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Journal of Chromatography B, 851 (2007) 172–185
JOURNAL OF CHROMATOGRAPHY B
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Review

Methods using stable isotopes to measure nitric oxide (NO) synthesis in the L-arginine/NO pathway in health and disease☆

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Received 1 June 2006; accepted 29 August 2006
Available online 16 October 2006

Abstract

Nitric oxide (NO) is an important gaseous radical involved in many physiological processes. It is produced from the amino acid L-arginine by the action of nitric oxide synthases (NOS) in what is called the L-arginine/NO pathway. Tracking its metabolic fate in biological fluids is of particular interest as it may indicate how the human body responds in health and disease. However, due to its short life span (a few seconds) it is very difficult to accurately monitor any up- or down-regulation in body fluids in vivo. As a consequence, methods have been developed based on the measurement of the NO-derived products nitrite and nitrate or on the substrate of NO, L-arginine and its simultaneously generated product, L-citrulline. Considering only a fraction of the endogenous L-arginine pool is used for the synthesis of NO, NO-production cannot be estimated by measuring changes in the concentrations of L-arginine and/or L-citrulline alone. Instead, to estimate NO-related changes in the L-arginine and/or L-citrulline pools a form of tagging these metabolites for the NOS-mediated reaction is required. The application of stable isotopes is an elegant way to track NOS-mediated changes. The present paper is focussed on the application of various combinations of chromatography and mass spectrometry to measure isotopic enrichments resulting from the conversion of L-arginine to NO and L-citrulline in a one-to-one stoichiometry. In addition, the various aspects and principles involved in the application of stable isotopes in metabolic studies in general and the study of the activity of NOS in particular are discussed.
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Keywords: Reviews; Nitric oxide; Stable isotope; L-Arginine; Mass spectrometry

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☆ This paper is part of a special issue entitled "Analysis of the L-Arginine/NO pathway", guest edited by D. Tsikas.

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doi:10.1016/j.jchromb.2006.08.054

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1. Introduction—Synthesis, physiology and pathophysiology of NO

About 25 years ago the first paper appeared on the discovery of endothelium-dependent relaxation of arteries [1], caused by an endothelium-derived relaxing factor (EDRF) that was later identified as nitric oxide (NO) [2]. Since then, tens of thousands of papers have appeared on this topic. The L-arginine/NO pathway was found to be involved in many physiological processes, while changes in this pathway and NO synthesis were related to diseases. Many reviews cover this topic, which we only summarize briefly below.

Nitric oxide is synthesized from the amino acid L-arginine by the enzyme NO synthase (NOS) of which three isoforms are known. These enzyme isoforms are NOS1, NOS2, NOS3, previously known as neuronal, inducible and endothelial NOS, respectively. This also indicates the tissues in which the enzymes were first identified, with NOS2 present in macrophages and other tissues [3,4]. All isoforms produce NO under basal conditions [5], while NO production through NOS2 is up regulated by inflammatory mediators [6]. Moreover, interaction between the isoforms seems to exist as NOS2 may down-regulate NOS3 activity [7].

Synthesis of NO by the vascular endothelium is responsible for vasodilator tone, which is essential for blood pressure regulation. In the central nervous system, NO is a neurotransmitter with several functions, including memory. In the peripheral nervous system, NO is the neurotransmitter of the previously known non-adrenergic and non-cholinergic nerves and regulates gastrointestinal, respiratory and genitourinary tract functions and some forms of neurogenic vasodilatation. Moreover, NO contributes to control of platelet aggregation and is involved in leucocytes interaction with vessel walls. In addition, NO interacts with mitochondrial systems to regulate cell respiration and to augment the generation of reactive oxygen species with formation of peroxynitrite, and is a mediator of cell defence [3,4].

The pathophysiology of NO seems both related to an overproduction of NO, as well as a limited (local) availability of NO. Overproduction of NO, which is in general related to enhanced NOS2 activity leads to vasodilatation, hypotension, vascular leakage and disruption of cell metabolism and cell damage. Reduced NO availability through a reduction in NO generation or through NO inactivation, on the other hand, has been related to a number of cardiovascular diseases, including hypertension, atherosclerosis, hypercholesterolemia and diabetes [4]. These two extremes in NO availability and its pathophysiology have resulted in the therapeutic application of both NOS inhibitors and NO donors.

A difference in activity of the different NOS isoforms may also be involved in this apparent contradiction, which is for, e.g. demonstrated by the pathophysiology of sepsis, a severe inflammatory condition. During sepsis, NOS2 activity is up regulated, which is considered a main contributor to the severe vasodilatation and hypotension observed in these patients, while NOS1 and/or NOS3 are even down regulated [8,9]. The latter could compromise the microcirculation in these patients [10]. Besides NOS enzyme activity, the level of NO production may also be affected by the availability of its precursor L-arginine. Although L-arginine is considered a conditionally essential amino acid [11,12], it may become essential during growth and under several pathophysiological conditions when endogenous L-arginine production becomes insufficient [13]. In patients with hypercholesterolemia, a disease known for its vascular dysfunction, administration of L-arginine could normalize this dysfunction [14]. On the other hand, endogenous NO production is inhibited by methylated L-arginine derivatives, i.e. asymmetric dimethylarginine (ADMA), symmetric dimethylarginine and N^{G}-monomethyl-L-arginine (L-NMMA), which are endogenous NOS inhibitors that are present in human plasma and urine [3]. Accumulation of these inhibitors in plasma has been reported in chronic renal failure [15]. Finally, NO availability can become limited due to breakdown of available NO by reactive oxygen species, which may be counteracted by antioxidants [4]. For other conditions, like cancer, the role of NO is still conflicting, since both DNA damaging and protective effects against cytotoxicity have been reported [16,17].

Therefore, a very delicate balance seems to exist between NO production and NO bioavailability with pathophysiological consequences probably both related to NO deficiency and to NO access. Most evidence for NO production in vivo is based on the measurement of NOS enzyme expression or the levels of the stable end-products nitrate and nitrite in blood and urine, while the number of studies that measure NO production directly in vivo

is relatively limited. Stable-isotope techniques could therefore be useful here.

2. Probing NO synthesis—analytical aspects

2.1. General

The lifetime of the NO radical in blood is believed to be in the order of 3–5 s [18]. As a consequence, a direct measurement in physiological fluids through conventional methods is impossible. The collection of blood for example, already requires more time. As a consequence, attempts are made to determine NO production directly in the bloodstream by developing NO-sensitive catheters, which once placed allow the collection of “on-line” information about ongoing NO production [19]. Alternatively, exhaled air is frequently used to study the regulation of NO synthesis [20]. Providing relatively clean samples and access able in a non-invasive manner, important factors for an easy and accurate analysis, this approach gained an increasing popularity. Nevertheless, its relevance is limited as it mainly provides information on local processes involving the lung especially and thus alternatives are required to collect information about ongoing processes in other organs.

2.2. Indirect methods to estimate NO production rate

One way to gather information involving other organs is to study precursor or reaction products of NO synthesis, as depicted in Fig. 1. After its production, the NO radical is quickly converted into nitrite and nitrate, which are excreted into the urine. As a consequence these products have been targeted to estimate NO synthesis. Additionally, urine provides an easy sampling source and has therefore often been selected to determine nitrate concentrations [21–23]. Various analytical methods have been employed to reach this goal. Both chromatographic approaches as well as mass spectrometry have been applied in addition to methods based on the Griess reaction [24]. However, even though these procedures all enabled a correct measurement of both nitrate and nitrite by itself, it was soon recognized that calculation of NO production based on nitrate data could significantly differ from the same calculation based on nitrite data. In fact it was questioned to what extent nitrate and/or nitrite fluxes represent NO production at all [25]. As our own (unpublished) data confirmed this discrepancy, we decided to turn to stable isotope techniques described below.

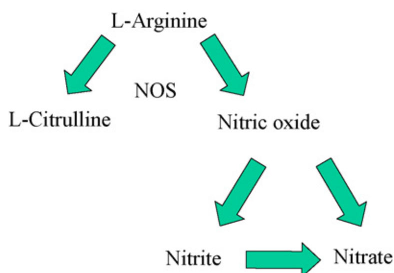

Fig. 1. Nitric oxide synthase (NOS)-catalyzed conversion of L-arginine to L-citrulline and NO. NO and NO-derived nitrite is oxidized to nitrate.

2.3. Stable-isotope approach in nitrate/nitrite analysis

Therefore, stable-isotope methods were developed, to enhance the level of specificity and estimate the NO derived part of nitrate or nitrite production only. One approach makes use of the inhalation of a stable-isotope labeled oxygen ($^{18}O_2$), which is subsequently converted to $N^{18}O$ and in addition to $N^{18}O_3{}^-$ [26]. Alternatively, the infusion of labeled nitrate [27] or labeled L-arginine [28–34] is applied with subsequent measurement of labeled nitrate in blood or urine.

While the advantage of these (indirect) methods for measuring NO production are that collection of exhaled air and urine are non-invasive and measurement of (labeled) nitrate relatively easy, the methods also have considerable disadvantages. One of the disadvantages is that the recovery of ingested labeled nitrate in the first 48 h urine collection is only 60% of the administrated dose, while the fate of the remaining 35% is still unknown [35]. Another disadvantage is the decay in nitrate production from labeled L-arginine of about 20 h [30], which makes phased changes in NO production difficult to determine. A compartmental model approach described by Avogaro et al. [33] deals with these disadvantages for conversion of arginine to nitrate by accounting for other source of nitrate than arginine, the residence time of plasma nitrates and the nitrate rate of appearance in urine. A more direct measurement of NO production therefore seems preferable and is the main focus of this review paper.

2.4. Estimation of NO production rate by measuring L-citrulline concentration

The production of citrulline runs through several routes. However, only the route involving NO synthase results in the formation of both NO and L-citrulline in a one-to-one molar ratio. However, the majority of citrulline (human studies: 90% [30], mice studies: 85–95% [36]) is produced from L-glutamine and also from ADMA after enzymatic hydrolysis by dimethylarginine dimethylaminohydrolase (DDAH) [37,38], while both these routes do not contribute to NO production. Therefore, quantitation of L-citrulline production alone cannot accurately provide an estimation of NO production.

2.5. Estimation of L-citrulline production rate using stable isotopes

So, to accurately estimate NO production, one must be able to discriminate the NOS-derived L-citrulline from L-citrulline derived from other routes. One way to do this is by tagging it with (a) stable isotope(s). In the present paper, we will focus on this approach and discuss the methods and calculations required to perform this type of study.

3. Theoretical background for stable-isotope enrichment studies

3.1. Principle of tracer technology

In nature, many elements have a natural abundance of stable isotopes. For biochemical molecules carbon provides the

most profound contribution to the isotopomeric pattern of a molecule, because the natural abundance of its ^{13}C-isotope is among the largest of the contributing elements (about 1.1%). This implies for instance that a six-carbon containing molecule like L-citrulline will exhibit an isotopomeric pattern containing six peaks of decreasing intensity. The abundance of these peaks, relative to the no heavy isotope containing "base peak" (in this context also referred to as the tracee) depends on the (cumulative) probability to incorporate one or more ^{13}C atoms (and of course stable isotopomers of other constituting elements like nitrogen or hydrogen) and can be calculated. Here we will refer to these peaks as the M + 0 (or M), M + 1, M + 2 ... M + n isotope. In the above example the intensity of the M + 1 peak (for the example here we will consider the incorporation of ^{13}C-isotopes only) will roughly be $6 \times 1.1\%$ equals 6.6% the intensity of the M + 0 peak (Fig. 2). Through the supplementation of a known amount of the M + 1 isotopomer of this component (here referred to as the tracer), the intensity of this isotopomeric peak will be enhanced relative to the M + 0 peak. As a consequence, the ratio between the no-heavy isotope containing M + 0 base peak (the tracee) and the one Dalton heavier M + 1 isotopomeric peak (the tracer), depicted as the Tracer–Tracee ratio (TTR) will increase according to:

$$\text{TTR}\,(\%) = \left(\frac{[\text{tracer}]}{[\text{tracee}]}\right) \times 100\%$$

However, to estimate this increase we have to correct for the natural abundance of each isotopomer (e.g. in a blood sample taken before supplementation of tracer) and in the case where amino acids have been derivatized, the abundance of the derivative molecule also has to be corrected for according to:

$$\text{Increase in TTR} = \text{TTR}\,(\text{measured}) - \text{TTR}\,(\text{natural})$$

Thus, to pick up a small change in TTR resulting from the supplementation of a tracer, the cumulative standard deviations and accuracy of both measurements {tracer (both natural and administrated) and tracee} are of importance. The concentration of the tracee usually will be much larger as compared to the concentration of the tracer and thus the variation in the latter determines most of the imprecision. Nevertheless, a small deviation in the estimation of the tracee concentration can easily cause some tenths of a percent change in the estimated TTR. As a consequence, the best data quality is obtained if both tracer and tracee measurements are combined in one measuring event (scan) to exclude inter-assay variability.

In order to prevent the infused tracer from interfering with the normal biological course for the tracee, the amount of tracer provided should be kept as low as possible. However, more tracers would provide a better precision in the measurement, and thus a delicate balance has to be considered. To pick up a meaningful increase of the traced isotopomer, one should consider the sensitivity and precision of the measuring system applied. With the exception of the much more precise dedicated isotope ratio mass spectrometer (IRMS), a modern gas chromatography–mass spectrometer (GC–MS) or liquid chromatography–mass spectrometry (LC–MS) instrument can realize a standard deviation in the order of 0.5% at a physiological level, which implies that a minimal enrichment of 0.5% should be aimed for, both when an M + 1 tracer or M + 2 or higher tracer are used. However, compared with the M + 1 tracer, the natural abundance of an M + 2 or higher isotopomer is considerably lower due to a

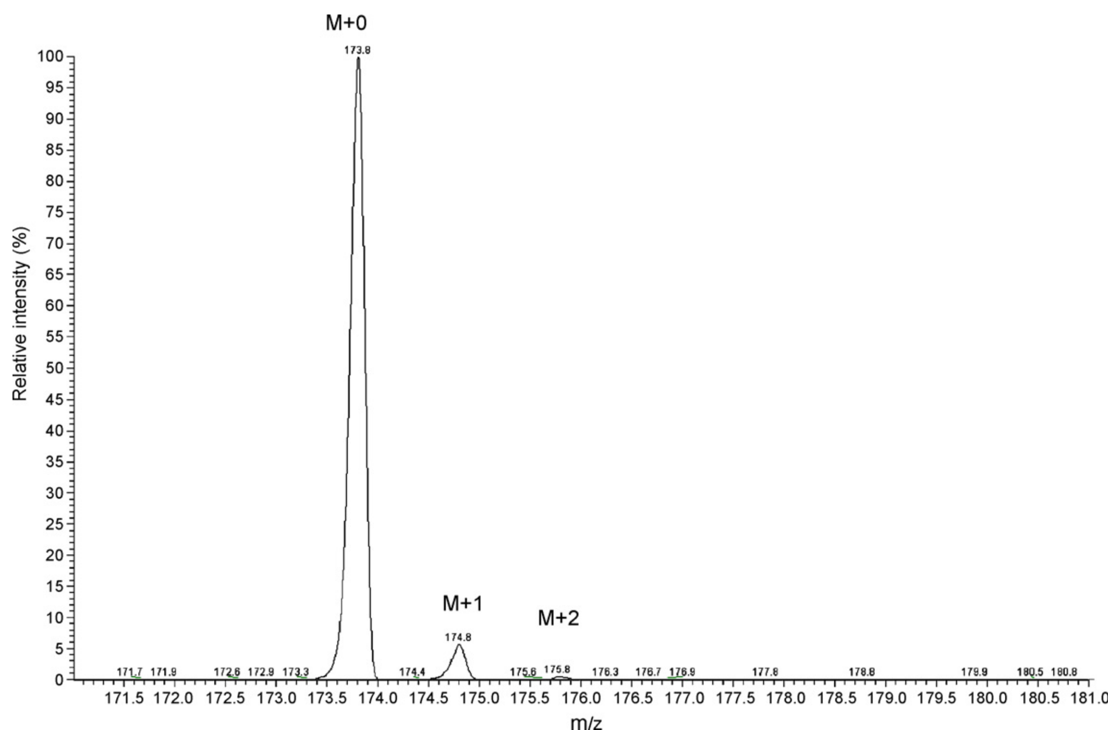

Fig. 2. Isotopomeric envelop of L-citrulline (m/z = 174) as measured in negative "zoomscan-mode" on a Thermo Electron model LCQ classic mass spectrometer, to indicate that a modern mass spectrometer is capable of measuring the complete isotopomeric distribution of an amino acid.

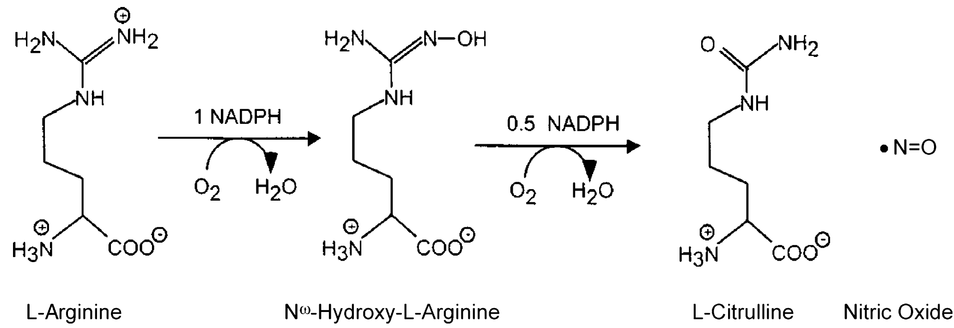

Fig. 3. Production of nitric oxide and L-citrulline from L-arginine.

reduced chance that one molecule would contain 2 or more ^{13}C atoms (Fig. 2). As a consequence the TTR (natural) for the (M + 2) or higher isotopomeric peaks is much lower than that for the (M + 1) peak. Therefore, its influence in the equation for calculating the increase in TTR is much lower, resulting in a lower standard deviation for the calculated increase in TTR and thus more accurate data. Consequently, an M + 2 or higher isotopomer requires a lower amount of tracer to pick up this enrichment.

3.2. Choice of labelling position

To determine which position in the citrulline and/or L-arginine best can be labeled to estimate NO production, we must first consider the conversion reaction (Fig. 3). Apart from this reaction however, the majority of L-citrulline production runs through the urea cycle, in which L-arginine is first converted to L-ornithine and next to L-citrulline (Fig. 4). In the first step of the urea cycle, the action of arginase removes the

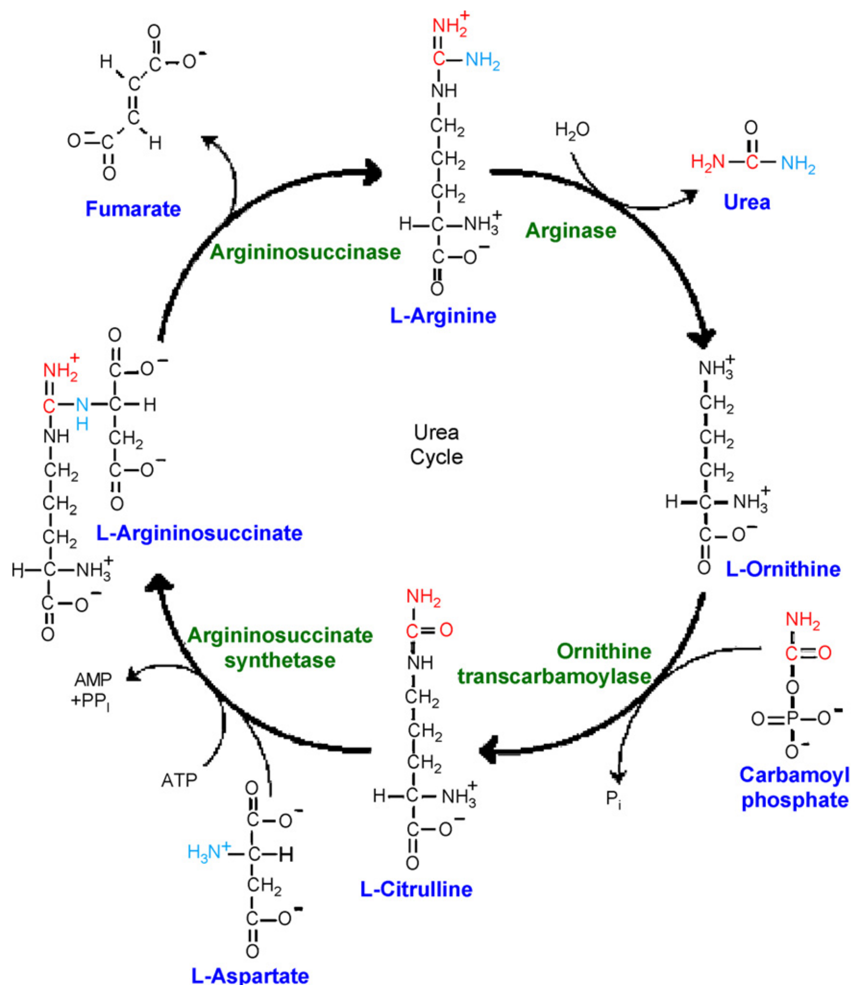

Fig. 4. The urea cycle as the major pathway leading to L-citrulline formation from L-arginine.

Table 1
Influence of tracer choice on stability of TTR measurement; details are described in the text

Mouse	Group	TTR CIT1	TTR CIT3	TTR ARG2	TTR ARG4	Ratio CIT1/ARG2	Ratio CIT3/ARG4
A	Lap	1.46	0.63	28.33	22.27	5.18	2.81
B	Lap	0.90	0.71	33.06	24.75	1.25	2.89
C	Lap	1.00	0.93	43.04	32.75	2.35	2.85
D	Con	1.13	0.67	34.18	26.42	4.79	2.55
E	Con	0.60	0.56	34.18	25.38	1.77	2.21
F	Con	1.01	0.90	34.28	26.55	2.57	3.41
	Mean	1.02	0.70	34.56	26.31	3.07	2.66
	S.E.M.	0.14	0.06	2.38	1.75	0.80	0.13

Lap, 24-h after laparotomy; con, control mice.

complete guanidine group of the L-arginine molecule to produce L-ornithine, whereas the conversion of L-arginine to L-citrulline through the action of NOS removes only one of the guanidine nitrogen atoms of the L-arginine molecule. This difference makes the guanidine group of the L-arginine molecule an ideal site to be targeted with isotopic labelling. Theoretically it would be sufficient to label only the nitrogen, which is preserved into the citrulline molecule to reach this goal, but due to conformational changes the guanidine L-arginine nitrogen atoms can shift their positions and as a consequence both nitrogen atoms should be labeled with a ^{15}N-tracer. Consequently, both citrulline and NO will incorporate labeled nitrogen. The amount of tracer incorporated in citrulline thus provides an estimate of the amount of NO generated. In addition, the gap between tracer and tracee mass can be enhanced by also incorporating deuterium atoms in the backbone of the molecule. Considering the above discussion on tracer technology indicating an increased accuracy of data obtained with an $M+2$ or higher isotopomer, it can be imagined that this addition of deuterium atoms in a molecule is of use to measure the tracer and therefore the TTR more accurately. The larger the concentration differences between tracer and tracee, the more these requirements matter.

In conclusion, although a multiply labeled tracer may be the better choice from a theoretical point of view, the available measuring system in fact determines if this benefit can be exploited. To explore if this theoretical benefit indeed provides a practical gain in our approach, we performed a pilot experiment in mice, where we infused L-[*guanidino*-$^{15}N_2$]-arginine (ARG2) and L-[*guanidino*-$^{15}N_2$-$^{2}H_2$]-arginine (ARG4) simultaneously for 30 min in 6 mice (unpublished). This resulted in formation of ^{15}N-citrulline (CIT1) and ^{15}N-$^{2}H_2$-citrulline (CIT3), respectively. The subsequent ratio between citrulline and L-arginine measured was more variable for the CIT1/ARG2 ratio than for the CIT3/ARG4 ratio (Table 1), illustrating that in this case the theoretical benefit of a multiply labeled tracer could indeed be exploited in the practical setup applied.

4. Methodology tracer studies

4.1. General principle of tracer studies

The calculation of whole body NO production is based on measuring the conversion of stable-isotope labeled L-arginine to L-citrulline using a single-pool model (e.g. blood/plasma) within which mixing is rapid [39]. When infusion of the substrate tracer into this pool starts the ratio between the tracer and the tracee (the unlabeled substrate) is low. But this ratio will increase during infusion until an isotopic equilibrium is reached. At this steady-state point the tracer is lost at the same rate as it appears. To accelerate the time to reach this isotopic equilibrium, a bolus of tracer is infused to prime the pool. The rate of tracer infusion needed to reach the desired TTR is determined by the production rate by the body of the substrate (e.g. L-arginine or L-citrulline). The desired TTR is mainly determined by analytical technical consideration (see appropriate sections) and costs of the tracer. Moreover, the infused tracer should not influence metabolism by acting as a food component or stimulating hormonal release, which is generally achieved when the enrichment is limited to about 10%.

A basic assumption in stable-isotope research is that the tracer behaves the same as the unlabeled compound. Therefore, when a stable-isotope labeled L-arginine is infused, it will (similar to the unlabeled L-arginine) be converted to L-citrulline and NO. For example, when a L-[*guanidino*-$^{15}N_2$]-arginine is used, this will yield a L-[^{15}N]-citrulline and ^{15}NO. Alternatively, L-[*guanidino*-$^{15}N_2$-$^{2}H_2$]-arginine will yield L-[^{15}N-$^{2}H_2$]-citrulline and ^{15}NO. Although the conversion itself does not occur in plasma, but takes place at the level of the NOS enzyme at different locations, the labeled L-citrulline formed will rapidly exchange with the plasma pool. Finally, an isotopic equilibrium in plasma is also reached for L-[^{15}N]-citrulline, although later than for the infused L-[*guanidino*-$^{15}N_2$]-arginine, as the pool of the latter is primed. Due to the small body pool size of citrulline (plasma concentration in healthy subjects varies between 25 and 40 μM [40]) steady state can be reached fast.

To calculate the absolute NO production rate, the citrulline production rate also needs to be measured. This is essential, since the citrulline production can be affected by diseases that increase its production, like renal disease [41] or decrease its production, like diseases with inadequate intestinal glutamine-to-citrulline turnover due to loss of intestinal mass [42]. L-Citrulline production rate can be measured by using a separate L-citrulline label, e.g. L-[*ureido*-^{13}C]-citrulline or L-[*ureido*-^{13}C-$^{2}H_2$]-citrulline or L-[*ureido*-^{13}C-$^{2}H_3$]-citrulline, but should not interfere with the L-citrulline isotopomer produced from the L-arginine tracer. All these aspects should be considered when designing the experiment.

4.2. Experimental design of tracer studies to measure NO production

As indicated above, the experimental design consists of the simultaneous infusion of an L-arginine and an L-citrulline tracer or the use of both tracers in a separate experiment. The latter can be applied when both isotopes interfere, which is the case when the L-citrulline isotopomer that is formed (from L-arginine) is identical to that of the L-citrulline tracer that is infused.

Both L-arginine and citrulline tracers are infused intravenously according to a primed-constant and continuous infusion protocol. For measurement on whole body level, arterial or arterialized blood samples [43,44] are taken before the tracer infusion (for background or natural isotope enrichment) and at several time points during the tracer infusion to assure a steady state and to reduce (during steady state) the variance of the measured TTRs. For measurement on organ level, the arterial-venous difference across the organ is measured by simultaneously sampling blood upstream and downstream of the organ. Moreover, flow across the organ needs to be measured. This organ measurement can relatively easily be applied in animals [45,46], but is more complicated in humans where organ measurements are often limited to the leg or arm (for muscle kinetics) [39], while most other organs require a surgical procedure to gain access. Blood samples are kept under cold conditions (ice), rapidly centrifuged to obtain plasma, and subsequently stored at −80 °C until further analysis.

In the first report on stable-isotope NO production measurement, using a 24-h primed-continuous infusion of L-[*guanidino*-$^{15}N_2$]-arginine with blood sampling at hourly intervals [30], observed a steady-state in L-[^{15}N]-citrulline enrichment within approximately 5–6 h which was maintained until the end of the tracer infusion period (both during fasting and feeding). This relatively long period to reach steady state for L-[^{15}N]-citrulline seems related to the long duration to reach steady state for the L-[*guanidino*-$^{15}N_2$]-arginine as well, although the authors do not mention this. In later studies, blood samples were taken at regular intervals from 3 h of infusion [41,47] when a steady state was reported. Lagerwerf et al. [48] used a 120-min infusion protocol and observed a steady state for infused L-[*guanidino*-$^{15}N_2$]-arginine and produced L-[^{15}N]-citrulline from 60-min of infusion. In our own human studies (unpublished) we use a 2-h primed-constant infusion protocol with a steady state reached for the labeled L-arginine after about 1 h and after about 1.5 h for the produced L-citrulline label. In mice studies, a steady state is even reached within 20 min [45], which makes it possible to limit the infusion protocol in mice to 30 min with only one blood sample taken during steady state.

On average about 0.26% [48] to 1% [30] of total L-arginine production is converted to NO (and L-citrulline). The priming dose of L-arginine used in fasted human adults varies between 5 μmol/kg, with continuous infusion at about 5 μmol/kg/h [30,41] to 11.2 μmol/kg prime and 11.2 μmol/kg/h continuous infusion [48]. This results in plasma L-arginine enrichments of around 5% [30] and 12% [48], respectively. Enrichments of produced L-citrulline in these studies were about 0.6% [30] and 0.3% [48]. During feeding or in patient studies, the infusion protocol may need some adaptation, as L-arginine and subsequent citrulline production may be different. However, in general the analytical technique used will be the major limitation for determining the amount of L-arginine tracer needed for the infusion protocol in order to determine the produced labeled L-citrulline adequately.

4.3. Calculation of NO production rate

Whole body plasma L-arginine and L-citrulline production rates (Q) are calculated from the arterial isotopic enrichment values of respectively infused L-arginine and L-citrulline tracers using the standard steady state isotope dilution equation [39]:

$$Q = \frac{I}{\mathrm{TTR}}$$

where TTR is the tracer-to-tracee ratio, and I is the rate of infusion of the tracer. TTR is corrected for background/natural enrichment, and, whenever multiple isotopes of one amino acid are supplied, their individual contribution to the measured isotopomeric distribution (TTR) are accounted for as described by Vogt et al. [49].

Calculation of the plasma L-arginine-to-L-citrulline flux (NO production) is performed as follows [30]:

$$Q_{\mathrm{Arg}\rightarrow\mathrm{Cit}} = \frac{Q_{\mathrm{Cit}} \times \mathrm{TTR}_{\mathrm{Cit(M+1)}}}{\mathrm{TTR}_{\mathrm{Arg(M+2)}}}$$

where Q_{Cit} is the plasma L-citrulline flux, estimated from the primed constant infusions of labeled L-citrulline. $\mathrm{TTR}_{\mathrm{Cit(M+1)}}$ and $\mathrm{TTR}_{\mathrm{Arg(M+2)}}$ are the respective TTR's of L-[*ureido*-^{13}C]citrulline and L-[*guanidino*-$^{15}N_2$]arginine, when the latter is infused. When L-[*guanidino*-$^{15}N_2$-$^2H_2$]arginine (Arg (M + 4) is infused, L-[*ureido*-^{15}N-$^2H_2$]citrulline (Cit (M + 3) is formed.

Similarly, the rate of organ NO synthesis can be calculated from the labeled L-arginine-to-L-citrulline conversion across that organ, by using the venous-arterial net balance of $\mathrm{TTR}_{\mathrm{Cit(M+1)}}$ divided by the arterial $\mathrm{TTR}_{\mathrm{Arg(M+2)}}$. The organ $\mathrm{TTR}_{\mathrm{Cit(M+1)}}$ net balance is corrected for the amount of synthesized citrulline that is extracted by the organ [50,51].

In summary, the stable-isotope technique is easy to perform on whole body level in humans and animals and provides a direct measurement of the metabolic pathway of L-arginine to NO. However, the major limitations will probably be analysis of the low L-citrulline enrichments and also the relatively high costs of the L-arginine and L-citrulline tracers. Another issue that has not yet been solved is the possible compartmentalization of arginine at both organ and sub cellular levels [52]. This also questions the real arginine precursor pool for citrulline and NO production. Moreover, as for all in vivo NO analyses techniques, NOS isoform specific NO production cannot be discriminated, since all NOS enzymes have the same metabolic pathway.

5. The analytical process

5.1. Chromatography

To measure the conversion of L-arginine to L-citrulline various combinations of chromatography and mass spectrometry have already been employed. In the present paper we will mainly focus on LC–MS, but will also provide some general aspects to consider when using GC–MS.

A key issue for any chromatographic approach is the wide believe that an MS system can address whatever analytical issues to generate desired data. Often however, a preceding chromatographic system is merely seen as an easy way to introduce samples into the MS system. Thus, the aim is not to enhance sensitivity, which in general is sufficient for the desired purpose, but to speed up the analytical cycle. However, when analytical sensitivity and/or accuracy are an issue, and for clinical metabolic research this is often the case when a complex matrix like blood or urine has to be analyzed, the chromatographic system may even be of more importance than the MS measurement itself. Chromatography can influence the ionization efficiency, exclude interfering contaminants, concentrate the target metabolites prior to the MS analysis or remove interfering salts.

Considering their physical properties, liquid chromatography is the first choice to separate amino acids in aqueous solutions like blood or urine. The first and still widely used method to separate and detect amino acids was based on the application of ion-exchange chromatography and ninhydrin detection [53]. However, up until the development of atmospheric pressure ionization (API) in the last decade, the direct coupling of a liquid

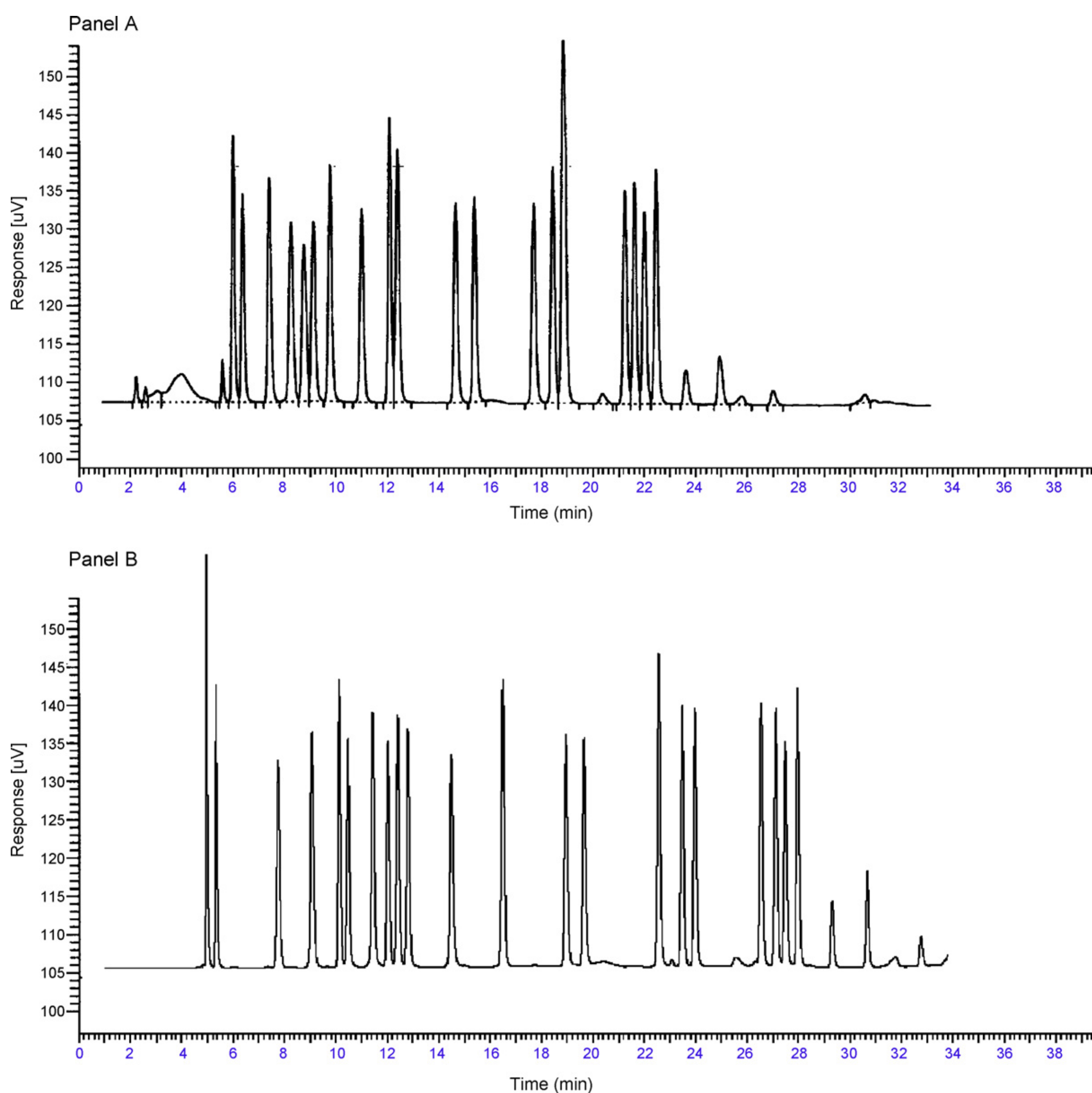

Fig. 5. Separation of *o*-phthaldialdehyde derivatives of amino acids. Panel A: Reprinted from H.M.H. van Eijk, D.R. Rooyakkers, P.B. Soeters, N.E.P. Deutz, Determination of amino acid isotope enrichment using liquid chromatography–mass spectrometry, Anal. Biochem. 271 (1999) 8, with permission of Elsevier [56]. Panel B: Reprinted from H.M.H. van Eijk, D.R. Rooyakkers, N.E.P. Deutz, Rapid routine determination of amino acids in plasma by high-performance liquid chromatography with a 2–3 μm Spherisorb ODS II column, J. Chromatogr. 620 (1993) 143, with permission of Elsevier [89].

chromatographic system to a mass spectrometer was impossible. In contrast, GC–MS had already been realized for quite some time before. As a consequence, the general approach to measure amino acid isotope enrichment was with GC–MS. But with the introduction of API, LC–MS became an option too. However, although API enables the application of flow rates usually applied in liquid chromatography, it still limits the choice of applicable solvents. Solvent buffers have to be volatile and their concentrations should be limited, as otherwise the API probe would become blocked. Thus, it is still impossible to combine a traditional ion-exchange method with mass spectrometry due to the required high solvent salt concentrations. The same problem is true for numerous other applications based on reversed phase chromatography after some kind of derivatization [54]. Although these methods often can be adapted to fit the requirements of an LC–MS approach, this often comes at the cost of a suboptimal separation (Fig. 5) [55,56]. Summarized, it can be concluded that although the LC–MS analysis of amino acids presently can be exploited, a thorough knowledge of the nature of the target analytes (either free or derivatized amino acids) is required to obtain the optimal compromise for both the chromatographic separation as well as the MS measurement. This may be illustrated by taking a carboxylic acid as an example. For the chromatographic separation it may be beneficial (or even essential) to apply acetic solvents, but (depending on the pH) this will cause the component to be protonated, a neutral form resisting negative ionization in the MS system. As a carboxylic acid by itself cannot be ionized in a positive mode, this solvent will thus decrease detection sensitivity or may even prohibit ionization at all.

The chromatographic process can also be used to exclude interfering contaminants, thus generating simpler mass spectra. As a mass spectrometer usually is by far the most expensive component of an LC–MS setup, the chromatographic process can be used to either enhance sensitivity and/or specificity, thus making it possible to generate the required data with a less expensive MS system. Additionally, derivatization is also an interesting option to consider. Although new methods are arising using ion-pairing reagents enabling the LC–MS analysis of underivatized amino acids [57–60], derivatization by itself may improve the quality of a separation [61], exclude co-eluting contaminants, or increase ionization efficiency and thus detection sensitivity [62].

Especially if the MS system is operated in electrospray ionization mode, the flow and column dimensions applied are also important factors to consider. A reduction of the flow rate from a standard of 1 ml/min to 100 μl/min may translate into a gain of 10 for the resulting MS signal [63]. As a consequence the same sensitivity can be reached with less sample material. Additionally, a chromatographic procedure preceding the MS analysis can also be used to automate the sample pre-treatment process. Next to a gain in accuracy (timing and the precision of sample handling are important factors influencing derivatization and/or extraction recoveries), it reduces the required operator labour and increases the system productivity, because samples now also can be run overnight. This may compensate (partly) for the time required for completing the chromatographic process.

For the present review we will focus on the determination of tracer–tracee ratios and more specifically on the TTRs of L-arginine and L-citrulline to calculate NO synthesis. To enable this, separation is of the utmost importance as their isotopomeric envelops otherwise would overlap, making it otherwise impossible to determine an enrichment in one of the isotopomers. To separate amino acids usually gas or liquid chromatography is applied. In the past, gas chromatography was the method of choice as this system could be coupled directly to a mass spectrometer. A draw back at that time was however that the derivatization procedures were sensitive with respect to derivatization recovery [48]. Presently, new derivatization techniques are applied which are supposed to have anticipated on this problem [64,65]. In between, the introduction of atmospheric pressure ionization techniques like electrospray and atmospheric pressure chemical ionization (APCI) liquid chromatography allowed the development of a LC–MS approach [56,66–68]. In this approach, separation and derivatization are easy, providing the opportunity to automate the complete analytical sequence. Nevertheless, the precision which can be realized for isotopic ratio measurements is by far not as good as can be obtained using a dedicated IRMS instrument, but for physiological experiments this does not need to be a problem, consider Fig. 6. For this experiment we added the L-[*ureido*-^{13}C]citrulline and L-[*guanidino*-$^{15}N_2$]arginine isotopes of L-citrulline and L-arginine to a 100 μM solution of naturally enriched L-arginine and L-citrulline to obtain TTR (%) ranging from 0 to 2.5% above natural abundance and measured these solutions according to ref. [56]. This resulted in straight calibration curves, with a regression equation for L-arginine $Y = 0.9816x + 0.0276$ and for L-citrulline $Y = 0.9913x + 0.0121$ with an R^2 for both amino acids = 0.9999. In practice, we found that using this method, we could pick up an increase of 0.1% in TTR above natural abundance using the described ion-trap LC–MS setup.

5.2. Choice of mass spectrometry equipment

Presently, mass spectrometer instruments are available in a great diversity. Choice can be made with respect to the ionization technique (electrospray, APCI, MALDI, CI, EI, etc), mass

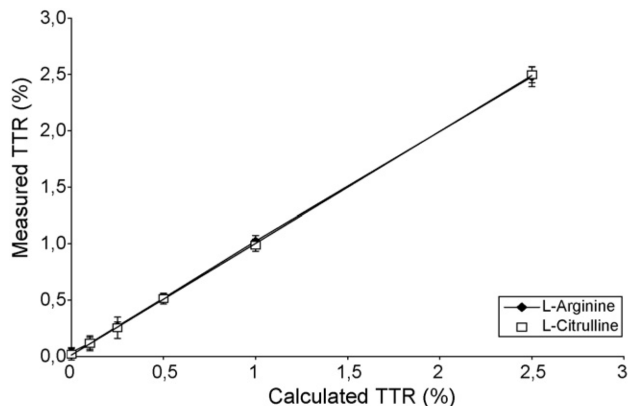

Fig. 6. Enrichment curve for L-arginine and L-citrulline. Details are described in the text.

analyzer (Sector instrument, IRMS, Quadrupole, ion-trap, TOF) and the combination of either of these in a single setup (e.g. GC-C-IRMS, ESI-Quad, MALDI-TOF) or in a consecutive (triple QUAD, TOF-TOF) or hybrid setup (QQ-TOF) [69,70].

5.2.1. *Isotope ratio mass spectrometry (IRMS)*

The traditional approach to measure isotope enrichments [71,72] using GC-C-IRMS provides a powerful method to measure very low amino acid enrichments. This is made possible by dedicating the setup of the MS system to this specific application only. Instead of using a much more sensitive electron multiplier for detection, these systems are equipped with faraday cups which provide a more stable signal, however at the cost of losing much of its sensitivity and spectral information. Additionally, detection is performed on the basis of isotopic pairs only. To enable this, the target molecules are combusted to carbon dioxide from which the isotopomeric distribution is determined. As a consequence, structural information is lost, including the position of the label. Moreover, the above instrument adaptations require extensive sample preparation procedures to provide sufficient (large) amounts of sample and this often can only be realized by off-line processing methods. Also, the in-run detection of different isotopomeric couples is impossible and because amino acid (derivatives) are combusted before analysis, one cannot apply multiply labeled tracer for one component either (1-^{13}C-leucine versus uniformly labeled ^{13}C leucine for instance). Nevertheless, still new IRMS approaches are being developed [73,74].

5.2.2. *Gas chromatography–mass spectrometry (GC–MS)*

Alternatively, the GC–MS approach can be applied. This method is by far not as precise as GC-C-IRMS, but counters many of the above-described problems. As a consequence, GC–MS is much more versatile [34]. In a GC–MS approach there are many choices possible as to the selection of the setup of the GC–MS system. Electron ionization (EI) and chemical ionization are presently the most popular choices to generate ions and are usually combined with either a quadrupole or ion-trap mass analyzer. The time of flight (TOF) analyzer presently provides an interesting alternative as its greatest limitation, a limited dynamic range, is recently expanded by one order of magnitude [75]. Further, the major benefit of the TOF analyzer is that all ions are collected without any sacrifice in sensitivity in contrast to scanning analyzers. This development now makes it possible to optimally use its superior resolution and fast scan rate, which is especially important in combination with a fast GC separation.

5.2.3. *Liquid chromatography–mass spectrometry (LC–MS)*

After the development of atmospheric pressure ionization techniques in the 90s, this technique became one of the most popular and powerful hyphenated techniques. The physical properties of many known biological components are such that liquid chromatography is the method of choice for their analysis rather than gas chromatography. Therefore, it is logical that once the coupling of LC with the MS was realized, LC–MS became by far the most versatile hyphenated technique applied ever. As a consequence, this is one of the few areas in instrumental analysis, which even now sees a continuous introduction of new developments, and not just a new design, but involving new capabilities [76].

Presently, the configuration of the desired MS system can be tailored to the specific needs. Modern systems usually make use of APCI or electrospray ionization. With respect to the mass analyzer, the available budget generally determines the choice. There is wide range of systems available in the market, each of them different in terms of sensitivity, resolution, linearity and MS/MS capabilities [77]. Next to triple stage quadrupole instruments available already for a long time, hybrid systems are becoming increasingly popular. From these the QQ-TOF configuration already gained a wide popularity. Next to this setup, ion trap-TOF, TOF–TOF and trap-FT combinations are presently available, each providing their specific benefits, either in terms of sensitivity, scan speed and/or precision. Usually, the desired configuration can be selected with or without additional features, which thus create the opportunity to fit a system into the available budget. In the case of isotope ratio measurements, the crucial factors determining the systems suitability, is the dynamic range in combination with the system sensitivity, instead of their mass accuracy, sampling speed and/or resolution in which they differ most. Considering the overwhelming choice in available systems, it would be beyond the scope of this paper to discuss all the advantages and limitations of the various mass spectrometers for the present application, especially as this also depends on the preceding sample preparation and chromatographic procedure. Nevertheless, we would like to indicate that when comparing representative instruments of different classes ranging from a purchase cost of 100,000–1000,000 €, only minimal differences in performance are found for this specific application [66].

5.2.4. *New mass spectrometry options*

From the above, it may be clear that the ideal MS system for an isotope ratio measurement presently does not yet exist. Although new developments are constantly claimed by manufacturers, they usually involve adaptations in the mass analyzer [76] and/or ionization system. However, only few reports investigate alternative detection options. In a recent review by Koppenaal et al. [78] the limitations of presently available mass detectors are described and new developments are discussed. From these, the focal-plane array detector seems very promising. Current prototypes are already capable of providing very low detection limits (10–100 ppq), a dynamic range of 7 orders of magnitude and very high precision (0.007% R.S.D.). This places this prototype with respect to precision already in between a scanning MS instrument and a dedicated IRMS system, while maintaining all of the possibilities of a scanning MS system. Application of such a detector would also greatly enhance the specificity of the citrulline enrichment measurement.

6. Review of studies using stable isotopes

The number of studies using stable isotopes to measure NO production is limited and based on conversion of stable-isotope labeled L-arginine to labeled L-citrulline or labeled nitrate, of

Table 2
Overview of studies in humans on NO production using stable isotopes

Condition	Subjects	Fasted				Fed				Reference
	Sex; mean age	Q_{ARG}	Q_{CIT}	$Q_{ARG \to CIT}$	$Q_{ARG \to NO3}$	Q_{ARG}	Q_{CIT}	$Q_{ARG \to CIT}$	$Q_{ARG \to NO3}$	
Healthy	5M; 26 years	72 ± 35			0.36 ± 0.1[a]	73 ± 5				[31]
Healthy	6M; 22 years	45 ± 5	13 ± 3		0.30 ± 0.07[b]	61 ± 6	10 ± 2			[32]
Healthy	7M; 23 years	70 ± 8	10.3 ± 1.2	1.00 ± 0.2	0.67 ± 0.5[c]	95 ± 6	8.8 ± 2.0	0.91 ± 0.3	1.23 ± 0.2[c]	[30]
Healthy	5M/3F; 22–24 years					56 ± 10			0.50 ± 0.07[c]	[29]
Healthy	13M/1F; 22–28 years	84 ± 13	9.5[d]	0.22 ± 0.07						[48]
Healthy	7M; 29 years	85 ± 15	9.5[d]	0.22 ± 0.06						[79]
Healthy	5M/2F; 62 years	86 ± 4	9.5[d]	0.23 ± 0.09						[79]
Healthy	5; 38 years	54 ± 11			0.62 ± 0.16[e]					[33]
PPHNa	$N = 9$; 3day					72 ± 6			0.13 ± 0.03[f]	[32]
PPHNc	$N = 9$; 7day					96 ± 13			0.59 ± 0.17[g]	[32]
Smokers	5M; 25 years	82 ± 8	9.5[d]	0.22 ± 0.09						[79]
FH	7M; 36 years	80 ± 13	9.5[d]	0.16 ± 0.04						[79]
CRF	6M/1F; 62 years	76 ± 26	9.5[d]	0.13 ± 0.02						[79]
ESRD	4M/4F; 54 years	98 ± 21	87 ± 10	9.40 ± 5.90		94 ± 23	75 ± 4	8.7 ± 4.8		[41]
DM II	6M; 41 years				0.52 ± 0.16[e]					[33]
Sepsis	6M/4F; 11 years	67 ± 21	25 ± 7	1.58 ± 0.7						[47]
Sepsis	7M/3F; 56 years	59 ± 23	4.5 ± 2.1	0.83 ± 0.56						[Luiking, unpublished]

Data as mean ± S.D. or S.E.M. in μmol/kg/h. M: male; F: female; PPHNa: patients with pulmonary hypertension of the newborn during acute pulmonary hypertension; PPHNc: patients with PPHN during convalescence; FH: familial hypercholesterolemia; CRF: chronic renal failure; ESRD: end stage renal disease (post hemodialysis); DM II: Type 2 diabetes.

[a] Average of fed and fasted state; measurement of labeled nitrate in urine, with correction for under recovery (factor 1.67); calculated from 208.2 ± 40.5 μmol/8 h (mean weight subjects is 71.9 kg).

[b] Average of fed and fasted state; measurement of labeled nitrate in urine, with correction for under recovery (factor 1.67); calculated from 194.8 ± 47 μmol/8 h (mean weight subjects is 80.9 kg).

[c] Measurement of labeled nitrate in urine, with correction for under recovery (factor 1.67).

[d] From ref. [31].

[e] Measurement of labeled nitrate in urine, with correction for under recovery (factor 1.67).

[f] Measurement of labeled nitrate in urine; calculated from 10.33 ± 2.2 μmol/day (mean weight subjects is 3.2 kg).

[g] Measurement of labeled nitrate in urine; calculated from 45.0 ± 13.2 μmol/day (mean weight subjects is 3.2 kg).

which the latter is mostly measured in urine. A series of studies that report NO production rates based on both methods is listed in Table 2.

6.1. Human studies

6.1.1. Health

Castillo et al. [30] introduced the labeled L-arginine-to-L-citrulline conversion as a measure of NO production in 1996. In the healthy young males studied, NO production rate was on average 0.96 μmol/kg/h for 24 h, not different between the fed and fasted state. About 1.2% of L-arginine was metabolized to NO under these normal healthy conditions. In contrast, both Lagerwerf et al. [48] and Wever et al. [79] reported a much lower NO production rate of about 0.22 μmol/kg/h in healthy subjects using the same tracer methodology, with only 0.26% of L-arginine being metabolized to NO. When four subjects were treated with the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) the NO production even decreased by about 66% [48]. Smoking as well as aging did not affect NO production in their studies [79].

Using the technique of labeled L-arginine infusion and subsequent measurement of labeled urinary nitrate as a measure of NO production, neither 6-day L-arginine supplementation at 10-fold normal daily intake [31], nor 6 days on an L-arginine-free diet [32] did affect conversion of L-arginine to NO in healthy subjects. Also typhoid vaccination of healthy subjects had no effect on NO production, despite a mild acute phase response [29]. Finally, a gender-related difference in whole body NO production was demonstrated, with enhanced urinary excretion of labeled nitrate in pre-menopausal women than in men [80]. This may contribute to the sex difference in endothelial function in normal and hypercholesterolaemic subjects [81].

6.1.2. Disease

A few studies used the labeled L-arginine-to-L-citrulline conversion method to measure NO production in disease. In end-stage renal disease patients receiving hemodialysis treatment, NO production was significantly elevated, comprising about 10% of total L-arginine disposal [41]. In contrast, Wever et al. [79] observed a significantly lower NO production in patients with chronic renal failure. This discrepancy could be explained by the fact that Wever et al. did not measure citrulline production in their study but used healthy subjects' values, while others reported much higher values of citrulline production in patients with renal failure [41]. Patients with familial hypercholesterolemia tended to have a lower NO production rate [79], which seems in line with the decreased NO bioavailability and impaired endothelium-dependent vasodilatation in this group of patients [82]. A significant higher NO production rate was observed in septic pediatric patients, resulting in 2.5% of L-arginine being metabolized through this pathway [47], while this increase was absent in septic adult patients (Luiking et al., unpublished) [32].

Using measurement of labeled urinary nitrate after L-arginine tracer infusion as a measure of NO production, non-complicated type 2 diabetes patients were found to have a decreased conversion of L-arginine to NO compared with healthy controls [33]. Enhanced urinary nitrate labelling after infusion of labeled L-arginine was observed in patients with infective gastroenteritis [80] and after interleukin-2 therapy in cancer patients [83], while nitrate labelling was lower in patients with congestive heart failure compared with healthy controls [84]. These studies, however, did not calculate the absolute arginine to nitrate flux as a measure of NO synthesis. In newborns with persistent pulmonary hypertension, NO production was reduced during acute pulmonary vasoconstriction to about 25% of the level measured during convalescence [32]. The authors therefore proposed the concept of reduced L-arginine availability related to diminished NO production in these patients.

6.2. Animal studies

Animal studies offer the opportunity to study several disease models, but also to measure metabolism across several organs or to study the role of specific enzymes in genetically modified mice. Application of the labeled L-arginine-to-L-citrulline conversion in mice revealed two peaks in whole body NO production after endotoxin challenge. An early peak at 4 h and a late peak at 12 h after lipopolysaccharide (LPS) infusion were apparent, of which the latter could be related to the catabolic phase of endotoxemia [85]. Moreover, using mice that specifically lacked NOS2 or NOS3, the increase in systemic NO production at 5 h after LPS appeared to be solely dependent on NOS2, whereas that mediated by NOS3 was reduced [9]. An acute reduction of circulating L-arginine through arginase treatment did not affect systemic NO production in both healthy and endotoxemia mice [86]. However, the level of NO synthesis also depends on the mice strain [36]. Using the same stable-isotope technique in pigs, a reduction of whole body NO production after 8 h of food deprivation was observed. NO production remained unchanged after an additional 24-h continuous LPS infusion in a hyperdynamic model of sepsis [51], while NO production by the portal drained viscera and liver were both increased in this sepsis model [50]. Infusion of L-arginine increased whole body NO production about 2.5-fold in this model, which could be accounted to an enhanced NO synthesis in the portal-drained viscera, the kidneys and most pronounced the liver [51]. This may suggest that L-arginine availability is rate limiting to sustain a maximal NO synthesis rate during endotoxemia in this model.

In another pig model of sepsis (9-h continuous LPS infusion with colloid infusion to maintain a normotensive state), whole body nitrate appearance rate measured with labeled nitrate infusion as an indicator of NO production was nearly doubled [27].

In rabbits on a high-cholesterol diet, conversion of L-[*guanidino*-$^{15}N_2$]arginine (administred via gastric cannulation) to ^{15}N-labeled urinary nitrate was reduced compared with rabbits on a normal diet [87]. Moreover, in that study a significant correlation was observed between urinary-labeled nitrate excretion and plasma levels of the endogenous NOS inhibitor ADMA. This suggests that NO synthesis in rabbits during early hypercholesterolemia is reduced, which may involve inhibition by ADMA [87]. Using a similar technique, experimental burn

injury in rats resulted in an increase in the L-arginine-dependent NO production [88].

The origin of nitrite in human urine is still unknown. Administration of L-[*guanidino*-$^{15}N_2$]arginine (given in drinking water at 10 mg/100 ml for 4 days) to mice and GC–MS analysis of urine samples for nitrite and nitrate revealed enrichment of both ^{15}N-nitrite and ^{15}N-nitrate in urine above the natural abundance suggesting that urinary nitrite derives from the L-arginine/NO pathway in mouse [65].

6.3. Discussion

It is obvious that NO production rate measured in healthy subjects is limited to a few studies and varies considerably, between 0.22 and 1.00 μmol/kg/h. This variability may partly depend on the stable-isotope infusion protocol and analytical techniques used. Therefore, when studying the effect of disease on NO production it seems valid to also include a control group instead of using literature values as a reference. In several diseases studied with the technique indications for reduced or enhanced NO production have been obtained, which may be attributed to reduced L-arginine availability or presence of NOS inhibitors on the one hand or enhanced (isoform-specific) NOS enzyme activity on the other hand.

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