expression in the hippocampi of diabetic and RAASi-treated rats. The expression of IL-1α, IL-6 and TNFα was found to be elevated in the hippocampi of diabetic rats and was reduced by RAASi. The

protein levels of the principal transcription factor NFκB, which is associated with IL-1α and TNFα signalling pathways, also increased. All RAASi treatments likewise decreased this parameter.

4.2.7. RAASi restore hippocampal BDNF production

We also examined the BDNF signalling pathway, as it plays a pivotal role in the pathophysiology of comorbid depression. The synthesis of both precursor and mature isoforms of BDNF in the hippocampus decreased in diabetic rats. These adverse changes were fully reversed by RAASi treatment. In our measurements of serine protease enzymes responsible for BDNF cleavage, we discovered that losartan and eplerenone increased the intracellular enzyme furin, while ramipril elevated the extracellular MMP3.

5. Conclusions

1. RAASi treatment reduces glucose-induced O-GlcNAcylation and alters profibrotic growth factor levels, along with a novel non-invasive biomarker of fibronectin turnover in human proximal tubular epithelial cells. Additionally, RAASi decrease both proliferative and fibrotic morphological changes in rat kidney fibroblast cells.
2. Monotherapeutic, non-depressor dose RAASi treatment is renoprotective and antifibrotic in T1DM rat kidneys, and can also be traced by urinary biomarkers.
3. Lyophilised sample processing effectively reduces data scatter while investigating focal fibrotic changes in rat kidney and heart. This is confirmed by lower variances of αSMA mRNA and protein levels in the lyophilized, homogenized samples than within the conventionally processed frozen samples.
4. As part of CV protection, without changes in blood pressure, RAASi attenuate vascular stiffness, intimal thickness, and medial fibrosis in diabetic rat aortas. Additionally, they decrease cardiac tissue damage, fibrotic protein accumulation, and the increase of profibrotic factors. Furthermore, low-dose RAASi treatment can also interfere with hypoxic and inflammatory pathways in diabetic hearts.
5. In DM-associated depression, RAASi can alleviate neuroinflammation via the NFκB inflammatory pathway and modify BDNF transformation by restoring cleavage enzymes.

6. Bibliography of the candidate's publications

Publications related to the thesis:

Gellai R, Hodrea J, Lenart L, Hosszu A, Koszegi S, Balogh D, Ver A, Banki NF, Fulop N, **Molnar A**, et al. Role of O-linked N-acetylglucosamine modification in diabetic nephropathy. Am J Physiol Renal Physiol. 2016;311(6):F1172-f81. **IF=3.611**

Koszegi S*, **Molnar A***, Lenart L, Hodrea J, Balogh DB, Lakat T, et al. RAAS inhibitors directly reduce diabetes-induced renal fibrosis via growth factor inhibition. J Physiol. 2019;597(1):193-209. **IF=4.547**

Balogh DB, **Molnar A**, Hosszu A, Lakat T, Hodrea J, Szabo AJ, et al. Antidepressant effect in diabetes-associated depression: A novel potential of RAAS inhibition. Psychoneuroendocrinology. 2020;118:104705. **IF=4.9**

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Balogh DB*, **Molnar A***, Degi A, Toth A, Lenart L, Saeed A, et al. Cardioprotective and Antifibrotic Effects of Low-Dose Renin-Angiotensin-Aldosterone System Inhibitors in Type 1 Diabetic Rat Model. Int J Mol Sci. 2023;24(23). **IF=4.9**

Publications not related to the thesis:

Hodrea J, Saeed A, **Molnar A**, Fintha A, Barczi A, Wagner LJ, et al. SGLT2 inhibitor dapagliflozin prevents atherosclerotic and cardiac complications in experimental type 1 diabetes. PLoS One. 2022;17(2):e0263285. **IF=3.7**