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Estimation of nitric oxide synthase activity via LC-MS/MS determination of $^{15}\text{N}_3$ -citrulline in biological samples

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Abstract

Rationale—We showed that the metabolite peaks of $^{15}\text{N}_3$ -citrulline ($^{15}\text{N}_3$ -CIT) and $^{15}\text{N}_3$ -arginine ($^{15}\text{N}_3$ -ARG) could be detected when $^{15}\text{N}_4$ -ARG was metabolized by nitric oxide synthase (NOS) in endothelial cells. The usefulness of these metabolites as potential surrogate indices of nitric oxide (NO) generation is evaluated.

Methods—A hydrophilic-interaction liquid chromatography electrospray tandem mass spectrometric assay (LC-MS/MS) was utilized for the simultaneous analysis of $^{15}\text{N}_4$ -ARG, ARG, CIT, $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG. $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG from impurities of $^{15}\text{N}_4$ -ARG were determined and corrected for the calculation of their concentration. $^{15}\text{N}_4$ -ARG-derived NO, i.e., ^{15}NO formation was determined by analyzing ^{15}N -nitrite accumulation by another LC-MS/MS assay.

Results—After EA.hy926 human endothelial cells were challenged with $^{15}\text{N}_4$ -ARG for 2 hours, the peak intensities of $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG significantly increased with $^{15}\text{N}_4$ -ARG concentration and positively correlated with ^{15}N -nitrite production. The estimated K_m values were independent of the metabolite (i.e., $^{15}\text{N}_3$ -CIT, $^{15}\text{N}_3$ -CIT+ $^{15}\text{N}_3$ -ARG or ^{15}N -nitrite) used for calculation. However, after correction for its presence as a chemical contaminant of $^{15}\text{N}_4$ -ARG, $^{15}\text{N}_3$ -ARG was only a marginal contributor for the estimation of NOS activity.

Conclusions—These data suggest that the formation of $^{15}\text{N}_3$ -CIT can be used as an indicator of NOS activity when $^{15}\text{N}_4$ -ARG is used as a substrate. This approach may be superior to the radioactive ^{14}C -CIT method which can be contaminated by ^{14}C -urea, and to the ^{14}N -nitrite method which lacks sensitivity.

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Conflict of interest

The authors have declared no conflict of interest.

Keywords

L-arginine; L-citrulline; liquid chromatography tandem mass spectrometry; nitric oxide; nitric oxide synthase

1 Introduction

As an endogenous signaling molecule, nitric oxide (NO) is involved in a variety of physiological processes including blood pressure regulation, platelet aggregation/adhesion, neurotransmission as well as cellular defense.^[1] Physiologically, NO is produced by the enzyme nitric oxide synthase (NOS) which converts L-arginine (ARG) into L-citrulline (CIT) and NO. NOS exists in a variety of isoforms including NOS1, NOS2 and NOS3 which are encoded by different genes.^[2] While NOS1 is constitutively expressed in neuronal and certain epithelial cells, NOS2 is inducible with lipopolysaccharide and cytokines in a multitude of different cells. The last isoform, NOS3 (endothelial NOS, eNOS) has been found to be constitutively expressed in endothelial cells. The activities of NOS1 and NOS3 are known to be regulated by Ca^{2+} and calmodulin. In endothelial cells, NO keeps blood vessels dilated, prevents the adhesion of platelets and white cells, and inhibits vascular smooth muscle proliferation.^[2] The loss of adequate production of NO in the endothelium is a major cause of endothelial dysfunction, which is a hallmark of a variety of cardiovascular diseases such as hypertension, atherosclerosis and diabetes.

Due to the pivotal role of NO in physiology, numerous analytical methods have been developed and used to quantify NO production via the NOS-mediated enzymatic conversion of ARG to NO.^[3] Direct measurement of NO in biological fluid is extremely difficult because NO is a short-lived molecule with an estimated in vivo half-life in human blood of 3–5 seconds.^[4] Therefore, oxidation end-products of NO, viz., inorganic nitrite and nitrate ions, are measured in blood and urine, and their accumulation has been used as an index of NO production. Various analytical approaches including colorimetric assay based on Griess reaction,^[5] fluorometric assay^[6] as well as gas chromatography-mass spectrometry^[7] have been applied to measure nitrite and nitrate. However, the extent to which these ion fluxes represent quantitative NO production under diverse experimental conditions is unknown.^[8] In addition, sources of nitrite and nitrate other than from ARG, e.g., dietary intake, would need to be accounted for.^[9]

In addition to NO, CIT is also produced from ARG by NOS. This amino acid, however, is also formed via other pathways, such as the urea cycle and the dimethylarginine dimethylaminohydrolase-mediated metabolism of asymmetric dimethylarginine (ADMA). Thus, a simple quantitation of total CIT production does not provide an accurate estimation of NO production. In order to discriminate the NOS-derived CIT from other sources, radiolabeled ARG, usually using ^3H or ^{14}C , has been utilized to enable measurement of radioactive CIT.^[10] In this assay, unreacted radiolabeled ARG is removed by retention on a cation exchange column which allows radiolabeled CIT to pass through the column, and NOS activity is determined simply by counting radioactivity in the effluent. However, it was pointed out that, without further chromatographic separation, arginase catalyzed conversion

of ^{14}C -ARG to ^{14}C -urea would be included erroneously as part of the radioactive signal of ^{14}C -CIT, as shown in rat liver mitochondria.^[11]

We have reported a liquid chromatography tandem mass spectrometric (LC-MS/MS) assay for the simultaneous bioanalysis of ARG, $^{15}\text{N}_4$ -ARG, CIT, and methylated arginines.^[12] Because discrete cellular compartments for ARG have been proposed,^[13, 14] the use of isotope-labeled ARG, e.g., $^{15}\text{N}_4$ -ARG, as an exogenous substrate is advantageous since it allows insights into the relative dispositional fates of exogenous vs. endogenous ARG.

When applying this assay to cellular studies, we observed two chromatographic peaks in cell lysate samples after cells were exposed to $^{15}\text{N}_4$ -ARG.^[12] These peaks were tentatively identified as those of $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG. Since $^{15}\text{N}_3$ -CIT can be formed with ^{15}NO from the metabolism of $^{15}\text{N}_4$ -ARG by NOS, and it can then be further metabolized to $^{15}\text{N}_3$ -ARG via the urea cycle, monitoring of these peaks may theoretically allow for an estimation of NOS activity. The objective of the present study, therefore, is to evaluate the use of peak intensity of $^{15}\text{N}_3$ -CIT as surrogate index of NO production when an exogenous ARG source ($^{15}\text{N}_4$ -ARG) is used. This approach was then compared to the extent of ^{15}N -nitrite production in cultured human endothelial cells.

2 Materials and Methods

2.1 Chemicals and reagents

ARG [as L-arginine HCl], CIT [as L-citrulline], 4-(2-Hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES), $\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$, bovine brain calmodulin, (6*R*)-5,6,7,8-tetrahydro-1-biopterin (BH_4), NADPH, ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and L- N^G -Nitroarginine methyl ester (L-NAME) were purchased from Sigma (St. Louis, MO). $^{15}\text{N}_4$ -ARG [as its HCl, (U- $^{15}\text{N}_4$, 98%)], the internal standard for ARG, $^{13}\text{C}_6$ -ARG [as ARG:HCl (U- $^{13}\text{C}_6$, 98%)], the internal standard for CIT, D_4 -CIT [as L-citrulline (4,4,5,5- D_4 , 96.5 %)], and ^{15}N -nitrite [as NaNO_2 (^{15}N , 98%+)] were obtained from Cambridge Isotope Laboratories, Inc. These compounds were used without further purification. Cell culture reagents were purchased from Invitrogen.

2.2 In vitro cell study

EA.hy926 human vascular endothelial cells, which is an immortalized cell line derived from human umbilical vein endothelial cells,^[15] were obtained as a gift from the University of North Carolina. Cells were grown in a low glucose-low ARG modified Dulbecco's modified Eagle's medium (DMEM) containing $0.9 \text{ g} \cdot \text{L}^{-1}$ of glucose and $21 \text{ mg} \cdot \text{L}^{-1}$ of ARG^[16] supplemented with 10 % fetal bovine serum, and $100 \text{ U} \cdot \text{mL}^{-1}$ penicillin and $100 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ streptomycin at 37°C in a 5 % CO_2 incubator for 7 days.

For intact cell experiments, cells were washed twice with phosphate-buffered saline and equilibrated in Locke's solution (LS; 154 mM NaCl, 5.6 mM KCl, 2 mM CaCl_2 , 1 mM MgSO_4 , 10 mM HEPES, 3.6 mM NaHCO_3 and 5.6 mM glucose) for 1 hour before the experiment. Different concentrations of $^{15}\text{N}_4$ -ARG (0, 20, 50, 100, 200, 500 μM , equivalent to 0, 3.6, 8.9, 17.8, 35.6, 89.1 $\text{mg} \cdot \text{L}^{-1}$) were added to the cells. After 2 hours, the cell

incubation medium was collected and cells were lysed and collected for analysis. For cell lysates experiments, cells were lysed with lysis buffer before the experiment. Different concentrations of $^{15}\text{N}_4$ -ARG (0, 5, 10, 20, 50, 100 μM , equivalent to 0, 0.9, 1.8, 3.6, 8.9, 17.8 $\text{mg}\cdot\text{L}^{-1}$) were added to the cell lysates. L-NAME (100 μM) was added to the reaction mixture to examine NOS inhibition. After 30 min, cell lysate samples were collected for analysis. Protein concentrations in the cell lysates were determined by Lowry assay.^[17]

2.3 Rat liver mitochondria study

Mitochondria were isolated from an adult male Sprague Dawley rat liver according to Raha et al.^[18] The isolated liver was finely minced and gently homogenized in homogenization buffer (50 mM HEPES pH 7.4, 70 mM sucrose, 220 mM mannitol, and 1 mM EGTA). Following homogenization the mixture was centrifuged at $3000 \times g$ for 10 min. The supernatant was then centrifuged at $15,000 \times g$ for 10 min. The pellet was gently solubilized in homogenization buffer and centrifugation steps were repeated. The pellet was resuspended and recentrifuged at $15,000 \times g$ for a final time and the final mitochondrial pellet was resuspended in buffer (0.34 M sucrose, 100 mM KCl, 10 mM Tris-Cl, 1 mM EDTA). The protein concentration was determined and adjusted to $5 \text{ mg}\cdot\text{mL}^{-1}$. The cytochrome c oxidase activity of the prepared mitochondria was measured by a colorimetric assay using a Cytochrome c Oxidase Assay Kit (Sigma, MO). The decrease in the absorbance at 550 nm was monitored by a spectrophotometer.

To assess metabolite production from exogenous ARG ($^{15}\text{N}_4$ -ARG) in the mitochondria, reactions were run according to Venkatakrishnan et al.^[11] An aliquot of 150 μg of isolated mitochondria was added to the reaction mixtures which consisted of 50 mM HEPES (pH 7.6), 400 μM NADPH, 400 μM CaCl_2 , 5 μM tetrahydrobiopterin, 50 μM $^{15}\text{N}_4$ -ARG, and 1.5-fold molar excess of calmodulin in a total volume of 0.25 mL. After incubation at 23°C for 10 min, samples were collected for analysis.

2.4 LC-MS/MS assay for $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG

$^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG were analyzed by hydrophilic-interaction liquid chromatography electrospray tandem mass spectrometry along with ARG, $^{15}\text{N}_4$ -ARG, CIT, ADMA, and SDMA.^[12] Briefly, an aliquot of 20 μL of the cell lysates or cell medium was mixed with 3 internal standards (20 μL of $^{13}\text{C}_6$ -ARG 1 μM , 20 μL of D_4 -CIT 1 μM , and 20 μL of D_7 -ADMA 250 nM) and 120 μL of mobile phase B (acetonitrile containing 0.5% acetic acid and 0.025% trifluoroacetic acid) was added for protein precipitation. After centrifugation at $13,000 \times g$ for 20 min, the supernatant was collected for analysis.

The liquid chromatography consists of Shimadzu LC-20AD delivery pump, SIL-20AC autosampler and CBM-20A system controller (Shimadzu Scientific Instruments; Columbia, MD). Analytes were separated by liquid chromatography on a $150 \times 2.1 \text{ mm}$ Alltima HP HILIC 3 μm column with an isocratic elution with 15 % mobile phase A (water containing 0.5 % acetic acid and 0.025 % trifluoroacetic acid) and 85 % mobile phase B (acetonitrile containing 0.5 % acetic acid and 0.025 % trifluoroacetic acid) at a flow rate of $0.25 \text{ mL}\cdot\text{min}^{-1}$ for 6 min.

[M+H]⁺ ions were analyzed in the multiple reaction monitoring mode of the ABI/Sciex API3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an electrospray ion (ESI) source. To identify isotopomeric CIT and ARG metabolites of ¹⁵N₄-ARG, MRM transitions of 179.2→71.1, 178.2→71.0, and 177.2→71.0 were added in the MS setting and monitored. The predicted MRM transitions of ¹⁵N₃-CIT and ¹⁵N₃-ARG are m/z 179.2→71.1 and 178.2→71.0, respectively. Due to the unavailability of authentic standards, ¹⁵N₃-CIT and ¹⁵N₃-ARG concentrations were estimated by assuming identical behaviors of isotopomeric compounds, using established calibration curves for unlabeled CIT and ¹⁵N₄-ARG, respectively. D₄-CIT and ¹³C₆-ARG were used as internal standards, respectively. The MRM transitions of D₄-CIT and ¹³C₆-ARG are m/z 180.2 → 74.1 and 181.2 → 74.2, respectively.

2.5 LC-MS/MS assay for ¹⁵N-nitrite

¹⁵N-nitrite was determined by an LC-MS/MS assay.^[19] An aliquot of cell lysates and cell medium sample 100 µL was first incubated with 10 µL of 0.5 mM 2,3-diaminonaphthalene (DAN, in 0.5 M HCl) for 10 min at room temperature to convert ¹⁵N-nitrite in the sample to ¹⁵N-2,3-naphthotriazole (¹⁵N-NAT) using the procedure reported by Misko et al.^[6] After addition of acetonitrile for protein precipitation, samples were centrifuged at 13,000 × g for 5 min. An aliquot of 20 µL of the supernatant was then mixed with 5 µL of the internal standard, 1H-naphth[2,3-d]imidazole (5 ng·mL⁻¹), and diluted with 170 µL of water and injected to LC-MS/MS. The liquid chromatography consisted of a Shimadzu LC-20AD delivery pump, SIL-20AC autosampler, and CBM-20A system controller (Shimadzu Scientific Instruments; Columbia, MD). Chromatographic separation was accomplished on Agilent XDB-C18, 2×50mm, 5 µ column by a gradient elution of 5% mobile phase B (0.1 % v/v formic acid in acetonitrile) in mobile phase A (0.1 % v/v formic acid in water), rising linearly to 95 % mobile phase B in 4 min. The flow rate was 0.3 mL/min. [M+H]⁺ ions were analyzed in the multiple reaction monitoring (MRM) mode of the ABI/Sciex API3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an electrospray ion (ESI) source. The MRM transitions were m/z 170.1→115.0 for ¹⁴N-NAT, m/z 171.1→115.0 for ¹⁵N-NAT, and m/z 169.2→115.0 for the IS.

2.6 Kinetic calculations

NOS activity was assessed based on the production of various products, by fitting the observed data to the Michaelis-Menten equation.

$$V = \frac{V_{\max} \times S}{K_m + S}$$

where V is the reaction rate, V_{max} is the maximum rate, S is the concentration of the substrate, i.e., ¹⁵N₄-ARG, and K_m is the substrate concentration at which the activity is half-maximal. The kinetic parameters, V_{max} and K_m were estimated by nonlinear regression of substrate dependence.

V was estimated by the accumulated amounts of either ^{15}N -nitrite, $^{15}\text{N}_3\text{-CIT}$, or $^{15}\text{N}_3\text{-CIT} + ^{15}\text{N}_3\text{-ARG}$ over the incubation time of 120 min, and corrected for protein content. Individual data were fitted to the Michaelis-Menten equation using WinNonlin 5.0 (Pharsight Corporation, Mountain View, CA).

2.7 Statistical analysis

Statistical analyses were performed using one-way ANOVA. Comparisons among different groups were followed using Tukey post hoc analysis. Differences with $p < 0.05$ were denoted as statistically significant.

3 Results

3.1 Observation of new chromatographic peaks from cell samples after exposure to $^{15}\text{N}_4\text{-ARG}$

After EA.hy 926 human endothelial cells were exposed to $^{15}\text{N}_4\text{-ARG}$ for 2 hours, cell lysate samples were analyzed to examine peaks representing a series of isotopomeric CIT and ARG compounds in the ion chromatogram. Based on the theoretical molecular masses and fragmentation patterns of possible metabolites, selected MRM transitions of $179.2 \rightarrow 71.1$, $178.2 \rightarrow 71.0$, and $177.2 \rightarrow 71.0$ were performed. Under each MRM transition, peaks with the retention time of CIT, 2.83 min, and that of ARG, 3.33 min were examined.

Fig. 1 shows the observed ion chromatograms with MRM transition of m/z $179.2 \rightarrow 71.1$ (peak A), $178.2 \rightarrow 71.0$ (peak B), and $177.2 \rightarrow 71.0$ (peak C) with retention times of 2.83 min (that of CIT) and 3.33 min (that of ARG). The intensity of peak A and peak B increased in a saturable manner when EA.hy926 human endothelial cells were exposed to increasing extracellular $^{15}\text{N}_4\text{-ARG}$ concentrations (Supplementary Fig. S1A and B). However, the intensity of peak C was only marginally dependent on extracellular $^{15}\text{N}_4\text{-ARG}$ concentrations in appearance only, and statistical significance could not be demonstrated ($p > 0.05$, Supplementary Fig. S1C).

3.2 Tentative assignments of observed peaks

Table 1 shows the theoretical MRM transition and chromatographic properties of various CIT and ARG isomers. It is apparent that the observed characteristics of peak A were consistent with that of $^{15}\text{N}_3\text{-CIT}$, and those of peak B with $^{15}\text{N}_3\text{-ARG}$. Both of these compounds are generated as a result of metabolism of $^{15}\text{N}_4\text{-ARG}$ to CIT, and the back conversion of CIT to ARG (Fig. 2). Thus, peaks A and B were then tentatively assigned as those for $^{15}\text{N}_3\text{-CIT}$ and $^{15}\text{N}_3\text{-ARG}$, respectively. The chromatographic characteristics of peak C are consistent with those of $^{15}\text{N}_2\text{-ARG}$ (Table 1). However, formal identity of these chromatographic peaks can only be made when the relevant authentic standard becomes available or when high resolution mass spectrometry technique is applied.^[20]

3.3 Other sources of $^{15}\text{N}_3\text{-CIT}$ and $^{15}\text{N}_3\text{-ARG}$ in samples

To determine possible sources of these compounds from chemical contamination and blank cells, an experiment was carried out to examine their presence using a broad range of $^{15}\text{N}_4\text{-ARG}$ standard solutions (0, 1, 4, 10, and 20 μM , $n=2$ each) in blank cell lysates without

incubation. The concentrations of $^{15}\text{N}_4$ -ARG standard solutions were chosen to cover the achievable intracellular $^{15}\text{N}_4$ -ARG concentration range after cells were challenged with 0 – 500 μM of $^{15}\text{N}_4$ -ARG.

When these samples were analyzed directly without incubation, there was no peak of $^{15}\text{N}_3$ -CIT found in the ion chromatogram, suggesting that this compound was generated by metabolism, rather than as an impurity. On the other hand, significant $^{15}\text{N}_3$ -ARG peaks were observed. The peak intensity of $^{15}\text{N}_3$ -ARG increased proportionally with the added $^{15}\text{N}_4$ -ARG concentration, with a mean value of 2.4 ± 0.3 % of $^{15}\text{N}_4$ -ARG (n=8), over the concentration range studied. This percentage was interpreted as arising from the presence of $^{15}\text{N}_3$ -ARG as an impurity in the $^{15}\text{N}_4$ -ARG commercial sample. In subsequent data analysis, therefore, all observed $^{15}\text{N}_3$ -ARG peak intensities were corrected for the presence of this compound as an impurity in $^{15}\text{N}_4$ -ARG.

3.4 Correlation between $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG vs. ^{15}N -nitrite

To evaluate whether $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG production can estimate NOS activity, the peak intensities of $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG were compared to another index of NOS activity, ^{15}N -nitrite production, by using cell samples after EA.hy926 human endothelial cells were exposed to different concentrations of $^{15}\text{N}_4$ -ARG for 2 hours. After incubation, an aliquot of the sample was used to determine peak intensities of $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG and an aliquot of the same sample was used to determine ^{15}N -nitrite concentrations and two measurements were compared. The peak intensities of $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG increased with an increase in the observed ^{15}N -nitrite concentration (Supplementary Fig. S2).

To test whether the combined $^{15}\text{N}_3$ -CIT + $^{15}\text{N}_3$ -ARG concentrations would correlate better to ^{15}N -nitrite than $^{15}\text{N}_3$ -CIT alone, we estimated the concentrations $^{15}\text{N}_3$ -CIT and $^{15}\text{N}_3$ -ARG assuming the calibration curves. Based on this assumption, the contribution of $^{15}\text{N}_3$ -ARG, after correction as an impurity from added $^{15}\text{N}_4$ -ARG, was relatively small to the total estimated concentration of $^{15}\text{N}_3$ -CIT + $^{15}\text{N}_3$ -ARG. In principle, the metabolism of ARG to CIT and NO should produce a 1:1 ratio of these metabolites. However, results showed that the estimated concentrations of $^{15}\text{N}_3$ -CIT were 14.5-fold higher than those of ^{15}N -nitrite (Supplementary Fig. S3). Statistical analyses of the data indicate significant correlations between $^{15}\text{N}_3$ -CIT vs. ^{15}N -nitrite (Pearson correlation coefficient, $p = 0.862$, $R^2 = 0.740$), $^{15}\text{N}_3$ -CIT + $^{15}\text{N}_3$ -ARG vs. ^{15}N -nitrite ($p = 0.865$, $R^2 = 0.744$), and $^{15}\text{N}_3$ -CIT vs. $^{15}\text{N}_3$ -ARG ($p = 0.998$, $R^2 = 0.931$).

3.5 Comparison of kinetic parameters of NOS activity

Michaelis-Menten plots of estimated NOS activity based on the formation of different metabolic products as a function of $^{15}\text{N}_4$ -ARG concentration in the intact cells and cell lysates are shown in Fig. 3 and Fig. 4, respectively. In intact cells, the estimated K_m values were independent of the metabolic product used. The K_m in the intact cells were higher than that in the cell lysates. On the other hand, the estimates of V_{max} varied depending on the metabolic product used. The estimated V_{max} values were about 10-fold higher using either $^{15}\text{N}_3$ -CIT or $^{15}\text{N}_3$ -CIT + $^{15}\text{N}_3$ -ARG, as compared to using ^{15}N -nitrite as an indicator of metabolite formation in the intact cells (Table 2). Unlabeled nitrite and nitrate were also

measured in the cell lysates. However, the $^{15}\text{N}_4\text{-ARG}$ concentration dependent production of unlabeled nitrite and nitrate was not clear, which hampered to estimate kinetic parameters likely due to the high baseline values (Supplementary Fig. S4).

3.6 Effects of NOS inhibitor on $^{15}\text{N}_3\text{-CIT}$ production

Fig. 5 shows the effects of L-NAME on the production of $^{15}\text{N}_3\text{-CIT}$ in the cell lysates. In the presence of L-NAME at 100 μM in the reaction mixture, $^{15}\text{N}_3\text{-CIT}$ formation was significantly diminished ($p < 0.05$).

3.7 Application of $^{15}\text{N}_3\text{-CIT}$ assay to rat liver mitochondria

Arginase catalyzed conversion of $^{14}\text{C-ARG}$ to $^{14}\text{C-urea}$ has been suggested to present a confounding factor in using radioactive $^{14}\text{C-CIT}$ as an index of NOS activity. This was pointed out by studies that showed apparent $^{14}\text{C-CIT}$ conversion in rat liver mitochondria, which did not have NOS activity.^[11, 21] Using the same rat liver mitochondria preparation, we examined whether $^{15}\text{N}_3\text{-CIT}$ formation can be observed.

Using cytochrome c oxidase activity as an index, we found that the isolated mitochondria fraction had a 5.5-fold enrichment in enzyme activity compared to whole homogenate, and a 17.0-fold enrichment compared to the supernatant fraction. Significant presence of $^{15}\text{N}_3\text{-CIT}$ was found to be formed when the whole liver homogenate was incubated with $^{15}\text{N}_4\text{-ARG}$ (Fig. 6). However, with the isolated rat mitochondria fraction, 2 of the 4 samples did not show any measurable $^{15}\text{N}_3\text{-CIT}$, while the other two samples revealed marginal peak intensity. The mean $^{15}\text{N}_3\text{-CIT}$ peak intensity found in the mitochondria preparation was not statistically different with or without added $^{15}\text{N}_4\text{-ARG}$. No appreciable $^{15}\text{N}_3\text{-ARG}$ was found in any of these samples.

4 Discussion

In the present study, metabolite formation ($^{15}\text{N}_3\text{-CIT}$ and $^{15}\text{N}_3\text{-ARG}$) from stable isotope labeled ARG ($^{15}\text{N}_4\text{-ARG}$) was evaluated as a possible index of NOS activity in endothelial cells and cell lysates. When $^{15}\text{N}_4\text{-ARG}$ is used as a substrate for NOS, $^{15}\text{N}_3\text{-CIT}$ and ^{15}NO should be produced at equal molar amounts (Fig. 2). After $^{15}\text{N}_3\text{-CIT}$ is produced, two urea cycle enzymes, i.e., argininosuccinate synthase and argininosuccinate lyase, convert it back to $^{15}\text{N}_3\text{-ARG}$ via $^{15}\text{N}_3\text{-argininosuccinate}$, which couples with an unlabeled nitrogen from aspartate.^[22, 23] Further metabolism of $^{15}\text{N}_3\text{-ARG}$ by NOS produces $^{15}\text{N}_3\text{-CIT}$ and unlabeled NO. Rapid equilibrium of the guanidine group possibly generate $^{15}\text{N}_2\text{-CIT}$ and ^{15}NO from $^{15}\text{N}_3\text{-ARG}$. However, $^{15}\text{N}_2\text{-CIT}$ was not detected (Table 1), indicating $^{15}\text{N}_3\text{-CIT}$ and unlabeled NO are dominantly produced from $^{15}\text{N}_3\text{-ARG}$ in our experimental system. Thus, after the first metabolic step of $^{15}\text{N}_4\text{-ARG}$, subsequent urea cycle lead to repeated formation of $^{15}\text{N}_3\text{-CIT}$ and $^{15}\text{N}_3\text{-ARG}$ in a futile cycle (Fig. 2). Note that with the added substrate $^{15}\text{N}_4\text{-ARG}$, only isotopically labeled $^{15}\text{N-NO}$ (measured as $^{15}\text{N-nitrite}$) is produced. However, through the subsequent urea cycle ($\text{CIT} \rightarrow \text{ARG}$ recycling system), the NO comes from the unlabeled nitrogen atom derived from aspartate, and unlabeled NO was formed as a consequence.

While we could not determine the true concentrations of $^{15}\text{N}_3\text{-CIT}$ and $^{15}\text{N}_3\text{-ARG}$ in this study because authentic compounds are not yet available, we estimated their concentrations based on the assumption that the calibration curves of CIT and $^{15}\text{N}_4\text{-ARG}$ could be applied. A partial justification of this assumption is that the LC-MS/MS peak intensity responses of $^{14}\text{N-ARG}$, $^{15}\text{N}_4\text{-ARG}$, $^{13}\text{C}_6\text{-ARG}$ were identical, and the calibration curves of $^{14}\text{N-ARG}$ and $^{15}\text{N}_4\text{-ARG}$ gave the same slopes when $^{13}\text{C}_6\text{-ARG}$ was used as an internal standard.^[12] Similarly, the LC-MS/MS peak intensity responses of $^{14}\text{N-CIT}$ vs. $\text{D}_4\text{-CIT}$ were also identical. Thus, isotopic labeling of CIT and ARG did not appear to affect their chromatographic responses dramatically. Naturally, the validity of the assumption used must be confirmed experimentally using authentic compounds of $^{15}\text{N}_3\text{-CIT}$ and $^{15}\text{N}_3\text{-ARG}$, if and when they become available.

Based on this estimation, ^{15}N -nitrite production was positively related to either $^{15}\text{N}_3\text{-CIT}$ production alone, or to the combined $^{15}\text{N}_3\text{-CIT} + ^{15}\text{N}_3\text{-ARG}$ accumulation (Supplementary Fig. S3). However, ^{15}N -nitrite levels were 14.5 ± 4.4 and 18.7 ± 5.6 -folds less than those of $^{15}\text{N}_3\text{-CIT}$ alone, and combined $^{15}\text{N}_3\text{-CIT} + ^{15}\text{N}_3\text{-ARG}$, respectively. Higher levels of $^{15}\text{N-CIT}$ compared to ^{15}N -nitrite and ^{15}N -nitrate were also reported when L-[guanidino- $^{15}\text{N}_2$]-ARG was used as a substrate.^[24] The use of ^{15}N -nitrite as a surrogate measure of produced ^{15}NO also assumes that the predominant route of ^{15}NO disappearance occurs through conversion to nitrite. However, it is possible that ^{15}NO may be lost via evaporation and/or oxidized to other product, i.e. ^{15}N -nitrate. Direct nitration of cellular proteins, e.g., to form nitrated tyrosine, is also possible. Thus, ^{15}N -nitrite accumulation may represent an under-estimate of ^{15}NO production. It is also possible that potential abundant interference and/or additional pathways are contributory to labeled CIT in cell systems.^[24]

Analysis of NOS activity using isotopic-labeled ARG typically has been obtained via determination of $^{14}\text{C-CIT}$, formed from $^{14}\text{C-ARG}$. Flam et al.^[25] reported that NO production which was measured by nitrite concentration, exceeded apparent $^{14}\text{C-CIT}$ formation at least by 8-fold in bovine aortic endothelial cells, indicating that radioactive CIT formation may significantly underestimate the actual NO production. The favored formation of unlabeled nitrite over $^{14}\text{C-CIT}$ is opposite to our present finding that ^{15}N -nitrite levels were 14.5-folds lower than that of $^{15}\text{N}_3\text{-CIT}$ (Supplementary Fig. S3). This discrepancy, however, can be explained by tracking the fate of $^{14}\text{C-CIT}$ and NO in the radioactive assay (Supplementary Fig. S5). Here, since the isotope labeling is made on the carbon atom, rather than the nitrogen atom, metabolism of $^{14}\text{C-ARG}$ would generate unlabeled NO and urea cycle would form a futile cycle of $^{14}\text{C-CIT}$ and $^{14}\text{C-ARG}$ along with new formation of NO. Thus, utilization of $^{14}\text{C-CIT}$ as an index of NOS production only measures NO production from the added substrate, and not from urea cycle. In contrast, use of unlabeled nitrite measures NO production from both the added substrate alone and from urea cycle.

Our results also indicate that the apparent production of $^{15}\text{N}_3\text{-ARG}$, after correction for its presence as a chemical contaminant of $^{15}\text{N}_4\text{-ARG}$, was only a marginal contributor for the estimation of NOS activity (Supplementary Fig. S3). Unlike $^{15}\text{N}_3\text{-CIT}$, which has only one major metabolic pathway operating through urea cycle, $^{15}\text{N}_3\text{-ARG}$ can be metabolized by several other enzymes besides NOS, viz., arginine decarboxylase, arginine:glycine

amidinotransferase and arginase.^[26] Thus, $^{15}\text{N}_3\text{-ARG}$ may be partially removed from the futile $^{15}\text{N}_3\text{-CIT} \rightarrow ^{15}\text{N}_3\text{-ARG}$ cycle through these enzymes.

Kinetic analysis using the various metabolic products of $^{15}\text{N}_4\text{-ARG}$ showed that the K_m values estimated from $^{15}\text{N}_3\text{-CIT}$ or $^{15}\text{N}_3\text{-CIT} + ^{15}\text{N}_3\text{-ARG}$ were similar to those from ^{15}N -nitrite, suggesting that all these metabolites utilize the same enzyme. The magnitude of K_m we observed in the intact endothelial cells is consistent with a value of $29 \pm 6 \mu\text{M}$ in bovine aortic endothelial cells, using radiolabeled ARG to CIT conversion as an index.^[27] The K_m estimates in the cell lysates in the present study were also comparable with the literature reports of $\sim 3 \mu\text{M}$ which was determined in the isolated enzyme preparation.^[27] The higher K_m in the intact cells compared with that in the cell lysates may be contributed to the $^{15}\text{N}_4\text{-ARG}$ transport into the cell before its metabolism. As discussed, the different V_{max} estimated values obtained likely reflected the underestimation of ^{15}NO production when ^{15}N -nitrite was used as the metabolite index.

Finally, we tested the application of this LC-MS/MS method to estimate NOS activity in the rat liver mitochondria preparation.^[11, 21] The presence of mitochondrial NOS has long been disputed. Several investigators have pointed out errors in methodologies used in documenting this activity^[28–30] including the presence of apparent activity which could not be blocked by NOS inhibitors. Recently, it was shown that the apparent ^{14}C -CIT radioactivity signal may have included ^{14}C -urea, which is formed via arginase-mediated metabolism of ARG.^[11, 21] Here, we showed that insignificant $^{15}\text{N}_3\text{-CIT}$ formation in rat liver mitochondria, while significant accumulation of this metabolite can be observed in rat liver homogenates.

The use of $^{15}\text{N}_3\text{-CIT}$ as an index of NOS activity in cell culture studies may possess several technical advantages over that of ^{14}C -CIT. First, stable isotopes are used in place of radioactive compounds, enabling better safety and easier disposal of experimental waste. Second, the LC-MS/MS method offers additional assay capabilities as other ARG-related compounds such as the dimethylarginines can also be simultaneously determined. Third, the identity of the studied compounds can be made with better certainty because the LC-MS/MS method incorporates chromatographic separation and mass identification along with fragmentation patterns.

Compared to the use of inorganic nitrite as an index of generated NO activity, the present method may also possess several advantages. First, endogenous (unlabeled) nitrite levels in biological systems are high (in μM 's), so the detection of generated NO (as measured by nitrite) lacks sensitivity. Second, as mentioned previously, the conversion of NO to nitrite in biological systems may not be complete because of NO evaporation and other reactions. The present method suggests that the sum of $^{15}\text{N}_3\text{-CIT}$ and $^{15}\text{N}_3\text{-ARG}$ would represent the total NO produced from the added $^{15}\text{N}_4\text{-ARG}$ substrate.

However, the current method also carries some disadvantages. First, an authentic standard of $^{15}\text{N}_3\text{-CIT}$ is not yet available to allow for unambiguous estimation of the concentrations of the compound. Second, LC-MS/MS methods are more costly to run, and require more extensive instrumentation and resources. However, the present study points out the potential

utility of LC-MS/MS methodology as an alternative for the existing ^{14}C -CIT assay in determining NOS activity.

5 Conclusions

When $^{15}\text{N}_4$ -ARG is used as a substrate in cell culture studies, metabolite formation of $^{15}\text{N}_3$ -CIT may be used as a surrogate indicator of NO generation and assessment of NOS activity. Determination of this isotopic metabolite may allow for the simple and sensitive examination of NO production from the exogenous ARG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representative ion chromatograms under MRM transitions of 179.2→71.1 (peak A), 178.2→71.0 (peak B), and 177.2→71.0 (peak C) in a human endothelial cell lysate sample obtained after cells were incubated with 100 μM of $^{15}\text{N}_4\text{-ARG}$ for 2 hours.

Figure 2.

Schematic representation of $^{15}\text{N}_3\text{-CIT}$ and $^{15}\text{N}_3\text{-ARG}$ formation from $^{15}\text{N}_4\text{-ARG}$. When $^{15}\text{N}_4\text{-ARG}$ is used as a substrate for NOS, $^{15}\text{N}_3\text{-CIT}$ is produced via NOS and subsequent urea cycle enzymes, argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL), form $^{15}\text{N}_3\text{-ARG}$. Further metabolism of $^{15}\text{N}_3\text{-ARG}$ by NOS produces unlabeled NO and $^{15}\text{N}_3\text{-CIT}$.

Figure 3.

Michaelis-Menten plots of estimated NOS activity in EA.hy926 cells based on the formation of different metabolic products after exposure to 0 – 500 μM of $^{15}\text{N}_4\text{-ARG}$ for 2 hours ($n=6$ for each concentration). The drawn lines are fits to the observed data using the Michaelis-Menten equation.

Figure 4.

Michaelis-Menten plots of estimated NOS activity in EA.hy926 cell lysates based on the formation of $^{15}\text{N}_3\text{-CIT}$ after cell lysates were exposed to 0 – 100 μM of $^{15}\text{N}_4\text{-ARG}$ for 30 min ($n=3$ for each concentration). The drawn lines are fits to the observed data using the Michaelis-Menten equation.

Figure 5.

Production of $^{15}\text{N}_3\text{-CIT}$ in the human endothelial cell lysates after cell lysates were incubated with $^{15}\text{N}_4\text{-ARG}$ (100 μM) in the presence of NOS inhibitor, L-NAME (100 μM) for 30 min. Data are presented as the mean $\pm$ SD (n=3). *, p<0.05 vs. control (i.e., without $^{15}\text{N}_4\text{-ARG}$). #, p<0.05 vs. $^{15}\text{N}_4\text{-ARG}$ (i.e., without L-NAME).

Total homogenate	+	-	+	-
Mitochondria	-	+	-	+
$^{15}\text{N}_4\text{-ARG}$	-	-	+	+

Figure 6.

Production of $^{15}\text{N}_3\text{-CIT}$ in the whole rat liver homogenate or purified rat liver mitochondria fraction after incubation with 50 μM of $^{15}\text{N}_4\text{-ARG}$ for 10 min. Data are presented as the mean $\pm$ SD (n=4). ***, p<0.001 vs. control (i.e., without $^{15}\text{N}_4\text{-ARG}$).

Table 1

LC-MS/MS properties of stable isotope-labeled isomers of CIT and ARG originating from the metabolism of $^{15}\text{N}_4$ -ARG.

Compounds		Expected MRM transition (m/z)	Retention time (min)	Results
CIT analogs	$^{15}\text{N}_3$ -CIT	179.2→71.1	2.83	Peak A in Fig. 1
	$^{15}\text{N}_2$ -CIT	178.2→71.0	2.83	Not detected
	$^{15}\text{N}_1$ -CIT	177.2→71.0	2.83	Not detected
ARG analogs	$^{15}\text{N}_3$ -ARG	178.2→71.0	3.33	Peak B in Fig. 1
	$^{15}\text{N}_2$ -ARG	177.2→71.0	3.33	Peak C in Fig. 1

Table 2

Estimated parameters of NOS activity by using different metabolic products (n=6 for each $^{15}\text{N}_4$ -ARG concentration).

Indicator	Vmax (pmol·mg ⁻¹ ·min ⁻¹)	Km (μM)
	Estimate (CV%)	Estimate (CV%)
Intact cells		
^{15}N -Nitrite	0.506 (11.9)	46.6 (29.5)
$^{15}\text{N}_3$ -CIT	4.22 (12.6)	59.6 (28.1)
$^{15}\text{N}_3$ -CIT+ $^{15}\text{N}_3$ -ARG	5.11 (13.1)	68.0 (27.6)
Cell lysates		
$^{15}\text{N}_3$ -CIT	3.18 (9.3)	5.33 (44.1)