Effects of non-physiological tyrosine isomers on macrophage function and their possible role in the development of kidney disease

Ph.D. thesis summary

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Abbreviations

Akt: Protein kinase B; Protein kináz B

ANOVA: Analysis of Variance; Varianciaanalízis

Arg1: Arginase-1; Argináz-1

BSA: Bovine Serum Albumin; Szarvasmarha szérumalbumin

CRF: Corticotropin-releasing factor: Kortikotropin felszabadító faktor

DMEM: Dulbecco's Modified Eagle Medium

DOPA: Dihydroxy-phenylalanine; Dihidroxi-fenilalanin

EMT: Epithelial–Mesenchymal Transition; Epiteliális–mezenchimális átmenet

FPE: Foot Process Effacement; Podocita lábnyúlvány-fúzió

FSGS: Focal Segmental Glomerulosclerosis; Fokális szegmentális glomeruloszklerózis

HEK-293: Human Embryonic Kidney cells; Humán embrionális vesesejtek

iNOS: Inducible nitric oxide synthase; Indukálható nitrogén-monoxid-szintáz

IRS-1: Insulin receptor substrate 1; Inzulinreceptor-szubsztrát-1

pAkt: Phosphorylated Akt; Foszforilált Akt

PAST: Paleontological Statistics Software; PAST statisztikai szoftver

PBS: Phosphate-buffered saline; Foszfát-pufferelt sóoldat

Phe: Phenylalanine; Fenilalanin

ROI: Region of Interest; Vizsgált régió

STAT5: Signal Transducer and Activator of Transcription 5; Jelátvivő és transzkripciós aktivátor 5

T2DM: Type 2 Diabetes Mellitus; 2-es típusú diabetes mellitus

WT1: Wilms' Tumor Protein; Wilms-tumor fehérje

1. INTRODUCTION

Phenylalanine (Phe) is one of the essential amino acids and is enzymatically converted into para-tyrosine (para-Tyr), dihydroxyphenylalanine (DOPA), catecholamines, melanin, and thyroid hormones. Due to the vulnerability of the aromatic ring of Phe, in addition to enzymatic modifications, non-enzymatic modifications are also possible. The hydroxyl radical can hydroxylate the aromatic ring of Phe, which—besides the physiological para-tyrosine (para-Tyr)—can lead to the formation of non-physiological meta-tyrosine (meta-Tyr) and ortho-tyrosine (ortho-Tyr).

The amount of para-Tyr, meta-Tyr, and ortho-Tyr formed non-enzymatically is, however, three orders of magnitude lower than that of the amount of para-Tyr generated in the enzymatic process.

Increased levels of meta-Tyr and ortho-Tyr reflect enhanced production of hydroxyl radicals, which indicates oxidative stress. Tyr isomers have long been studied mainly as markers of oxidative stress, but increasing evidence suggests that non-physiological Tyr isomers may also act as causative agents of harmful effects [1].

We observed that a correlation can be demonstrated between serum ortho-Tyr levels and erythropoietin resistance in dialyzed patients [2]. In our previous study, meta-Tyr and ortho-Tyr inhibited erythropoietin-dependent erythroblast proliferation in a time- and dose-dependent manner and practically prevented erythropoietin-induced ERK and STAT5 phosphorylation [3].

According to our earlier investigations, non-physiological Tyr isomers (meta- and ortho-Tyr) were markers of oxidative stress in type 2 diabetes mellitus (T2DM) occurring with or without chronic kidney disease [4].

Our further results showed that, due to its antioxidant effect, resveratrol reduced urinary excretion of ortho-Tyr in patients with type 2 diabetes mellitus, and in parallel, an improvement in insulin resistance was also observed [5].

Culturing different cell lines (HEK, podocyte, macrophage, and adipocyte) on meta-Tyr and ortho-Tyr led to insulin resistance in these cells, similar to what is observed in a high-glucose environment. In these cells cultured on meta- and ortho-Tyr, insulin-induced phosphorylation of insulin receptor substrate-1 (IRS-1) decreased [6].

Our earlier Western blot studies showed that the unstimulated, basal phosphorylation of Akt (Protein Kinase B) in 3T3-L1 adipocytes, HEK-293 cells (human embryonic kidney cells), and podocytes was significantly higher following meta- and ortho-Tyr treatment compared with

para-Tyr treatment. The increased phosphorylation resulted in resistance in these cells, as insulin-stimulated phosphorylation did not become significant.

We also observed that when the phosphorylated tyrosine was meta-Tyr or ortho-Tyr, the dephosphorylation of the phosphorylated polypeptide by the tyrosine phosphatase 1B enzyme was practically impossible, while phosphorylated para-Tyr was rapidly dephosphorylated [6].

Inflammatory processes play an important role in the development of T2DM, but the mechanism has not yet been fully clarified [7]. It is likely that inappropriate activation of immune cells and the resulting altered inflammatory processes contribute to the development of T2DM [7]. Insulin resistance may affect macrophage function, since insulin influences macrophage M1 and M2 polarization through the activation of Akt signaling [8,9].

Based on these findings, we concluded that non-physiological tyrosine isomers are not only markers of oxidative stress but may also play a role in the pathogenesis of several diseases [3,4,6,10].

2. OBJECTIVES

1. Our aim was to determine the temporal and quantitative uptake of Tyr isomers by kidney cells.
2. We aimed to investigate the effects of non-physiological Tyr isomers on insulin signaling in macrophages by measuring the intensity of immunofluorescently labeled pAkt (phosphorylated Akt).
3. We intended to examine how para-, meta-, and ortho-Tyr treatments influence macrophage M1/M2 polarization by determining the arginase-1/iNOS (inducible nitric oxide synthase) ratio.
4. Our goal was to demonstrate how insulin affects M1/M2 macrophage polarization, and how abnormal Tyr isomers influence insulin's effect in this regard.
5. We aimed to compare patients in the control and FSGS (focal segmental glomerulosclerosis) groups based on their clinical data during the analysis of kidney biopsy samples.
6. We intended to compare kidney biopsy samples from control and FSGS groups based on the staining intensity of WT1 (Wilms tumor protein), vimentin, and CRF (corticotropin-releasing factor) in the glomeruli and the tubulo-interstitium using immunofluorescence.
7. Our goal was to investigate how meta- and ortho-Tyr affect the expression of WT1, vimentin, and CRF in embryonic kidney cells.

3. METHODS

Cell culture

For the cultivation and maintenance of J774A.1 mouse BALB/C monocyte-macrophages and HEK-293 cells used in our experiments, we used Dulbecco's Modified Eagle's Medium (DMEM), supplemented with the following components: 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 µg/ml fluconazole, and 1 µg/ml insulin. Depending on the experiment, the medium contained either 5 or 25 mmol/l glucose. Cells were cultured on coverslips placed in 6-well plates, in a humidified incubator at 37 °C with 5% CO_2.

3.1. CHRONIC INSULIN RESISTANCE IN MACROPHAGES INDUCED BY NON-PHYSIOLOGICAL TYROSINE ISOMERS

Treatment and analysis of macrophage cells

During treatments, para-, meta-, or ortho-Tyr was added to the medium at a concentration of 72 mg/l. For para-Tyr treatments, both 5 and 25 mmol/l glucose were used, whereas for meta- and ortho-Tyr treatments, the medium contained exclusively 5 mmol/l glucose. Treatments were performed for 5 days. At the end of day 5, cells were kept overnight in serum-free medium. For insulin signaling studies, insulin doses of 6, 25, 100, and 400 nmol were applied for 10 minutes, whereas for M1/M2 polarization studies, only 400 nmol insulin concentration was used for 48 hours.

The effects of Tyr isomers on insulin signaling in macrophages were assessed by determining the amount of pAkt via immunofluorescent labeling and intensity measurement. Macrophage M1/M2 polarization was assessed by calculating the arginase-1/iNOS ratio, measuring the immunofluorescently labeled arginase-1 and iNOS intensities. Negative control medium contained only para-Tyr, while positive control medium contained para-Tyr and high glucose (25 mmol/l). After removing the medium and rinsing with ice-cold PBS, cells were fixed on ice in ice-cold 4% paraformaldehyde (in PBS) for 20 minutes. Permeabilization was performed with 0.1% Triton X-100 in PBS for 15 minutes. For blocking, 5% bovine serum albumin was used for 60 minutes at room temperature. Primary antibody incubation was carried out overnight at 4 °C, followed by 60 minutes at room temperature. Secondary antibody incubation was performed for 4 hours at room temperature. Cell nuclei were stained with DAPI, and coverslips were mounted with DPX.

Tyrosine uptake in HEK cells

We prepared a medium containing para-, meta-, and ortho-Tyr in equal amounts. HEK cells were incubated in this medium for 0, 10, 20, 30, 40, 50, and 60 minutes.

The amounts of Tyr isomers taken up by cells were determined using an HPLC system. Derivatization or labeling was not required, as para-, meta-, and ortho-Tyr were quantified via their autofluorescence using a Shimadzu Class 10 HPLC system with an RF-10 AXL fluorescence detector.

3.2. WT1, VIMENTIN, AND CRF EXPRESSION IN FOCAL SEGMENTAL GLOMERULOSCLEROSIS (FSGS) IN HUMAN KIDNEYS AND THE EFFECT OF META- AND ORTHO-TYROSINE ON WT1, VIMENTIN, AND CRF EXPRESSION IN HEK-293 CELLS

Patients involved in the study

We examined kidney biopsy samples and clinical parameters of 42 patients diagnosed with FSGS. As controls, kidney biopsy samples from 7 patients with thin basement membrane nephropathy were used. All patients were over 18 years of age; the average age in the FSGS group was 39.9 ± 18.6 years (23 male, 19 female), while in the control group it was 51.1 ± 15.6 years (3 male, 4 female). All kidney biopsies were performed at the 2nd Department of Internal Medicine and Nephrology-Diabetes Center, Medical School, University of Pécs.

Kidney biopsy samples

In kidney biopsy samples, the degree of podocyte foot process effacement (FPE) was determined by electron microscopy. WT1, vimentin, and CRF expression levels were assessed by immunofluorescent labeling and measurement of labeling intensity.

Sections obtained from the samples were permeabilized with 0.6% Triton X-100 and blocked with 1% bovine serum albumin (BSA) for 30 minutes. For primary antibody labeling, sections were incubated with primary antibodies for 90 minutes. For secondary antibody labeling, incubation was performed for 60 minutes. Finally, sections were mounted with VECTASHIELD Antifade Mounting Medium.

Treatment and analysis of HEK-293 cells

For HEK-293 cell treatments, the medium contained para-, meta-, or ortho-Tyr at a concentration of 72 mg/l. Treatments with tyrosine isomers were applied for 5 days, after which cells were kept overnight in serum-free medium.

Cells were then rinsed with PBS to remove residual medium, followed by fixation with 4% paraformaldehyde in PBS for 20 minutes. Permeabilization was performed for 10 minutes with 0.3% Triton X-100 in PBS. Blocking was done with 2.5% bovine serum albumin for 45 minutes. During primary antibody labeling, cells were incubated for 60 minutes with primary antibodies dissolved in PBS. For secondary antibody labeling, cells were incubated for 60 minutes with secondary antibodies, also in PBS. Finally, cells were mounted with VECTASHIELD Antifade Mounting Medium.

Intensity measurement

Images of immunofluorescent labeled samples were captured using a confocal laser scanning microscope. Labeling intensity was measured using Nikon NIS Elements software. Regions of interest (ROI) were selected manually. After duplicating all ROIs (with the duplicated ROI area matching the original), duplicates were placed over background areas. From the total pixel intensity measured within the ROI area, the background ROI intensity was subtracted. The resulting intensity value was then corrected for the area of the ROI.

Statistical analysis

The Shapiro–Wilk test was used to verify the distribution of variables. For normally distributed variables, analysis of variance (ANOVA) was applied; for non-normally distributed variables, the Kruskal–Wallis test and the Mann–Whitney U test were used. Data trends were tested using the Mann–Kendall trend test. Correlations between treatments were analyzed using Spearman's rho correlation test. A p value less than 0.05 was considered statistically significant. Statistical analyses were performed using Paleontological Statistics (PAST) software, version 3.21.

4. RESULTS

4.1. CHRONIC INSULIN RESISTANCE IN MACROPHAGES INDUCED BY NON-PHYSIOLOGICAL TYROSINE ISOMERS

Tyrosine uptake in HEK cells

When examining the uptake of Tyr isomers, we found that in the control group, the cells contained only negligible amounts of meta- and ortho-Tyr compared to para-Tyr. From 20 minutes onwards, the amount of meta-Tyr was not significantly different from that of para-Tyr, whereas the amount of ortho-Tyr, despite increasing, remained lower than that of para-Tyr even after 60 minutes. Thus, HEK-293 cells take up meta- and ortho-Tyr in amounts close to those of para-Tyr.

Effect of insulin treatment on pAkt levels in J774A.1 macrophages in different media

In the untreated control groups, we observed the following:
In high-glucose (25 mmol/l) medium, the unstimulated pAkt levels in macrophages were higher compared to treatment with 5 mmol/l glucose. Treatment with meta- and ortho-Tyr alone also increased unstimulated pAkt levels compared to the para-Tyr control.

Upon insulin stimulation, pAkt levels changed as follows:

In medium containing physiological para-Tyr, insulin increased pAkt levels in a dose-dependent manner. High glucose concentration abolished the insulin-induced increase in pAkt levels. Both meta- and ortho-Tyr prevented or reversed the effect of insulin on pAkt.

Effect of insulin treatment on the arginase-1/iNOS ratio in J774A.1 macrophages incubated in different media

High-glucose treatment decreased the arginase-1/iNOS ratio in macrophages. Similarly, both meta- and ortho-Tyr reduced the arginase-1/iNOS ratio compared to physiological para-Tyr treatment. Under para-Tyr control conditions, insulin decreased the arginase-1/iNOS ratio. High-glucose medium abolished the effect of insulin. Ortho-Tyr treatment also abolished the insulin-induced decrease in the arginase-1/iNOS ratio, while meta-Tyr reversed this effect, leading to insulin dependent increase of the arginase-1/iNOS ratio in macrophages.

4.2. WT1, VIMENTIN, AND CRF EXPRESSION IN FOCAL SEGMENTAL GLOMERULOSCLEROSIS (FSGS) IN HUMAN KIDNEYS AND THE EFFECT OF META- AND ORTHO-TYROSINE ON WT1, VIMENTIN, AND CRF EXPRESSION IN HEK-293 CELLS

Baseline characteristics of control and FSGS patients

Comparing the baseline characteristics of the control and FSGS patient groups, we found, as expected, that FPE was significantly higher in the FSGS group compared to the controls. Furthermore, erythrocyte sedimentation rate (ESR) was also higher in the FSGS group than in the controls, and the use of diuretics was greater in the FSGS group compared to that recorded in the control group.

Kidney biopsy samples

In kidney biopsy samples, we found that with respect to WT1 labeling intensity, there was no significant difference between glomeruli in the FSGS and control groups. However, in the tubulo-interstitium, WT1 expression was lower in the FSGS group compared to controls. Vimentin labeling intensity in the glomeruli was lower in the FSGS group compared to controls, while in the tubulo-interstitium, vimentin expression was higher compared to the control group. CRF labeling intensity was lower in glomeruli than in the tubulo-interstitium. The degree of FPE in the glomeruli correlated with vimentin and CRF labeling intensity.

HEK-293 cells

In HEK-293 cells, we observed that both meta- and ortho-Tyr treatment reduced WT1 levels compared to physiological para-Tyr treatment. Furthermore, meta-Tyr decreased vimentin expression in HEK-293 cells compared to the para-Tyr control.

5. DISCUSSION

When examining the uptake of Tyr isomers, we found that HEK-293 cells take up all three isomers to nearly the same extent. In macrophages not treated with insulin, 25 mmol/l glucose concentration, meta-Tyr, and ortho-Tyr increased pAkt levels and decreased the arginase-1/iNOS ratio. Under physiological para-tyrosine treatment, insulin dose-dependently increased pAkt levels and decreased the arginase-1/iNOS ratio. Both meta- and ortho-Tyr treatment abolished or reversed insulin's enhancing effect on pAkt and its reducing effect on the arginase-1/iNOS ratio.

In glomeruli, WT1 staining intensity was similar in FSGS and control groups, whereas in the tubulo-interstitium WT1 intensity was lower in FSGS patients. Vimentin intensity was lower in glomeruli and higher in the tubulo-interstitium of FSGS patients compared to controls. The degree of FPE determined by electron microscopy correlated with vimentin and CRF levels in the glomeruli. In HEK cells, WT1 labeling intensity was lower in the meta- and ortho-Tyr groups, and vimentin intensity was lower in the meta-Tyr group compared to para-Tyr.

5.1. EXAMINATION OF TYR ISOMER UPTAKE

The harmful effects of non-physiological Tyr isomers on living organisms have long been studied [11]. From our experiments, we can conclude that cells are capable of taking up pathological Tyr isomers to the same extent as physiological para-Tyr. It is likely that Tyr isomers can be incorporated into proteins, thus exerting harmful effects on living organisms [12].

5.2. EFFECT OF ABNORMAL TYR ISOMERS ON INSULIN SIGNALING IN MACROPHAGES

Beside our research group's studies, we found no other research investigating the effects of Tyr isomers on pAkt or other proteins involved in insulin signaling. However, we identified publications examining the impact of oxidative stress on Akt phosphorylation in macrophages and other tissues [13–17]. Since the quantity of non-physiological tyrosines increases with oxidative stress, these studies can be indirectly compared with ours. Among these studies, three examined macrophages, and all three support our finding that increased levels of non-physiological Tyr isomers and glucose raise pAkt levels.

These studies did not assess the impact of oxidative stress on insulin. In our group's previous work, we examined the effect of non-physiological Tyr isomers on insulin signaling via IRS-1 and pAkt measurements [6]. These findings support our current results, namely that in the presence of meta- and ortho-Tyr, insulin's enhancing effect on pAkt levels is abolished.

5.3. EFFECT OF ABNORMAL TYR ISOMERS ON M1/M2 POLARIZATION OF MACROPHAGES

Unfortunately, the effect of insulin resistance on macrophage polarization has been little studied. We found two relevant publications in the literature examining the effect of insulin and insulin resistance on macrophage M1/M2 polarization [18,19]. These studies, which also analyzed iNOS and Arg1 (arginase-1) expression, support our findings that insulin resistance decreases iNOS and increases Arg1 levels in macrophages. The reliability of our study is further strengthened by the consistency between our results obtained from examining insulin signaling and M1/M2 polarization.

5.4. ANALYSIS OF KIDNEY BIOPSY SAMPLES AND HEK-293 CELLS

In diagnosing FSGS, the presence of FPE detected by electron microscopy plays a key role, as it represents an initial step in podocyte injury [20,21]. As expected, in the samples of patients diagnosed with FSGS that we examined, the ratio of FPE was higher than in the control samples.

WT1 is indispensable for podocyte function and thus for maintaining glomerular function. Reduced WT1 expression can lead to acute glomerulonephritis and mesangial sclerosis [22]. Based on these data, WT1 plays a cardinal role in kidney function, and its loss (primarily) leads to the development of glomerulopathies. According to our results, WT1 levels did not differ between glomeruli of FSGS and control groups. Although podocytes cannot regenerate, studies confirm that parietal epithelial cells can migrate and differentiate toward the podocyte lineage following injury [23,24]. These cells can proliferate after podocyte injury and replace damaged podocytes [22]. This regenerative process may account for unchanged WT1 levels in the early stages of FSGS.

Chronic tubulo-interstitial changes observed in FSGS may reduce WT1 labeling intensity, which could explain our findings.

Our findings show that WT1 labeling intensity decreases in media containing meta- and ortho-Tyr. From this, we may infer that abnormal Tyr isomers may play a role in suppressing WT1 protein expression and thus contribute to the development of FSGS.

Vimentin is an intermediate filament mainly found in connective tissue cells of mesenchymal origin and in smooth muscle cells, and it plays an important role in forming the cytoskeleton of podocytes. It is involved in maintaining podocyte integrity and mediating the response of podocytes to injury [26,27]. Moreover, vimentin can indicate tubular injury in the case of epithelial–mesenchymal transition (EMT), where neo-expression of vimentin can be observed [28]. Based on the above, vimentin plays an important role in kidney function, and changes in its levels may serve as a marker of kidney injury.

The significantly lower levels of vimentin in glomeruli of FSGS patients can be explained by the increased number of injured podocytes, as supported by the strong negative correlation between podocyte FPE and vimentin (glomeruli), as well as WT1 (glomeruli) and vimentin (glomeruli).

Vimentin expression was significantly higher in the tubulo-interstitium of patients with FSGS, which may mark EMT as a response to chronic injury [29–31]

In HEK-293 cells, our results show that meta-Tyr significantly decreases vimentin staining intensity, consistent with our findings in kidney biopsy samples.

We found no studies examining CRF expression in FSGS, nor did we find data on CRF expression in healthy kidneys. In our study, using immunofluorescence labeling, we detected CRF expression in both FSGS and control groups. CRF staining intensity was significantly lower in glomeruli of the FSGS group and showed no difference in the tubulo-interstitium between the two groups.

HEK-293 cells showed CRF positivity, but treatment with meta-Tyr or ortho-Tyr did not affect CRF labeling intensity, suggesting that CRF likely does not play a significant role in the pathogenesis of FSGS.

5.5. CONCLUSIONS

Based on the above, pathological Tyr isomers play a significant role in the development of insulin resistance in macrophages, through which they influence their M1/M2 polarization. Furthermore, abnormal Tyr isomers contribute indirectly, via insulin resistance, and according to our results, directly as well, to the development of various kidney diseases.

6. THESES OF THE DISSERTATION

1. In macrophages, under physiological para-tyrosine treatment, insulin increased pAkt levels and decreased the arginase-1/iNOS ratio.

2. In macrophages, under meta- and ortho-Tyr treatment, insulin decreased pAkt levels and increased the arginase-1/iNOS ratio.

3. In macrophages, in the absence of insulin treatment, 25 mmol/l glucose concentration, meta- and ortho-Tyr treatment increased pAkt levels and decreased the arginase-1/iNOS ratio.

4. In the glomerulus, WT1 intensity did not differ between the FSGS and control groups, whereas in the tubulo-interstitium, WT1 intensity was lower in the FSGS group compared to controls.

5. In the glomerulus, vimentin intensity was lower in the FSGS group compared to controls, while in the tubulo-interstitium, vimentin intensity was higher in FSGS compared to controls.

6. CRF intensity measured in the glomerulus was lower in the FSGS group compared to controls.

7. In HEK-293 cells, meta- and ortho-Tyr treatment decreased WT1 intensity compared to controls.

8. Meta-Tyr treatment decreased vimentin intensity in HEK-293 cells.

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8. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

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IF: 2,3

2.:Csurgyók, R., Sütő, G., Engelmann, P., Wittmann, I., Vas, T. (2024). Kóros tirozinizomerek hatása a krónikus inzulinrezisztencia kialakulására és az M1/M2 polarizáció irányára makrofágokban. DIABETOLOGIA HUNGARICA, 32(3), 231–245. doi:10.24121/dh.2024.16

9. LIST OF PUBLICATIONS NOT RELATED TO THE DISSERTATION

1. Bali, Z.K.; Inkeller, J.; Csurgyók, R.; Bruszt, N.; Horváth, H.; Hernádi, I. Differential Effects of A7 Nicotinic Receptor Agonist PHA-543613 on Spatial Memory Performance of Rats in Two Distinct Pharmacological Dementia Models. Behav. Brain Res. 2015, 278, 404–410, doi:10.1016/j.bbr.2014.10.030. IF: 3,0

2. Bali, Z.K.; Bruszt, N.; Tadepalli, S.A.; Csurgyók, R.; Nagy, L.V.; Tompa, M.; Hernádi, I. Cognitive Enhancer Effects of Low Memantine Doses Are Facilitated by an Alpha7 Nicotinic Acetylcholine Receptor Agonist in Scopolamine-Induced Amnesia in Rats. Front. Pharmacol. 2019, 10, doi:10.3389/fphar.2019.00073. IF: 3,8

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