Study of the reactive metabolites in type 2 diabetes and the effect of antidiabetic treatment on their metabolism

Ph.D. theses

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I. Introduction

Diabetes mellitus (DM) is a complex heterogenous disorder and a major global health concern with approximately 9% of the world population. In addition, diabetes is a leading risk factor for cardiovascular disease and stroke. Moreover, mircrovascular complications like diabetic nephropathy, neuropathy and retinopathy are associated with diabetes. Type 1 diabetes is characterised by absolute insulin deficiency caused by an autoimmun mechanism. On the other hand, type 2 diabetes is generally defined as a relative insulin deficiency and characterised by a combination of insulin resistance, hyperglycaemia and obesity. Current clinical management of diabetes focuses on surrogate parameters such as blood glucose and HbA1c. These correlate with the incidence of diabetic complications. However, multiple interventional studies showed that intensive glycaemic control had limited, or even detrimental effects on diabetic complications compared standard therapy. Furthermore, several patients with type 2 diabetes developed typical diabetic complications before manifestation and diagnosis of diabetes. Since our therapeutic strategies are neither very successful nor preventing diabetic complications we hypothesize that late diabetic complications are associated with other metabolic pathologies also. In this context, reactive metabolites or dicarbonyls like methylglyoxal (MG), and the advanced glycation end products (AGEs) play an important role. MG is a highly reactive, and hence toxic α-oxoaldehyde which is formed by the

degradation of triosephosphates during the glycolysis. MG reacts directly and rapidly with proteins to form AGEs leading to several pathological consequences. An accumulation of AGEs has been shown to contribute to the pathogenesis of diabetic macro- and microvascular complications. Moreover, within the the process of energy production of cells, reactive oxigen specieses (ROSs) and oxidative stress are more pronounced leading to infalmmation and DNA-damage. Recent clinical studies have suggested that reactive metabolites such as MG may play a causal role in the development of diabetes and its complications. Thus understandig the mechanism regulating formation and matabolism of reactive metabolites and AGEs is an important step in defining new therapeutic targets of diabetes and its complications. Several animal and clinical trials have been carried out with the aim to target the reactive metabolites and AGE-pathway in diabetes. Dicarbonyl scavangers were the first drugs developed to alleviate dicarbonyl stress. Unfortunately none of these compounds could be approved for treatment of diabetic complications.

The biguanide, metformin is currently the drug of first choice for the treatment of type 2 diabetes. Its primary mechanism of glucose lowering action is to increase hepatic and peripherial tissue insulin sensitivity. Moreover, an MG-lowering effect of metformin has been also reported. However, the exact mechanism of this effect is not completly understood. In addition, metformin has gained attention for its pleiotropic effects such as positive influence cardiovascular risk factors like plasma lipid parameters. However, the potential

beneficial effect of metfomin on cardiovasciular risk in patients with type 2 diabetes is not entirely clear.

II. Objectives

The effect of metformin on MG and AGE metabolism was studied in a prospective pilot trial in patients with newly diagnosed type 2 diabetes (study I.). Furthermore, we investigated the effect of metformin therapy on lipid parameters and on cardiovascular risk in a retrospective study of patients with type 2 diabetes without lipid lowering therapy (study II). Finally, we studied the role of an arginine-rich, cyclic peptiede (CycK(Myr)R4E) on the plasma MG levels and an MG-induced hyperalgesia in mice (study III).

II.1. Objectives of study I.

1. The effect of metformin on the plasma MG level.
2. The effect of metformin on the formation of trioses phosphate intermediates in red blood cells.
3. The effect of metformin on the Glyoxalase I and Glyoxalase II activity in red blood cells and peripheral mononuclear cells.
4. The effect of metformin on the plasma concentration of D-lactate, the stable end-product of glyoxalase metabolism.

5. The effect of metfomin on the plasma concentration of the AGE product, N(ε)-(Carboxymethyl)-Lysine (CML).

II.2. Objectives of study II.

1. The effect of metformin therapy on the lipid parameters in patients with type 2 diabetes without statin therapy.
2. The effect of metformin therapy on the cardiovascular risk in patients with type 2 diabetes without a history of cardiovascular disease.

II.3. Objectives of study III.

1. The effect of an arginine-rich, cyclic peptide (CycK(Myr)R4E) on the plasma MG levels in mice.
2. The effect of (CycK(Myr)R4E) on the MG-induced hyperalgesia in mice.

III. Methods

III.1. Methods of the study I.

In a prospective obeservational clinical trial 12 patients with newly diagnosed type 2 diabetes were treated with high-dose metformin for 24 weeks. Lifestyle intervention was performed monthly by a dietetic counselor. Blood was sampled in the fasting state. Blood was immediately processed in the Central Laboratory of the Semmelweis University, and in the Molecular Biology Laboratory of the 2nd Department of Medicine, Semmelweis University under standardized conditions. Red blood cells (RBC), peripheral mononuclear cells (pBMC) and plasma were isolated from venipuncture EDTA anticoagulant. The concentration of MG in deproteinized plasma was measured by HPLC. The concentration of trioses phosphate intermediates in deproteinised haemolysate was determined by endpoint enzymatic assay. The activity of Glyoxalase 1 and Glyoxalase 2 in RBCs and pBMCs was calculated using spectrometric methods. The concentration of D-lactate in plasma samples was measured using the spectophotometric method, which monitors the initial rate of change in absorbance at 340nm caused by a formation of NADH. The measurement of CML-modified protein concentration in plasma samples was performed by direct ELISA using an antibody against CML. Patients were recruited from the 2nd Department of Medicine, Semmelweis University. The laboratory tests were

performed in the research laboratory of the Department of Internal Medicine I and Clinical Chemistry, University of Heidelberg.

III.2. Methods of the study II.

In a cross-sectional study, 102 patients with type 2 diabetes without lipid lowering therapy were analysed for lipid profile and cardiovascular risk depending on metformin therapy. Blood was drawn in the morning under fasting conditions and immediately processed in the Central Laboratory of the Heidelberg University Hospital under standardized conditions. Cardiovascular risk was calculated using the United Kingdom Prospective Diabetes Study Risk Calculator. Patients were recriuted in the outpatient clinic of the Heidelberg University Hospital.

III.3. Methods of study III.

A total of 36 C57BL/6 mice were randomly assigned to three groups. One group received the peptide CycK(Myr)R_4E (0.25 mg/mouse in 0.9 % NaCl) intraperitoneal (ip.) while the two other groups received saline ip. One of the saline and the peptide treated group received MG (5 µg/g) iv. 30 min after peptide/saline injection while the third group received saline iv. Mice were housed with a 12-hour/12-hour light/dark cycle and had free access to water and food. Blood was collected through the submandibular vein into EDTA tubes 30 min after MG injection. Samples were spun for 5 min at 6000 rpm at 4 °C, the

supernatant frozen in liquid N_2 and stored at -80 °C until analysis. MG content was determined by LC-MS/MS analysis. Pain response was tested using a hot plate analgesia meter (Columbus Instruments, Ohio, USA) with the plate set to 50 °C 3 h after MG injection. Mice were removed from the plate when hind pare lifting, licking, shaking or jumping occurred. Mice were removed after a maximal cut-off time of 60 s. Pain response for each animal was measured in triplicates. All procedures in this study were approved by the Animal Care and Use Committees at the Regierungspräsidium Tübingen and Karlsruhe, Germany (35-9185.81/G-3/15) and were performed in the research laboratory of the Department of Internal Medicine I and Clinical Chemistry, University of Heidelberg.

IV. Results

IV.1. Results of the study I.

Metformin treatment significantly reduced fasting glucose ($p<0.05$) and HbA_{1c} ($p<0.01$). Metformin treatment significantly reduced plasma MG levels ($p<0.01$). Metformin treatment had no effect on the levels of trioses phosphate intermediates in red blood cells. The reduction in plasma MG was paralleled by an increase in the activity of Glyoxalase 1 ($p<0.01$) in pBMCs while in RBC only shown a trend towards reduction. No significant differences were found in Glyoxalase 2 activity in either the pBMCs or RBCs. Metformin had no effect on plasma D-lactate concentration. Metformin treatment reduced significantly the plasma CML-modified protein concentration ($p<0.05$). Multivariable regression analysis demonstrated that the observed reduction in plasma MG was dependent only on changes in Glyoxalase 1 activity in pBMCs ($p<0.01$) and RBCs ($p<0.05$).

IV.2. Results of the study II.

Patient with metformin therapy had significantly lower total cholesterol ($p<0.01$) and LDL-cholesterol ($p<0.05$) levels than patients without metformin. This effect was independent form glycaemic control and other medication. No effect of metformin could be found on cardiovascular risk.

IV.3. Results of the study III.

Control mice had MG plasma levels of approximately 150 mmol/l whereas MG injection resulted in increased MG plasma levels ($p<0.001$) which was lowered by treatment with CycK(Myr)R_4E ($p<0.001$). Mice which received MG displayed hyperalgesia compared to the control group ($p<0.01$) while treatment with the MG-scavenging peptide CycK(Myr)R_4E prior to MG injection resulted in an almost complete normalization of hyperalgesia ($p<0.01$).

V. Conclusions

V.1. Conclusions of the study I.

1. Metformin has a lowering effect on plasma MG in patients with type 2 diabetes.

2. Metformin treatment has no effect on the formation of trioses phosphate intermediates in RBS in patients with type 2 diabetes.

3. Metfomin increase the Glyoxalase 1 activity but not Glyoxalase 2 activity in RBCs and pBMCs.

4. Metformin has no effect on the plasma D-lactate levels, the stable end-product of glyoxalase metabolism.

5. Metfomin treatment reduces the concentration of advanced glycation end products in plasma

V.2. Conclusions of study II.

1. Metformin treatment has beneficial effects on cholesteol and LDL-cholesterol levels in patients with type 2 diabetes without statin therapy.

2. Using a multivariable risk assessement score metformin had not any treatment effect on the cardiovascular risk in patients with type 2 diabetes.

V.3. Conclusions of study III.

1. The MG-scavanger CycK(Myr)R4E peptide is effective to reduce plasma MG levels in mice.

2. The MG-scavanger CycK(Myr)R4E peptide is effective to reduce MG-induced pain in mice.

VI. Publications

VI.1. Publications related to the thesis

1. Kender Z, Groener J, Reismann P, Kopf S. (2019) The effect of Metformin Therapy on Cholesterol, LDL Levels, and Cardiovascular Risk in Patients with Type 2 Diabetes without Statin Therapy. Orv Hetil, 160: 1346-1352

2. Brings S, Fleming T, De Buhr S, Beijer B, Lindner T, Wischnjow A, Kender Z, Peters V, Kopf S, Haberkorn U, Mier W and Nawroth PP. (2017) A Scavenger Peptide Prevents Methylglyoxal Induced Pain in Mice. Biochim Biophys Acta Mol Basis Dis, 1863: 654-662.

3. Kender Z, Fleming T, Kopf S, Torzsa P, Grolmusz V, Herzig S, Schleicher E, Racz K, Reismann P and Nawroth PP. (2014) Effect of Metformin on Methylglyoxal Metabolism in Patients with Type 2 Diabetes. Exp Clin Endocrinol Diabetes, 122: 316-319.

4. Kender Z, Torzsa P, Grolmusz KV, Patocs A, Lichthammer A, Veresne Balint M, Racz K and Reismann P. (2012) [The Role of Methylglyoxal Metabolism in Type-2 Diabetes and Its Complications]. Orv Hetil, 153: 574-585.

VI.1. Other Publications

1. Jende JME, Groener JB, Rother C, Kender Z, Hahn A, Hilgenfeld T, Juerchott A, Preisner F, Heiland S, Kopf S, Pham M, Nawroth P, Bendszus M and Kurz FT. (2019) Association of Serum Cholesterol Levels with Peripheral Nerve Damage in Patients with Type 2 Diabetes. JAMA Netw Open, 2: e194798.

2. Groener JB, Gelen D, Mogler C, Herpel E, Toth C, Kender Z, Peichl M, Haufe S, Haberkorn U, Sulaj A, Zemva J, Kopf S, Nawroth PP, Brune M and Rudofsky G. (2019) Braf V600e and Retinoic Acid in Radioiodine-Refractory Papillary Thyroid Cancer. Horm Metab Res, 51: 69-75.

3. Kopf S, Groener JB, Kender Z, Fleming T, Bischoff S, Jende J, Schumann C, Ries S, Bendszus M, Schuh-Hofer S, Treede RD and Nawroth PP. (2018) Deep Phenotyping Neuropathy: An Underestimated Complication in Patients with Pre-Diabetes and Type 2 Diabetes Associated with Albuminuria. Diabetes Res Clin Pract, 146: 191-201.

4. Kopf S, Groener JB, Kender Z, Fleming T, Brune M, Riedinger C, Volk N, Herpel E, Pesta D, Szendrodi J, Wielputz MO, Kauczor HU, Katus HA, Kreuter M and Nawroth PP. (2018) Breathlessness and Restrictive Lung Disease: An Important Diabetes-Related Feature in Patients with Type 2 Diabetes. Respiration, 96: 29-40.

5. Muller-Krebs S, Nissle K, Tsobaneli J, Zeier M, Kihm LP, Kender Z, Fleming T, Nawroth PP, Reiser J and Schwenger V. (2015) Effect of Benfotiamine in Podocyte Damage Induced by Peritoneal Dialysis Fluid. Front Med (Lausanne), 2: 10