Norfuraneol dephosphorylates eNOS at threonine 495 and enhances eNOS activity in human endothelial cells

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Aim Pentoses are widely abundant in organic food. Thermal treatment of pentoses leads to the formation of norfuraneol (NF). The aim of this study was to show whether NF, which is taken up regularly, for example with cooked food, affects the human endothelial nitric oxide synthase (eNOS) system.

Methods and results The study was performed using cultured human umbilical vein endothelial cells (HUVEC), HUVEC-derived EA.hy926 cells, and bovine aortic endothelial cells. Nitric oxide (NO) release and eNOS activity were measured using diaminofluorescein-2 and [14C]L-arginine/[14C]L-citrulline conversion. Levels of (phospho-)eNOS were detected by western blotting. Reactive oxygen species (ROS) production was assessed using 2′,7′-dichlorodihydrofluorescein diacetate. Pharmacokinetic parameters of NF were calculated by VolSurf software. NF dose dependently increased eNOS activity and NO release (30–300 μM), but did not affect total eNOS protein or cellular ROS levels. The increase in eNOS activity coincided with specific dephosphorylation of eNOS-Thr495, known to enhance eNOS activity. Inhibition of protein phosphatase 1 (PP1) by calyculin A, tautomycetin, or siRNA against PP1 reversed NF-induced eNOS-Thr495 dephosphorylation. Phosphorylation at eNOS-Ser1177 was not significantly altered by NF. Inhibition of protein kinase C with bisindolylmaleimide I (GFX) or calphostin C mimicked the effect of NF. In contrast to GFX, however, NF had no effect on phorbol-12-myristate-13-acetate-induced endothelial ROS formation. In silico, NF is stable towards CYP3A4 metabolism, shows low protein binding, and high tissue distribution.

Conclusion NF enhances endothelial NO release most likely by promoting specific dephosphorylation of eNOS-Thr495 via PP1 in vitro and may be a promising compound to enhance endothelial function in vivo.

1. Introduction

A key event in the development of atherosclerosis is endothelial dysfunction, characterized by a reduced capacity of endothelial cells to suppress processes of inflammation, thrombosis, and oxidative stress. Clinical and experimental data have established the production of nitric oxide (NO) by the endothelial nitric oxide synthase (eNOS) as a critical factor for the maintenance of a healthy endothelium and vascular homeostasis: NO regulates vasomotor tone and exerts anti-atherogenic effects on vascular smooth muscle cells, platelets, and inflammatory cells.1,2 Increasing NO bioavailability in the vasculature is considered a strategy to prevent the onset of cardiovascular diseases and is therefore of pharmacological interest.3

It is well known that dietary habits are playing an important role in the development of cardiovascular diseases.4 While a ’Western diet’ is associated with increased cardiovascular risk, a ’Mediterranean diet’ can lower it,5 suggesting that the daily diet has the potential to modulate eNOS function. Indeed, positive effects on endothelial NO production have been demonstrated for compounds found in red wine, green tea, pomegranates, or soy beans.6–11

Pentoses are naturally occurring monosaccharides ubiquitously present in organic food. D-ribose, for example, serves as a linker component within RNA in all living organisms. In the form of pentosans, pentoses can also be found in plants reaching concentrations of 2–6% in wheat or rye flour.12 Thermal decomposition of pentoses, e.g. during cooking, leads to the formation of the conversion product norfuraneol (4-hydroxy-5-methyl-3(2H)-furanone, NF, Figure 1A). NF has been described as a major component of meat broth.13 It is also found in other foods, such as in soy sauce or coffee,14 and is used as a flavour component. Chemically, NF can be considered as a reductone and is thus potentially redox-active, a feature NF may share with known eNOS modulators such as plant-derived polyphenols.6–11 Since NF is present in...
relevant amounts in the daily diet, we examined its putative effect on the human eNOS system.

2. Methods

2.1 Chemicals and cell culture reagents

Dulbecco’s modified Eagle’s medium (DMEM) without phenol red containing 4.5 g/L glucose, endothelial growth medium EBMTm (EBM™ SingleQuots, HAT supplement (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), glutamine, benzylpenicillin, streptomycin, and amphotericin B were bought from Lonza (USA). Trypsin and fetal bovine serum (FBS) were obtained from Invitrogen (USA). G418 sulfate was purchased from PAA Laboratories GmbH (Austria). Phorbol-12-myristate-13-acetate (PMA) (2 nM) served as a positive control and L-nitro-arginine (L-NNA) (200 μM) was used as eNOS inhibitor. [*]C]-citrulline production was normalized to the control (*P < 0.05; **P < 0.01) (mean ± SEM, n = 3). (C) EA.hy926 cells were incubated with the indicated concentrations of NF for 18 h. NO release was quantified by incubation with the NO-sensitive fluorescent probe diaminofluorescein-2 for 30 min. Fluorescence values were normalized to cell count and to the activity of control cells. Phorbol-12-myristate-13-acetate (PMA) (2 nM) served as a positive control and L-NNA (200 μM) was used as eNOS inhibitor (*P < 0.05; **P < 0.01) (mean ± SEM, n = 3). (D) Bovine aortic endothelial cells (BAoEC) were treated with 100 μM NF for 24 h. Endothelial NO release was measured as in Figure 1C (*P < 0.05) (mean ± SEM, n = 4).

2.2 Synthesis of norfuraneol (4-hydroxy-5-methyl-(2H)-furanone, NF)

Three grams of xylose and 3 g of beta-alanine were dissolved in 50 mL water containing 2 g of disodium phosphate and 1 g of potassium phosphate. After boiling for 30 min the solution was extracted with ethyl acetate and the organic phase was purified using a silica gel column. Structure and purity (≥99%) were confirmed by high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and melting point analysis, which corresponded to literature.

2.3 Cell culture

The endothelial cell line EA.hy926 (kindly provided by Dr C.-J.S.E., University of North Carolina, Chapel Hill, NC), which is derived from human umbilical vein endothelial cells (HUVECs),17 was cultivated as described previously (see Supplementary data for detailed information).18 HUVEC were isolated from umbilical cords obtained from a local hospital as described19 and cultivated in EBM growth medium supplemented with 10% FBS, EBM SingleQuots, 100 U/mL benzylpenicillin, 100 μg/mL streptomycin, and 1% amphotericin until passage six. For experiments, cells were seeded in gelatin-coated six-well plates at a density of 8 × 10^5 cells/well. Bovine aortic endothelial cells (BAoEC) were purchased from Genlantis (USA) and cultured according to the supplier’s instructions. Basic cell parameters were routinely checked using a cell viability analyzer (ViCell™, Beckman Coulter, CA, USA).

2.4 [*]C]-arginine/[*]C]-citrulline conversion assay

Citrulline is produced from arginine by eNOS in equimolar amounts to NO and can thus serve as a surrogate marker of NO production. The assay was performed as previously described.18 Briefly, cells were equilibrated in (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer, then 0.32 μM [*]C]-arginine (313 Ci/mmol) and 1 μM Ionomycin were added. 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). After separation from [*]C]-arginine by thin layer chromatography (Polygram SIL N-HR, Machery Nagel, Austria), [*]C]-citrulline was quantified by autoradiography in a phosphorimager (BAS-1800II, Fujifilm, Japan). AIDA software (raytest USA Inc., USA) was used for densitometric analysis.

2.5 Quantification of nitric oxide release by diaminofluorescein-2

Quantification of NO release by EA.hy926 or BAoEC was performed using the NO-sensitive fluorescent probe diaminofluorescein-2 (DAF-2) as described previously.18 Briefly, cells were equilibrated in phosphate buffered saline (PBS) containing calcium, magnesium, and arginine (100 μM) before addition of Ionomycin (1 μM) and DAF-2 (0.1 μM) followed by incubation at 37°C for 30 min. Fluorescence of the supernatant was measured in a desktop fluorometer (RF 1501, Shimadzu, Japan).
2.6 Determination of total reactive oxygen species production

Total cellular reactive oxygen species (ROS) production was analysed by flow cytometry after loading of cells with the fluorogenic probe H<sub>2</sub>DCF-DA and performed essentially as described (see Supplementary data for detailed information). The geometric mean of log fluorescence of 10 000 cells (corrected for autofluorescence) of each treatment group was taken as a measure for the total ROS load.

2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting

Sample preparation, electrophoresis, and western blot were performed essentially as described. Detailed information can be found in the Supplementary data.

2.8 Silencing of protein phosphatase 1

Cells were seeded into six-well plates and at a confluence of 60% (EA.hy926) and 90% (HUVEC), respectively, transfected with 200 nM siRNA using the OptiMEM/Oligofectamine system (Invitrogen, USA) according to the manufacturer’s instructions. siRNA targeting PP1 (sc-43545) was obtained from Santa Cruz (USA), unspecific scrambled control RNA from Invitrogen (USA). Seventy-two hours after transfection, cells were used for experiments. Knockdown of PP1 was examined by western blot analysis employing an antibody directed against the catalytic subunit of PP1 (sc-443; Santa Cruz).

2.9 Ribonucleic acid analysis

Total RNA was isolated with peqGOLD total RNA Kit (peqlab, Germany) according to the manufacturer’s protocol. cDNA was synthesized from 5 µg of total RNA using Superscript<sup>TM</sup> II Reverse Transcriptase (Invitrogen, USA), purified on Micro Bio-Spin 30 Tris Columns (BIO-RAD, USA), and assayed by quantitative real-time PCR on a LightCycler<sup>TM</sup> 480 (Roche Diagnostics, Switzerland) using Lightcycler<sup>TM</sup> 480 SYBR Green I Master Mix (Roche Diagnostics, Switzerland). Detailed information can be found in the supplementary methods section.

2.10 In silico predictions of the pharmacokinetic profile of norfuraneol

VolSurf<sup>23</sup> is a computational procedure to produce 2D molecular descriptors from 3D molecular interaction energy grid maps. VolSurf working examples are extensively reported. The use of multivariate statistics coupled with interactive 2D and 3D plots, insights for drug design, pharmacokinetics profiling, and screening are obtained. For NF, we used the protein binding (PB), volume distribution (VD), and CYP3A4 metabolic stability (MetStab) models. For details of each model please refer to the supplemental methods section.

2.11 Statistical methods

Statistical analysis was done using GraphPad Prism software version 4.03 (GraphPad Software Inc., USA). Normalized data were transformed logarithmically [Y=log(Y)] before analysis. One-way analysis of variance combined with Dunnett’s post-test was used for the comparison of multiple treatment groups with the control. If two groups were compared, two-tailed paired t-test was applied. P<0.05 was considered significant. All graphs are showing means ± SEM.

3. Results

3.1 Norfuraneol enhances eNOS activity and endothelial nitric oxide release

To investigate a putative effect of NF (Figure 1A) on eNOS activity, we first performed an [14C]-arginine/14C]-citrulline conversion assay using HUVEC-derived EA.hy926 cells, which are characterized as a stable endothelial cell line. EA.hy926 cells treated with 30–300 µM NF for 18 h showed a significantly higher [14C]-citrulline production than untreated control cells (P<0.01, Figure 1B). This effect was concentration-dependent and could be blocked by the specific eNOS inhibitor N<sub>ω</sub>-nitro-<i>L</i>-arginine (L-NNA). As a positive control we used ascorbic acid, which is known to stabilize the eNOS cofactor tetrahydrobiopterin and thereby to enhance eNOS activity.

Since NO can be degraded intracellularly by superoxide derived from enzymatic sources, we quantified NO released by endothelial cells using the NO-sensitive fluorogenic probe DAF-2. Incubation of cells with NF (30–300 µM) for 18 h significantly increased NO release compared with control cells (P<0.01, Figure 1C). Addition of L-NNA again abolished the effect of NF, indicating the specificity of the obtained fluorescence signal for eNOS-derived NO. Incubation with PMA (2 nM) for 18 h, which increases NO release due to upregulation of eNOS as well as downregulation of protein kinase C (PKC), was used as a positive control. Similar results were obtained in BAoEC, in which 100 µM NF increased endothelial NO release by approximately 40%. Cell viability—determined by trypsin blue exclusion—remained higher than 95% after NF treatment (data not shown).

3.2 Norfuraneol enhances eNOS activity in a time-dependent manner

Increased NO production may be the consequence of enhanced eNOS gene transcription resulting in higher eNOS protein levels. However, we were not able to detect any significant differences in eNOS protein levels after NF-treatment for 24 h (see Supplementary material online, Figure S1). Next we examined the kinetics of the NF-induced eNOS activation. EA.hy926 cells stimulated with NF for different periods of time (30 min to 24 h) were subjected to the [14C]-L-arginine/[14C]-citrulline conversion assay (Figure 2A). As early as 2 h after NF treatment endothelial [14C]-citrulline production was significantly increased (P<0.05) and remained elevated for at least 24 h (P<0.01). Cycloheximide (10 µg/mL), an inhibitor of protein biosynthesis, was not able to block this increase (Schmitt, unpublished observation, 2008) indicating that de novo synthesis of eNOS and/or an activating eNOS-associated protein, such as heat shock protein 90, is not required for the action of NF. These results suggest that NF has a direct and immediate effect on eNOS activity.

3.3 Norfuraneol promotes dephosphorylation at eNOS-threonine 495

eNOS is tightly regulated by phosphorylation of specific amino acid residues. Phosphorylation at serine<sup>1477</sup> (human sequence) as well as dephosphorylation at the constitutively phosphorylated threonine<sup>495</sup> are both known to increase...
Norfuranol and eNOS

3.4 Effect of norfuranol in the presence of protein kinase C inhibitors

PKC is considered to be the predominant kinase acting on eNOS-Thr495.1,34 To study whether inhibition of PKC may account for the observed decrease in eNOS-Thr495 phosphorylation we treated endothelial cells with the pan-PKC inhibitors CAC35 and GFX.36 Incubation with CAC (0.25–1 μM) for 1.5 h led to gradual dephosphorylation at eNOS-Thr495 (Figure 3A). Addition of NF during the last 60 min decreased the phosphorylation level even further (Figure 3A), suggesting that NF either intensified PKC inhibition or activated an alternative pathway promoting eNOS-Thr495 dephosphorylation. Similarly, treatment with GFX (10 μM) mimicked the effect of NF although co-stimulation with NF did not lower eNOS-Thr495 phosphorylation additionally (Figure 3A). Pre-treatment of EA.hy926 cells with NF also significantly decreased the phosphorylation of eNOS-Thr495, completely abolished the effect of NF and completely prevented NF-induced dephosphorylation (Figure 4A). Basal phosphorylation levels were lowered by 200 nM OKA at eNOS-Thr495 but did not enhance NF-induced dephosphorylation (Figure 4B). CYA is a phosphatase inhibitor with specificity towards PP1.40 Pretreatment of cells with 1 nM CYA, which did not affect basal phosphorylation levels at eNOS-Thr495, completely abolished the effect of NF (Figure 4A). Consistent results were obtained using 5 nM CYA, which strongly increased basal eNOS-Thr495 phosphorylation (Figure 4B) and TAU (0.1–1 μM) as an alternative PP1 inhibitor (Figure 4C).41 Since these results indicate that PP1 might be the mediator of NF-induced dephosphorylation at eNOS-Thr495, we further investigated the role of PP1 in this process by the silencing of PP1 in HUVEC and EA.hy926 cells using siRNA. The silencing of PP1 enhanced basal phosphorylation levels at eNOS-Thr495, confirming that PP1 may mediate the effect of NF and underlining the important role of PP1 in the regulation of eNOS-Thr495 phosphorylation.40

3.5 Inhibition and silencing of protein phosphatase 1 blocks norfuranol-induced eNOS-threonine 495 dephosphorylation

Since we have seen that NF can synergize with PKC inhibitors regarding the phosphorylation of eNOS-Thr495, we further investigated whether NF promotes dephosphorylation via activation of phosphatases acting on eNOS-Thr495. Protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and calcineurin (PP2B) have been described to be involved in the dephosphorylation of this site (reviewed in Mount et al.34). We applied specific phosphatase inhibitors and investigated whether these affect the ability of NF to induce dephosphorylation of eNOS-Thr495. CSA, an inhibitor of PP2B,39 did neither alter the basic phosphorylation level of eNOS-Thr495 nor could pre-treatment with CSA block the effect of NF (data not shown) denying a major role of PP2B for the action of NF. OKA (100 nM), which selectively inhibits PP2A,32,40 was not able to block the action of NF (Figure 4A). Basal phosphorylation levels were lowered by 200 nM OKA at eNOS-Thr495 but did not enhance NF-induced dephosphorylation (Figure 4B). CYA is a phosphatase inhibitor with specificity towards PP1.40 Pretreatment of cells with 1 nM CYA, which did not affect basal phosphorylation levels at eNOS-Thr495, completely abolished the effect of NF (Figure 4A). Consistent results were obtained using 5 nM CYA, which strongly increased basal eNOS-Thr495 phosphorylation (Figure 4B) and TAU (0.1–1 μM) as an alternative PP1 inhibitor (Figure 4C).41 Since these results indicate that PP1 might be the mediator of NF-induced dephosphorylation at eNOS-Thr495, we further investigated the role of PP1 in this process by the silencing of PP1 in HUVEC and EA.hy926 cells using siRNA. The silencing of PP1 enhanced basal phosphorylation and completely prevented NF-induced dephosphorylation of eNOS-Thr495, confirming that PP1 may mediate the effect of NF and underlining the important role of PP1 in the regulation of eNOS-Thr495 phosphorylation.40

3.6 Norfuranol does not alter cellular reactive oxygen species production or NAD(P)H oxidase 4 expression

There are data indicating that dephosphorylation of eNOS-Thr495 may lead to eNOS uncoupling, forcing eNOS to

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Figure 3  Effect of norfuraneol (NF) in the presence of protein kinase C (PKC) inhibitors. (A) EA.hy926 cells were pretreated with the pan-PKC inhibitors calphostin C (CAC, 0.25–1 μM) or bisindolylmaleimide I (GFX, 10 μM) for 30 min. Control cells received an equal amount of solvent [0.1% dimethyl sulphoxide (DMSO)]. NF (100 μM) was added for 1 h before subjecting cells to western blot analyses for (phospho-)eNOS and tubulin. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and detected by immunoblot. One representative blot is shown. Comparable results were obtained in five separate experiments. (B) EA.hy926 cells were pretreated with 100 μM NF for 1–2 h, stimulated with the PKC activator Phorbol-12-myristate-13-acetate (PMA) (300 nM) or DMSO (0.1%) for 15 min and harvested for detection of eNOS protein by western blot analyses. One representative blot is shown. Band intensities were normalized to tubulin (*P < 0.05; ns, not significant) (mean ± SEM, n = 4). (C) Human umbilical vein endothelial cells were pretreated with NF (100 μM) or GFX (10 μM) for 1 h before stimulation with PMA (2 nM) for further 60 min. During the last 30 min, cells were loaded with 2',7'-dichlorodihydrofluorescein diacetate [H2DCF-DA (20 μM)] to allow assessment of intracellular reactive oxygen species (ROS) levels by subsequent flow cytometric analysis (*P < 0.05; ns, not significant) (mean ± SEM, n = 3).

Figure 4  Influence of phosphatase inhibitors and silencing of protein phosphatase 1 (PP1) on norfuraneol (NF)-induced eNOS-Thr495 dephosphorylation. (A) EA.hy926 cells were pretreated with the PP1 inhibitor calyculin A (CYA, 1 nM), the protein phosphatase 2A (PP2A) inhibitor okadaic acid (OKA, 100 nM) or dimethyl sulphoxide (DMSO, 0.1%) for 30 min. NF (100 μM) was added for 1 h and cells were harvested for the detection of eNOS protein by western blot analyses. One representative blot is shown. Consistent results were obtained in five separate experiments. (B) EA.hy926 cells were treated as in panel A but with higher concentrations of CYA (5 nM) or OKA (200 nM). One representative blot is shown. Comparable results were obtained in five separate experiments. (C) EA.hy926 cells were pretreated with the PP1 inhibitor tautomycin (TAAU, 0.1–1 μM) for 30 min. Control cells received an equal amount of solvent (0.1% DMSO). NF (100 μM) was added for 1 h before subjecting cells to western blot analyses of (phospho-)eNOS and tubulin levels. One representative blot is shown. Similar results were obtained in five separate experiments. (D) Human umbilical vein endothelial cells or EA.hy926 cells were treated with siRNA against PP1 (siPP1) or scrambled control siRNA (scr). 72 h after transfection cells were incubated with NF (100 μM) for 2 h and cells were harvested for the detection of (phospho-)eNOS, PP1, and tubulin protein levels by western blot.
produce superoxide instead of NO. As a result, essential eNOS cofactors as well as NO itself are degraded and a vicious circle of further eNOS uncoupling follows. We therefore determined whether long-term treatment with NF affected cellular ROS production. Incubation of cells with NF for 24 h did not alter total cellular ROS production (Supplementary material online, Figure S2A). PMA, which is known to enhance ROS production in endothelial cells, significantly increased, whereas the flavoprotein inhibitor diphenyleneiodonium chloride decreased ROS (P < 0.01 and P < 0.001, respectively). NAD(P)H oxidases are major sources of ROS and isoform 4 (Nox4) is the most abundantly expressed in endothelial cells. Since Nox4 can be upregulated under conditions of oxidative stress, we assessed Nox4 mRNA expression. In line with the previous findings, treatment with NF for 24 h did not affect Nox4 expression in endothelial cells (Supplementary material online, Figure S2B), whereas PMA more than doubled Nox4 mRNA levels (P < 0.05).

3.7 Norfuraneol shows promising pharmacokinetic properties in silico

In order to get a first insight in the pharmacokinetic profile of NF we ran in silico calculations using VoSurf + software. It can predict certain pharmacokinetic parameters of compounds based on the comparison with profiles of a collection of >500 related, but chemically diverse entities. For NF, the software predicted a high concentration of free drug in the plasma because only 31% of it may bind to human serum albumin (HSA). Usually, the range of HSA binding lies between 10% and 100% depending on the compound. In a VD model NF scored −0.38 which translates into high achievable concentrations in tissues. In the metabolic stability model, NF obtained a value of 0.98. This means that NF remained practically unmetabolized after 60 min of incubation at 37°C with a defined concentration of CYP3A4-enriched microsomal proteins (Table 1).

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<th>PB (%)</th>
<th>−log (VD)</th>
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<td>31</td>
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NF was subjected to in silico calculations using VoSurf software. Results obtained with models for protein binding (PB), volume distribution (VD), and metabolic stability (MetStab) are depicted. Details concerning the software and its models can be found in the (supplemental) methods section.

Table 1 in silico predictions for the pharmacokinetic profile of norfuraneol (NF)

NF-mediated dephosphorylation at Thr495, however, was not accompanied by a significantly increased phosphorylation status at Ser1177, a feature that is—to the best of our knowledge—not yet reported for a compound modulating eNOS activity.

Chemical inhibition of PP1 with CYA and TAU or silencing of PP1 protein via siRNA completely abolished NF-induced eNOS-Thr495 dephosphorylation, suggesting a critical role of PP1 in this process. A complete reversal of the effect of NF was achieved at a moderate CYA concentration which did not affect basal eNOS phosphorylation. Although both PP1 and PP2A are found to associate with eNOS, each phosphatase appears to act preferentially on a specific eNOS phosphorylation site. Whereas PP2A is mainly responsible for dephosphorylation of Ser1177, PP1 is the dominant phosphatase acting on Thr495. It therefore seems plausible that the effect caused by NF may involve an increase of PP1 activity which contributes to the ensuing dephosphorylation at Thr495. The crucial role of PP1 for the regulation of eNOS-Thr495 phosphorylation is also supported by our experimental data, as downregulation of PP1 or its inhibition had profound effects on basal phosphorylation levels at this amino acid residue.

Inhibition of PP2A by OKA did not prevent dephosphorylation at Thr495 in response to NF. Application of OKA without NF even lowered basal Thr495 phosphorylation. Although this may seem surprising because PP2A has been implicated in the dephosphorylation at Thr495, similar findings have been reported before.

PKC is considered to be the predominant kinase acting on eNOS-Thr495, although the specific PKC isoform is unknown. Inhibition of PKC with CAC or GFX mimicked the effect of NF, raising the question whether PKC inhibition contributes to the action of NF. However, as opposed to the PKC inhibitor GFX, NF failed to block the PMA-induced and PKC-mediated ROS production in HUVEC and EA.hy926, arguing against a direct inhibitory action of NF on PKC, although we cannot exclude the possibility of a PKC isoform-specific effect of NF. Furthermore, addition of NF to CAC-treated cells potentiated the decrease in eNOS-Thr495 phosphorylation level, which may also indicate the induction of an alternative pathway promoting eNOS-Thr495 dephosphorylation, such as activation of PP1. Although our experiments clearly demonstrate that NF decreases eNOS-Thr495 phosphorylation likely via a PP1-mediated mechanism, we cannot exclude that NF is also affecting other phosphorylation sites known to regulate eNOS activity.

It remains open how NF promotes PP1 activation. It is important to emphasize that at this point we cannot discriminate between a direct increase of the enzymatic activity of PP1 by NF or any indirect effect that increases the impact of PP1 on eNOS phosphorylation (e.g., alteration of interaction partners, cellular localization, or substrate availability by NF). PP1 activity is regulated via interaction of the catalytic subunit with over 50 regulatory subunits, but no redox-sensitive way of PP1 regulation has been identified so far. Interestingly, dephosphorylation at eNOS-Thr495 is also observed upon treatment with hydrogen peroxide, suggesting that a yet unknown redox-sensitive
pathway may regulate the phosphorylation status at that site. In this context we also tested whether NF might alter endogenous endothelial hydrogen peroxide production but did not detect any changes within the first 2 h after treatment with NF, the time frame when dephosphorylation of eNOS-Thr495 occurred (data not shown).

In a study by Lin et al.,34 dephosphorylation of Thr495 has been associated with eNOS uncoupling, although these results have been challenged recently.46 The resulting vicious circle of increased superoxide and decreased NO production plays an important role in the development of vascular diseases.1 We have demonstrated that treatment over 24 h with NF had no influence on total ROS production and did not alter the expression of Nox4, a prominent source of ROS in endothelial cells. Furthermore, we measured increased endothelial NO release 24 h after incubation with NF. Thus, we consider it unlikely that a treatment with NF and the ensuing dephosphorylation of eNOS-Thr495 lead to eNOS uncoupling or enhanced oxidative stress under our culture conditions.

Although NF is produced by thermal treatment of certain pentoses and has the potential to be taken up regularly with food, there are no data available yet regarding its absorption or metabolism. In contrast to some other bioactive dietary compounds, such as resveratrol, NF seems to be more abundantly present, at least in cooked foods since it is a breakdown product of ubiquitously present pentoses. In silico quantitative modelling predicts NF to possess low affinity for HSA and to resist metabolic conversion by CYP3A4. These properties suggest a good availability of NF in blood plasma potentially leading to high concentrations in tissues. A strong tendency towards tissue accumulation was further confirmed in a VD model. We therefore believe that the NF concentration used in our study (as low as 30 µM) is not necessarily beyond achievable levels in humans. Although very promising, such theoretical calculations cannot substitute a thorough pharmacokinetic analysis in vivo. It further remains to be seen whether NF also exerts the described effects in isolated vessels or has beneficial effects on endothelial function in vivo.

Taken together, we have shown that NF, a compound found in relevant amounts in cooked food, is able to increase endothelial NO production and eNOS activity in cultured human endothelial cells presumably due to specific dephosphorylation of eNOS at Thr495 via a mechanism involving predominantly the activation of PKP. An additional inhibition of PKC by NF, however, cannot be ruled out at the moment. The specific targeting of eNOS-Thr495 seems to be a so far unique pharmacological property. In silico, NF exerts stability towards CYP3A4 metabolism, low PB, and high tissue distribution. If these findings can be confirmed in vivo, NF might represent a promising dietary compound with vaso-protective properties.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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