Redox modulation of the vascular function.

A novel mechanism leading to impaired insulin-induced vasorelaxation.

Ph.D. theses summary

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1. Introduction

Type 2 diabetes mellitus is a complex metabolic disorder accompanied by vascular complications. Failure to maintain good glucose homeostasis is associated with increased risk of micro- and macrovascular complications. Development of insulin resistance is the hallmark of diabetes mellitus and the pathogenesis of vascular complications. Vascular insulin resistance is an early event in the development of hypertension, which could explain why hypertension is associated tightly with type 2 diabetes mellitus. [1] Endothelial dysfunction is associated with insulin resistance and increased aortic stiffness. Deterioration of insulin- but not acetylcholine induced vasorelaxation is prior in hypertensive rats, underscoring the hypothesis, that vascular insulin resistance is an early event in the development of hypertension. [1]

Insulin is one of the major hormonal regulators of tissue metabolism, but it has also a pivotal role in regulating vasomotor activity. [2, 3] Vascular effects of insulin could be manifested in dilation and/or constriction. [4] The vasodilator action of insulin is primarily attributed to the release of nitric oxide (NO) from the endothelium produced via the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway. [5, 6] However, insulin is able to cause rapid release of endothelin-1 (ET-1) via ERK activation. [7] Also reactive oxygen and nitrogen species thought to play an important role in the physiological insulin signaling; oxidants like hydrogen peroxide have insulin-mimicking effects, on the other hand insulin induces ROS and RNS production. [8]

There is a huge body of evidence in involvement of ROS in diabetic vascular disease. ROS influence various downstream signaling pathways, resulting in altered vascular reactivity, expression of pro-inflammatory mediators and structural changes in vascular wall. [9] Accumulation of ROS, loss of NO bioavailability, metabolic alterations, impaired insulin signaling and inflammatory responses of the endothelium leading to endothelial dysfunction are thought to be the hallmark of vascular insulin resistance.[10, 11]

Early intensive glycemic control and induction of normoglycemia in type 2 diabetes resulted in an improved insulin sensitivity. Furthermore, short term intensive insulin therapy results in improved glycemic control, endothelial function, decrease of insulin resistance,

and leads to extended remissions. [12, 13] However, the exact mechanisms of the disease modifying effects of transient intensive insulin therapy are still unclear.

A wealth of evidence implicates that increased oxidative stress plays a major role in the pathogenesis of insulin resistance. One target of the oxidative damage is proteins, which could lead to cellular dysfunction. In addition to direct damage by reactive oxidative metabolites, however, alternative pathomechanisms have been proposed, but they have received little attention. For example, superoxide can be metabolized to H_2O_2, which can give rise to the production of more reactive intermediates, such as hydroxyl radical (•OH) [14], which – among others - can modify phenylalanine residues to form para-, meta- and ortho-tyrosine (p-, m- and o-Tyr). [15-18]

Modified amino acids could originate from protein-bound amino acids [15] or may be incorporated into proteins during their synthesis resulting in a polypeptide without direct oxidative damage of the protein itself, which then can exert cytotoxic actions. [19-21]

Furthermore, o-Tyr and m-Tyr levels were found to be significantly higher in the aortic tissue of hyperglycemic cynomolgus monkeys. [15] Misincorporation of o-Tyr and m-Tyr into structural or catalytic proteins could also contribute to impaired cellular function, such as erythropoietin-hyporesponsiveness in erythroblasts and inhibition of tumor growth *in vivo*, possibly by interfering with MAP/ERK signaling. [22, 23]

However, incorporation of o-Tyr into vascular proteins and the physiological consequences of exogenous administration of these tyrosine isomers on vasomotor function have not yet been investigated.

2. Aims

We hypothesized that redox state of consecutive segments of the arterial tree decreases toward the periphery and concomitantly, NO-mediated insulin-induced relaxations increases toward the periphery. Our work was also designed to study the in vivo effects of oxidized amino acid (o-Tyr) supplementation on vascular function.

In order to test our hypotheses:

- We determined and modulated the redox state of three consecutive segments of the arterial tree, the thoracic and abdominal aorta, and the femoral artery.

- We also measured the relaxation of these consecutive arterial segments under control conditions in response to insulin.

- Vasorelaxation due to insulin was tested after modulating their redox state by aortic banding and H_2O_2-aminotriazole, both of which are known to increase vascular redox state.

- Also, we studied the role of ERK pathway in mediating the insulin-induced vasomotor responses.

- Furthermore, we determined the effects of o-Tyr and p-Tyr supplementation on vascular insulin resistance, and

- The mechanism of action using endothelial cells *in vitro* and vascular segments *ex vivo*, in both acute and chronic conditions.

3. Methods

3.1 Animals and tissue preparation

Animal experiments were carried out with the permission of the Animal Experiment Committee of the University of Pécs, Hungary. Adult (11-13 week-old, 320-380 g), male Sprague-Dawley rats fed with regular diet *ad libitum* were employed in the non-interventional experiments. Prior to studies, rats were anesthetized with intraperitoneal ketamine injections (50 mg/ bw kg, i.p.; Richter Gedeon, Budapest, Hungary) and sacrificed by decapitation.

As previously described [24], aortic banding was conducted using male Sprague-Dawley rats (8 week-old) with an average weight of 220 g. Under general anesthesia with ketamine (50 mg/ bw kg, i.p.) and diazepam (5 mg/ bw kg, i.p.; Richter Gedeon, Budapest, Hungary), the abdominal cavity was opened, and the abdominal aorta was surgically dissected from the inferior vena cava at a site slightly above the iliac bifurcation. A 21-gauge needle was then placed along the side of the isolated segment of the aorta. Thereafter, a 2-0 suture was tightly tied around the aorta and the overlying needle. The needle was then gently removed, generating thus severe aortic banding above the iliac bifurcation. After four weeks, rats were anesthetized and decapitated as described above. Aortic banded rats exhibited elevated mean arterial blood pressure proximally from the banding. [24]

Male Sprague-Dawley rats (5-6 week-old, 100-140 g) were used in the tyrosine isomer incorporation studies. Two-hours fasted rats were orally supplied by gavage with either 1.76 mg/die of p-Tyr or o-Tyr (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline or vehicle (saline only) during six days per week for four weeks. At the end of four-week treatment, one group of rats was sacrificed after anesthesia with ketamine, and HPLC (high-performance liquid chromatography) and vasomotor studies were performed. In another group of rats, p-Tyr/o-Tyr supplementation was discontinued for four weeks (“washout” period) after which they were sacrificed for HPLC and vasomotor studies.

The descending thoracic aorta, the abdominal aorta, and the femoral arteries were removed, cleaned from connective tissue followed by their dissections into two sections. The

proximal sections were immediately hydrolyzed for HPLC analyses. The distal parts were used for vasomotor studies.

3.2 Assessment of oxidative status and tyrosine isomer incorporation in the consecutive arterial segments

Due to the fact that hydroxyl free radical has extremely short half-life and its detection is very limited [25]; we used an alternate approach to detect the stable end-product of oxidative reactions: o-Tyr, which is an isomer of the natural amino acid L-tyrosine.[15-18] The proximal sections of the given vascular segments were hydrolyzed in well-closing, O-ring protected polypropylene tubes. Desferrioxamine and butylated hydroxytoluene (at final a concentration of 3.6 mM and 45 mM, respectively) were added to the samples to avoid a possible free radical formation during hydrolysis. Then 200 µl of 12N hydrochloric acid was added, and we performed an overnight acid hydrolysis of the proteins at 120°C.[17] The hydrolyzates were then filtered through a 0.2 µm filter (Millipore Co., Billerica, MA, USA), and 20 µL of the filtrate was injected onto the HPLC column of a Shimadzu Class LC-10 ADVP HPLC system (Shimadzu USA Manufacturing Inc., Canby, OR, USA) using a Rheodyne manual injector. Quantitative analysis of the amino acids was carried out upon their autofluorescence using a LiChroCHART 250-4 column (Merck KGaA, Darmstadt, Germany), in an isocratic run using aqueous solution containing 1% acetic acid and 1% sodium acetate as the mobile phase. The tyrosine isoforms were measured at 275 nm excitation and 305 nm emission wavelengths, so were the phenylalanine levels at 258 nm excitation and 288 nm emission wavelengths using a Shimadzu RF-10 AXL fluorescent detector (Shimadzu USA Manufacturing Inc., Canby, OR, USA) upon their autofluorescence. Therefore, no pre-column or post-column staining or derivatization was required. [17, 18] The area under-the-curve (AUC) was determined for the amino acids, and exact concentrations were calculated using external standard calibration. In some cases the elution time of the substances was also verified by standard peak-addition method. The amino acid concentrations were corrected for phenylalanine concentrations.

3.3 Assessment of vasomotor function of the consecutive arterial segments

The modified method described by Fésüs et al. [26] was used. The distal parts of the vessels were dissected into 2 mm long segments in ice-cold Krebs buffer, and rings were mounted on two stainless steel wires (40 µM in diameter) in a Danish Multimyograph Model 610M (DMT-USA Inc., Atlanta, GA, USA).

Vessels were bathed at 37˚C in Krebs buffer (pH 7.4) containing (in mM) NaCl 119.0, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25.0, Mg_2SO_4 1.2, glucose 11.1, $CaCl_2*2H_2O$ 1.6 and gassed with 5% CO_2 and 95% O_2. The resting tension/internal circumference relationship for each vessel was determined and then the internal circumference was set to 0.9 x L100, where L100 is the internal circumference of the vessel that would have *in vivo* when being relaxed under a transmural pressure of 100 mmHg. After this normalization procedure, vessels were allowed to stabilize for 30 min, then isometric tension was continuously recorded. Rings were pre-constricted with 100 nM epinephrine. After reaching a stabile contraction plateau, relaxant responses to increasing doses of acetylcholine (ACh), insulin, and sodium nitroprusside (SNP) were assessed. The magnitude of relaxation caused by ACh, insulin, and SNP was expressed as the percentage of the contraction evoked by epinephrine which was taken 100%.

Further enhancement of antioxidant capacity was achieved by catalase (CAT; 1000 U/ml) and superoxide dismutase (SOD; 200 U/ml). The pro-oxidant effect of H_2O_2 (50µM) was enhanced with the CAT-inhibitor aminotriazole (AT; 1 mM).[27] On the other hand, in the presence of inhibition of endogenous catalase activity we were able to investigate the effects of peroxidases. [28] SOD and CAT (SOD+CAT) and H_2O_2 and AT (H_2O_2+AT) were added to the vessel chamber 20 min before epinephrine to modulate vascular redox state. To test the role of ERK pathway in mediating the H_2O_2+AT-induced vasoconstriction and the attenuation of insulin-evoked vasorelaxation, we used PD98059 (PD; 10 µM and 50 µM) – an inhibitor of the mitogen-activated protein kinase kinase (MAPKK) – to inhibit ERK activation.[29] PD was added to the vessel chamber 30 min prior to the addition of epinephrine.

Acetylcholine, sodium nitroprusside, insulin, epinephrine, H_2O_2, aminotriazole, superoxide dismutase, catalase, PD98059, and Mg_2SO_4 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The NaCl, KCl, KH_2PO_4, $NaHCO_3$, $CaCl_2*2H_2O$, and glucose were purchased from Merck (Merck KGaA, Darmstadt, Germany).

3.4 Cell culture

Primary cultures of mouse endothelial cells (ECs) from endothelioma were purchased from LGC Promochem (Taddington, UK). ECs were grown in Dulbecco's modified Eagle medium (DMEM; Gibco, Csertex, Budapest, Hungary) supplemented with 10% Fetal Bovine Serum (Gibco) and 2% mixture of penicillin-streptomycin (Gibco) in a humidified incubator at 37°C and 5% CO_2. The medium was changed every 2 days. ECs cultures were randomly assigned into three groups and were incubated in media containing 1) 400 µM p-Tyr (Control); 2) 800 µM p-Tyr (p-Tyr); and 3) 400-400 µM p-Tyr and o-Tyr (o-Tyr); for 8 days or 30 min to detect chronic vs. acute effects. ECs were incubated with insulin (400 µM, 5 min) to assess the changes of its downstream effects on eNOS phosphorylation. At the end of experiments, ECs were scraped off mechanically and processed for further analyses.

3.5 Assessment of tyrosine isomer incorporation in endothelial cells

The total protein-bound cellular tyrosine content of ECs was measured by a method described by Molnár et al. [17] After adding 200 µl of distilled water, samples were sonicated for 2 min with ultrasonic homogenizer to obtain cell lysates. After the addition of 100 µL of 60% trichloroacetic acid, samples were centrifuged (4000 rpm, 10 min) and the sediment was resuspended in 200 µL of 1% trichloroacetic acid. After resuspension, 100 µl of 60% trichloroacetic acid was added to the lysates followed by a second centrifugation (4000 rpm, 10 min) then previous steps were repeated once again. Finally, 4 µl of 400 mM desferrioxamine and 40 µL of 500 mM butylated hydroxytoluene were added to the sediments to avoid a possible free radical formation during hydrolysis. Then 400 µl of 6N hydrochloric acid was added to the samples followed by processing steps for HPLC method as described above.

3.6 Western blot analyses

To assess eNOS phosphorylation, immunoblot analyses as we previously described were used. [30] ECs were solubilized in Tris-Triton extraction buffer [1 M Tris-HCl (pH 7.4), 1.15% Triton X-100, 500 mM EDTA, 200 mM EGTA supplemented with a mixture of protease

and phosphatase inhibitors] on ice for 30 min. Cell lysates were centrifuged (13,000 rpm, 10 min) then protein content of the supernatants was determined with the Bio-Rad protein assay kit (Hercules, CA, USA) using bovine serum albumin (BSA) as the standard. Equal amounts of proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Equal protein loading was confirmed by Ponceau-S staining. Membranes were blocked in Tris base saline containing 0.1 v/v % Tween and 5 w/v % BSA (TBS-T-5% BSA) for 60 min at room temperature. The blots were then probed with primary antibody against phospho-(Ser1177)-eNOS (1:1000; Cell Signaling, Beverly, MA, USA) diluted in TBS-T-5% BSA for overnight at 4°C, followed by washing steps and incubation with secondary, HRP-conjugated anti-rabbit IgG antibody (1:2000; Cell Signaling) for 60 min at room temperature. Immunoblots were the visualized by enhanced chemiluminescence (ECL; Super-Signal West Pico, Thermo Fisher Scientific, MA, USA) and developed on X-ray films (Kodak XAR, Sigma-Aldrich). For densitometric analyses the Scion Image for Windows Software (Frederick, MD, USA) was used. Phospho-(Ser1177)-eNOS levels were corrected for total eNOS which was detected by reprobing the blots after stripping as described elsewhere.[30]

3.7 Statistical analyses

Data are expressed as means ± SEM. All distributions were tested by Kolmogorov-Smirnov test. Statistical analyses were performed with ANOVA, extra sum-of-squares F test, non-parametric tests as appropriate using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism5 (GraphPad Software Inc., La Jolla, CA, USA). Statistically significant differences were defined as $P \leq 0.05$.

4. Results

4.1 Redox state, as indicated by ortho-Tyrosine (o-Tyr) levels in the wall of consecutive arterial segments isolated from untreated rats

The highest o-Tyr content was measured in the thoracic aorta, followed by the abdominal aorta, and the lowest amount was measured in the femoral artery, indicating that the redox state of consecutive arterial segments assessed by o-Tyr decreases toward the periphery. In addition, in the presence of H_2O_2+aminotriazole (H2O2+AT) the o-Tyr content was higher in the thoracic aorta, but there was no difference in the o-Tyr content of the abdominal aorta and femoral artery compared to control. In case of aortic banding, changes in the o-Tyr content closely mirrored that seen with H_2O_2+AT treatment, showing significantly higher levels in the thoracic aorta of rats and with no difference in the abdominal aorta or the femoral artery compared to controls. Addition of superoxide dismutase and catalase (SOD+CAT) resulted in significantly lower o-Tyr content in the thoracic aorta and the femoral artery, but not in the abdominal aorta compared to control vessels.

4.2 Vasomotor responses of consecutive arterial segments isolated from untreated rats

In response to insulin, we found clear differences in the magnitude of relaxation of the consecutive arterial segments. The EC_{50} value of insulin was lower in the femoral artery compared to both the thoracic and abdominal aorta. Moreover, the EC_{50} value of insulin was lower in the abdominal aorta compared to thoracic aorta. Together, the results indicate that insulin-induced vasorelaxation increases towards the periphery. We also found similar differences in the relaxation of the same arterial segments in response to acetylcholine and sodium-nitroprusside. Insulin induced vasodilatation is mediated mainly through the PI3K/AKT/eNOS pathway. Inhibition of eNOS with L-NAME almost abolished insulin-evoked vasodilatation. However, the partipication of eNOS derivered NO in the insulin-induced vasorelaxation decreases towards the periphery. Morover, a major proportion of the vasorelaxant effect in response to insulin is endothelium-independent in the femoral artery.

4.3 Effects of the redox state modulation by SOD+CAT and H_2O_2+AT on vasomotor responses of consecutive arterial segments isolated from untreated rats

In order to test the hypothesis that redox state plays an important role in the modulation of the magnitude of vasomotor responses of different vascular segments to insulin, the vessels were incubated with SOD+CAT to decrease, or with H_2O_2+AT to increase redox state, respectively. In the thoracic aorta, insulin-induced relaxation was significantly enhanced in the presence of SOD+CAT. In the abdominal aorta, SOD+CAT did not change the relaxation in response to insulin, whereas in the femoral artery SOD+CAT treatment reduced relaxation to insulin. However, the magnitude of aortic responses to insulin+SOD+CAT did not reach the level of the untreated femoral artery.

Addition of H_2O_2+AT significantly attenuated the insulin-induced relaxation in the thoracic aorta, but did not alter the response of the abdominal aorta. In contrast, presence of H_2O_2+AT significantly augmented the insulin-induced relaxation in the femoral artery.

4.4 Effects of the redox state modulation by aortic banding on vasomotor responses of consecutive arterial segments isolated from untreated rats

We also used aortic banding to modulate the redox state and hence the vascular ortho-tyrosine content, which we showed earlier in rats to increase blood pressure proximally from the banding in both the thoracic and abdominal segments of the aorta.[24, 31] Here we have found that aortic banding significantly diminished the insulin-induced relaxations in both the thoracic and abdominal aortic segments. However, distal to the banding, relaxation of the femoral artery remained unaltered in response to insulin.

4.5 Effects of the ERK pathway inhibition on insulin-induced vasomotor responses of consecutive arterial segments isolated from untreated rats

To test the hypothesis that ERK signaling pathway contributes to the vasomotor responses to insulin, vessels were pre-incubated with the ERK-inhibitor PD98059 (PD; 10 μM, 30 min). In the thoracic aorta, we found that PD98059 completely prevented the H_2O_2+AT-induced impairment of insulin-induced relaxations. Similar effects of PD98059 were

observed in aortic banding-induced impairment of insulin-induced relaxations. In the abdominal aorta, PD98059 did not change the insulin-induced relaxation in the presence of H_2O_2+AT, but in the aortic banding model PD98059 partially reversed its diminished relaxation in response to insulin. In contrast, PD98059 completely inhibited the epinephrine-induced vasoconstriction in the femoral artery, thus enabling studies with femoral arteries under the preexisting, endogenous conditions, which are not shifted to another redox state by exogenous factors, i.e. by H_2O_2+AT or aortic banding.

4.6 Effects of H_2O_2+AT and the ERK pathway inhibition on vasomotor responses of consecutive arterial segments isolated from untreated rats

In a subsequent set of experiments, H_2O_2+AT-induced transient constrictions were detected in all consecutive arterial segments in the absence of insulin. Results revealed the greatest constriction in the thoracic aorta, followed by a reduced response in the abdominal aorta, and a relatively weak constriction in the femoral artery. Addition of the ERK pathway inhibitor PD98059 dose-dependently prevented the H_2O_2+AT-induced constrictions in all consecutive arterial segments.

4.7 Effects of chronic oral o-Tyr supplementation of rats on the o-Tyr level in the vascular wall of isolated arterial segments

After one month oral supplementation of rats with p-Tyr, o-Tyr or vehicle, we measured the o-Tyr content in the thoracic and abdominal parts of the aorta and the femoral artery. Sustained administration of o-Tyr resulted in significant increases in the o-Tyr content of all three arterial segments, including the thoracic and abdominal aorta and the femoral artery compared to the control vessels of vehicle-treated rats.

Four weeks after the termination of tyrosine supplementation (at the 8^{th} week) we also assessed the o-Tyr content of the same arterial segments and found no differences in the o-Tyr content between any of the treatment (o-Tyr, p-Tyr and vehicle) group.

4.8 Effects of chronic p-Tyr and o-Tyr supplementation of rats on insulin-induced relaxation of isolated arterial segments

We determined whether sustained *in vivo* supplementation of rats with o-Tyr had any impact on insulin-induced relaxation *ex vivo*. We obtained significantly diminished response to insulin in the abdominal aorta and femoral artery of o-Tyr-treated rats compared to the vehicle- or the p-Tyr-treated groups.

In the thoracic aorta, there was no significant difference between the control and o-Tyr-treated groups. In contrast, we found increased relaxation in response to insulin in the thoracic aorta isolated from p-Tyr-treated rats compared to other groups. Four weeks after the termination of tyrosine isomer supplementation (at the 8^{th} week) we also assessed vasomotor responses of the arterial segments to insulin and found comparable insulin-induced relaxations in each arterial segment regardless of treatments (i.e. o-Tyr, p-Tyr and vehicle).

4.9 Acute effects of tyrosine isomers on insulin-induced relaxation of the isolated rat arterial segments

In an additional set of experiments, we demonstrated that insulin-induced relaxations of each arterial segment remained unchanged after acute treatments (30 min) with p-Tyr, o-Tyr or vehicle.

4.10 Incorporation of p-Tyr and o-Tyr into proteins of cultured endothelial cells

To investigate the incorporation of different tyrosine isomers into proteins, we measured the relative concentration of protein-bound o-Tyr levels in ECs cultured in control and p-Tyr or o-Tyr supplemented media for 30 minutes (acute experiment) or 8 days (chronic experiment). In acute experiments, we found no differences between protein-bound o-Tyr content of ECs incubated with different tyrosine isomers. In chronic experiments, proteins from o-Tyr-cultured ECs showed a higher relative o-Tyr content

compared to control and p-Tyr-cultured ECs. The o-Tyr content of control and p-Tyr-cultured ECs were comparable.

4.11 Effects of tyrosine isomers on insulin-induced eNOS phosphorylation in cultured endothelial cells

To determine whether decreased relaxation in response to insulin seen with increased o-Tyr levels was also associated with decreased eNOS activity, we assessed the activating phosphorylation of eNOS in response to insulin in endothelial cells cultured for 8 days under normal condition (Control) or increased p-Tyr and o-Tyr isomer levels. Immunoblot analyses showed that activating eNOS phosphorylation in response to insulin was significantly attenuated in endothelial cells cultured with o-Tyr compared to control cells.

5. Discussion

Previous studies showed that redox state of vessels is an important determinant of their vasomotor function. [32] Activation of ERK signaling by H_2O_2 is a well-known mechanism to impair the insulin action on vasomotor activity. [33] Our results demonstrate that different redox state levels along the arterial tree concur and are responsible for the variability of insulin-induced vasomotor responses. We demonstrated here for the first time that sustained treatment with o-Tyr markedly impaired the insulin-induced relaxation, at least in part, by eliciting endothelial dysfunction, as we found that sustained o-Tyr treatment also decreased the insulin-induced activating eNOS phosphorylation in endothelial cells.

5.1 Vasomotor responses of consecutive arterial segments to insulin: role of vascular redox state

We observed that the thoracic aorta showed the highest o-Tyr content compared to other distal consecutive vessels examined. Moreover, aortic banding or H_2O_2+AT treatment increased significantly the o-Tyr content of the thoracic aorta, but not in abdominal aorta and femoral arteries. Our data support the view that abdominal aorta, and in particular, the femoral artery may have higher antioxidant capacity compared to the thoracic aorta. [34]

We found that decreased redox state of the vascular wall towards the periphery is associated with a concomitant increase of vascular relaxations in response to insulin. Constrictions evoked by H_2O_2+AT were dose-dependently abolished with the ERK-inhibitor PD98059, which supports the notion that ERK pathway is crucial in mediating the ROS-induced vasomotor responses. These changes were associated with increased o-Tyr levels, both of which were accompanied by reduced insulin-evoked relaxation, and this was completely prevented by the ERK-inhibitor PD98059. Together, the data indicate that H_2O_2 and subsequent ERK activation could play a key role in vascular insulin resistance. While H_2O_2+AT treatment had no effect on insulin-induced relaxation in the abdominal aorta, it enhanced the insulin-induced relaxation in the femoral artery, suggesting that H_2O_2 is

involved in eliciting relaxation to insulin in arterial vessels with smaller caliber, as we found earlier.[35]

In the thoracic aorta, insulin-evoked relaxation was enhanced with lowering redox state by SOD+CAT and could be inhibited by L-NAME indicating an important role of NO in mediating the response. Thus NO inactivation with superoxide can be reduced by SOD+CAT [36-38] and this may be responsible for the enhanced insulin-evoked relaxation. Interestingly, the femoral artery showed an opposite response to antioxidant treatment where SOD+CAT resulted in impaired insulin-evoked relaxation, suggesting again the possible involvement of H_2O_2 in eliciting relaxation of this artery in response to insulin. [32, 39, 40]

5.2 Incorporation of o-Tyr into the vascular wall of supplemented rats and the proteins of endothelial cells alters vascular and endothelial functions

We found that chronic oral administration of o-Tyr resulted in significant increases of the vascular o-Tyr content. In addition, chronic oral o-Tyr administration of rats resulted in severely reduced insulin-induced relaxations in the abdominal aorta and the femoral artery. These findings support our hypothesis that chronic o-Tyr production via increased vascular redox state impairs vasomotor functions to insulin, which is typically an early sign of vascular insulin resistance. [34]

However, no differences in insulin-induced relaxations of the thoracic aorta were found between the o-Tyr and control group. This apparent discrepancy can be explained by a higher basal o-Tyr content in conjunction with greater vascular dysfunction in the thoracic aorta of non-supplemented rats, which could explain the lack of differences seen after o-Tyr supplementation. In addition, we found that there were increased protein-bound o-Tyr levels in o-Tyr-cultured endothelial cells accompanied by attenuated eNOS phosphorylation in response to insulin, indicating that incorporation of o-Tyr could impair endothelial function. Our data demonstrate that adverse effects of o-Tyr are not mediated through an acute mechanism.

5.3 Clinical significance

Understanding the molecular pathomechanisms of vascular insulin resistance can have tremendous beneficial implications for interfering and treating diabetes related vasomotor dysfunction, such as macro- and micorangiopathies. Our results could also advance explanation why hypertension is associated tightly with type 2 diabetes mellitus. Specifically, as a consequence of increased redox state related to diabetes mellitus, the accumulation of o-Tyr in the vascular wall impairs insulin-signaling by interfering with the production of vasodilator NO by eNOS, contributing to the development of high blood pressure.

Patients with type 2 diabetes and chronic kidney disease (CKD) have an increased o-Tyr burden [17, 18, 41, 42], which may contribute to the development of vascular complications. Transient, two-three weeks long intensive insulin therapy improves insulin sensitivity. This could be the consequence of diminished oxidative stress and subsequent decline in o-Tyr generation. Interfering with this pathway could represent a promising future strategy for the prevention and/or treatment of vascular complications in diabetes mellitus.

6. List of the Ph.D. theses:

1. The oxidative state, as assessed by ortho-tyrosine was higher in thoracic aorta of rats, followed by the abdominal aorta, and was the lowest in the femoral artery. **(A)**

2. Insulin-induced relaxations increased toward the periphery along the arterial tree. **(A)**

3. Hydrogen peroxide/aminotriazole treatment and aortic banding increased oxidative state of the thoracic aorta. **(A)**

4. Increased oxidative state of the thoracic aorta that was accompanied by ERK activation and decreased relaxation to insulin. **(A)**

5. Acutely lowered oxidative state by superoxide dismutase/catalase (SOD/CAT) improved insulin-induced relaxation in the thoracic aorta. **(A)**

6. Insulin-induced relaxation of the femoral artery could be enhanced with higher, and reduced with lower oxidative state. **(A)**

7. Sustained oral supplementation of rats with ortho-tyrosine increased the ortho-tyrosine content and reduced the relaxations to insulin in all arterial segments. **(B)**

8. Incorporation of ortho-tyrosine in endothelial cells mitigated eNOS phosphorylation *in vitro* to insulin. **(B)**

List of publications used for the thesis:

A. **István András Szijártó**, Gergő A. Molnár, Esztella Mikolás, Viktória Fisi, Boglárka Laczy, Maik Gollasch, Akos Koller, István Wittmann. Increase in insulin-induced relaxations of consecutive arterial segments toward the periphery. Role of vascular oxidative state. Free Radical Research 48(7):749-57 (2014), **IF: 2.976**

B. **István András Szijártó**, Gergő A. Molnár, Esztella Mikolás, Viktória Fisi, Judit Cseh, Boglárka Laczy, Tibor Kovács, Katalin Böddi, Anikó Takátsy, Maik Gollasch, Ákos Koller, István Wittmann. Elevated vascular level of ortho-tyrosine contributes to the impairment of insulin-induced arterial relaxation. Hormone and Metabolic Research 46(11):749-752 (2014), **IF: 2.121**

Cumulative impact factor: 5.097

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