Quenching of Nitric Oxide by an Oral Carbonaceous Adsorbent

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ABSTRACT—The ability of carbonaceous particles (AST-120), originally developed as an enteral adsorbent of uremic toxins, to quench nitric oxide (NO) was tested. NO in solutions prepared by two methods [NO gas bubbling and NO generating system, i.e., decomposition of 1-hydroxy-2-oxo-3-(aminopropyl)-3-isopropyl-1-triazene] were determined by a NO-specific reduction of carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide using an electron paramagnetic resonance spectrometry. NO concentrations were less in samples containing increasing concentrations of AST-120. In a separate study, nitrite concentrations in lipopolysaccharide-treated RAW264 cells were significantly less in incubation medium containing AST-120. Thus, AST-120 may be applicable as an enteral anti-NO agent.

Keywords: Carbonaceous adsorbent, Nitric oxide, Inflammatory bowel disease

Nitric oxide (NO) mediates various pathophysiological processes including inflammation in addition to its physiological role (1). Several agents such as analogs of the precursor, N\textsuperscript{G}-monomethyl-L-arginine, have been used to reduce NO production (1). However, many of these agents are not applicable for clinical use. Carbonaceous molecules such as activated charcoal are an adsorbent of gaseous and non-gaseous molecules (2). AST-120 (Cremedin\textsuperscript{®}), which consists of spherical porous carbonaceous particles with a diameter of 0.2 to 0.4 mm, has been used clinically as an enteral adsorbent of uremic toxins in progressive renal disease (3, 4). We hypothesized that such a non-specific adsorbent may quench NO because AST-120 is an analog of activated charcoals that are widely used as an adsorbent of NO\textsubscript{x} in industry.

In the present study, we tested the potency of AST-120 to quench NO in vitro by two sets of experiments. First AST-120-dependent adsorption of NO in solution was examined by determining NO-mediated reduction of carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (c-PTIO) to carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl (c-PTI) by electron paramagnetic resonance (EPR) spectrometry. Then, the amount of NO released by a biological system, lipopolysaccharide (LPS)-stimulated RAW264 cells, an established cell line of human macrophages, was compared between AST-120-treated and -nontreated cells.

In the first set of experiments, the ability of AST-120 to quench NO in solution was determined by the rate of reduction of c-PTIO. NO in solution was prepared by two methods: 1) NO gas and 2) a chemical NO generating system, reduction of 1-hydroxy-2-oxo-3-(aminopropyl)-3-isopropyl-1-triazene (NOC5). In the pilot study, NO-saturated Hanks balanced salt solutions (HBSS) was made by a continuous bubbling of pure NO gas (Yamato Sanki, Tokyo) in HBSS (Gibco, Grand Island, NY, USA) (for >30 min) under an anaerobic condition. Although NO in solutions are stable in the absence of oxygen, the experiments required mixing of solution in the atmosphere. Therefore, the rate of disappearance of NO was determined. For this purpose, 20 μl NO-saturated solution was added to 1 ml degassed HBSS and mixed continuously at room temperature. At specific times with the period of 5–30 sec after the additions, a 500-μl aliquot of the solution was mixed with high grade c-PTIO (the final concentration: 10 μM) (LabTech, Tokyo). A 200-μl sample of this mixture was transferred to a quartz sample tube for the subsequent EPR spectrometry as previously reported (5, 6). The settings for EPR measurements (by JES-RE1X; Joel, Akishima) were: microwave frequency: 9.42 GHz, microwave power: 8.0 mW; time constant: 0.1 msec; sweep time: 60 sec; center field: 335.8 mT; scan range: 5 mT; modulation frequency: 100 kHz; field modulation width: 0.063 mT; receiver gain: ×500.
pilot study demonstrated that concentration of NO was consistent at 15 sec. Using this protocol, the relationship between the amount of NO-saturated solution added to 10 μM c-PTIO in HBSS and the NO concentration was examined. Thus, 2–30 μl of NO-saturated solution was added to 1 ml of HBSS and EPR spectrometry was performed. Concentration of NO was determined by the height of the c-PTI signal (a product of NO-dependent reduction of c-PTIO), which was proportional to that of the original c-PTIO signal (5, 6). There was a linear relationship between the amount of NO solution added and calculated NO concentrations in the assay mixture (P < 0.0001, r = 0.998).

Based on the above pilot studies, one hundred microliters of NO-saturated solution was added to a tube containing 5 ml of HBSS with 1, 10, 50 or 100 mg/ml AST-120 (Kureha Chemical Industry, Tokyo). The tubes were placed on a shaker to keep the AST-120 agitated. Exactly 15 sec after the addition of NO-saturated solution, a portion of the solution (500 μl) was mixed with c-PTIO (10 μM) to measure the EPR spectrum. As shown in Fig. 1A, c-PTIO alone exhibited 5-line pattern of EPR signal. When NO-saturated solution was added, c-PTI signal gave a 9-line pattern. The c-PTI signal was substantially less when AST-120 was present in the mixture. Thus, when NO-saturated solution was added to HBSS containing 1 to 100 mg/ml AST-120, the c-PTI signals (i.e., NO concentration) decreased in proportion to the concentrations of AST-120 (Fig. 1B).

Then, the ability of AST-120 to quench NO produced by a chemical NO-generating system was tested. NOC5 (Dojindo, Kumamoto) was dissolved in 0.1 N NaOH and mixed with Dulbecco's-PBS, pH 7.2 to a final concentration of 100 μM. It has been shown that NOC5 at neutral pH is decomposed to generate NO spontaneously over an hour (7). The NOC5 containing D-PBS was incubated for 30 min at room temperature, and 5 ml of the solution was transferred to a tube with AST-120 (concentrations of AST-120 were 20 or 100 mg/ml). After 5, 15 and 30 sec, while the tube was agitated, a portion of the solution (500 μl) was mixed with 100 μM c-PTIO. The EPR spectrum of the solution was then obtained as described above except that the receiver gain was set at × 100. As shown in

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Fig. 1. EPR analysis of nitric oxide adsorption by AST-120. A) EPR spectrum of NO-specific reduction of c-PTIO and effect of AST-120. Top: EPR spectrum of c-PTIO: 200 μl of 10 μM c-PTIO in HBSS was subjected to EPR spectrometry as described in the text. c-PTIO gave 5-line signals in between MnO, an internal reference of the spectrometer. Middle: EPR spectrum of c-PTIO mixed with NO-containing HBSS: 100 μl of NO-saturated HBSS was added to 500 μl of HBSS, and c-PTIO was mixed into this solution 15 sec later. The EPR spectrometry showed a total of nine lines [5 lines of c-PTIO plus 4-lines (indicated by arrows) of c-PTI (the product of NO-dependent reduction of c-PTIO)]. As described in the text, the height of one c-PTI signal (the far left signal of c-PTI) was used to determine the concentration (6). Bottom: Attenuation of NO-dependent reduction of c-PTIO by AST-120: When 100 μg/ml of AST-120 was premixed in the mixture described in the middle, c-PTI signals were substantially attenuated, indicating that the concentration of NO in the mixture was less compared to that without AST-120. B) Adsorption of NO by AST-120: A 100-μl aliquot of NO-saturated HBSS was added to 5 ml of HBSS containing 0 (Control) to 100 μg/ml AST-120. Fifteen seconds after the addition of NO-saturated HBSS, NO remaining in the mixture was determined by EPR spectrometry using c-PTIO. The c-PTI signal was markedly reduced in the mixture containing increasing concentrations of AST-120. Values are each the mean ± S.E. of 3 determinations. *: Significantly (P < 0.05) different from the value for the control (no AST-120) (by the two-tailed unpaired t-test).
Fig. 2. Effect of AST-120 on NO concentrations in a solution with NO generating system. NOC5, the NO generating system, was prepared as described in the text. A 50-μl sample of NOC5 solution was transferred to a tube with 5 ml of HBSS containing 0 to 100 mg/ml AST-120. Five, 15 and 30 sec after the transfer, NO concentrations were determined. Closed squares: control (no AST-120), closed circles: AST-120 20 mg/ml, open circles: AST-120 100 mg/ml. Values are each the mean ± S.E. of 3 determinations. * and †: Significantly (P<0.05) different from the corresponding values for the control (no AST-120) and those for AST-120 (20 mg/ml), respectively (by the two-tailed unpaired t-test).

Fig. 3. AST-120-dependent adsorption of NO produced by RAW264 cells. RAW264 cells were stimulated with different concentrations (0, 1.0, 3.0 and 10.0 μg/ml) of lipopolysaccharide (LPS) (O26:B6) with or without AST-120 (0, 3, 10 and 30 mg/ml). Cells were incubated for 17 hr in a shaking incubator, and the culture media were assayed for nitrite concentration. LPS induced a dose-dependent increase in nitrite concentrations, and AST-120 reduced nitrite concentration dose-dependently. Closed squares: control (no AST-120), open circles: AST-120 3 mg/ml, closed circles: AST-120 10 mg/ml, open squares: AST-120 30 mg/ml. Values are each the mean ± S.E. of 3 determinations. *: Significantly (P<0.05) different from the corresponding values for the control (no AST-120) (by the two-tailed unpaired t-test).

The second set of experiment studied NO-quenching by the carbonaceous adsorbent in a biological system: the ability of AST-120 to reduce NO released from inflammatory cells was tested with cultured RAW264 cells (Riken Chemical Lab., Tokyo). For this purpose, RAW264 cells were cultured in RPMI1640 (Gibco) with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Gibco) and propagated. Semiconfluent cells were resuspended into a modified Eagle's medium (Gibco) with 10% FBS and 2 mM glutamine, and plated onto 24-well microtest plates with an insert (Falcon, Franklin Lakes, NJ, USA) at a concentration of 10⁶ cell/ml/well. Six hours later, when cells were attached to the plate, different concentrations (0 to 10 μg/ml) of E. coli LPS (O26: B6; Sigma, St. Louis, MO, USA) were added. In some wells, the insert containing 3 to 30 mg/ml of AST-120 was placed, while the insert without AST-120 was placed in the controls. In this setting, the AST-120 particles were not in direct contact with the cells. LPS-stimulated cells were incubated for 17 hr, and the culture media were assayed for nitrite concentrations using photometric measurements of diazo chromophore (Griess method) as previously reported (8). As shown in Fig. 3, increasing concentrations of AST-120 resulted in decreased concentrations of NO₂ in the culture media of RAW264 cells stimulated with different doses of LPS. When these cells were treated with 3-[4,5-dimethylthioazol-2-yl]-2,5-diphenyl-tetrazolium bromide to determine cell viability, there was no difference among cells treated with different concentrations of AST-120 (data not shown). Therefore, AST-120 reduced NO produced by cells without apparent effects on the viability of cells.

The above results indicated that the carbonaceous adsorbent prepared for clinical use is an effective quencher of NO. Thus, in two chemical systems and one biological one to produce NO, AST-120 reduced NO. Molecular mechanisms for NO adsorption by carbonaceous particles...
used in the present study remain to be elucidated. However, gaseous molecules such as NO and carbon monoxide were shown to be adsorbed by carbonaceous particles used for industrial purposes (2). Those materials have been shown to adsorb molecules by physical adsorption (van der Waals force) and chemical adsorption (2, 9, 10). Similar mechanisms may be involved in NO quenching by AST-120.

AST-120 has been used as an adsorbent of uremic toxins (3). It has been shown to adsorb uremic molecules by an enteral exchange of molecules through the epithelium. The agent is not absorbed or decomposed during the passage through the gastrointestinal tract, does not affect digestive enzymes, and is excreted unaltered when orally administered (4). These characteristics of AST-120 have shown to be advantageous over conventional charcoal powder for use as a luminal adsorbent for uremic toxins. Such characteristics may also benefit when AST-120 is used as an NO quencher. The pathophysiological role of NO in the inflammatory disease of bowel diseases, including peptic ulcer, ulcerative colitis or Crohn’s disease has been demonstrated (11–13). In models of these diseases, the administration of LNMA, which reduces the production of NO dependent on nitric oxide synthases, was variably effective in reducing severity of the disease. Infiltrations of polymorphonuclear cells, monocytes and macrophages are often observed on the surface of the epithelium in those pathophysiological conditions (12). Our results from RAW264 cells demonstrated that AST-120 reduces NO produced by infiltrating cells; therefore, AST-120 in the enteral lumen may reduce local concentrations of NO by a direct adsorption of the molecules. In some biological circumstances, NO is formed by non-enzymatic mechanisms (6, 14, 15). Direct quenching of NO by AST-120 may be effective in such circumstances, as shown in the present study. Since NO in a biological system modulates both physiological and pathophysiological processes, further in vivo studies to examine benefits of NO quenching under each pathophysiological condition are required.

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