RESEARCH ARTICLE

A Maillard reaction product enhances eNOS activity in human endothelial cells

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Received: July 13, 2009
Revised: August 12, 2009
Accepted: August 28, 2009

Nitric oxide (NO) produced by the endothelial nitric oxide synthase (eNOS) is an important signaling molecule in the cardiovascular system. Although dietary factors can modulate eNOS activity, putative effects of processed food are barely investigated. We aimed to examine whether the model Maillard reaction product 3-hydroxy-2-methyl-1-propyl-4(1*H*)-pyridone (HMPP), formed from maltol or starch and propylamine, affects the eNOS system. Incubation of EA.hy926 endothelial cells with 30–300 μM HMPP for 18 h enhanced endothelial NO release measured with the fluorescent probe diaminofluorescein-2 and eNOS activity determined by the [14C]L-arginine-[14C]L-citrulline conversion assay. HMPP increased NO production also in two different types of primary human endothelial cells. Protein levels of eNOS and inducible NO synthase remained unaltered by HMPP. HMPP inhibited eNOS activity within the first 2–4 h, whereas it potently increased eNOS activity after 12–24 h. Levels of eNOS phosphorylation, expression of heat-shock protein 90, caveolin-1 and various antioxidant enzymes were not affected. Intracellular reactive oxygen species remained unchanged by HMPP. This is the first study to demonstrate positive effects of a Maillard reaction product on eNOS activity and endothelial NO production, which is considered favourable for cardiovascular protection.

Keywords:
Endothelial nitric oxide synthase / Endothelium / Hydroxypyridone / Nitric oxide / Maillard reaction

1 Introduction

Nitric oxide (NO) produced by the endothelial nitric oxide synthase (eNOS) is an important signaling molecule in the cardiovascular system. NO exerts a direct vasodilatory effect on blood vessels, inhibits the proliferation of vascular smooth muscle cells, attenuates the adhesion of inflammatory cells to the endothelium and inhibits platelet aggregation. Reduced eNOS activity is associated with the development of atherosclerosis [1]. Since the endothelium constitutes the predominant tissue to which any given molecule is exposed after absorption into the blood stream, this makes it a potential site of action for compounds taken up with the daily diet.

An increasing number of studies show that natural products are able to positively affect eNOS function. Such compounds are found, for example, in red wine, green tea, gingko, or pomegranate juice, among others [2–6]. However, whereas many investigators are studying plant extracts or purified phytochemicals, studies addressing putative effects of processed food on eNOS are scarce.

The ubiquitous Maillard reaction between carbohydrates and amines is regarded as a prototype reaction of food processing. Maillard reaction products (MRP) are present in considerable amounts in cooked food. Besides being the main determinants of color and aroma, some MRP also possess

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Abbreviations: Cav-1, caveolin-1; **DAF-2,** 4,5-diaminofluorescein; **eNOS,** endothelial nitric oxide synthase; **HMPP,** 3-hydroxy-2-methyl-1-propyl-4(1*H*)-pyridone; **Hsp90,** heat-shock protein 90; **iNOS,** inducible NO synthase; **L-NAA,** N_{ω}-amino-L-arginine; **MRP,** Maillard reaction product; **NO,** nitric oxide; **PB,** protein binding; **PMA,** phorbol-12-myristate-13-acetate; **ROS,** reactive oxygen species; **VD,** volume distribution

pharmacological activity [7]. Given the chemical diversity of MRP in food, we synthesized a model compound of a dietary MRP, which is formed by the reaction between maltol or starch with propylamine [8] (Fig. 1). The aim of this study was to examine whether the model MRP 3-hydroxy-2-methyl-1-propyl-4(1*H*)-pyridone (HMPP) affects the endothelial eNOS system.

2 Materials and methods

2.1 Chemicals and cell culture reagents

DMEM without phenol red containing 4.5 g/L glucose, HAT supplement (100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine), glutamine, benzylpenicillin and streptomycin were purchased from Lonza (Belgium), trypsin from Invitrogen (USA). G418 sulfate was purchased from PAA Laboratories GmbH (Austria). Fetal bovine serum was obtained from Gibco *via* Invitrogen (UK). Phorbol-12-myristate-13-acetate (PMA), 4,5-diaminofluorescein (DAF-2) and ionomycin were bought from Alexis Biochemicals (Switzerland) and (^{14}C)L-arginine (313 Ci/mmol) from New England Nuclear (USA). Antibodies were obtained from the following companies: anti-eNOS from BD (USA), anti-tubulin and horseradish peroxidase-conjugated goat anti-mouse secondary antibody from Santa Cruz (USA). All other chemicals were bought from Sigma-Aldrich (Austria). TLC plates were bought from Machery-Nagel (Austria). The luciferase assay system was obtained from Promega (USA).

2.2 Synthesis of HMPP

The synthesis was performed essentially as described previously [9]. In brief, maltol and propylamine were dissolved in water and heated under reflux for 3 h. Then the solvent was evaporated under reduced pressure. The product was purified *via* column chromatography on silica gel using ethylacetate/methanol (4:1) as eluent followed by recrystallization from ethylacetate. Structure and purity were confirmed by TLC and NMR.

2.3 Cell culture

The human endothelial cell line EA.hy926 [10] (kindly provided by Dr. C.-J. S. Edgell, University of North Carolina, Chapel Hill, NC, USA) was grown in DMEM without phenol red supplemented with 584 mg/mL glutamine, 100 U/mL benzylpenicillin, 100 μg/mL streptomycin, HAT supplement and 10% fetal bovine serum until passage 30. For experiments, cells were seeded in six-well plates at a density of 5×10^5 cells *per* well and treated with test compounds at confluence after approximately 72 h. EA.hy926 cells stably transfected with a luciferase reporter gene construct as described below (EA.hy926-heNOS-Luc; kindly provided by Dr. Paulus Wohlfahrt, Sanofi-Aventis, Germany) were cultured as EA.hy926 cells, besides additional medium supplementation with G418 (400 μg/mL), until passage 60. Stably transfected cells were seeded into 24-well plates at a density of 1.2×10^5 cells *per* well and, when reaching confluence, the medium was renewed 4 h before treatment. HMPP was dissolved in ultra-pure water, filtered through a 0.22 μm filter and stored at –80°C as 1000-fold stock solutions. Basic cell parameters were routinely checked using a cell viability analyzer (ViCell, Beckman Coulter, USA) and cells were tested for the absence of mycoplasms.

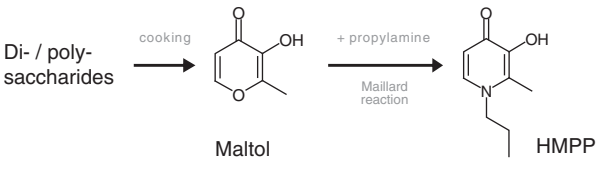

Figure 1. Simplified synthesis of HMPP.

2.4 Luciferase reporter gene assay

EA.hy926 endothelial cells stably transfected with the plasmid p-eNOS-3500-Hu-Luc-neo [11], containing 3500 bp of the human eNOS promoter driving a luciferase reporter gene (EA.hy926-heNOS-Luc), were treated with the respective compounds for 18 h. Cells were then washed with PBS, lysed and the assay was performed according to the instructions of the manufacturer (Promega). Luminescence was detected with a plate reader (Genios Pro, Tecan, Switzerland) and normalized to the protein content of the lysates determined by the Bradford method.

2.5 (^{14}C)L-arginine/(^{14}C)L-citrulline conversion assay

The assay was performed as described previously [12]. Briefly, cells were equilibrated in HEPES buffer (pH 7.4), then (^{14}C)L-arginine (313 Ci/mmol) and ionomycin were added subsequently to a final concentration of 0.32 μM and 1 μM, respectively. After lysing and extracting cells with ethanol/water, extracts were dried under vacuum (SPD 1010 SpeedVac, Thermo Savant) and resolved in water/methanol (1:1). Following separation on a TLC plate (Polygram SIL N-HR, Machery-Nagel), (^{14}C)L-citrulline was quantified by autoradiography in a phosphorimager (BAS-1800II, Fujifilm, Japan). AIDA software (raytest USA, USA) was used for densitometric analysis.

2.6 Quantification of NO release by diaminofluorescein (DAF)-2

Quantification of NO released by EA.hy926 endothelial cells was performed using the NO-sensitive fluorescent probe DAF-2 [13] as described previously [12].

SDS-PAGE and Immunoblotting were performed as described previously [14].

2.7 *In silico* predictions of the pharmacokinetic profile of HMPP

VolSurf [15] is an established computational procedure to produce 2-D molecular descriptors from 3-D molecular interaction energy grid maps employed for drug design, pharmacokinetics profiling, and screening [16–20]. For HMPP, we used the protein binding (PB), volume distribution (VD) and cytochrome P3A4 metabolic stability model. For details of each model refer to the Supporting Information "Materials and methods" section. Molecular Discovery Limited kindly provided free access to software VolSurf+.

2.8 Statistical methods

Statistical analysis was done using GraphPad Prism software version 4.03 (GraphPad Software, USA). Normalized data were transformed logarithmically ($Y = \log(Y)$) before analysis. One-way ANOVA combined with Dunnett posttest was used for comparison of different concentrations against the control. If two groups were compared, two-tailed paired *t*-test was applied, $p < 0.05$ was considered significant. All graphs are shown as means ± SEM.

3 Results

3.1 HMPP enhances endothelial NO release and eNOS activity

To examine whether HMPP, a model compound for a MRP (Fig. 1), has an effect on NO release from endothelial cells, we employed the NO-sensitive fluorescent probe DAF-2 [13]. Incubation of EA.hy926 human endothelial cells with 100 μM HMPP increased DAF-2 fluorescence in the cell culture supernatant threefold compared to baseline levels ($p < 0.01$) (Fig. 2A). The effect was blocked by coincubation with the eNOS inhibitor N_ω-amino-L-arginine (L-NAA). Incubation with PMA for 18 h, which increases eNOS activity and NO release due to downregulation of protein kinase C [21], was used as a positive control. An increase of 60–100% of bioavailable NO could also be observed in primary human umbilical vein and aortic endothelial cells, ruling out a cell-type-specific effect of HMPP on EA.hy926 cells (Supporting Information Figs 1A and B). In an alternative approach, we assessed eNOS activity by performing a (^{14}C)L-arginine/(^{14}C)L-citrulline conversion assay. NO is produced *via* NOS-mediated conversion of L-arginine into L-citrulline, which renders radiolabeled L-citrulline a marker for NO production. Treatment of EA.hy926 human endothelial cells with HMPP for 18 h dose-dependently increased eNOS activity ($p < 0.01$) (Fig. 2B). Addition of the eNOS inhibitor L-NAA completely abolished this effect ($p < 0.01$). A measure of 300 μM HMPP raised L-citrulline levels by approximately 25%, whereas ascorbic acid, known to enhance eNOS activity *via* stabilization of the essential eNOS cofactor tetrahydrobiopterin [22], enhanced L-citrulline production by 50%.

Throughout the experiments cell viability remained higher than 95% as assessed by trypan blue exclusion.

3.2 HMPP does not influence eNOS promoter activity and protein levels

Although eNOS is a constitutively expressed enzyme, it has been shown to be dynamically regulated at the transcriptional and posttranscriptional level [23]. We therefore addressed a putative effect of HMPP on eNOS gene tran-

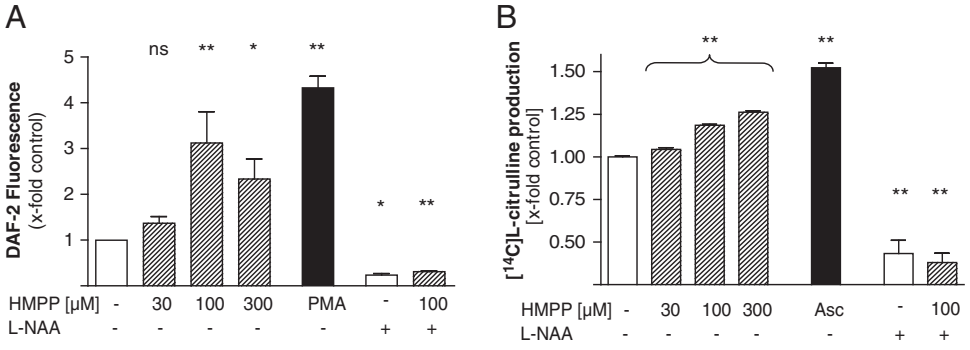

Figure 2. HMPP enhances endothelial NO release and eNOS activity. (A) Quantification of endothelial NO release by DAF-2. Cells were treated with the indicated concentrations of HMPP for 18 h. DAF-2 fluorescence was normalized to cell count and expressed as *x*-fold control (ns, not significant; $^*p < 0.05$; $^{**}p < 0.01$) (mean ± SEM, $n = 3$). The eNOS inhibitor L-NAA (200 μM) blocked the signal. PMA (2 nM) served as a positive control. (B) EAhy.926 endothelial cells were incubated with 30–300 μM HMPP for 18 h. ENOS activity was measured with a [^{14}C]L-citrulline/[^{14}C]L-arginine conversion assay ($^{**}p < 0.01$) (mean ± SEM, $n = 3$). Ascorbic acid (Asc; 100 μM) was used as positive, the eNOS inhibitor L-NAA (200 μM) as a negative control.

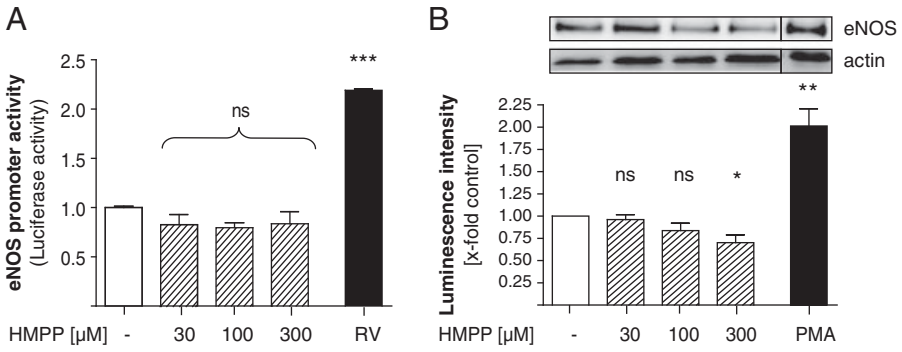

Figure 3. Effects of HMPP on eNOS promoter transactivation and eNOS protein levels. (A) EA.hy926-heNOS-Luc endothelial cells were incubated with 30–300 μM HMPP for 18 h and assayed for luciferase activity. Luminescence was normalized to the protein concentration of cell lysates and to the activity of untreated control cells (ns, not significant) (mean ± SEM, $n = 3$). Resveratrol (RV, 5 μM) served as positive control for eNOS promoter activation (***$p < 0.001$). (B) Effect of HMPP on eNOS protein levels. Cells were treated with 30–300 μM HMPP for 24 h before detection of eNOS protein *via* immunoblotting. Band intensities were normalized to actin and expressed as *x*-fold control (ns, not significant; *$p < 0.05$) (mean ± SEM, $n = 5$). One representative blot is shown. PMA was used as a positive control for upregulation of eNOS protein (**$p < 0.01$).

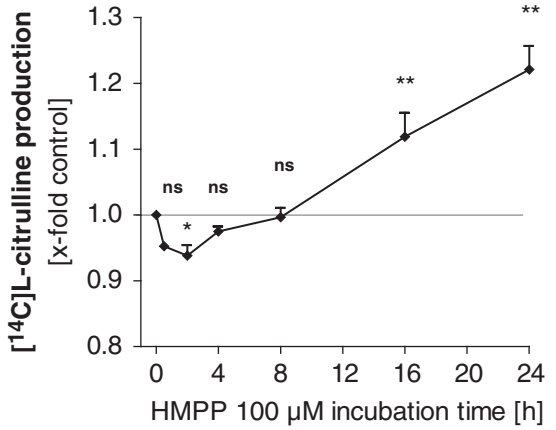

Figure 4. An initial inhibition of eNOS precedes the HMPP-induced increase in eNOS activity. EAhy.926 endothelial cells were incubated with 100 μM HMPP for different time intervals (0.5–24 h) and assayed for eNOS activity in a [^{14}C]L-citrulline/[^{14}C]L-arginine conversion assay (ns, not significant; *$p < 0.05$; **$p < 0.01$) (mean ± SEM, $n = 3$).

scription using EA.hy926-heNOS-Luc cells [11], stably transfected with a plasmid containing 3500 bp of the human eNOS promoter coupled to a luciferase reporter gene. Incubation of these cells with 30–300 μM HMPP for 18 h did not influence luciferase activity, suggesting that eNOS gene transcription was not altered by HMPP (Fig. 3A). Resveratrol, a well-known inducer of eNOS gene transcription [24], potently enhanced luciferase activity ($p < 0.001$).

When addressing cellular eNOS protein levels, we found that treatment with 30–100 μM HMPP for 24 h had no significant effect (Fig. 3B). At a concentration of 300 μM, however, eNOS protein levels were slightly decreased ($p < 0.05$) compared with those of untreated control cells. As a positive control we treated cells with PMA for 24 h, which robustly increased cellular eNOS protein content ($p < 0.01$) [23]. In assessing a potential contribution of NO produced by inducible NOS (iNOS), we found that iNOS protein was almost undetectable in EA.hy926 cells by Western blot and its expression was unaffected by treatment with HMPP (Supporting Information Fig. 2). LPS-stimulated macrophages showed a marked expression of iNOS protein demonstrating the functionality of the antibody used.

3.3 A transient inhibition of eNOS precedes the HMPP-induced increase in eNOS activity

To gain insight into the kinetics underlying the observed increase in eNOS activity, we performed a time-course experiment. Endothelial cells were treated with 100 μM HMPP for different time intervals ranging from 0.5 to 24 h and then harvested for a (^{14}C)L-arginine/(^{14}C)L-citrulline conversion assay (Fig. 4). We found that eNOS activity was significantly inhibited at early time points ($p < 0.05$ after 2 h) but then started to rise. Basal levels were reached after approximately 8 h and eNOS activity continued to increase steadily up to 24 h ($p < 0.01$).

3.4 HMPP neither affects eNOS phosphorylation at Ser-1177 or Thr-495, nor alters protein levels of heat-shock protein 90 (Hsp90), caveolin-1 (Cav-1) or intracellular reactive oxygen species (ROS) production

Since the positive effect of HMPP on eNOS activity cannot be explained by increased expression of eNOS protein, HMPP may influence the posttranslational regulation of eNOS activity. Increased phosphorylation of Ser-1177 and/or decreased phosphorylation at Thr-495 are known to enhance eNOS enzymatic activity [25]. However, neither of

the phosphorylation sites is affected by HMPP after 8 and 24 h (Supporting Information Fig. 3A). Binding partners of eNOS also determine the final activity of eNOS. Cav-1 hereby acts as a negative regulator whereas binding of Hsp90 to eNOS facilitates full enzymatic activity of the enzyme [26]. Treatment of EA.hy926 cells with 100 μM HMPP did not change cellular protein expression of Hsp90 or Cav-1 (Supporting Information Fig. 3B). In addition, coimmunoprecipitations did not reveal altered recruitment of Hsp90 or Cav-1 to eNOS in the presence of HMPP (data not shown). Thus, HMPP does not appear to alter eNOS activity *via* changes in eNOS phosphorylation at its predominant regulatory sites or by modulating binding of eNOS to Hsp90 or Cav-1.

Enzymatic activity of eNOS and bioavailability of NO can further be modulated by ROS. Increased levels of ROS impair availability of tetrahydrobiopterin, a pivotal cofactor of eNOS, and inactivate NO by formation of peroxynitrite [27, 28]. We therefore checked whether HMPP affects cellular ROS production. We determined total intracellular ROS levels in HMPP-treated EA.hy926 cells employing the redox-sensitive dye dihydrodichlorofluorescein. Incubation with 100 μM HMPP did not elicit any significant changes in the total cellular ROS after 0.5, 2, 8 and 24 h (Supporting Information Fig. 3C). This is consistent with the observed unchanged protein expression levels of major antioxidant enzymes, namely heme-oxygenase 1, catalase, Cu/Zn superoxide dismutase and Mn superoxide dismutase. Moreover, we were unable to detect any alteration in expression of GTP cyclohydrolase-1, the rate-limiting enzyme in the synthesis of tetrahydrobiopterin [26] (Supporting Information Fig. 3D).

3.5 HMPP shows satisfactory pharmacokinetic properties *in silico*

Additionally, to get an insight into the pharmacokinetic profile of HMPP, we ran *in silico* calculations using VolSurf+ software, which predicts certain pharmacokinetic parameters based on comparison with profiles of a collection of over 500 chemical entities [15].

Table 1. VolSurf-based pharmacokinetic predictions

	PB	Volume distribution −log(VD)	Metabolic stability (MetStab) %
HMPP	54.45	0.15	100
HMACP	56.53	0.09	96

In silico predictions for the pharmacokinetic profile of HMPP. HMPP was subjected to *in silico* calculations using VolSurf software. Results obtained with models for, VD and metabolic stability (MetStab) are depicted. Details concerning the software and its models can be found in the Supporting Information ''Materials and methods'' section.

For HMPP, the software predicted considerable amounts of free drug in the plasma due to 54% binding of HMPP to HSA. In a VD model, HMPP scored −0.15, which translates into medium to high achievable concentrations in tissues. In the metabolic stability model, HMPP obtained a value of 1.0. This suggests that HMPP would remain unmetabolized after 60 min of incubation at 37°C with cytochrome P3A4-enriched microsomal proteins. Since HMPP merely represents a model compound for MRP, we performed the same calculations for the MRP 3-hydroxy-2-methyl-1-amino-capronyl-4(1*H*) pyridine (HMACP), in which the propylamine moiety is replaced by the essential amino acid L-lysine. The calculations yielded comparable values (Table 1).

4 Discussion

In this study we show that HMPP, a model compound for MRP [7], is able to increase eNOS activity and NO release from human endothelial cells *in vitro*. Upon treatment with HMPP (100 μM) eNOS activity was transiently inhibited during the first 2–4 h, whereas longer incubation for 12–24 h potently enhanced eNOS activity and NO release. This increase is neither due to augmented eNOS (or iNOS) gene transcription, nor based on altered eNOS phosphorylation at its main regulatory sites.

When measuring NO release from endothelial cells with the NO-sensitive fluorescent probe DAF-2, we detected a threefold increase by treatment with HMPP (100 μM) for 18 h. This value is mirrored by an approximately 25% rise of endothelial L-citrulline accumulation measured by (^{14}C)L-arginine/(^{14}C)L-citrulline conversion. The numerical discrepancy between those two results may be explained by constant recycling of L-citrulline to L-arginine *via* argininosuccinate, which is pivotal for full enzymatic activity of eNOS, and may give rise to L-citrulline values lower than the actual amount of NO produced [29]. In bovine aortic endothelial cells, NO production was found to exceed L-citrulline accumulation by at least eightfold [30]. If this stoichiometry was applied to EA.hy926 endothelial cells used in our study, a 25% increase in L-citrulline would correspond to at least a doubling of endothelial NO production, which is by and large consistent with our experimental data. A measure of 30–300 μM HMPP elicited a strictly dose-dependent activation of eNOS as monitored *via* L-arginine/L-citrulline conversion. However, when measuring endothelial NO release using DAF-2, 300 μM HMPP resulted in lower DAF-2 fluorescence than 100 μM HMPP. The reason for this discrepancy is not entirely clear. One possible explanation may be linked to the interference of antioxidants with DAF-2-based NO detection methods [31]. Since the chemical structure of HMPP suggests that it possesses some reducing activity, high HMPP concentrations may have inhibited the reaction of NO with DAF-2, resulting in lower fluorescence signals.

The initial inhibition and subsequent increase of eNOS activity in response to HMPP might bring up the notion of a cellular stress response that is effective 8 h after cell exposure to HMPP, a time frame sufficient to induce *de novo* protein expression. Indeed, eNOS is known to directly interact with a multitude of proteins, which can enhance its activity [32]. Increased expression and/or recruitment of such proteins in the context of an adaptive stress response could account for the induction of eNOS activity in response to HMPP. The group of Pischetsrieder recently showed that MRP induce nuclear NF-κB translocation in macrophages *via* H_2O_2, which may possibly influence intestinal immune function [33]. Moreover, Somoza *et al.* [34] observed an increase of antioxidant parameters *in vivo* by feeding MRP-rich bread crust extract to male Wistar rats indicating that some MRP may trigger an antioxidant response element-dependent antioxidant response. All these observations fit into the concept of ''hormesis,'' suggesting that in response to moderate doses of ''stress'' cells may activate compensatory mechanisms protecting against repeated exposure to this ''stress'' [35, 36]. In this sense, the initial eNOS inhibition upon treatment with HMPP might trigger a hormetic response, which increases eNOS activity for at least 24 h. In the course of our study, we investigated a series of candidate proteins that could function as mediators of such an adaptive stress response. However, we were not able to detect significant changes in expression levels of Hsp90, heme-oxygenase 1, catalase or superoxide dismutases. Alterations of cellular ROS levels, which can have a knock-on effect on eNOS activity and NO availability, are unlikely since we did not observe any changes upon HMPP administration. Thus, at this point, the precise mechanism underlying HMPP-induced eNOS induction remains elusive. Future investigations preferentially in form of an unbiased comparative protein expression profiling of untreated *versus* HMPP-treated cells could provide an unambiguous answer. Besides a hormetic action, HMPP could act on a variety of posttranslational modifications that knowingly modulate eNOS activity, including tyrosine phosphorylation, nitrosylation, acetylation or subcellular localization of eNOS, some of which have only recently been discovered [37–40].

It is important to emphasize that HMPP, which has been shown to be taken up readily into cells [41], is only a model compound for a chemically diverse class of MRP derived from maltol or starch. Owing to the absence of propylamine in the daily diet, basic amino acids such as lysine are likely to replace propylamine in this Maillard reaction [7]. The chemical nature of the amino acids will greatly affect the polarity of the resulting MRP and therefore influence its membrane permeability [42]. The favorable effect of MRP on eNOS activity, as pointed out in our study, would thus have to be confirmed for other MRP on an individual basis. *In silico* calculations, however, predicted comparable values for plasma binding, tissue distribution and metabolic stability for HMPP and its lysine derivative (Table 1). At this point it needs to be stressed that such *in silico* predictions cannot substitute thorough *in vivo* pharmacokinetic studies, but are rather to be seen as first indications of satisfactory bioavailability of HMPP and possibly related MRP. The potential for regular uptake of such MRP due to their ubiquitous presence in the daily diet supports future investigations in the context of MRP and endothelial function both *in vitro* and *in vivo*. The beneficial impact of particular processed food on eNOS function is supported by our previous study demonstrating that norfuraneol, a pentose-breakdown product found in cooked food, increases eNOS activity by protein phosphatase-1-mediated dephosphorylation of eNOS at Thr 495 [14].

Overall, our study is the first to demonstrate positive effects of a MRP on eNOS activity and endothelial NO production. Besides the reported antifibrotic potential of MRP [43] our findings suggest the possibility of a favorable impact of certain MRP in regard to cardiovascular protection that warrants further investigations.

The authors thank Dr. C.-J. Edgell (University of North Carolina, Chapel Hill, NC) for providing EA.hy926 cells and Dr. Paulus Wohlfahrt (Sanofi-Aventis, Germany) for EA.hy926-heNOS-Luc-cells. Additionally, we thank Dr. Steffen Hering and Dr. Oskar Hoffmann (University of Vienna, Austria) for lab space to perform experiments with radioactive isotopes and Dr. Thomas Erker and Dr. Norbert Handler (University of Vienna) for NMR measurements. The expert technical assistance of H. Beres and R. Leitner is gratefully acknowledged.

The authors declare they have no conflict of interest.

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