Elevated Vascular Level of *ortho*-Tyrosine Contributes to the Impairment of Insulin-Induced Arterial Relaxation

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- insulin
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- eNOS

Abstract

Previous studies have shown that in diabetes mellitus, insulin-induced relaxation of arteries is impaired and the level of *ortho*-tyrosine (*o*-Tyr), an oxidized amino acid is increased. Thus, we hypothesized that elevated vascular level of *o*-Tyr contributes to the impairment of insulin-induced vascular relaxation. Rats were fed with *o*-Tyr for 4 weeks. Insulin-induced vasomotor responses of isolated femoral artery were studied using wire myography. Vascular *o*-Tyr content was measured by HPLC, whereas immunoblot analyses were preformed to detect eNOS phosphorylation. Sustained oral supplementation of rats with *o*-Tyr increased the content of *o*-Tyr in the arterial wall and significantly reduced the relaxations to insulin. Sustained supplementation of cultured endothelial cells with *o*-Tyr increased the incorporation of *o*-Tyr and mitigated eNOS Ser (1177) phosphorylation to insulin. Increasing arterial wall *o*-Tyr level attenuates insulin-induced relaxation – at least in part – by decreasing eNOS activation. Elevated level of *o*-Tyr could be an underlying mechanism for vasomotor dysfunction in diabetes mellitus.

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Introduction

Insulin is one of the major hormonal regulators of tissue metabolism, but it plays also an important role in regulating vasomotor activity [1,2]. Vascular effects of insulin could be manifested in dilation and/or constriction depending on tissues of origin [3]. The vasodilator action of insulin is primarily attributed to the release of nitric oxide (NO) produced by NO synthase in the endothelium (eNOS), which is activated via the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway [4–6].

In diabetes, increased levels of reactive oxygen species (ROS) play an important role in the development of impaired vasomotor response to insulin [7–9]. 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) [10], which – among others – can modify phenylalanine residues to form *para*-, *meta*- and *ortho*-tyrosines (*p*-, *m*- and *o*-Tyr) [11–14]. Modified amino acids could originate from protein-bound amino acids [11] or may be incorporated into proteins during their synthesis resulting in a polypeptide without direct oxidative damage of the protein itself [15–17]. In line with these findings, free *m*-Tyr was shown to be incorporated into cellular proteins, possibly via protein synthesis, which then can exert cytotoxic actions [17]. Furthermore, *o*-Tyr and *m*-Tyr levels were found to be significantly higher in the aortic tissue of hyperglycemic cynomolgus monkeys and that of rats with aortic banding-induced hypertension [11,18]. 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 [19,20]. Of note, cytotoxicity of *m*-Tyr can be blocked in vitro with phenylalanine [15], suggesting that L-phenylalanine tRNA synthase recognizes *m*-Tyr to affect incorporation of ROS-damaged amino acids into cellular proteins [15]. However,

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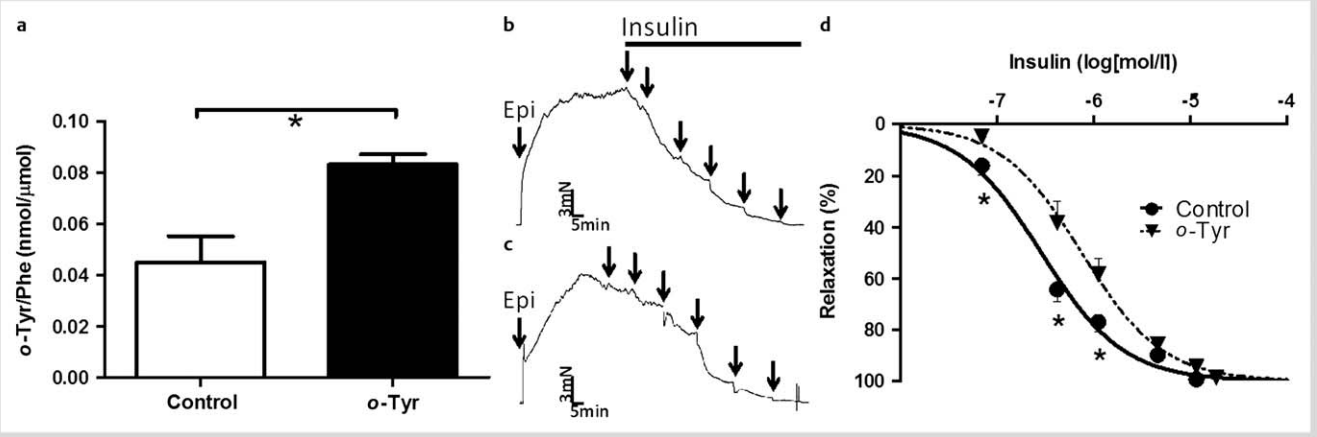

Fig. 1 **a** o-Tyr levels in the vascular wall of femoral arteries isolated from nonsupplemented and *o*-Tyr supplemented rats. There was a significantly increased *o*-Tyr level in the femoral artery isolated from the *o*-Tyr supplemented compared to the non-supplemented rats. *o*-Tyr levels are normalized to the phenylalanine (Phe) levels. Recordings of relaxation of femoral arteries isolated from **b** nonsupplemented, and **c** *o*-Tyr supplemented rats in response to increasing concentrations of insulin. **d** The dose-response curve of insulin-induced relaxations shows a rightward shift for femoral arteries of *o*-Tyr supplemented rats compared to femoral arteries of untreated control rats. Control n = 4; *o*-Tyr n = 4. Data are means ± SEM (Mann-Whitney test) *p < 0.05.

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

On the basis of the aforementioned findings and because tyrosine kinases are known to be also important in the activation of eNOS by phosphorylation of eNOS Ser (1177) [6] we hypothesized that accumulation of *o*-Tyr in the vascular wall can contribute to impaired relaxation of arteries to insulin by interfering with the PI3K/Akt/eNOS/NO signaling pathway. Thus, we determined the effects of oral supplementation of *o*-Tyr on the vasomotor responses of isolated arteries to insulin and investigated the phosphorylation of eNOS in response to *o*-Tyr and *p*-Tyr treatment in cultured endothelial cells.

Material and Methods

Animals and experimental design

Animal experiments were carried out with the permission of the Animal Experiment Committee of the University of Pécs, Hungary. Male Sprague-Dawley rats (4–5 week-old; 90–120 g) were used in the tyrosine isomer incorporation studies. Rats were orally supplied with either 1.76 mg/day of *o*-Tyr or vehicle during 6 days per week for 4 weeks. In general, there were no observable differences between the groups. At the end of 4-week treatment, HPLC measurements and vasomotor studies were performed.

Assessment of *ortho*-tyrosine and tyrosine isomer incorporation into arterial segments

Proximal sections of the isolated femoral artery were hydrolyzed and then levels of phenylalanine and tyrosine isoforms were analyzed by HPLC using a fluorescent detector [13].

Assessment of vasomotor function

The modified method described by Fésüs et al. [21] was used. Femoral rings were pre-constricted with 100 nM epinephrine. The magnitude of relaxation caused by insulin was expressed as the percentage of the isometric (Danish Multimyograph Model 610 M) contraction evoked by epinephrine, which was taken as 100 %.

Endothelial cell treatments

Primary cultures of mouse endothelial cells (ECs) were randomly assigned into 2 groups and were incubated in media containing 1) 400 µM *p*-Tyr (Control) and 2) 400–400 µM *p*-Tyr and *o*-Tyr for 8 days. Then the uptake of *o*-Tyr was analyzed. Prior to assessing eNOS phosphorylation, ECs were incubated with insulin (400 nM for 5 min) to activate endothelial nitric oxide synthase (eNOS) phosphorylation.

Assessment of tyrosine isomer incorporation in endothelial cells

The levels of protein-bound *o*-Tyr of ECs were analyzed by HPLC method as described by Molnár et al. [13].

Immunoblot analyses

Immunoblot analyses to detect eNOS phosphorylation were performed as we previously described [22]. Phospho-(Ser1177)-eNOS levels were corrected for total eNOS.

Statistical analyses

Data are expressed as means ± SEM. Statistical analyses were performed with unpaired *t*-test, and nonparametric tests as appropriate. Statistically significant differences were defined with $p \leq 0.05$.

Results

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

Compared to the control vessels of vehicle-treated rats, vascular *o*-Tyr content significant increased as a result of 4 weeks of oral supplementation of rats with *o*-Tyr (**Fig. 1a**).

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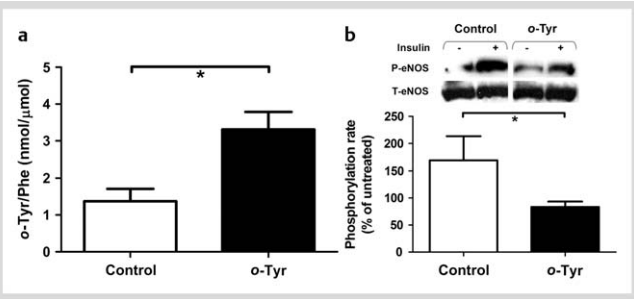

Fig. 2 **a** Incorporation of *o*-Tyr into proteins of cultured endothelial cells. Protein-bound *o*-Tyr levels are shown after 8 days of incubation of endothelial cells with normal medium or *o*-Tyr supplemented media. As a result of *o*-Tyr treatment cellular incorporation significantly increased. Cellular *o*-Tyr levels are expressed relative to phenylalanine (Phe) levels (Control n = 7; *o*-Tyr n = 7). **b** Effect of *o*-Tyr administration on insulin-induced eNOS phosphorylation in cultured endothelial cells. Upper panel: Immunoblots showing changes in eNOS Ser (1177) phosphorylation (P-eNOS) in untreated and insulin-treated (400 nM) endothelial cells cultured in normal medium (Control) or *o*-Tyr supplemented media. Lower panel: Densitometry shows that insulin-induced eNOS phosphorylation was significantly (*$p < 0.05$) reduced in *o*-Tyr-cultured cells compared to untreated control cells (Control). The phospho-eNOS level is expressed as the percentage of insulin-untreated cells after normalization to total eNOS (T-eNOS). Control n = 5; *o*-Tyr n = 4. Data are means ± SEM (**a** Mann-Whitney test; **b** Unpaired *t*-test). Data are means ± SEM (**a** Unpaired t-test; **b** Mann-Whitney test). *$p < 0.05$.

Effects of chronic *o*-Tyr supplementation of rats on insulin-induced relaxation of isolated femoral arteries

Original recordings of isometric tension of femoral arteries of control (nonsupplemented) rat show that insulin elicited substantial relaxation (**Fig. 1b**), whereas a vessel of *o*-Tyr-treated rats exhibited reduced relaxation (**Fig. 1c**). Summary data show a significantly diminished relaxation of the femoral artery of *o*-Tyr-treated rats in response to insulin compared to arteries of vehicle-treated rats (**Fig. 1d**).

Incorporation of *o*-Tyr into proteins of cultured endothelial cells

To investigate the incorporation of *o*-Tyr into endothelial proteins, we measured the relative concentration of protein-bound *o*-Tyr levels in endothelial cells (ECs) cultured in control conditions and *o*-Tyr supplemented media for 8 days. Proteins from *o*-Tyr-cultured ECs showed a higher *o*-Tyr content compared to control ECs (**Fig. 2a**).

Effects of *o*-Tyr on insulin-induced eNOS phosphorylation in cultured endothelial cells

Immunoblot analyses show that activating eNOS phosphorylation [eNOS Ser (1177)] in response to insulin was significantly reduced in ECs cultured in the presence of *o*-Tyr compared to untreated control cells (**Fig. 2b**).

Discussion

In the present study, we have shown for the first time that vaso-incorporation of *o*-Tyr reduces arterial relaxation to insulin and decreases eNOS phosphorylation in ECs. We interpret these finding to mean that increased levels of vascular *o*-Tyr inhibit eNOS phosphorylation and thus reduced synthesis of NO can result in impaired relaxation to insulin.

Previous studies have shown that *o*-Tyr levels are increased in aortic tissues in diabetes mellitus [11,23], which raised the possibility that *o*-Tyr content contributes to insulin-induced vasomotor dysfunction. However, diabetes is a complex disease and it is likely that many pathomechanisms are activated simultaneously. In order to elucidate the potential role of elevated cellular level of *o*-Tyr in modulation of insulin-induced arterial relaxation we used a model of chronic oral administration of *o*-Tyr. We have found that sustained oral treatment of rats with *o*-Tyr markedly increased vascular content of *o*-Tyr and at the same time impaired the insulin-induced relaxation in isolated femoral arteries.

The findings that vascular *o*-Tyr content modulates the magnitude of vasomotor responses to insulin and that increasing *o*-Tyr content attenuates insulin-induced vasorelaxations suggest a novel pathophysiological mechanism, which could be important in the development of not only vasomotor dysfunction, but in general, macro- and microangiopathy known to be present in diabetes mellitus. Moreover, increased level of *o*-Tyr due to exogenous (e.g., nutrition) [24,25] or endogenous sources other than diabetes mellitus, such as hypertension [18] or inflammation [26] (known to be associated with oxidative stress) could promote *o*-Tyr incorporation into vascular wall and impair vasomotor function.

It is known that insulin-induced relaxation is primarily mediated by eNOS derived NO (Supplementary **Fig. 1S**) [4–6]. Thus, to elucidate possible underlying mechanisms responsible for the reduced arterial relaxation to insulin, we cultured endothelial cells and investigated the effects of *o*-Tyr treatment on eNOS phosphorylation. We found that protein-bound *o*-Tyr levels significantly increased in *o*-Tyr-cultured endothelial cells, which was accompanied by attenuated eNOS phosphorylation in response to insulin. These findings suggest that incorporation of *o*-Tyr impairs eNOS function, that is, synthesis of NO, which could explain how *o*-Tyr interferes with NO mediated relaxation to insulin. On the basis of the findings of the present and previous studies, we also propose that deterioration of the PI3K/Akt/eNOS/NO pathway that mediates the vasorelaxation to insulin could contribute to development of vascular insulin resistance [4,5].

Clinical significance

Impaired regulation of tissue blood flow in diabetes mellitus and its consequences are major clinical problems being one of the major health burdens world-wide [1,2]. Thus, 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. Specifically, our study suggests that accumulation of *o*-Tyr in the vascular wall may contribute to the impaired insulin-induced relaxation by interfering with the production of vasodilator NO by eNOS. Patients with type 2 diabetes and chronic kidney disease (CKD) have an increased *o*-Tyr burden [13,14,23,27], which may contribute to the development of vascular complications. Interfering with this pathway could represent a promising future strategy for the prevention and/or treatment of vascular complications in diabetes mellitus.

Limitations and future perspectives

Although, the present study demonstrates that chronic oral supplementation of *o*-Tyr can increase levels of *o*-Tyr in arterial wall tissue with functional consequences of impaired vasomotor

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responses to insulin, the detailed molecular signaling pathways in the vasculature need to be elucidated by further studies.

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Conflict of Interest

The authors declare that there is no duality of interest associated with this manuscript.

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