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Journal of Chromatography B, 814 (2005) 1–9
JOURNAL OF CHROMATOGRAPHY B
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Review

Recent methodological advances in the mass spectrometric analysis of free and protein-associated 3-nitrotyrosine in human plasma

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Received 5 July 2004; accepted 4 October 2004
Available online 11 November 2004

Abstract

L-Tyrosine and L-tyrosine residues in proteins are attacked by various reactive-nitrogen species (RNS) including peroxynitrite to form 3-nitrotyrosine (NO_2Tyr) and protein-associated 3-nitrotyrosine (NO_2TyrProt). Circulating NO_2Tyr and NO_2TyrProt have been suggested and are widely used as biomarkers of oxidative stress in humans. In this article the mass spectrometry (MS)-based analytical methods recently reported for the quantification of circulating levels of NO_2Tyr and NO_2TyrProt are discussed. These methodologies differ in sensitivity, selectivity, specificity and accessibility to interferences with the latter mainly arising from artifactual formation of NO_2Tyr and NO_2TyrProt during sample treatment such as acidification and chemical derivatization. Application of these methodologies to healthy normal humans revealed basal circulating levels for NO_2Tyr which range between 0.7 and 64 nM, i.e. by two orders of magnitude. Application of gas chromatography–tandem mass spectrometry (GC–tandem MS) methods by two independent research groups by using two different protocols to avoid artifactual nitration of L-tyrosine revealed almost identical mean plasma levels of the order of 1.0 nM in healthy humans. The lower limits of quantitation (LOQ) of these methods were 0.125 and 0.3 nM, respectively. This order of magnitude for basal NO_2Tyr is supported by two liquid chromatography–tandem mass spectrometry (LC–tandem MS) methods with LOQ values of 4.4 and 1.4 nM. On the basis of the data provided by GC–tandem MS and LC–tandem MS the use of a range of 0.5–3 nM for NO_2Tyr and of 0.6 pmol/mg plasma protein or a molar ratio of 3-nitrotyrosine to tyrosine in plasma proteins of the order of $1{:}10^6$ for NO_2TyrProt in plasma of healthy humans as reference values appear reasonably justified. Recently reported clinical studies involving 3-nitrotyrosine as a biomarker of oxidative stress are discussed in particular from the analytical point of view.

Keywords: Reviews; Biomarker; Oxidative stress

Contents

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doi:10.1016/j.jchromb.2004.10.003

1. Introduction

1.1. Circulating 3-nitrotyrosine as a biomarker of oxidative stress caused by reactive-nitrogen species

Reactive-nitrogen species (RNS), in particular oxides of nitrogen (NO_x) such as nitrogen monoxide (NO; nitric oxide) and nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and nitryl chloride (NO_2Cl), can readily react with L-tyrosine (Tyr) and protein-associated tyrosine (TyrProt) to form 3-nitro-L-tyrosine, i.e., NO_2Tyr and NO_2TyrProt, respectively (Fig. 1) (discussed in [1]). Besides the uncertainty of what species actually nitrates tyrosine in vivo [2], detection of NO_2Tyr and/or NO_2TyrProt may provide evidence for generation of RNS. RNS are continually formed in the human organism under physiological conditions. Our understanding of the biological significance of RNS in vivo in humans is mainly based on the determination of the concentration of NO_2Tyr and/or NO_2TyrProt in plasma. The highly divergent values so far reported for NO_2Tyr and NO_2TyrProt in human plasma at the basal state, even those provided by mass spectrometry (MS)-based methods which are generally accepted to have inherent specificity and accuracy, make establishment of standard values for NO_2Tyr and NO_2TyrProt very difficult ([3]; Table 1).

Accurate quantification of substances present in human plasma at very low concentrations is a challenging analytical task, but in particular NO_2Tyr offers special concerns [19]. Approaches to the analysis of NO_2Tyr and/or NO_2TyrProt and concerns of particular importance for their accurate quantitative determination have been critically discussed by Duncan [19]. In the present review the recent developments in the quantitative analysis of NO_2Tyr and NO_2TyrProt in human plasma at the basal state by MS-based analytical approaches are critically reviewed. The overall discussion ([19] and present work) leads to the conclusions: (1) that our knowledge of the physiological and pathological significance of tyrosine nitration in vivo in humans is confounded by severe methodological problems; (2) that at present only the tandem MS methodology provides reliable values for circulating NO_2Tyr, and that the basal levels obtained by this analytical approach may serve as reference values; (3) that some of the high levels and changes in plasma levels of NO_2Tyr and/or NO_2TyrProt observed in early work may not prove real; (4) and that only the use of thoroughly validated analytical approaches will ensure generation of reliable data which are indispensable in evaluating the reliability of 3-nitrotyrosine as a biomarker of oxidative stress caused by RNS.

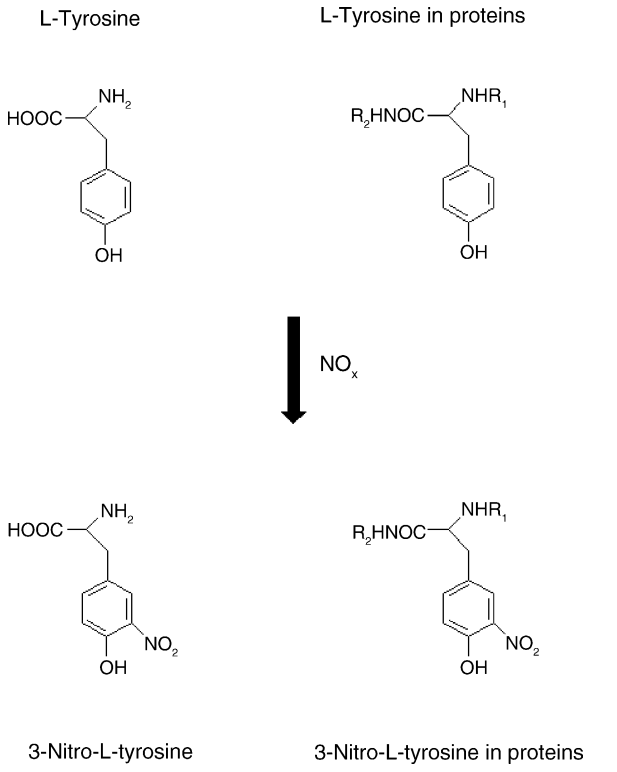

Fig. 1. Reaction of reactive nitrogen species (NO_x) with the aromatic ring of the free amino acid L-tyrosine (left panel) and L-tyrosine residues in proteins (right panel) to form free 3-nitro-L-tyrosine and protein-associated 3-nitro-L-tyrosine, respectively. R_1 and R_2 are neighbour amino acid residues in proteins. NO_x may comprise nitrosating and nitrating agents such as nitric oxide (NO), peroxynitrite ($ONOO^-$), and nitrylchloride (NO_2Cl).

Table 1
Summary of basal mean levels of NO_2Tyr and NO_2TyrProt and of the molar ratio of NO_2TyrProt/TyrProt measured in plasma of healthy humans and rats reported mostly in methodological articles in the literature cited in chronological order

NO_2Tyr (nM)	NO_2TyrProt (pmol/mg)	NO_2TyrProt/TyrProt ($\times 1{:}10^6$)	Species	Method	Reference
(A) Mass spectrometry based					
2.8	N.M.	N.M.	Human ($n=8$)	GC–tandem MS	Schwedhelm et al. (1999) [4]
64	N.M.	35	Human ($n=8$)	GC–MS	Frost et al. (2000) [5]
<4.4 (LOQ)	N.M.	N.M.	Human	LC–tandem MS	Yi et al. (2000) [6]
11	N.M.	N.M.	Human ($n=3$)	GC–MS	Gaut et al. (2002) [7]
≤1.4 (LOQ)	N.M.	N.M.	Rat ($n=4$)	LC–tandem MS	Delatour et al. (2002) [8]
	N.M.	4–18	Rat ($n=6$)	LC–tandem MS	Delatour et al. (2002) [9]
0.73	0.1 (or 24 nM)	1.55	Human ($n=18$)	GC–tandem MS	Tsikas et al. (2003) [10]
0.74	0.6	N.M.	Human ($n=12$)	GC–tandem MS	Söderling et al. (2003) [11]
(B) Non-mass spectrometry based					
31	N.M.	N.M.	Human	HPLC–fluorescence	Kamisaki et al. (1996) [12]
N.M.	N.M.	0–1	Rat/human	HPLC–ECD–UV	Shigenaga et al. (1997) [13]
N.M.	N.D.	N.D.	Human	HPLC–UV	Fukuyama et al. (1997) [14]
N.M.	5.1	N.M.	Human	HPLC/GC–TEA	Petruzzelli et al. (1997) [15]
N.D.	N.D.	N.M.	Human	ELISA	ter Steege et al. (1998) [16]
N.D.	120 nM	N.M.	Human	ELISA	Khan et al. (1998) [17]
N.M.	170	N.M.	Human	Immunology/WB	Pignatelli et al. (2001) [18]

N.M.: not measured; N.D.: not detected; LOQ: limit of quantitation.

1.2. Methodological problems with the quantification of circulating 3-nitrotyrosine

Numerous analytical methods based on different methodologies have been reported for the quantitative determination of NO_2Tyr and NO_2TyrProt in human plasma and other biological matrices (Table 1). The wide range of basal plasma levels of NO_2Tyr and NO_2TyrProt in humans is a crystal clear disclosure for the existence of numerous methodological problems in the quantification of circulating NO_2Tyr and NO_2TyrProt. These difficulties mainly originate from the physiological occurrence of NO_2Tyr and NO_2TyrProt at very low basal concentrations in human plasma. This forces indirect detection with the consequence of analytical methods becoming complex, time-consuming, costly, and most importantly susceptible to interferences. The most serious methodological problems in the measurement of NO_2Tyr and NO_2TyrProt in human plasma include the well-known and thoroughly investigated artifactual formation during sample treatment, and the lack in specificity as well as in sensitivity of the analytical approaches used [3,19]. These points are discussed below in detail.

2. Methodological approaches

2.1. Assurance of accurate basal levels for circulating free 3-nitrotyrosine by gas chromatography–tandem mass spectrometry

2.1.1. Avoidance of protein denaturation

NO_2TyrProt is more abundantly present in human plasma than NO_2Tyr (Table 1). Denaturation of plasma proteins during generation, storage, thawing, and treatment of plasma samples may erroneously contribute to NO_2Tyr levels. The contribution of NO_2TyrProt to NO_2Tyr is best avoided: (a) by thawing plasma samples stored at −80 °C once only [4,10]; (b) by using mild conditions for plasma generation from blood and by immediate analysis of the plasma ultrafiltrate samples [4,10,11].

2.1.2. Avoidance of artifactual acid-catalyzed formation

Artifactual formation of NO_2Tyr and NO_2TyrProt from tyrosine and nitrate and/or nitrite may occur from acidification of biological samples and during sample derivatization. This problem was effectively overcome by two different strategies (Fig. 2). In the first method suggested by Schwedhelm et al. [4], NO_2Tyr is separated from tyrosine, nitrite and nitrate by HPLC, so that artifactual nitration of tyrosine within the consecutive derivatization procedures does not occur. In the second method reported by Söderling et al. [11], NO_2Tyr is reduced by dithionite to 3-aminotyrosine, so that even if artifactual formation of NO_2Tyr after reduction would occur, it would not contribute to endogenous NO_2Tyr.

2.1.3. Specificity

Schwedhelm et al. convincingly demonstrated by GC–tandem MS [4] that the *n*-propyl-pentafluoropropionyl (PFP)-trimethylsilyl (TMS) derivative of an unknown compound coelutes in GC with the *n*-propyl-PFP-TMS derivative of NO_2Tyr and has the same parent ion, i.e. *m/z* 396, which is monitored in GC–MS, but has different product ions, which are monitored by GC–tandem MS. This observation strongly suggests that GC–MS does not possess the necessary specificity to selectively measure plasma NO_2Tyr despite preceding HPLC analysis [4,10]. We compared the concentrations of NO_2Tyr measured by GC–MS to those measured by GC–tandem MS in the same plasma samples of 18 healthy volunteers by means of different statistical methods. Linear regression analysis revealed only a very weak statistically

non-significant correlation between GC–MS and GC–tandem MS (Fig. 3). Data analysis by the generally accepted method of Bland and Altman [20] revealed an unacceptable difference of 3.14 ± 3.72 nM between GC–MS and GC–tandem MS. Also, the ratio of the concentrations of NO_2Tyr measured by GC–MS to those measured by GC–tandem MS in the same plasma samples was unacceptable, i.e. 3.92 ± 3.69 [4]. Finally, paired *t*-test analysis of these data revealed statistically significantly different means ($P = 0.0023$) from GC–MS (4.79 nM) and GC–tandem MS (1.65 nM). These results convincingly demonstrate that use of simple GC–MS in the method by Schwedhelm et al. [4]/Tsikas et al. [10] will reproducibly yield too high false values for NO_2Tyr. It is, therefore, likely that similar interferences may also occur in other GC–MS methods and may result in erroneous, highly overestimated levels for NO_2Tyr, for instance of the order of 11 nM [7] and even 64 nM [5]. Indeed, Söderling et al. reported that in their method accurate quantification of NO_2Tyr in human plasma was not possible by GC–MS [11].

2.1.4. Sensitivity

Several reported methods such as HPLC and even LC–tandem MS lack sufficient sensitivity to detect basal plasma levels of NO_2Tyr of the order of 1 nM. Yi et al. [6] reported that their LC–tandem MS method, which detects underivatized 3-nitrotyrosine, does not apply to the quantification of NO_2Tyr in human plasma at the basal state due to insufficient sensitivity, i.e. due to a limit of quantitation (LOQ) of 4.4 nM. The findings by Yi et al. suggest that basal NO_2Tyr levels in human plasma are below 4.4 nM and are, therefore, supportive of the levels measured by GC–tandem MS [4,10,11]. LC–tandem MS analysis of derivatized NO_2Tyr, i.e. as its butyl ester, has been reported by Delatour et al. [8,9] to increase sensitivity, i.e. to lower the LOQ to 1.4 nM. This group has not determined NO_2Tyr in human normal plasma but in rat plasma and found NO_2Tyr at 1.5 nM in only four of eight animals, a value which is virtually the LOQ value of the method [8]. This finding is also strong supportive of the NO_2Tyr levels of the order of 1 nM measured by GC–tandem MS [10,11]. The works by Yi et al. [6] and Delatour et al. [8,9] also suggest that the LC–tandem MS methodology is not suitable, at least at present, for the quantification of NO_2Tyr in plasma of humans due to lacking sensitivity.

Although derivatization of 3-nitrotyrosine, e.g. to its butyl ester [8,9], may be utilized to lower LOD and LOQ of the LC–tandem MS approach, incorporation of a derivatization step, especially the acid-catalyzed, artifact-rich esterification of 3-nitrotyrosine, would nullify the clear advantages of the LC–tandem MS methodology. Obviously, only considerable advances in the instrumentation, notably in ion-source/interface devices for LC–tandem MS apparatus, may enable advantageous quantification of NO_2Tyr in human plasma by LC–tandem MS. Recently Svatikova et al. reported that mean basal levels of NO_2Tyr in plasma of healthy humans amounted to 0.7 nM as measured by means of a LC–tandem MS approach [21]. This enhancement of sensitivity could be due to the use of an advanced API interface, i.e. API 3000

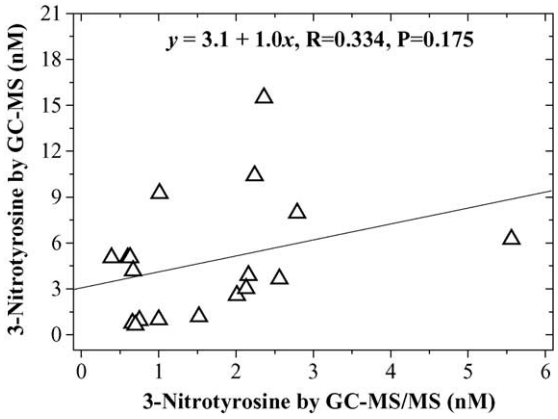

Fig. 3. Relationship between the levels of free 3-nitro-L-tyrosine measured in plasma of 18 healthy volunteers by GC–MS and GC–tandem MS. Note that each 1-μl aliquots of the same samples were analyzed by the same GC–tandem MS instrument (TSQ 7000) in the selected-ion monitoring mode (i.e. by GC–MS) and in the selected-reaction monitoring mode (i.e. by GC–tandem MS). The original study has been reported in ref. [10].

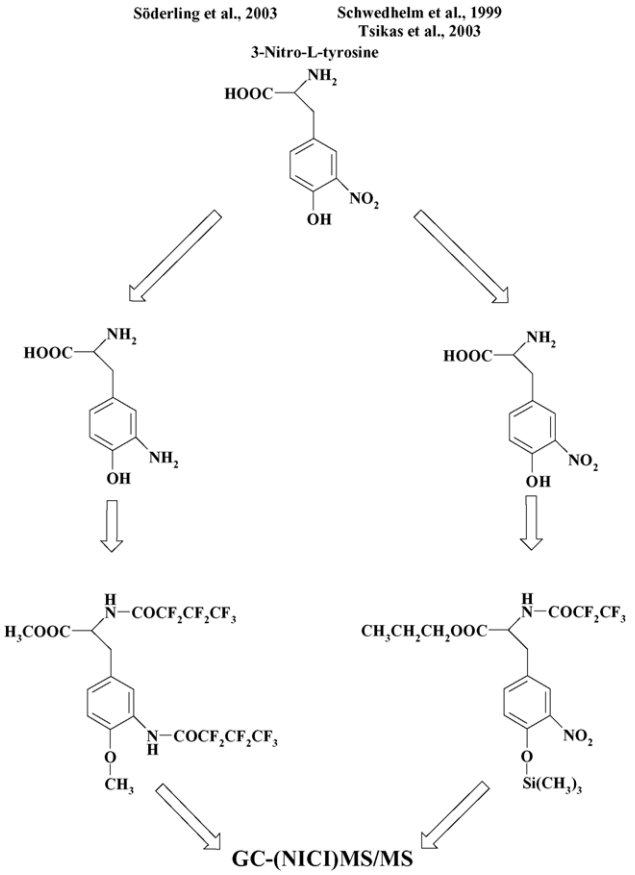

Fig. 2. Schematic of the quantification of 3-nitro-L-tyrosine by gas chromatography–negative-ion chemical ionization tandem mass spectrometry (GC–(NICI)MS/MS) after derivatization as described by Söderling et al. [11] (left panel) and Schwedhelm et al. [4]/Tsikas et al. [10] (right panel).

versus API 2000 [8]. However, the LC–tandem MS approach used in the study of Svatikova et al. [21] has not yet been described in detail [22].

2.2. A closer comparison of mass spectrometry methods

In the GC–tandem MS methods of the Göteborg group [11] and the Hannover group [4,10], many experimental procedures, including the derivatization reactions used, are very similar. This and the fact that in both studies the same GC–tandem MS instrument model was used under almost identical MS conditions, allow a more close comparison of analytical methods. Söderling et al. [11] analyzed 3-nitrotyrosine as its methyl ester–diheptafluorobutyl amide–methyl ether (Me–HFB–Me) derivative (Fig. 2) and reported a limit of detection (LOD) value of 30 amol of 3-nitrotyrosine at a signal-to-noise (S/N) ratio of 4:1, and an estimated LOQ value of 0.3 nM in plasma. Schwedhelm et al. [4] reported an LOD value of 4 amol at an S/N ratio of 11:1 and a measured LOQ value of 0.125 nM. This comparison indicates that GC–tandem MS quantification of 3-nitrotyrosine is more sensitive when it is analyzed as *n*-propyl-PFP-TMS derivative [4,10] (Fig. 2) than as Me–HFB–Me derivative [11], at least when the former is preceeded by HPLC.

A more practice-relevant evaluation of these methods is the comparison of reported chromatograms from the GC–tandem MS quantification of endogenous 3-nitrotyrosine in human plasma. In the method of the Hannover group, endogenous 3-nitrotyrosine at an approximate plasma concentration of 1 nM is typically detected at an S/N ratio of 200:1 (Fig. 4). In the method of the Göteborg group, endogenous 3-nitrotyrosine at an approximate plasma concentration of 0.9 nM is typically detected at an S/N ratio of 11:1 (see Fig. 7C in ref. [11]). The higher sensitivity of the Hannover's group method is in part due to the lower LOD value and in part to the lower LOQ value, with the latter most likely resulting from the use of an additional HPLC method which minimizes matrix effects, i.e. suppresses the "background" signal. On the one hand, however, the conversion of 3-nitrotyrosine to 3-aminotyrosine (Fig. 2) by dithionite saves time by omitting the HPLC step.

Gaut et al. used both GC–MS (in the negative-ion chemical ionization (NICI) mode) and LC–tandem MS (in the electrospray ionization mode) to quantify free 3-nitrotyrosine as well as 3-chlorotyrosine and 3-bromotyrosine in human plasma [7]. This group reported that GC–MS was 100-fold more sensitive than LC–tandem MS for analyzing authentic 3-nitrotyrosine. However, the reported basal levels of the order of 11 nM in plasma samples from only three individuals are several times greater than those obtained by GC–tandem MS and LC–tandem MS (Table 1). This fact and the solid evidence of interferences in the GC–MS analysis despite use of a HPLC step for sample purification (Fig. 3) suggest that the reported GC–MS methods do not provide accurate values for free 3-nitrotyrosine.

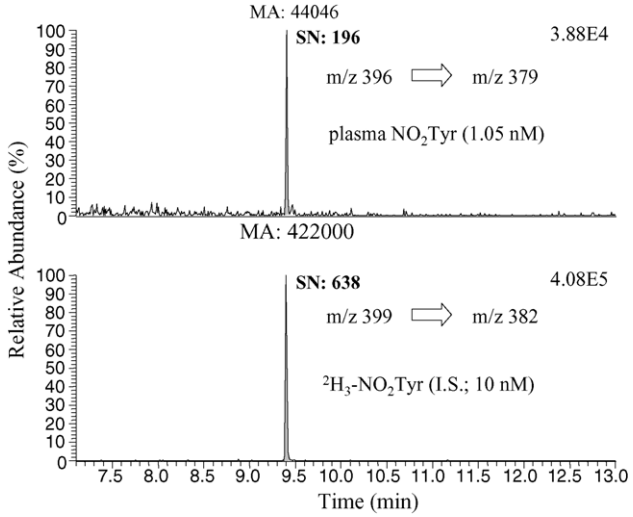

Fig. 4. Typical partial chromatogram from the GC–tandem MS analysis of a plasma sample from a healthy volunteer for free 3-nitro-L-tyrosine (NO_2Tyr) [10]. One milliliter of plasma was spiked with the internal standard (I.S.) d_3-NO_2Tyr to a final concentration of 10 nM and ultrafiltered by centrifugation. A 200-μl aliquot of the ultrafiltrate was analyzed by HPLC, endogenous NO_2Tyr and d_3-NO_2Tyr present in the HPLC fraction eluting with the retention time of synthetic 3-nitro-L-tyrosine were isolated by solid-phase extraction. NO_2Tyr and d_3-NO_2Tyr were subsequently converted to their *n*-propylester-*N*-pentafluoropropionyl-trimethylsilyl ether derivatives. Selected-reaction monitoring of *m/z* 379 from *m/z* 396 for endogenous NO_2Tyr (upper trace) and *m/z* 382 from *m/z* 399 for d_3-NO_2Tyr (lower trace) was performed. MA, peak area; SN, signal-to-noise ratio. The peaks shown were generated from the injection of approximately 4 fmol of NO_2Tyr and 40 fmol of d_3-NO_2Tyr (a recovery of 100% was assumed).

3. Reference values for circulating 3-nitrotyrosine

The inherent accuracy of analytical methods based on the MS-methodology, e.g. GC–MS, GC–tandem MS, LC–MS, and LC–tandem MS, has led to the proposal of its use as a reference methodology and for accuracy control in clinical chemistry [23]. MS-based analytical approaches, in particular GC–tandem MS and LC–tandem MS, are generally accepted as the Gold Standard in defining reference values of endogenous substances in biological fluids including inborn errors of metabolism [24]. In consideration of the highly divergent values so far reported for NO_2Tyr and NO_2TyrProt in plasma or serum of healthy volunteers, even from the use of the MS-methodology (Table 1), the important and crucial issue of reference values for circulating 3-nitrotyrosine should also be addressed here. Regarding 3-nitrotyrosine as a biochemical parameter for quantifying the state of oxidative stress in vivo in humans, reference values are indispensable in evaluating the impact not only of clinical studies, but also that of analytical methods. Establishment of standard values for NO_2Tyr and NO_2TyrProt in human plasma, which would reflect the status of physiological production rate of RNS, is an indispensable prerequisitive for: (1) the definition of pathological conditions; (2) the decision on the necessity for taking pharmacological or nutritional measures or change of

Table 2
Basal mean levels of NO_2Tyr and NO_2TyrProt and molar ratio of NO_2TyrProt/TyrProt measured by GC–tandem MS in plasma of healthy humans in various studies and their characteristics

Gender	Age (years)	Number	NO_2Tyr (nM)	NO_2TyrProt (pmol/mg)	NO_2TyrProt/TyrProt ($\times 1{:}10^6$)	Reference
Female	31 (24–43)	6	2.90 ± 1.00	N.M.	N.M.	[4]
Male	33 (27–43)	5	2.80 ± 0.56	N.M.	N.M.	[4]
Female/male	51 ± 10	12	1.14 ± 0.73	N.M.	N.M.	[10]
Female/male	25 ± 3	6	2.68 ± 1.54	N.M.	0.42 ± 0.07	[10]
Male	26 ± 3	18	0.73 ± 0.12	N.M.	1.55 ± 0.13	[10,25]
Female	46 ± 11	6	0.95 ± 0.29	0.48 ± 0.16	N.M.	[11]
Male	40 ± 8	6	0.54 ± 0.17	0.71 ± 0.58	N.M.	[11]
Male	24.2 ± 2.5	15	1.10 ± 0.56	N.M.	1.20 ± 0.69	[26]
Female/male	51 (42–74)	10	0.75 ± 0.44	N.M.	1.40 ± 0.71	Present study
		84 (total)	0.54–2.90 (range)		0.42–1.55 (range)	

N.M.: not measured.

habit; and (3) the judgment of therapy success on the basis of a biochemical laboratory parameter.

For the last 5 years, two independent research groups from Göteborg [11] and Hannover [4,10] have determined basal values for circulating NO_2Tyr and NO_2TyrProt in the frame of several studies including well-documented clinical studies in 84 healthy humans who had no history of cardiovascular, renal and other diseases or smoking (see refs. in Table 2). The range of the mean value (1.5 ± 1.0 nM) for basal NO_2Tyr in those studies was 0.5–2.9 nM as measured by the fully validated GC–tandem MS methods reported elsewhere in detail [4,10,11]. Both groups used almost the same procedure to generate blood plasma and plasma ultrafiltrate, but they used different strategies to eliminate potential interferences by artifactual formation of NO_2Tyr. The Hannover group used HPLC for the separation and isolation of NO_2Tyr from human plasma [4,10], whereas the Göteborg group reduced the 3-nitro group of NO_2Tyr into the 3-amino group by dithionite [11]. Mean and range for circulating NO_2Tyr measured by the Göteborg and Hannover groups are supported by the findings of two other groups, i.e. by Yi et al. [6] and Delatour et al. [8,9], who used the LC–tandem MS methodology. These values for NO_2Tyr are, therefore, candidates for use as reference values.

As to NO_2TyrProt, the reported data are still inhomogenous, because different NO_2TyrProt have been quantified and the ratio of 3-nitrotyrosine to tyrosine has not been always determined (Tables 1 and 2). For albumin-associated 3-nitrotyrosine, i.e. NO_2TyrALB, a mean concentration of 24 nM was estimated and a mean 3-nitrotyrosine-to-tyrosine molar ratio of $1.5 \times 1{:}10^6$ at the basal state was determined [10]. These data correspond to 0.1 pmol of 3-nitrotyrosine per mg albumin and are in agreement with those reported by Söderling et al. for plasma total proteins, i.e. 0.6 pmol of 3-nitrotyrosine per mg protein [11]. The 3-nitrotyrosine-to-tyrosine molar ratio of $1.5 \times 1{:}10^6$ is supported by the findings of Shishehbor et al. [27]. This group has used the LC–tandem MS methodology, in particular the ion trap approach, but did not measure the 3-nitrotyrosine-to-tyrosine molar ratio in healthy humans. In the control group comprising of patients with no coronary artery disease or peripheral arterial disease, 35% of the patients had ratios smaller than $3.6 \times 1{:}10^6$, and 27% of the patients had ratios in the range $3.6–6.3 \times 1{:}10^6$ [27].

Prerequisite for the MS analysis of protein-associated 3-nitrotyrosine in plasma proteins is the acid-, base- or enzyme-catalyzed hydrolysis. The major problem in the analysis of NO_2TyrProt is the artifactual formation of 3-nitrotyrosine during chemical and enzymatic digestion of proteins. This issue has been addressed and thoroughly investigated by all investigators, and different strategies have been developed to minimize, control and quantify artifactual formation of 3-nitrotyrosine [4–6,9–11,13]. The analytical difficulties associated with sample handling, which may include extraction, derivatization, and artifactual formation, have been recently detailed discussed by Duncan [19].

In protein hydrolysates, tyrosine may reach mM-concentrations [10]. As nitrate and nitrite are ubiquitous, acid-catalyzed proteolysis may artifactually produce 3-nitrotyrosine. By using a sophisticated method Delatour et al. found by LC–tandem MS that in plasma the contribution of acid-catalyzed hydrolysis of proteins ranged from 16 to 40% [9]. Addition of phenol has been shown to reduce but not to completely avoid artifactual formation of 3-nitrotyrosine during acid-catalyzed proteolysis [13]. Yi et al. adopted a gas phase HCl hydrolysis and demonstrated that no artifatcs arose during this step [6]. Frost et al. performed proteolysis by alkaline hydrolysis [5]. However, the usefulness of alkali-catalyzed proteolysis as performed by Frost et al. [5] is difficult to be evaluated, because this group reported the highest basal levels for protein-associated and free 3-nitrotyrosine despite use of GC–MS (Table 1). On the other hand, enzyme-catalyzed proteolysis may contribute to 3-nitrotyrosine by self-digestion of the proteolytic enzymes, provided they are also nitrated on tyrosine. Indeed, Yi et al. [6] and Tsikas et al. [10] have shown that the proteolytic enzyme pronase may considerably contribute to tyrosine and 3-nitrotyrosine by self-digestion in the absence of other proteins. By contrast, Shigenaga et al. reported that the proteolytic enzyme pronase used contributed to tyrosine by less than 2% [13].

4. Clinical studies

4.1. β-Blockers and oxidative stress

β-Blockers such as nebivolol, carvedilol, and metoprolol have been associated with additional antioxidative effects in vitro. In healthy humans with normal clinical history we investigated the effects of these β-blockers at standard antihypertensive doses on the oxidative stress [26,28]. The status of oxidative stress was assessed by measuring the urinary excretion of the lipid peroxidation biomarker 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) and 3-nitrotyrosine, i.e. NO_2Tyr and NO_2TyrProt, by GC–tandem MS. Nebivolol decreased significantly urinary excretion of 8-*iso*-$PGF_{2\alpha}$ [28], but had no effect on NO_2Tyr and NO_2TyrProt as compared with the placebo group (Fig. 5A). Carvedilol and metoprolol showed a non-significant trend toward reduction of 8-*iso*-$PGF_{2\alpha}$ [26], NO_2Tyr, and NO_2TyrProt (Fig. 5B).

4.2. Organic nitrates and oxidative stress

Increased vascular superoxide production is currently discussed as a mechanism leading to nitrate tolerance in nonintermittent therapy. Healthy humans were administered therapeutically relevant doses of the organic nitrates pentaerythrityl tetranitrate (PETN) or isosorbide dinitrate (ISDN), and the biomarkers of oxidative stress 8-*iso*-$PGF_{2\alpha}$, NO_2Tyr, and NO_2TyrProt as well as their major urinary metabolites were measured by GC–tandem MS [25]. Neither urinary excretion of 8-*iso*-$PGF_{2\alpha}$ and plasma NO_2Tyr and 3-nitrotyrosinoalbumin (Fig. 5C) nor their respective urinary metabolites changed significantly after PETN or ISDN administration as compared to the data before drug administration [25].

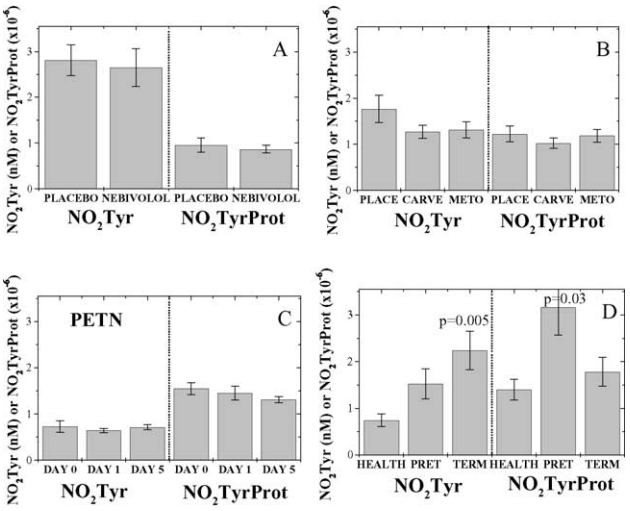

Fig. 5. Plasma concentration of free 3-nitro-L-tyrosine (NO_2Tyr expressed in nM) and protein-associated 3-nitrotyrosine (NO_2TyrProt expressed as the molar ratio of 3-nitro-L-tyrosine to L-tyrosine) in various clinical studies. (A) Effect of the β-blocker nebivolol and placebo [28]. (B) Effect of the β-blockers carvedilol (CARVE) and metoprolol (METO) and placebo (PLACE) [26]. (C) Effect of pentaerithrityl tetranitrate (PETN) before (DAY 0), after one day (DAY 1), and after 5 days (DAY 5) of administration [25]. (D) NO_2Tyr and NO_2TyrProt in healthy humans (HEALTH) and in patients suffering from end-stage renal disease (PRET, pre-terminal; TERM, terminal). Values are shown as mean ± S.E.M. For more details see the text and the respective articles.

4.3. 3-Nitrotyrosine in end-stage renal disease

In plasma samples from 27 patients suffering from end-stage renal disease and from 10 sex- and age-matched (mean age, 51.2 years) healthy volunteers serving as the control group we determined the concentration of NO_2Tyr and NO_2TyrProt by GC–tandem MS [10]. Patients were divided into two groups in accordance with the serum creatinine levels. Patients with serum creatinine levels above 530 μM but not on dialysis comprised the group pre-terminal (mean age 59.1 years, $n = 15$). The terminal group comprised of patients on dialysis and serum creatinine levels above 700 μM (mean age 52.8 years, $n = 12$). In comparison with the control group comprising of healthy volunteers, the patients had higher NO_2Tyr and NO_2TyrProt levels (Fig. 5D). Statistically significant higher NO_2Tyr levels were found in the terminal group, whereas NO_2TyrProt levels were significantly higher in the pre-terminal group.

4.4. Age-dependence of 3-nitrotyrosine

In plasma samples of a group comprising of healthy non-smoking volunteers (age, 43 ± 9 years; six females, six males) NO_2Tyr and NO_2TyrProt were determined by GC–tandem MS [11]. With respect to NO_2Tyr a statistically insignificant correlation was found between NO_2Tyr and age, whereas no correlation at all was found for NO_2TyrProt and age (Fig. 6).

4.5. Other studies

Shishehbor et al. have determined by LC–tandem MS the levels of protein-associated 3-nitrotyrosine, i.e.

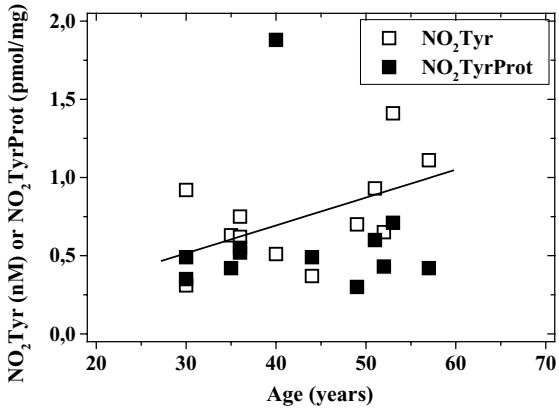

Fig. 6. Relationship between age and plasma NO_2Tyr ($R = 0.54$, $P = 0.07$) or NO_2TyrProt ($R = 0.04$, non-significant) in 12 healthy volunteers (six females and six males). This figure was constructed by using the data of Table 2 from ref. [11].

NO_2TyrProt, in patients with established coronary artery disease (CAD) and in patients with no clinically evident CAD [27]. NO_2TyrProt median levels were $9.1 \times 1{:}10^6$ in the patients plasma with CAD ($n = 100$) and $5.2 \times 1{:}10^6$ in the patients' plasma without CAD ($n = 108$). In an interventional study Shishebor et al. have found by LC–tandem MS that treatment with atorvastatin (10 mg/d) for 12 weeks of hypercholesterolemic subjects ($n = 35$) with no known CAD led to a significant reduction (by 25%) in NO_2TyrProt (mean ± standard deviation) levels from $15 \pm 7 \times 1{:}10^6$ at baseline to $11 \pm 5 \times 1{:}10^6$ after 12 weeks [27,29]. Unfortunately, Shishehbor et al. did not report on NO_2TyrProt levels in healthy volunteers nor on NO_2Tyr levels in the patients investigated [27,29]. Furthermore, the range of the NO_2TyrProt values reported by Shishehbor et al. is unusually wide, i.e. it covers more than order of magnitude, and the reported levels are considerably greater than those measured in the patients suffering from end-stage renal disease, in whose we found the highest NO_2TyrProt and NO_2Tyr levels so far (present study; Fig. 5D). By contrast, the urinary and circulating concentrations of the generally accepted biomarker of oxidative stress, i.e. 8-*iso*-$PGF_{2\alpha}$, have been shown to be moderately elevated in diseases associated with increased oxidative stress including hypercholesterolemia (reviewed in [30]), and also to moderately change upon pharmacological intervention [25,26,28].

Obstructive sleep apnea (OSA) has been linked to cardiovascular disease, endothelial dysfunction and oxidative stress, generated by repetitive nocturnal hypoxemia and reperfusion. Svatikova et al. [21] measured NO_2Tyr levels in plasma of OSA patients and healthy normal subjects by means of the LC–tandem MS methodology ([22]; discussed above). This group reported that OSA patients and healthy controls had almost identical plasma levels of NO_2Tyr (e.g. morning levels: 0.66 nM in the OSA group and 0.62 nM in the control group) [21].

Non-MS analytical assays such as HPLC, GC, and ELISA have been applied much more frequently to determine 3-nitrotyrosine in biological fluids than MS-based methods [19]. However, because of lack in sensitivity and specificity the results of the studies, in which such methods have been applied, should be treated cautiously. These methods either cannot detect even the more abundantly present NO_2TyrProt at the basal state or they provide very high levels (see references in Table 1). Pignatelli et al. investigated nitrated and oxidized proteins in smokers and lung cancer patients [18]. In the plasma of healthy non-smokers, Pignatelli et al. have detected NO_2TyrProt at 170 pmol/mg protein [18]. This value is at least 280 times higher than the NO_2TyrProt basal levels provided by GC–tandem MS (Table 1).

4.6. Summary of the clinical studies

Circulating NO_2Tyr and NO_2TyrProt are widely used as biomarkers of oxidative stress in humans. However, serious methodological problems cast a cloud on the potential significance of NO_2Tyr and NO_2TyrProt as biomarkers of RNS-mediated oxidative stress in vivo in humans. Clinical studies, in which the GC–tandem MS methodology has been used to quantify NO_2Tyr and NO_2TyrProt in human plasma, suggest that NO_2Tyr and NO_2TyrProt may indeed be useful to quantify RNS-mediated oxidative stress even in healthy humans. However, the application of the GC–tandem MS methodology to clinical studies clearly shows that the basal levels of NO_2Tyr and NO_2TyrProt are relatively low, and suggests that the changes resulting from therapeutical interventions should be considered rather very moderate. Therefore, some of the extremely high levels and dramatic alteration of circulating NO_2Tyr and NO_2TyrProt levels observed in various clinical studies may not prove true. The highest plasma levels of NO_2Tyr and NO_2TyrProt measured by GC–tandem MS so far were observed in patients suffering from end-stage renal disease. Although this disease may be associated with elevated oxidative stress, it may be assumed that the elevated plasma levels in particular of NO_2Tyr are largely due to accumulation rather than due to highly elevated oxidative stress. At present there is limited good quality data available on 3-nitrotyrosine, and the usefulness and reliability of circulating 3-nitrotyrosine as a biomarker of RNS-mediated oxidative stress in vivo in humans remain to be established.

5. Concluding remarks

Since the recognition of 3-nitro-L-tyrosine as a potential biomarker of nitrosative/nitrative oxidative stress in vivo, serious effort has been made to develop analytical methods for the quantification of NO_2Tyr as well as NO_2TyrProt in plasma of humans and animals. However, the analytical methods available to date yielded highly divergent values for circulating NO_2Tyr and NO_2TyrProt at the basal state, ranging from 1 to 64 nM for NO_2Tyr and 0 (i.e. not detectable) to 120 nM for NO_2TyrProt. Regarding basal levels of NO_2Tyr and NO_2TyrProt best agreement was observed between methods based on the GC–tandem MS and LC–tandem MS technologies, with LC–tandem MS being at present considerably less sensitive than GC–tandem MS. On the basis of the data provided yet by the tandem MS methodologies, the range of 0.5–3 nM for free 3-nitrotyrosine, i.e. NO_2Tyr, and the range of $0.4–1.6 \times 1{:}10^6$ for protein-associated 3-nitrotyrosine, i.e. the 3-nitrotyrosine-to-tyrosine molar ratio in plasma proteins, seem to be justified for use as reference ranges in healthy normal subjects.

Acknowledgements

The authors would like to thank the members of the Caidahl and Tsikas research groups for their contributions.

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