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Nitro-*L*-Arginine Interferes with the Cadmium Reduction of Nitrate/*Griess* Reaction Method of Measuring Nitric Oxide Production[1])

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Summary: Nitro-*L*-arginine is used as an inhibitor of nitric oxide synthase in many biological systems. Nitric oxide (NO) is unstable and degrades to nitrite (NO_2^-) and nitrate (NO_3^-). The colorimetric reaction of NO_2^- with *Griess* reagent is commonly used to measure NO_2^-. NO_3^- may be measured as NO_2^- following reduction by cadmium or cadmium/copper. We found that bradykinin increased the formation of NO_2^- by bovine coronary endothelial cells. Nitro-*L*-arginine further increased the formation of NO_2^-. This increase is due to the interference of nitro-*L*-arginine in determination of NO_3^- by the cadmium reduction to NO_2^- and *Griess* reagent reaction. Incubation of nitro-*L*-arginine with cadmium or cadmium/copper produced a product that reacts with *Griess* reagent to form a compound that has an absorption spectrum identical to the product formed by NO_2^- and *Griess* reagent. Caution must be exercised when using the NO_2^-/NO_3^- measurement by the *Griess* reaction to assess inhibition of nitric oxide synthase by nitro-*L*-arginine.

Introduction

Nitric oxide (NO) is now recognized as an endothelium-derived vasodilator and a possible neurotransmitter (1). In biological systems, it is produced in very low concentrations, and it has a very short half-life (< 6 s) (2). Under physiological conditions, NO may degrade to nitrite (NO_2^-) and also nitrate (NO_3^-). This, however, represents only one of its inactivation pathways. Detection of NO_2^- and NO_3^- is commonly used as an index of nitric oxide synthase[2]) activity. A number of methods for NO, NO_2^- and NO_3^- detection have been reported. Measurements of reactive NO are made by chemiluminescence using the reaction of NO with ozone (3, 4) or luminol-hydrogen peroxide (5, 6) and by spectrophotometric assays based on the reaction with oxyhaemoglobin (7) or Fe-S proteins (8). For measuring the more stable NO_2^- and NO_3^-, a chromatographic method (7), a reaction of NO_2^- with *Griess* reagent followed by a spectrophotometric detection (9, 10), an automated system with a NO_2^--*Griess* reagent reaction (11, 12), and fluorometric method (13) have been reported. With these spectrophotometric and fluorometric methods, NO_3^- has to be reduced to NO_2^- by nitrate reductase or more commonly by a metal such as cadmium prior to the reaction with the reagents.

During the study of the effects of nitro-*L*-arginine on NO release from cultured endothelial cells, the NO_2^-/NO_3^- concentration was determined by cadmium (Cd) reduction and *Griess* reagent reaction. We found that the samples always produced more colour or apparent NO_2^- in the presence than in the absence of nitro-*L*-arginine. Preliminary data showed that the bradykinin stimulation of bovine coronary artery endothelial cells increased the production of NO_2^-. In the presence of nitro-*L*-arginine, the production of NO_2^- increased further rather than decreasing. This prompted us to investigate the effects of nitro-*L*-arginine on this assay method and the production of the interfering species in the assay.

Materials and Methods

Materials

Sulphanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride, and Cd powder (−100 mesh) were purchased from Aldrich Chemical Co. (Milwaukee, WI); $NaNO_2$, $NaNO_3$, *D*-arginine and *L*-arginine from Sigma Chemical Co. (St. Louis, MO); nitro-*L*-arginine from Sigma Chemical Co., Serva Feinbiochemica (Heidelberg, Germany), and Schweizerhalle (South Plainfield, NJ); bradykinin from BACHEM Bioscience Inc. (Philadelphia, PA); [^{3}H]*L*-arginine from Amersham (Arlington Heights, IL); all other chemicals and reagents from Sigma Chemical Co. Amino-*L*-arginine was a gift from Dr. *Owen Griffith* (Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI). Distilled-deionized water was used in this study. Precleaned plasticware with methanol and deionized water was used for all solutions.

Determination of nitrite in bovine coronary artery endothelial cells

Bovine coronary artery endothelial cells were cultured onto 6-well plates as previously described (14). The cells were washed with Hepes buffer and then incubated in 3 ml of buffer containing nitro-*L*-arginine (30 µmol/l) or vehicle for 10 min at 37 °C. Then, bradykinin (1 µmol/l) was added, and the incubation continued for 2 h. Following the incubation, the supernatant was removed for NO_2^-/NO_3^- measurement and the cells were frozen for protein determina-

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[2]) Enzyme: Nitric oxide synthase

tion (15). Total nitrite (NO_2^- and NO_3^-) was measured by the *Griess* reaction (see below). The results were expressed as total nitrite (pmol) and were normalized to the total amounts of protein. Cell-free samples did not have protein content and they were compared at the same volumes and concentrations of chemicals.

Nitric oxide synthase assay

Bovine coronary artery endothelial cells were grown to confluence on 225 cm^2 flasks and lysed with a pH 7.4 buffer containing: Hepes (11 mmol/l), sucrose (0.35 mol/l), EDTA (0.1 mmol/l), dithiothreitol (1 mmol/l), leupeptin (10 mg/l), aprotinin (2 mg/l), soybean trypsin inhibitor (10 mg/l), phenylmethylsulphonyl fluoride (10 mg/l), NP-40 (10 g/l), and glycerol (100 g/l). The conversion of [^{3}H]arginine to [^{3}H]citrulline was used as an index of nitric oxide synthase activity. The reaction volume was 100 µl in pH 7.5 buffer containing: Tris-HCl (50 mmol/l), EDTA (0.1 mmol/l), EGTA (0.1 mmol/l), mercaptoethanol (1 g/l), leupeptin (100 µmol/l), phenylmethylsulphonyl fluoride (1 mmol/l), soybean trypsin inhibitor (10 mg/l), aprotinin (2 mg/l), calmodulin (10 nmol/l), NADPH (1 mmol/l), tetrahydrobiopterin (3 µmol/l), *L*-arginine (10 µmol/l), [^{3}H]*L*-arginine (7.4 kBq, 2.52 TBq/mmol), calcium chloride (2.5 mmol/l), and 100 µg protein. Nitro-*L*-arginine was added to some tubes at a final concentration of 30 µmol/l. All samples were incubated for 15 min at 37 °C in a shaking water bath and the reaction was stopped by the addition of 1 ml of ice-cold pH 5.5 STOP buffer containing: Hepes (20 mmol/l), EDTA (2 mmol/l), and EGTA (2 mmol/l). The [^{3}H]citrulline was separated from the [^{3}H]arginine by passing the sample over an equilibrated Dowex cation exchange column (Na^+ form). Nitric oxide synthase activity is expressed as Bq of citrulline.

Nitrate/nitrite determination

All NO_3^-, NO_2^- and NO_3^-/NO_2^- standards and samples were prepared in pH 7.4 Hepes buffer consisting of Hepes (10 mmol/l), NaCl (150 mmol/l), KCl (5 mmol/l), $CaCl_2$ (2 mmol/l), $MgCl_2$ (1 mmol/l) and glucose (5 mmol/l). Nitrate was reduced to NO_2^- by incubating 1 ml of the solution with approximately 0.05 g of Cd powder for different periods of time, generally 15 min. To this incubation, 5 µl of 10% HCl were added, and the solution was centrifuged for a few minutes to separate the Cd. The supernatant of 800 µl was transferred to a small tube. A 25 µl of 5 g/l sulphanilamide in 2 mol/l HCl was added and mixed. Then, 25 µl of 3 g/l naphthylethylenediamine dihydrochloride in 0.1 mol/l HCl were added, mixed and allowed to react for 15 min. Two aliquots of 350 µl were transferred to 96-well microplate and the absorbance was measured at 540 nm on Microplate Autoreader (Bio-Tek Instrument, Winooski, VT). The absorbance was the average of two wells for each sample. Nitrite was treated the same way as NO_3^- but without the Cd. Absorbance of Hepes buffer blanks treated with Cd were subtracted from all measurements. Results were expressed as apparent NO_2^- (µmol/l).

High performance liquid chromatography

Chromatography was performed on a 1090 Series II Hewlett Packard Liquid Chromatograph (Hewlett Packard Co., Palo Alto, CA). Three types of columns and gradient programmes were used for separation of nitro-*L*-arginine and its Cd catalyzed product(s);

(a) an ODS C_{18} column (5 µm, 100 × 2.1 mm) (Hewlett Packard Co., Palo Alto, CA) with a 3 min 0 to 15% acetonitrile in water elution gradient;

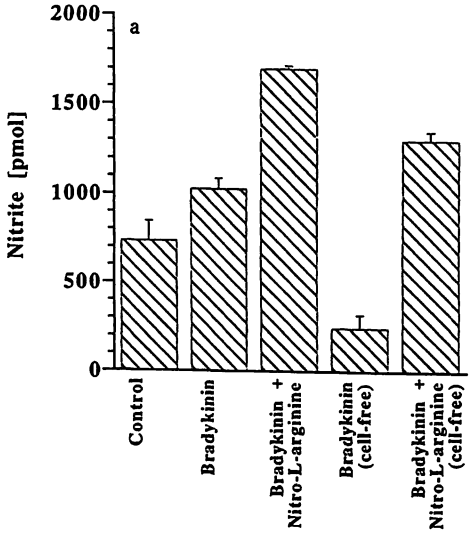
(b) a Nucleosil C_{18} column (5 µm, 250 × 10 mm) (Phenomenex, Torrance, CA) with a 5 min 0 to 10% acetonitrile in water elution gradient; and

(c) a Nucleosil 5 NH_2 column (5 µm, 250 × 4.6 mm) (Phenomenex) with 60% acetonitrile/40% water with 0.1 mol/l ammonium acetate elution solvent.

The absorbance was detected by a Hewlett Packard model 1040 photodiode array detector, and the chromatograms were recorded at 275 nm with 10 nm bandwidth and reference wavelength of 450 nm with 20 nm bandwidth. Full UV absorbance spectra (200–400 nm) were obtained during the HPLC analysis every 6 s and stored on a Hewlett Packard ChemStation for later analysis. The product peak fraction was collected and reacted with *Griess* reagent or otherwise analyzed.

Results and Discussion

It has been shown that bradykinin stimulated the NO production of endothelial cells (16) and the nitro-*L*-arginine was a potent inhibitor of the nitric oxide synthase (17, 18). Preliminary data show that bradykinin stimulated total NO_2^- (NO_2^- + NO_3^-) production by cultured endothelial cells (fig. 1a). However, preincubation of the



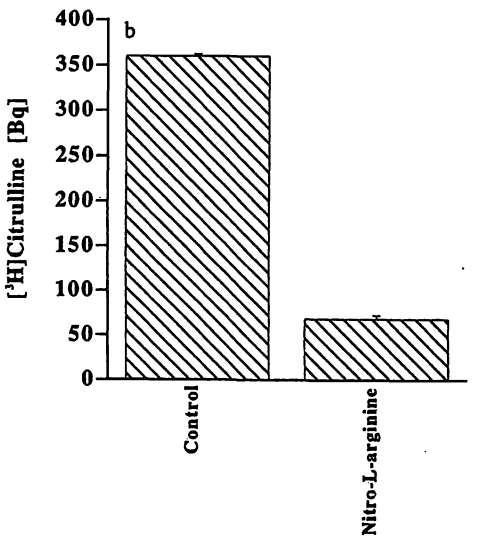


Fig. 1 a) Effect of nitro-*L*-arginine and bradykinin on total NO_2^- (NO_2^- + NO_3^-) by bovine coronary endothelial cells. These data are normalized to the total protein content. Also shown are cell-free incubations with bradykinin. The data for cell-free incubations are compared at the same volumes and concentrations of chemicals. Each value represents the mean ± S. E. (n = 6). b) Nitric oxide synthase activity (expressed as Bq per volume unit) of endothelial cells (control) and in the presence of nitro-*L*-arginine. (n = 3).

cells with the nitric oxide synthase inhibitor, nitro-*L*-arginine, increased instead of decreased NO_2^- production. In contrast, nitro-*L*-arginine markedly inhibited the nitric oxide synthase activity in the cells (fig. 1b). The total NO_2^- (NO_2^- + NO_3^-) in media (cell free) was also increased by nitro-*L*-arginine (fig. 1a). These data indicate that nitro-*L*-arginine actually inhibits NO production by endothelial cells and suggests that nitro-*L*-arginine may interfere with the assay for NO_3^- by cadmium reduction and *Griess* reaction.

Standards of NO_2^-, NO_3^- or a mixture of equimolar amounts of NO_2^- and NO_3^- were prepared. The samples containing NO_3^- were treated with Cd and reduced to NO_2^-. The samples were then reacted with *Griess* reagent, and the absorbance was measured. The standard curves are shown in figure 2a. The absorbance of the three sets of standards were superimposable for every concentration indicating that the experimental conditions were acceptable for the assay of NO_3^-. The possible interference of impurities in Hepes buffer and the Cd was investigated by incubating various amounts of Cd powder in water or Hepes buffer for 15 min, adding *Griess* reagent to the aliquots and measuring absorbance. More apparent NO_2^-, i. e., greater absorbance, was measured with increasing amounts of Cd. The absorbance was slightly greater in Hepes buffer than in water. However, the amount of colour detected as NO_2^- in the water and Hepes buffer was far less than (over 100 times) that measured in subsequent experiment with nitro-*L*-arginine. *D*-Arginine, *L*-arginine, amino-*L*-arginine, and nitro-*L*-arginine from several sources were incubated in Hepes buffer with and without Cd. Arginine analogs without the nitro-group did not produce measurable NO_2^- with *Griess* reagent when incubated in the presence or absence of Cd (fig. 2b). In contrast, large amounts of apparent NO_2^- were measured when nitro-*L*-arginine was incubated with Cd. No NO_2^- was detected when nitro-*L*-arginine was not treated with Cd. Nitro-*L*-arginine from different suppliers gave similar results. The nitro-*L*-arginine-Cd incubation solution was purified by HPLC to eliminate any possible impurity, including NO_3^-, and the peak fractions were collected and reacted with *Griess* reagent. The fraction containing unreacted nitro-*L*-arginine did not produce the colour while the fraction containing the reaction product gave the colour with *Griess* reagent. This shows that an impurity in nitro-*L*-arginine could not account for the colour production. The concentrations of apparent NO_2^- increased with increasing amounts of nitro-*L*-arginine. Incubation of nitro-*L*-arginine in Hepes buffer with Cd produced apparent NO_2^- immediately (data not shown) and the apparent NO_2^- concentration did not increase with a longer incubation time up to 3 h. The apparent NO_2^- was also observed in the incubation of nitro-*L*-arginine with Cd/Cu amalgam or by adding nitro-*L*-arginine to the flow through of a Cd column used with an HPLC or an automated NO_2^-/NO_3^- flow injection analyzer (19).

To identify the product(s) from the incubation of nitro-*L*-arginine with Cd, the incubation solution was analyzed by HPLC, and the compounds were separated on a C_{18} or amino propyl column. Nitro-*L*-arginine gave a single peak on HPLC that eluted on the ODS C_{18} column at 1 min and on the Nucleosil C_{18} column at 7.8 min. Following Cd treatment, two peaks were observed, the nitro-*L*-arginine peak and a Cd-derived reaction product peak. The reaction product was more polar than nitro-*L*-arginine and eluted at 0.6 min on the ODS C_{18} column and at 5.5 min on the Nucleosil C_{18} column. The retention time of the reaction product was greater than nitro-*L*-arginine on the amino propyl column (17.3 min

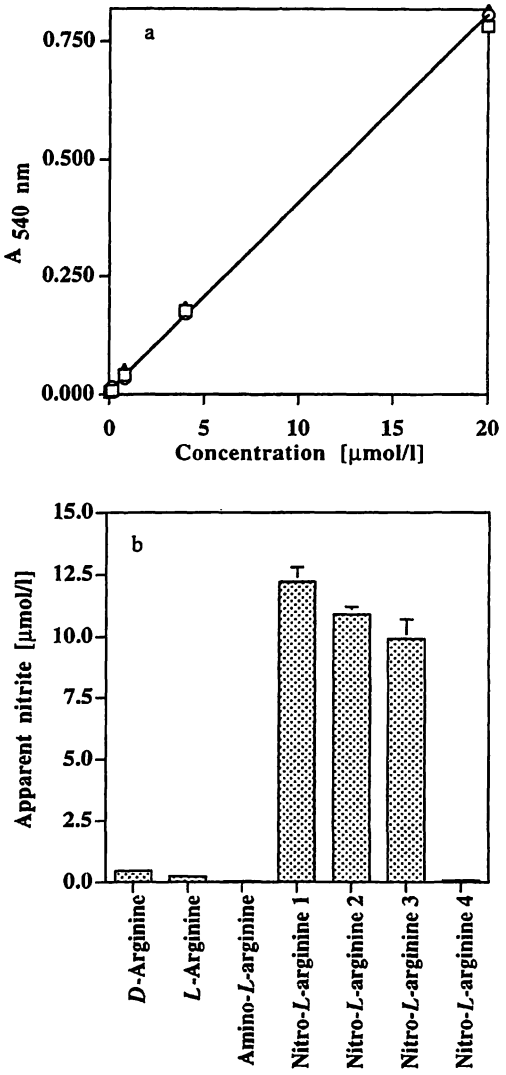
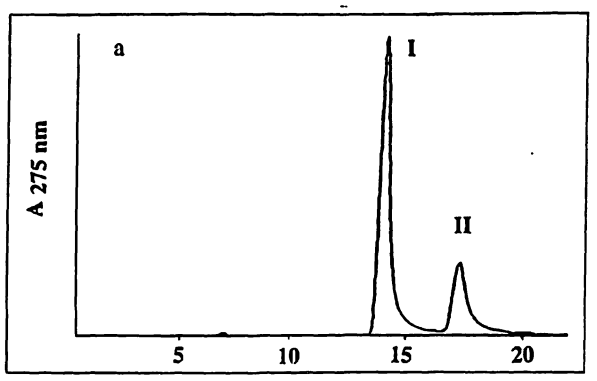


Fig. 2 a) Typical standard curves of NO_2^- (square), NO_3^- (circle), and an equimolar mixture of NO_2^- and NO_3^- (triangle). Nitrate was reduced to NO_2^- with Cd and measured by the *Griess* reaction. Nitrite was measured directly by the *Griess* reaction.
b) Apparent NO_2^- production from incubation of arginines (1.0 mmol/l) with Cd in Hepes buffer. Shown are *D*-arginine, *L*-arginine, amino-*L*-arginine, nitro-*L*-arginine 1 (Schweizerhalle), nitro-*L*-arginine 2 (Serva Feinbiochemica), nitro-*L*-arginine 3 (Sigma Chemical Co.), nitro-*L*-arginine 4 (Serva Feinbiochemica without Cd). Each value represents the average of 2 sets of duplicate determinations.



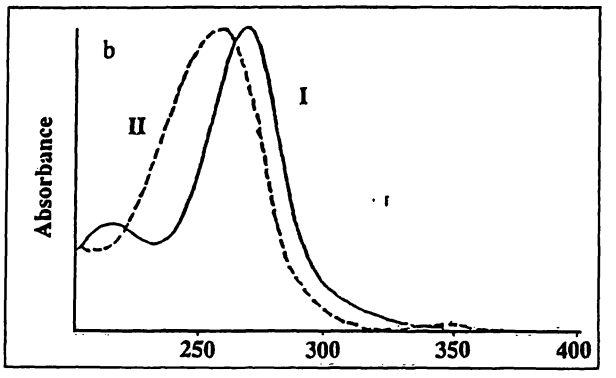


Fig. 3 a) Chromatogram of the incubation of nitro-*L*-arginine with Cd separated on the Nucleosil 5 NH_2 column (see text). The first peak (I) at 14.2 min corresponds to nitro-*L*-arginine retention time, the second peak (II) at 17.3 min is the reaction product. b) UV absorption spectra taken from the peaks of chromatograms during the analysis. The solid line (I) is the spectrum of nitro-*L*-arginine (the first peak) and the dashed line (II) is the spectrum of the reaction product (the second peak). The spectra are normalized at the absorption maxima.

versus 14.2 min for nitro-*L*-arginine) (fig. 3a). The absorption maximum of the reaction product was shifted from 270 nm for nitro-*L*-arginine to 255 nm with a weak absorption peak at 350 nm (fig. 3b). This product also had a light yellow colour. The retention time and UV absorbance of this product did not match *L*-arginine or any of the arginine analogs used in this study. The reaction product of nitro-*L*-arginine with Cd was isolated by HPLC and added to the *Griess* reagent. It formed a coloured adduct that had the same visible spectrum as the one formed in the reaction of NO_2^- with the *Griess* reagent (absorbance maxima of 543 nm). We therefore call the colour or absorbance produced by the Cd-derived reaction product "apparent NO_2^-". The identity of this reaction product is not known.

In conclusion, the incubation of nitro-*L*-arginine with Cd generates a product, possibly products, that appears responsible for the apparent NO_2^- that was determined by the reaction with *Griess* reagent. These data indicate that nitro-*L*-arginine will interfere with the measurement of NO_3^-. Detection of NO_2^- and NO_3^- is commonly used as an index of nitric oxide synthase activity even though these ions are not the only products of NO degradation (12, 20). Interference of nitro-*L*-arginine with the detection of NO_3^- will further complicate this relationship. For this reason, caution should be used when using the NO_2^-/NO_3^- measurement by the *Griess* reaction to assess inhibition of nitric oxide synthase by nitro-*L*-arginine.

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