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Serum nitrite sensitively reflects endothelial NO formation in human forearm vasculature: evidence for biochemical assessment of the endothelial L-arginine–NO pathway

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Abstract

Objective: A reduced bioactivity of endothelial nitric oxide (NO) has been implicated in the pathogenesis of atherosclerosis. In humans, the endothelial L-arginine–NO pathway has been indirectly assessed via the flow response to endothelium-dependent vasodilators locally administered into the coronary, pulmonary or forearm circulation. However, biochemical quantification of endothelial NO formation in these organ circulations has been hampered so far because of the rapid metabolism of NO. Therefore, we aimed to work out a reliable biochemical index to assess endothelial NO formation in human circulation. **Methods:** In 33 healthy volunteers, forearm blood flow (FBF) was measured by standard techniques of venous occlusion plethysmography at rest, after local application of the endothelium-dependent vasodilator acetylcholine (ACH), the endothelium-independent vasodilator papaverine (PAP), the stereospecific inhibitor of endothelial NO synthase (eNOS) L-NMMA, and L-arginine (ARG), the natural substrate of eNOS. In parallel, nitrite and nitrate concentrations in blood samples taken from the antecubital vein were measured by HPLC using anion-exchange chromatography in combination with electrochemical and ultraviolet detection following a specific sample preparation method. **Results:** ACH dose-dependently increased resting FBF (from 3.0±0.3 to 10.4±0.9 ml/min per 100 ml tissue) and serum nitrite concentration (from 402±59 to 977±82 nmol/l, both $p<0.05$, $n=12$). A significant correlation was observed between the changes in FBF and the serum nitrite concentration ($r=0.61$, $p<0.0001$). L-NMMA reduced resting FBF and endothelium-dependent vasodilation by 30% and this was paralleled by a significant reduction in serum nitrite concentration at the highest dose of ACH ($n=9$, $p<0.001$). PAP increased FBF more than fourfold, but did not affect serum nitrite concentration ($n=11$), whereas ARG significantly increased both FBF and nitrite. Basal serum nitrate amounted to 25±4 μmol/l and remained constant during the application of ACH, PAP and L-NMMA. **Conclusions:** The concentration of serum nitrite sensitively reflects changes in endothelial NO formation in human forearm circulation. This biochemical measure may help to characterize the L-arginine–NO pathway in disease states associated with endothelial dysfunction and to further elucidate its pathophysiological significance for the development of atherosclerosis in humans.

Keywords: Endothelium-derived factors; Nitrite oxide; Acetylcholine; L-Arginine

1. Introduction

Nitric oxide (NO) is a widespread signalling molecule involved in numerous biological functions, such as regulation of vascular tone, immune response and neurotransmission (for review, see [1]). At baseline, NO is formed continuously by endothelial NO synthase (eNOS) in the low nanomolar concentration range. Disturbance of eNOS is suggested to play a key role in vascular dysfunction and the development of atherosclerotic lesions associated with arterial hypertension, hypercholesterolemia and diabetes mellitus [2–5]. For this, the measurement of endothelium-derived NO or its metabolites in the human circulation appears highly desirable. However, the rapid metabolism of NO and its major metabolite nitrite as well as technical

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limitations in the analysis of these short-lived molecules has hampered biochemical assessment of the endothelial L-arginine NO pathway in humans to date.

In the presence of oxygen, NO is rapidly oxidized, via dinitrogen trioxide, to nitrite following pseudo-first order kinetics, with a strict 1:1 stoichiometry [6,7]. Therefore, nitrite, which is stable for hours in aqueous solutions, has frequently been used as a marker of NO production in saline perfused experimental settings. We and others have shown that, in human blood, nitrite, although stable for up to several hours in plasma, is rapidly converted to its end product nitrate within the erythrocytes ([8,9] and for review, see [10]). As yet, this rapid conversion hampered the detection of nitrite in whole blood, although it might represent a specific, though short-lived, intermediate metabolite of NO. Therefore, we aimed to measure nitrite levels in blood samples taken from the antecubital vein with a recently developed analytical technique [11], which allows one to detect nitrite highly sensitively, down to the low nanomolar concentration range, in combination with a sample preparation method that prevents further degradation of nitrite during the sampling procedure. In order to investigate whether nitrite represents an active measure of eNOS activity in human forearm circulation, forearm blood flow and nitrite levels were determined at baseline, after stimulation and stereospecific inhibition of the endothelial L-arginine NO pathway.

2. Methods

2.1. Subjects

The final study sample consisted of 33 healthy volunteers who were screened by clinical history, physical examination and routine chemical analysis. None of these subjects revealed present or past evidence of cardiovascular diseases, known to affect endothelial function such as hypertension, hypercholesterolemia, chronic heart failure, or diabetes mellitus. Participants in the study were instructed to refrain from smoking cigarettes or drinking alcohol and beverages containing caffeine for at least 12 h before the investigation. The protocol of the study was approved by the ethics committee of the Heinrich-Heine-University and all subjects gave written informed consent before participating in the study.

2.2. Forearm blood flow measurements

Forearm blood flow (FBF in ml/min per 100 ml tissue) was measured simultaneously in both arms by standard techniques using mercury-in-rubber strain-gauge plethysmography (Periquant 833, Gutmann, Eurasburg, Germany), described in detail elsewhere [12,13]. For each determination, five measurements of FBF were performed and the results were averaged. A 2F catheter was inserted into the brachial artery for local drug infusion and a 4F catheter was inserted into the antecubital vein for blood sampling. After measurement of resting control values of FBF (>20 min after cannulation), acetylcholine (1 to 10 μg/min) was infused, to stimulate, and L-NMMA (8 μmol/min), to competitively inhibit, endothelial NO synthase in the forearm circulation. Bolus injections of papaverine (3 μmol) were used as a negative control to achieve maximal dilation of resistance arteries independent of the activity of endothelial NO synthase. The natural substrate of eNOS, L-arginine (200 μmol/min), was infused to increase endogenous NO availability and D-arginine served as a stereospecific negative control.

2.3. Measurement of nitrite and nitrate

Analysis of nitrite and nitrate was performed with a HPLC system using anion-exchange chromatography, which is described in detail elsewhere [11]. Briefly, blood samples taken from the deep antecubital vein were immediately mixed with equal amounts of sodium hydroxide (0.1 mol/l) and thereafter the pH was adjusted to 7.0 by the addition of a standard volume of phosphoric acid (1 mol/l). After centrifugation (14 000 *g*), the supernatant was ultrafiltered (cut-off 10 kDa). The resulting ultrafiltrate was injected into the HPLC system, which consisted of a Sykam S1000 pump, a Jasco 851-AS autosampler (Gross-Zimmern, Germany) with a Rheodyne injection valve 9010 (Cotati, CA, USA), and an anion-exchange column LC A08 125×4.6 mm I.D. (Sykam, Gilching, Germany). Within the same analytical run, nitrate was detected by an UV detector (Linear Instruments UVIS 204, Reno, NE, USA) and nitrite by an electrochemical detector, connected in line (ESA Coulochem 5200A, Chelmsford, MA, USA). Data acquisition and integration were performed using the Pyramid Chromatography System (version 1.923, Axxiom, Moorepark, CA, USA). The specificity (peak identification), reproducibility (coefficient of variance <8%) and sensitivity (10 nmol/l for nitrite and 1 μmol/l for nitrate) of this assay has been evaluated previously [11].

2.4. Statistical analysis

Data processing was performed with the software packages SPSS® (Statistical package for analysis in social sciences, release 5.0.1., SPSS Inc., Chicago, IL, USA). Data are expressed as the mean±SEM unless otherwise stated and two-sided p-values of <0.05 were considered to be significant. A two-way analysis of variance for repeated measures with consecutive post-hoc tests was used to test for differences from control concerning the dose-dependent changes in FBF and nitrite during infusion of acetylcholine and L-NMMA. Univariate linear regression models (Spearman rank) were performed to evaluate the relationship between changes in FBF and nitrite.

3. Results

The study population consisted of 31 men and two women (age: 24±2 years, body length: 177±8 cm, body weight 69±8 kg). At the time of investigation, heart rate was determined to be 75±7 min^{-1} and mean arterial blood pressure to be 86±10 mmHg. At the concentrations indicated, neither acetylcholine, papaverine or L-NMMA exerted systemic vasoactive effects, as was assessed by the measurement of blood pressure, heart rate and FBF in the control arm. The resting level of serum nitrate amounted to 25±4 μmol/l. This concentration did not significantly change during infusion of acetylcholine, L-NMMA or papaverine.

In a first series of experiments, baseline levels for nitrite were measured in parallel in the brachial artery and in the antecubital vein and were determined to be 411±62 and 402±59 nmol/l, respectively (n=12, total range of nitrite levels: 127–748 nmol/l). In four of these twelve individuals, the venous concentration of nitrite was higher compared to the arterial levels, whereas in the other eight individuals, the levels of nitrite were found to be higher in the brachial artery compared to the antecubital vein. Acetylcholine (ACH) dose-dependently increased FBF from 3.0±0.3 to 10.4±0.9 ml/min per 100 ml tissue (see Fig. 1). In parallel, the venous concentration of serum nitrite increased to 977±82 nmol/l. Serum nitrite concentration correlated significantly with the FBF; individual data points are given in Fig. 2 (r=0.61, p<0.0001). In two further individuals, the kinetics of ACH-induced changes in nitrite concentration and FBF were determined at 15 s intervals during infusion of 10 μg/min ACH for 1 min. The time course of changes in nitrite concentration closely paralleled the changes in FBF (see Fig. 3).

Arterial nitrite levels remained unaffected during infusion of ACH. Consequently, the arteriovenous difference for nitrite across the forearm circulation, that is, venous nitrite corrected for nitrite entering from the arterial side, increased to 134±41, 276±47 and 557±51 nmol/l at doses of 1, 3 and 10 μg/min of ACH, respectively. The ACH-induced formation of nitrite was estimated as the product of FBF (normalized to 100 ml of tissue) and the arteriovenous difference in nitrite concentration with 0.6±0.2, 1.9±0.4, 5.8±0.6 nmol/min nitrite. The correlation between ACH-induced changes in FBF and the formation of nitrite in the forearm circulation amounted to r=0.89, p<0.0001.

In a separate series of investigations, L-NMMA reduced FBF and serum nitrite concentration at baseline: from 2.7±0.6 to 1.8±0.4 ml/min per 100 ml of tissue and from 268±41 to 157±38 nmol/l (p=0.056 and 0.059, respectively, n=9; total range of nitrite levels, 85–631 nmol/l). ACH-induced changes in FBF and nitrite concentration were significantly blunted (see Fig. 4). The maximum flow response significantly decreased from 8.5±0.75 to 6.2±0.9 ml/min/100 ml of tissue and, in parallel, the increase in nitrite concentration was reduced by more than 51% (n=9, p<0.001). FBF was significantly correlated with nitrite concentration in the presence and absence of L-NMMA. The slope of regression between changes in FBF and in serum nitrite was not statistically altered by L-NMMA. The intravenous application of acetylic acid (500 mg) did not affect changes in either FBF or in nitrite (n=3, data not shown).

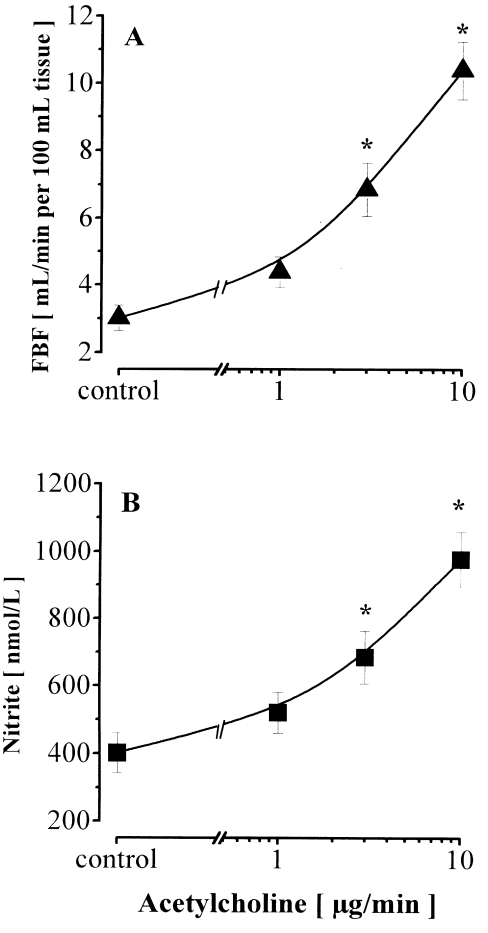

Fig. 1. Changes in forearm blood flow (FBF) (A) and serum nitrite concentration (B) in blood samples taken from the antecubital vein at baseline (control) and during increasing doses of acetylcholine, n=12, * indicates a significant difference from control.

In another set of experiments, papaverine increased FBF by more than fourfold, which was comparable to the maximum vasodilation observed upon infusion of acetylcholine (n=11). In contrast, the venous nitrite concentration remained almost identical (see Fig. 5). Thus, the rate of NO formation in forearm vasculature was calculated to be only 0.06 nmol/min during administration of papaverine, but 4.6 nmol/min during administration of acetylcholine (n=11). L-Arginine (200 μmol/min) increased significantly both FBF (2.5±0.3 ml/min per 100 ml of tissue) by 75±15% and venous nitrite concentration (398±53 nmol/l) by 322±72% (n=3), whereas D-arginine (200 μmol/min) did not affect either parameter (n=2).

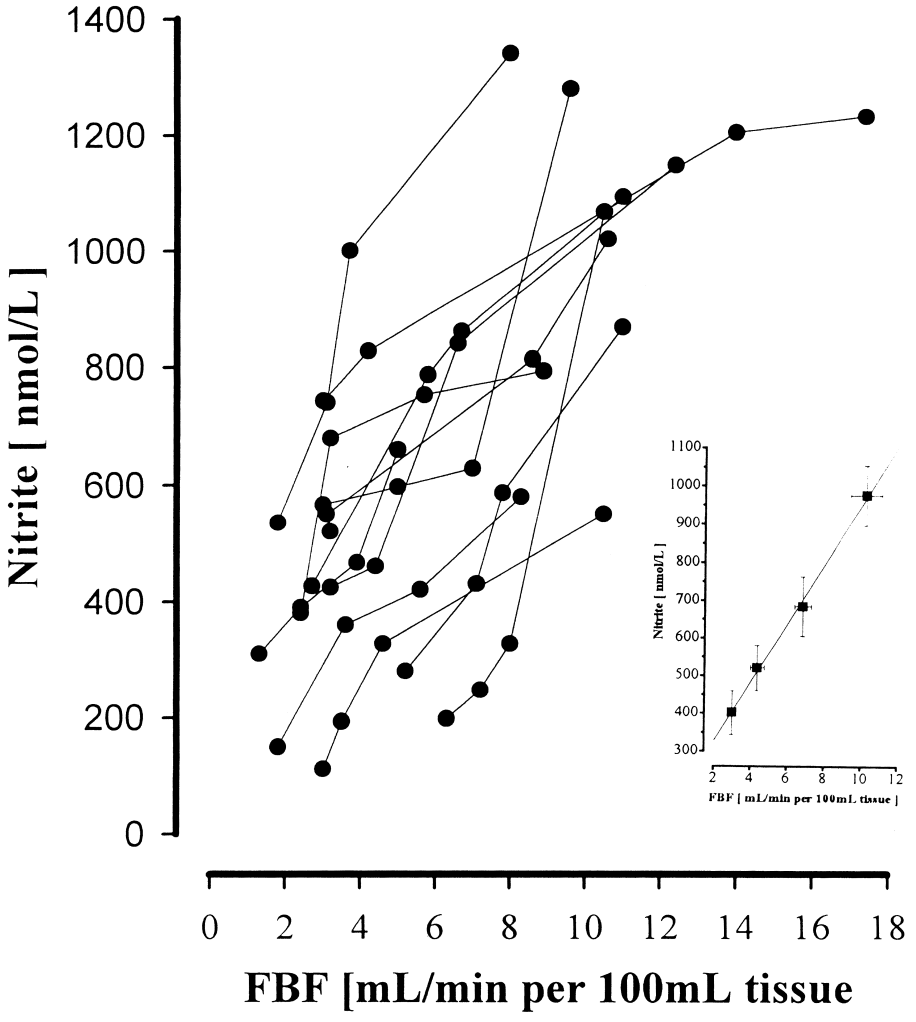

Fig. 2. Relationship between forearm blood flow (FBF) and serum nitrite concentration. Responses in individual subjects during increasing doses of acetylcholine (1, 3, and 10 μg/min), as given in Fig. 1 ($n=12$), are depicted. Regression analysis for single data points: $r=0.61$, $p<0.0001$. Inset: relationship between mean values of FBF and nitrite levels.

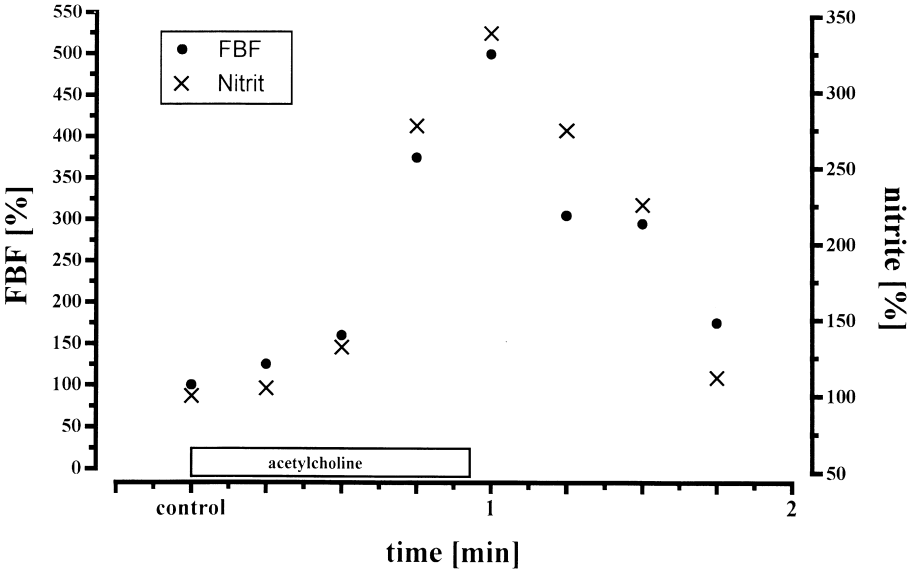

Fig. 3. Kinetics of ACH-induced changes in FBF and serum nitrite concentration in a representative individual. ACH was infused for 1 min at a dose of 10 μg/min.

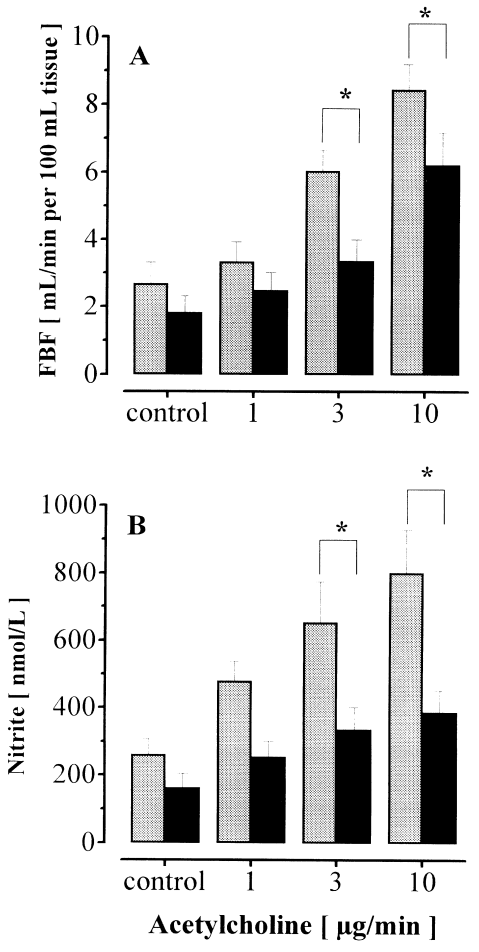

Fig. 4. Effects of inhibition of endothelial NO formation by L-NMMA on forearm blood flow (FBF) (A) and serum nitrite concentration (B) at baseline and during infusion of increasing doses of acetylcholine (n=9). Open bars indicate measurements without L-NMMA and closed bars those in the presence of 8 µmol/min L-NMMA; * indicates a significant difference from control.

4. Discussion

The present data clearly demonstrate that serum nitrite reliably reflects changes in endothelial NO formation in the human forearm vasculature. Thus, nitrite is likely to be a reliable biochemical measure of the activity of the endothelial L-arginine–NO pathway in human circulation. Several criteria for chemical transmitters involved in the regulation of blood flow have been established [14]. Accordingly, a diagnostic marker for the L-arginine NO pathway should fulfill the following criteria: (i) the marker should be quantitatively detectable in the expected physiological concentration range of the signalling molecule, (ii) the marker should sensitively reflect the substrate and the receptor-mediated stimulation and the stereospecific inhibition of the L-arginine NO pathway and (iii) it should not be affected by unspecific vasodilation.

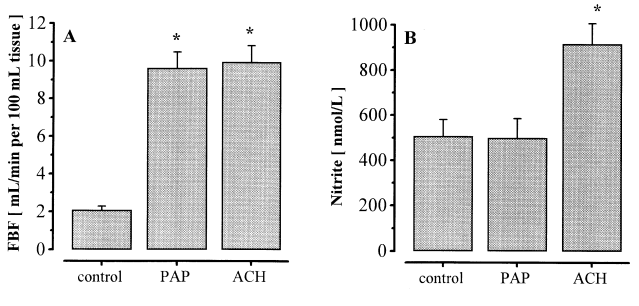

Fig. 5. Comparison of the effects of papaverine (3 µmol) and acetylcholine (10 µg/min) on forearm blood flow (FBF) (A) and serum nitrite concentration in the antecubital vein (B), n=11, * indicates a significant difference from control.

4.1. Nitrite as an index of endothelial NO formation

In experimental settings using either the hemoglobin assay, the chemiluminescence or the porphyrinic sensor, the concentration of NO at the luminal site of vascular endothelium has been reported to be in the low micromolar concentration range in different vascular beds or isolated vessels [15–17]. Our own data on the level of serum nitrite in human forearm vasculature are in line with the concentration range of NO expected from these animal models. Recently, the local concentration of NO in hand veins has been determined in a comparable range of 0.1 to 0.3 µmol/l [18].

Basal NO formation in endothelial cells has been shown to participate in setting the resting tone of resistance vessels and thus regulating arterial blood pressure [19]. The specificity of serum nitrite as an indicator of basal NO formation in human forearm circulation was tested by either application of L-NMMA, to inhibit the basal activity of eNOS, or by application of L-arginine, the natural substrate for eNOS. Inhibition of eNOS resulted in a reduction of the basal serum nitrite concentration, which was paralleled by a marked vasoconstriction, suggesting that at least a substantial proportion of the basal nitrite level is attributable to endothelial NO formation. This finding complements previous work by Stamler et al. [20] showing that the L-arginine:NO pathway is involved in the regulation of resting vascular tone in human circulation. In addition, we have demonstrated that the application of L-arginine, the natural substrate of eNOS, stereospecifically increased basal serum nitrite and in parallel dilated resistance arteries in forearm circulation. The only modest increase in FBF in response to L-arginine complements previous findings in human forearm vasculature [21].

Based on the pioneering work of Furchgott and Zawadzki [22], the so-called acetylcholine test was introduced in various clinical studies to assess endothelial function in vivo [23]. In human forearm circulation, acetylcholine is widely accepted as an endothelium-dependent vasodilator, which is assumed to dilate resistance arteries via the receptor-mediated activation of eNOS [24–26]. In other species and vascular beds, it has been demonstrated that, apart from NO, prostaglandins and the

endothelium-derived hyperpolarizing factor (EDHF) are involved in acetylcholine-induced vasodilation [27–29]. In our study, acetylcholine dose-dependently increased serum nitrite and this was paralleled by a dose-dependent vasodilation. The finding of a significant correlation between changes in FBF and serum nitrite concentration supports the view that serum nitrite reliably reflects changes in endothelial NO formation. This notion is further strengthened by the observation that the time course of acetylcholine-induced changes in nitrite closely paralleled the changes in FBF. The specificity of serum nitrite as an index of eNOS activity is further complemented by our finding that the competitive, stereospecific inhibitor of eNOS (L-NMMA) reduced both FBF and serum nitrite. The acetylcholine-induced changes in FBF and nitrite were not entirely inhibited by L-NMMA, most likely because the applied dose was not sufficiently high to completely block endothelial NO synthesis. Alternatively, a part of the acetylcholine-induced vasodilation could be mediated not only by NO, but also by EDHF. The regression analysis between FBF and serum nitrite was similar in the presence and absence of L-NMMA in human forearm vasculature, which underlies the competitive mode of eNOS inhibition by L-NMMA described previously [30]. The fact that the nitrite level increased more than twofold despite a threefold increase in flow almost entirely rules out the possibility that the changes in nitrite merely reflect a washout phenomenon due to increases in flow independent from the activity of eNOS. Interestingly, the ratio of maximum to basal serum nitrite as an index of acetylcholine-induced stimulation of eNOS amounts to about 2.5, which fits perfectly the ratio of basal and stimulated NO formation in cultured endothelial cells or in saline perfused isolated rodent hearts, in which NO was measured directly [6,15].

The interpretation that the serum concentration of nitrite reflects the activity of the endothelial L-arginine–NO pathway is further supported by the finding that the endothelium-independent vasodilator papaverine increased FBF to a comparable extent as seen in response to acetylcholine but without affecting the serum concentration of nitrite in the antecubital vein. If endothelium-derived NO was released at a constant rate into the vascular lumen, the venous nitrite concentration would be expected to decrease during papaverine administration. Theoretically, this effect could be counterbalanced by a reduced removal of nitrite in a given blood volume because of a shorter passage time through forearm vasculature during papaverine-induced vasodilation, compared to baseline. However, this appears rather unlikely, taking into account the half-life of nitrite in human blood (105 s), which exceeds the passage time through the forearm vasculature (at rest, less than 4 s) by a factor of >25.

4.2. Nitrate as an index of endothelial NO formation

Sophisticated gas chromatography/mass spectrometry techniques using labeling of the circulating nitrate pool with either ^{15}N or ^{18}O isotopes may be helpful in assessing whole-body NO production via urine and plasma analysis, although this approach does not allow one to distinguish between vascular and nonvascular sources of NO formation [31,32]. Furthermore, the serum level of nitrate appears to be an unreliable measure of regional changes in eNOS activity, as this parameter is influenced by a variety of eNOS-independent factors, such as dietary nitrate intake, formation of saliva, bacterial synthesis of nitrate within the bowel, denitrifying liver enzymes, inhalation of atmospheric gaseous nitrogen compounds and renal function [10,33,34]. Furthermore, the natural abundance of nitrate with a rather high background concentration in human blood would hamper the exact measurement of changes in eNOS activity. Moreover, circulating nitrate, in contrast to the short-lived nitrite, reveals a rather long half-life, in the range of 3–4 h [35]. Thus, simple measures of plasma nitrate will not accurately reflect eNOS activity in humans, whereas this might be possible in rodents [36]. In line with these literature data, we did not find any significant alteration of nitrate concentration during the application of either acetylcholine, papaverine, L-arginine or L-NMMA.

4.3. Study limitations

Using ^{15}N-labelled L-arginine, it has been demonstrated in fasted young volunteers that almost all of the circulating nitrite is derived from the L-arginine–NO pathway [32]. Several lines of experimental evidence on the oxidative chemistry of NO revealed that the biological effects of endothelium-derived factor may not be mediated exclusively by NO, but in part also via the formation of closely related, short-lived adducts, such as N_2O_3, nitrosium ion, nitroxyl anion, peroxynitrite and nitrosothiols, depending on the surrounding redox conditions [37–41]. At present, the proportion of nitrite, determined in our study, that was derived from NO directly or its related adducts cannot be distinguished: Nitrite may arise from the reaction of endothelium-derived NO with molecular oxygen. Alternatively, in the presence of oxygen-derived radicals, NO may be released from the endothelium as peroxynitrite, which, in turn, can react with circulating thiols to form nitrosothiols. Furthermore, following the autoxidation of NO to N_2O_3, endothelium-derived relaxing factor may react with redox-activated thiols to form circulating nitrosothiols [39,41–43]. Serum nitrosothiols are likely to be destabilized in part during our sampling procedure and consecutively to form nitrite. In our study, considerable variation in the interindividual nitrite levels was observed, whereas the intraindividual changes in nitrite levels in relation to flow response, that is, the individual slope of each subject's curve in response to acetylcholine was rather low (see Fig. 2). Future studies are warranted to define the impact factors on variance in individual baseline levels of nitrite.

The serum concentration of nitrite might be affected not only by the endothelial synthesis of NO and its aforementioned oxidative pathways but also by the further metabolism of nitrite. Serum nitrite rapidly diffuses into erythrocytes where it is oxidated to nitrate in a temperature-, hemoglobin- and pH-dependent manner [8]. Therefore, the simple measurement of plasma nitrite ex vivo without preventing its rapid conversion to nitrate during blood sampling is unlikely to be a reproducible and accurate measure of endogenous NO synthesis [44]. In contrast, using our analytical approach, the period for blood sampling and preparation, to stop the conversion of nitrite, takes less than 5 s and allows subsequent sensitive detection of nitrite with a reproducible recovery in ex vivo specimens of human blood [11].

In general, the NO detected at the luminal surface of the endothelium may reflect only a relative index of total NO synthesized in the vascular endothelium [45]. Thus, in the present study, the serum nitrite concentration is likely to reflect only a part of the NO released to the luminal, but not of the NO released to the abluminal, side of the vascular endothelium. At least in saline perfused vessels and organs, there is compelling experimental evidence that, upon bradykinin-mediated eNOS stimulation, the NO detected at the luminal side reflects a constant proportion of total endothelial NO synthesis [15,17,46]. Very recent reports suggest that eNOS might be localized in different cellular compartments (cytosol, membrane and Golgi region) with different couplings to membrane signal transduction mechanisms [47–49]. Therefore, different modes of eNOS stimulation, e.g. substrate-, receptor- or calcium-mediated, may influence the ratio of NO formed by either the cytosolic or the membrane-bound fraction of eNOS and, thus, potentially may influence the ratio of luminally and abluminally released NO. Caveolar co-localization of eNOS and the arginine transporter CAT1 allows direct and preferential transport of extracellular arginine to the membrane-bound fraction of eNOS [49]. Extrapolating these experimental findings to the in vivo situation might explain our, as yet preliminary, observation that the application of L-arginine preferentially increased the luminal release of NO/nitrite, whereas abluminal release, as indicated by increases in FBF, is rather modest compared to that observed upon application of acetylcholine.

We cannot determine the extent to which the different endothelial cells along the vascular tree contribute to the signal of serum nitrite measured in samples taken from the antecubital vein. Immunhistological and functional studies indicate that the major site of endothelial NO formation is located in conduit and resistance arteries, to a lesser extent, in the veins and to a negligible proportion in the capillaries [50,51]. In our study, the highly significant correlation of changes in FBF and nitrite concentration and the results of negative and positive controls obtained with L-NMMA, L- and D-arginine suggest that the changes in nitrite concentration most likely reflect changes in endothelial NO formation at the arterial site of human forearm circulation.

In conclusion, sensitive detection of nitrite with simultaneous inhibition of its conversion to nitrate during blood sampling provides an active measure to analyze endothelial NO formation in humans and thus to evaluate pharmacological interventions designed to specifically manipulate the endothelial L-arginine–NO pathway in patients with atherosclerotic diseases.

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