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The ACE Inhibitory Dipeptide Met-Tyr Diminishes Free Radical Formation in Human Endothelial Cells via Induction of Heme Oxygenase-1 and Ferritin[1]

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Abstract

Food sources such as soybeans and fish contain angiotensin I converting enzyme (ACE) inhibitory peptides with antihypertensive properties. Methionine-tyrosine (Met-Tyr) is an ACE inhibitory dipeptide derived from sardine muscle. The present study investigates the effect of Met-Tyr on the expression of the antioxidant stress proteins, heme oxygenase-1 (HO-1) and ferritin, in endothelial cells derived from the human umbilical vein and their contribution to the decrease in radical formation that occurs under the influence of this dipeptide. Preincubation of endothelial cells with Met-Tyr (10–300 μmol/L) followed by washout markedly diminished subsequently induced NADPH-mediated radical formation. This indirect protection was associated with a significant increase in protein expression of HO-1 and ferritin and abolished by the HO inhibitor zinc deuteroporphyrin IX 2,4-bis-ethylene glycol (ZnBG). The HO product bilirubin produced antioxidant effects comparable to those of Met-Tyr. Met-Tyr raised HO-1 mRNA levels by enhancing mRNA stability. Antioxidant effects were specific for Met-Tyr and not observed with other methionine-containing dipeptides or ACE inhibitory agents. Our results demonstrate that Met-Tyr protects endothelial cells from oxidative stress via induction of HO-1 and ferritin but independently of its ACE inhibitory properties. This pathway represents a novel, potentially antiatherogenic mechanism of Met-Tyr and dietary proteins releasing Met-Tyr during gastrointestinal digestion. J. Nutr. 136: 2148–2152, 2006.

Introduction

Cardiovascular disease is the leading cause of death in the Western world, despite the introduction of numerous preventive and therapeutic drug regimens over the last 2 decades. Recent dietary trials in secondary prevention of myocardial infarction reported a 70% decrease of the recurrence rate in patients consuming a Mediterranean diet (Lyon Diet Heart Study) (1–3). Consequently, increasing interest has focused on identifying dietary compounds that can diminish oxidative damage and therefore prevent the development of vascular diseases. Food-derived bioactive peptides represent one such source of health-enhancing components. These peptides are found in milk, meat, and fish of various kinds as well as in many plants. They are inactive within the sequence of their parent protein and can be released during gastrointestinal digestion or during food processing. Depending on the sequence of amino acids, these peptides can exhibit diverse activities, including opiate-like, immunomodulatory, antioxidant, antithrombotic, antimicrobial, and antihypertensive actions (4,5). Many of the known bioactive peptides reveal pleiotropic properties (6). Thus, the dipeptide Met-Tyr, which is derived from sardine muscle protein (7), possesses antihypertensive activities; along with similar dipeptides, it has received considerable attention for its antihypertensive effects in vivo (8–10). Met-Tyr and related dipeptides are thought to diminish blood pressure mostly by inhibition of angiotensin I converting enzyme (ACE)[2] in the cardiovascular system. However, the exact mechanisms underlying their vasoprotective actions, which may well extend beyond the established ACE inhibitory effects, have not been identified.

Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes the degradation of heme. This process leads to generation of carbon monoxide (CO), free iron, and biliverdin; the last mentioned is subsequently converted to bilirubin by biliverdin reductase. Bilirubin exerts strong antioxidant effects at physiological plasma concentrations. High-normal plasma levels of bilirubin were reported to be inversely related to atherogenic risk and to provide protection against endothelial damage (11,12). CO was similarly shown to produce antiapoptotic and cytoprotective actions (13,14). Furthermore, the HO-1–dependent release of free iron during heme catabolism results in the upregulation of ferritin protein expression. Ferritin was shown to provide marked antioxidant cellular protection by rapidly sequestering free cytosolic iron, the crucial catalyst of oxygen-centered radical formation via the Fenton reaction in biological systems (15,16).

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[2] Abbreviations used: ACE, angiotensin I converting enzyme; AD, actinomycin D; HO, heme oxygenase; L-NMMA, L-N^G-monomethyl arginine; ROS, reactive oxygen species; ZnBG, zinc deuteroporphyrin IX 2,4-bis-ethylene glycol.

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Thus, in addition to HO-1, ferritin plays an important role of its own as a fast-acting endogenous cytoprotectant in cellular antioxidant defense mechanisms (17,18).

We showed recently that single amino acids such as L-alanine and L-methionine are capable of inducing HO-1 and ferritin expression in endothelial cells (19,20). Therefore, induction of endothelial HO-1 followed by formation of its antioxidant enzymatic products could also contribute to the antihypertensive and tissue-protective properties of Met-Tyr and other food-derived ACE inhibitory peptides. The present study investigates whether HO-1 and ferritin are targets of Met-Tyr and act as functional antioxidant mediators for ACE inhibitory dipeptides in human endothelial cells (ECV304), which were established as a model cell line for endothelia (21).

Materials and Methods

Materials. Fetal bovine serum, cell culture media, streptomycin, and penicillin were obtained from Gibco. PeqGOLD TriFast was received from Peqlab. The chemiluminescence western blotting kit was from Amersham and the random primed DNA labeling kit was from Roche. HO-1 primary antibody was obtained from Alexis. The dipeptides methionine-tyrosine, methionine-phenylalanine, and methionine-methionine were from Bachem. All other chemicals were purchased from Sigma Chemical. For HO-1 probes, the template was an EcoRI restriction fragment of the human HO-1 cDNA (clone 2/10), which was kindly provided by Dr. Rex Tyrrell, School of Pharmacy and Pharmacology, University of Bath, UK (22).

Cell culture. The human endothelial cell line ECV304 was obtained from the European Collection of Cell Cultures (21). ECV304 endothelial cells were maintained and subcultured in M199 medium containing 10% fetal bovine serum, streptomycin (100 mg/L), and penicillin (100 kU/L). The cells were grown in a humidified incubator at 37°C and 5% CO_2.

Formation of ROS. The formation of NADPH-dependent reactive oxygen species (ROS) was measured by monitoring lucigenin-derived chemiluminescence at 37°C using the Berthold LB96V luminometer according to previously published protocols (19,23,24).

HO-1 and ferritin protein analysis. Endothelial cells were cultured in 100-mm dishes as described above. After a 24-h incubation with control media or the indicated compounds, cells were washed and extracted. A polyclonal antibody to HO-1 and ferritin was used to identify the protein content as described previously (19,25).

HO-1 mRNA analysis. Subconfluent endothelial cells in 100-mm dishes were incubated for 2–8 h in the presence of control media or Met-Tyr (300 μmol/L). For mRNA stability measurements, cells were incubated in the presence of the established HO-1 inducer cadmium chloride ($CdCl_2$, 10 μmol/L). After 4 h, the cells were washed with cold PBS and the medium was changed. Subsequently, the inhibitor of transcription, actinomycin D (AD), was added to the cell culture medium at the final concentration of 1 mg/L 15 min before Met-Tyr. Cells were harvested after 2, 4, and 6 h. Total RNA was extracted using peqGOLD TriFast according to the instructions of the supplier. Briefly, samples containing equal amounts of RNA (20–30 μg) were separated in a 1% denaturing formaldehyde gel. Separated RNA was transferred onto a positively charged nylon membrane by vacuum transfer (500 mbar). The transferred RNA was fixed by baking at 80°C for 30 min. After ^{32}P-labeling of a human HO-1 cDNA probe with a Random Primed DNA Labeling Kit, the membranes were hybridized overnight at 65°C. Equal loading was assessed by a second hybridization using ^{32}P-labeled β-actin cDNA probe. Quantification of HO-1 mRNA content was performed using computer-assisted videodensitometry.

Statistical analysis. Results are expressed as means ± SEM. Data were analyzed by ANOVA and subsequently by Bonferroni's correction for multiple comparisons. An unpaired t test was used for pair-wise comparisons. All statistical calculations were performed using GraphPad Prism 3.02. Differences were considered significant at $P < 0.05$. Analyses were based on 3–6 independent experiments using different cell passages on different days.

Results

Effect of Met-Tyr on HO-1 and ferritin. A 24-h incubation of endothelial cells with the ACE inhibitory dipeptide Met-Tyr (30–300 μmol/L) produced concentration-dependent increases in HO-1 protein levels (**Fig. 1**). HO-1 mRNA increased markedly and in a time-dependent manner after exposure of endothelial cells to Met-Tyr with a maximal stimulation to 1.5-fold ($P = 0.1$) of the control at 300 μmol/L Met-Tyr (**Table 1**). Therefore, we investigated whether Met-Tyr increases HO-1 protein expression through stabilization of HO-1 mRNA. After exposure of endothelial cells to the potent HO-1 inducer $CdCl_2$, transcription was blocked by adding AD 15 min before Met-Tyr. The HO-1 mRNA levels were higher in AD/Met-Tyr–treated cells than in AD-treated cells (**Fig. 2**). Previous studies showed that HO-1 protein expression is accompanied by the induction of a secondary antioxidant protein, ferritin. Thus, we examined the effects of Met-Tyr on ferritin protein expression in endothelial cells. Immunoblot analysis demonstrated a marked increase in ferritin protein expression after a 24-h exposure to Met-Tyr (**Fig. 3**).

Effect of Met-Tyr on ROS. NADPH-dependent ROS formation was diminished by up to 30% after a long-term preincubation of endothelial cells (24 h) with Met-Tyr (**Fig. 4**). To explore a potential involvement of HO-1 products such as bilirubin in the observed protection by Met-Tyr, the selective HO inhibitor zinc deuteroporphyrin IX 2,4-bis-ethylene glycol (ZnBG) was used. ZnBG abrogated cellular protection afforded by Met-Tyr against NADPH-mediated free radical formation (Fig. 4). In agreement with this, the HO-1 metabolite bilirubin, when added directly to the suspended cells, produced an almost complete inhibition of ROS formation (**Fig. 5**). Moreover, a Met-Tyr–induced blockade of free radical formation was prevented in the presence of the NO synthase inhibitor L-N^G-monomethyl arginine (L-NMMA; Fig. 4). ZnBG or L-NMMA alone did not affect NADPH-mediated radical formation under these conditions (not shown).

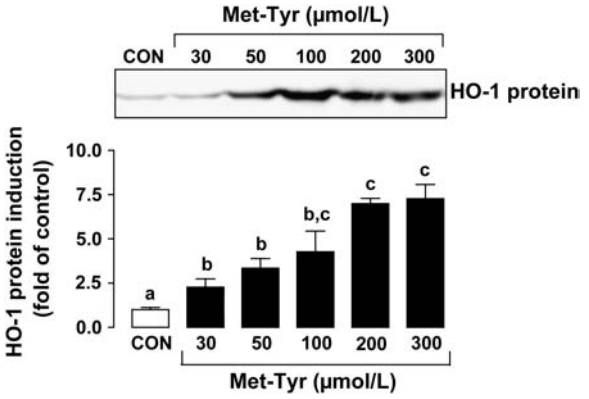

Figure 1 Met-Tyr increases HO-1 protein expression in endothelial cells. Values are means ± SEM, $n = 5$. Means without a common letter differ, $P < 0.05$. A representative Western blot analysis is shown in the upper panel.

TABLE 1 Effect of exposure to 300 μmol/L Met-Tyr on HO-1 mRNA levels in endothelial cells[1]

Exposure, *h*	CON	2	4	6	8
Fold of control	1.00 ± 0.07	0.87 ± 0.07	1.12 ± 0.16	1.35 ± 0.24	1.54 ± 0.31

[1] Values are means ± SEM, $n = 5$.

Effects of Met-Met, Met-Phe, and captopril on HO-1 and ROS. HO-1 protein induction by Met-Tyr was specific in that other ACE inhibitory compounds tested, such as the ACE inhibitory dipeptide Met-Phe and captopril, a sulfhydryl-containing clinically used ACE inhibitor, did not affect HO-1 protein levels (**Table 2**). The dipeptide Met-Met, which does not possess ACE inhibitory properties also did not induce HO-1 protein expression (Table 2). Met-Met, Met-Phe, or captopril did not diminish free radical formation in endothelial cells (**Table 3**).

Effects of single amino acids on HO-1 and ROS. Furthermore, we investigated the effect of the free amino acids L-methionine and L-tyrosine alone and in combination at concentrations comparable to those used for the dipeptide Met-Tyr. Under these conditions, HO-1 protein expression was not affected in the presence of the single amino acids alone or in combination (Table 2). Free radical formation in endothelial cells remained unaltered in the presence of the free amino acids L-methionine and L-tyrosine alone or in combination at micromolar concentrations (Table 3).

Discussion

A large variety of bioactive peptides are generated at relevant physiological levels in the gut lumen during normal digestion. These peptides enter the circulation intact and are capable of producing biologic actions. Several ACE inhibitory peptides that affect the cardiovascular system and exert antihypertensive actions were isolated (26–29). However, the exact vasoprotective mechanisms of these peptides in hypertensive subjects remain unclear.

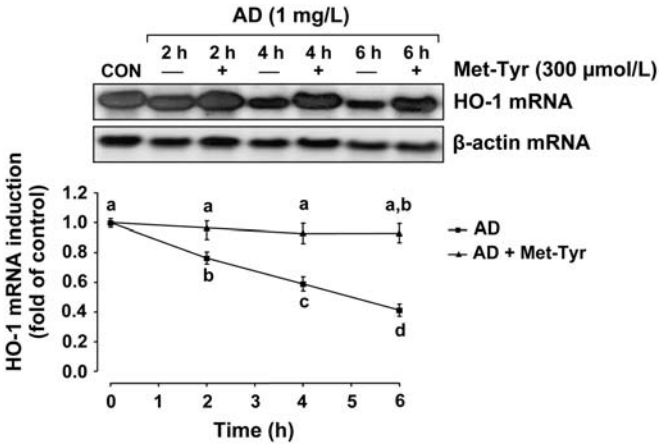

Figure 2 Met-Tyr stabilizes HO-1 mRNA in $CdCl_2$-treated cells in the presence of actinomycin D. Values are means ± SEM, $n = 5$. Means without a common letter differ, $P < 0.05$. A representative Northern blot analysis is shown in the upper panel.

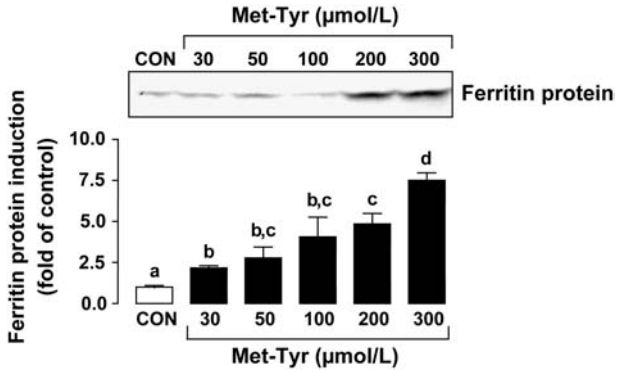

Figure 3 Met-Tyr increases ferritin protein expression in endothelial cells. Values are means ± SEM, $n = 5$. Means without a common letter differ, $P < 0.05$. A representative Western blot analysis is shown in the upper panel.

The present study demonstrates that the ACE inhibitory dipeptide Met-Tyr, derived from sardine muscle (7), stimulates expression of the antioxidant defense protein HO-1 in a concentration-dependent manner. Previous findings revealed that HO-1 protein expression is accompanied by the induction of a secondary antioxidant protein, ferritin. Met-Tyr produced increases in ferritin protein expression at concentrations that also resulted in HO-1 induction. The main physiological stimulus of ferritin protein synthesis is intracellular iron (30). Due to the Met-Tyr–mediated HO-1 induction, the amount of intracellularly available free iron is increased, which could facilitate the induction of ferritin protein expression. Pretreatment with Met-Tyr was associated with protection of endothelial cells from oxidative stress. Met-Tyr inhibited ROS formation, which was elicited by the addition of NADPH to the cells after washout of Met-Tyr. Based on these findings, it is plausible that Met-Tyr activates antioxidant signaling pathways resulting in a sustained protection even after removal of Met-Tyr. That HO-1 could mediate Met-Tyr activity under these conditions was demonstrated by the direct radical scavenging effect of the HO-1 product bilirubin. In agreement with this, we found that endothelial cell protection by Met-Tyr was abrogated in the presence of the HO inhibitor ZnBG, suggesting that HO-1 and its enzymatic products are indeed of functional relevance and responsible for the observed antioxidant actions. Recently, we demonstrated that the antioxidant defense protein HO-1 is an intracellular site of action for the amino acids L-alanine and L-methionine, which exert long-term cytoprotection (19,20). Moreover, it was shown that curcumin and caffeic acid

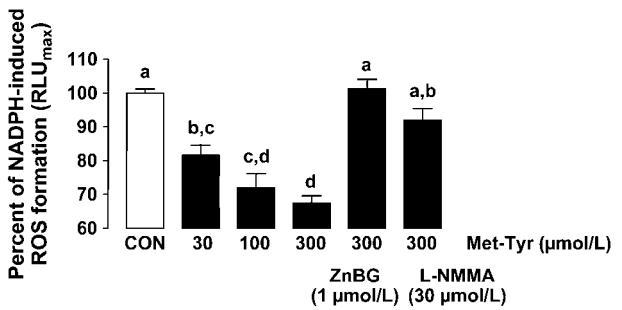

Figure 4 Met-Tyr diminishes NADPH-mediated superoxide formation in endothelial cells. This effect is reversed in the presence of the HO inhibitor ZnBG and the nitric oxide synthase inhibitor L-NMMA. Values are means ± SEM, $n = 4$. Means without a common letter differ, $P < 0.05$.

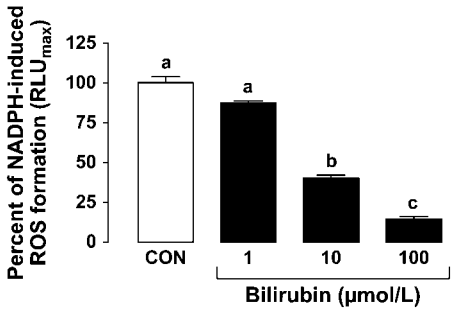

Figure 5 Effect of bilirubin on NADPH-dependent ROS formation in endothelial cells. Values are means ± SEM, $n = 3$. Means without a common letter differ, $P < 0.05$.

phenethyl ester, 2 plant-derived polyphenolic compounds with antioxidant, antitumor, and anti-inflammatory properties, are potent inducers of HO-1 in vascular endothelial and neuronal cells (31–33).

Although we were not able to detect significant changes in HO-1 mRNA levels on the basis of Northern blot analysis, we found strong evidence that Met-Tyr stabilizes HO-1 mRNA. Treatment of endothelial cells with Met-Tyr markedly prolonged the half-life of HO-1 mRNA after exposure to the potent HO-1 inducer $CdCl_2$. The induction of HO-1 expression by endogenous NO, an established HO-1–inducing signaling molecule (34–36), also involves post-transcriptional effects such as stabilization of HO-1 mRNA (37,38). Hence, NO could be a mediator of Met-Tyr–dependent antioxidant actions. This is further supported by our finding that an inhibitor of NO synthase (L-NMMA) abrogated Met-Tyr–induced radical scavenging. Interestingly, previously observed antioxidant effects of L-methionine at millimolar concentrations were also abrogated by a NO synthase inhibitor (19), pointing to the relevance of NO as a mediator for both functional dipeptides and single amino acids. Furthermore and in agreement with our observations presented here, it was reported that regulation of mammalian genes other than HO-1 by amino acids also has a post-transcriptional component affecting mRNA stability (39–41).

TABLE 2 Effects of Met-Tyr, Met-Met, Met-Phe, captopril, and single amino acids on HO-1 protein expression in endothelial cells[1]

	HO-1 protein induction, *fold of control*
CON	1.00 ± 0.01
Met-Tyr	
100 μmol/L	4.27 ± 1.17*
300 μmol/L	7.26 ± 0.81*
Met-Met	
100 μmol/L	1.14 ± 0.2
300 μmol/L	1.21 ± 0.19
Met-Phe	
100 μmol/L	1.11 ± 0.17
300 μmol/L	0.9 ± 0.19
Captopril	
100 μmol/L	1.33 ± 0.25
300 μmol/L	1.88 ± 0.1
Single amino acids	
L-Met 300 μmol/L	2.37 ± 0.55
L-Tyr 300 μmol/L	1.14 ± 0.31
L-Met 300 μmol/L + L-Tyr 300 μmol/L	0.91 ± 0.18

[1] Values are expressed as fold induction of control. The data are shown as means ± SEM of n = 3–6 independent observations. *Different from control, $P < 0.05$.

TABLE 3 Effects of Met-Tyr, Met-Met, Met-Phe, captopril, and single amino acids on ROS formation in endothelial cells[1]

	NADPH-mediated ROS formation, *% of control*
CON	100.0 ± 0.8
Met-Tyr 300 μmol/L	71.5 ± 1.9*
Met-Met 300 μmol/L	97.0 ± 1.9
Met-Phe 300 μmol/L	97.1 ± 1.6
Captopril 300 μmol/L	95.2 ± 4.9
Single amino acids	
L-Met 300 μmol/L	100.3 ± 3.0
L-Tyr 300 μmol/L	98.3 ± 2.4
L-Met 300 μmol/L + L-Tyr 300 μmol/L	96.7 ± 3.2

[1] Values are means ± SEM, $n = 5$. * Different from control, $P < 0.05$.

The effects of Met-Tyr on ROS formation and HO-1 expression were detected at micromolar concentrations, which are well within the range of the 50% inhibitory concentration for Met-Tyr–dependent ACE inhibitory activity (7,10). The single amino acids L-methionine and L-tyrosine, alone or in combination, did not produce any significant effects under these conditions. This points to a crucial role of the peptide bond in Met-Tyr and/or specific structural features of the dipeptide. Support for our observation comes from earlier studies demonstrating that the constituent amino acids of carnosine and related agents are far less effective antioxidants than their parent proteins (5,42).

The ACE inhibitory dipeptide Met-Phe, also derived from sardines (7), and the clinically used ACE inhibitor captopril did not affect HO-1 expression and ROS formation. Therefore, HO-1 induction by Met-Tyr can be assumed to occur independently of ACE inhibition. The methionine-containing dipeptide Met-Met, which does not inhibit ACE, also did not mimic the effects of Met-Tyr in the present study. These findings underline the unique role of Met-Tyr as a functional dipeptide with HO-1–inducing and antioxidant properties that are both unrelated to its ACE inhibitory action.

In summary, we demonstrated for the first time that the food-derived bioactive dipeptide Met-Tyr stimulates expression of the antioxidant defense proteins HO-1 and ferritin in endothelial cells. These genomic actions of Met-Tyr lead to sustained antioxidant cellular protection. In addition to its ACE inhibitory and blood pressure–lowering effects, HO-1 induction is a novel, potentially antiatherogenic mechanism of Met-Tyr and dietary proteins releasing Met-Tyr during gastrointestinal digestion.

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