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Formation of Guanidinosuccinic Acid, a Stable Nitric Oxide Mimic, from Argininosuccinic Acid and Nitric Oxide-Derived Free Radicals

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Guanidinosuccinic acid (GSA) is noted for its nitric oxide (NO) mimicking actions such as vasodilatation and activation of the N-methyl-D-aspartate (NMDA) receptor. We have reported that GSA is the product of argininosuccinate (ASA) and some reactive oxygen species, mainly the hydroxyl radical. We tested for GSA synthesis in the presence of NO donors. ASA (1 mM) was incubated with NOR-2, NOC-7 or 3-morpholinosydomine hydrochloride (SIN-1) at 37°C. GSA was determined by HPLC using a cationic resin for separation and phenanthrenequinone as an indicator. Neither NOR-2 or NOC-7 formed GSA. SIN-1, on the other hand, generates NO and the superoxide anion which, in turn, generated peroxynitrite which was then converted to the hydroxyl radical. Incubation of ASA with SIN-1 leads, via this route, to GSA. When ASA was incubated with 1 mM SIN-1, the amount of GSA produced depended on the incubation time and the concentration of ASA. Among the tested SIN-1 concentrations, from 0.5 to 5 mM, GSA synthesis was maximum at 0.5 mM and decreased with increasing concentrations of SIN-1. Carboxy-PTIO, a NO scavenger, completely inhibited GSA synthesis. SOD, a superoxide scavenger, decreased GSA synthesis by 20%, and catalase inhibited GSA synthesis only by 12%; DMSO, a hydroxyl radical scavenger completely inhibited GSA synthesis in the presence of SIN-1. These data suggest that the hydroxyl radical derived from a combination of NO and the superoxide anion generates GSA, a stable NO mimic. Meanwhile, synthesis of GSA by NO produces reactive oxygen and activates the NMDA receptor that generates NO from GSA, suggesting a positive feed back mechanism.

Keywords: Guanidinosuccinic acid, argininosuccinate, nitric oxide, NMDA receptor, vasodilator

INTRODUCTION

Synthesis of guanidinosuccinic acid (GSA) increases in the patients with renal failure and its concentration rises in urine, serum[1,2] and cerebro-spinal fluid.[3] Many biological activities of GSA such as strong inhibition of platelet aggregation,[4,5] induction of hemolysis,[6] erythrocytes transketolase inhibition[7] and induction of generalized clonic and tonic convulsions[8] have been reported. Because of these toxic effects, GSA

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has been implicated as an important uremic toxin. GSA, called by another name, N-amidino-L-aspartic acid, has been known to depolarize cat spinal motorneurones like most other analogues of aspartate and glutamate.[9] Recently, it was demonstrated that the convulsions induced by GSA were mediated by the activation of the N-methyl-D-aspartate (NMDA) receptor.[10,11] The NMDA receptor is a peptide receptor which is activated by glutamate, NMDA and its analogues and its activation increases nitric oxide (NO) synthesis through the nitric oxide synthetase activation. Therefore, the NMDA receptor has been implemented in various physiological and pathological processes such as memory, nerve system development, seizure disorders, and ischemic and excitotoxic brain damage.[12] In addition to this new action of GSA leading to the generation of NO, the presence of GSA in normal rat arteries and its NO mimicking actions were reported.[13] In that report, GSA (EC_{50} is around 8 μM) canceled the constriction of rat aorta by phenylephrine at 1×10^{-7} M accompaning by an increase of cGMP.

In addition, we demonstrated that GSA synthesis by isolated rat hepatocytes increased depending on the urea concentration in the incubation medium.[14] Taking a lead from the report that urea inhibits argininosuccinic acid lyase,[15] we also found that GSA is the product of argininosuccinic acid (ASA) and a reactive oxygen species.[16,17] ASA, an intermediate of urea synthesis, is a precursor of arginine. Recently, ASA increased in significance because of the role it plays in NO generation. The cycle called the citrulline–nitric oxide cycle or citrulline–arginine cycle is composed of nitric oxide synthetase, ASA synthetase and argininosuccinic acid lyase and was shown to operate in many tissues.[18,19] The existence of ASA in many tissues including endothelial cells,[20] smooth muscle[21] and nervous system[22] has been reported. Therefore, if NO generates GSA from ASA, the possibility arises that GSA amplifies NO synthesis via NMDA receptor.

In this paper, we report GSA synthesis *in vitro* from ASA stimulated by an NO derived reactive oxygen species.

MATERIALS AND METHODS

3-Morpholinosydomine hydrochloride (SIN-1), (±)-(E)-4-Methyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR-2), 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propamine (NOC-7) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide, sodium salt (carboxy-PTIO) were purchased from Dojin Laboratories (Kumamoto, Japan). SIN-1 (10 mM) was dissolved in 20 mM hydrochloric acid just before the experiments.

ASA was incubated in 1 ml of 50 mM potassium phosphate buffer (pH 7.4) with 1 mM SIN-1 with or without 1 mM carboxy-PTIO, superoxide dismutase (SOD) (42 U/ml) (Sigma Co.) or catalase (775 U/ml) (Sigma Co.). ASA (1 mM) was also incubated with NOR-2 or NOC-7 in the same buffer at 37°C. At the end of incubation, 1 ml of 20% (w/v) trichloroacetic acid was added to the incubation mixture and a part (0.1 ml) was used for GSA determination. GSA was determined by high-performance liquid chromatography (HPLC) using 9,10-phenanthrenequinone for the postlabeling after separation on a cation exchanging column according to Yamamoto *et al.*[23] as described previously.[14]

RESULTS

Investigation of GSA Synthesis from ASA with SIN-1, NOR-2 or NOC-7

The chromatograms of standard GSA and various incubations are shown in Figure 1. SIN-1 generates NO and the superoxide anion simultaneously, and these radicals form peroxynitrite which, in turn, generates the hydroxyl radical or a hydroxyl radical-like reactive oxygen species.[24] NOR-2[25] and NOC-7[26] generate NO. When

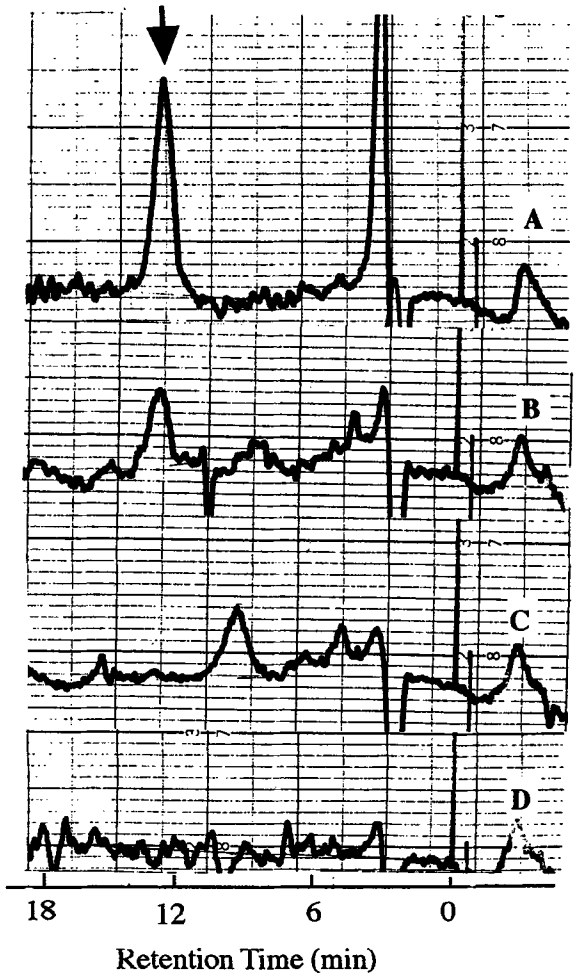

FIGURE 1 Chromatograms used in GSA analysis. Chromatogram A represents 0.5 nmole of authentic GSA as indicated by the arrow mark, B is the chromatograph obtained after 30 min incubation of 1 mM SIN-1 and 1 mM ASA, C is that following 30 min incubation of 1 mM ASA and 1 mM NOR-2. D is the chromatogram after 30 min incubation of 1 mM NOC-7 and 1 mM ASA. The horizontal axes show the retention time (min) and the vertical axes show the strength of fluorescence.

ASA was incubated with SIN-1, GSA was formed as shown in Figure 1(b). An unknown peak that had a shorter retention time than that of GSA appeared when ASA and NOR-2 were incubated as shown in Figure 1(c). This unknown peak appeared when NOR-2 was incubated without ASA. Therefore, we concluded that this peak was derived from NOR-2 reacting with the NO

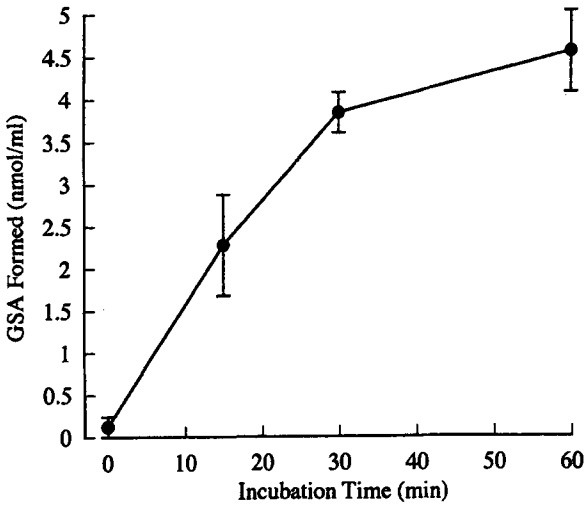

FIGURE 2 Time course of GSA production from ASA and SIN-1 *in vitro*. ASA (1 mM) was incubated with 1 mM SIN-1 as described in Materials and Methods. Each point represents the mean of duplicate incubations. Vertical bars depict the range of each determination.

generated by NOR-2. NOC-7 also did not form GSA from ASA as shown in Figure 1(d).

We have reported that the hydroxyl radical is much more effective than the superoxide anion in the synthesis of GSA from ASA.[16] When 1 mM ASA was incubated with 1 mM SIN-1 at 37°C, GSA increased almost linearly depending on the incubation period up to 60 min (Figure 2). This result corresponds well to the report that the half life of SIN-1 at pH 7.4 in phosphate buffered saline was 53 min.[27] GSA increased depending on the concentration of ASA when incubated with 1 mM SIN-1 as shown in Figure 3. Among the concentrations of SIN-1 tested, ranging from 0.5 to 5 mM, GSA synthesis from 1 mM ASA was maximum at 0.5 mM and decreased with increasing concentrations of SIN-1, as shown in Figure 4, despite the fact that the amount of GSA synthesized increased depending on the incubation time in this experiment (Figure 2). The reason for less GSA synthesis at higher concentrations of SIN-1 is not clear. Competition between SIN-1 and ASA for the hydroxyl radical may account in part for this. Another possible reason is that at high concentrations of SIN-1, the reactive oxygen species that

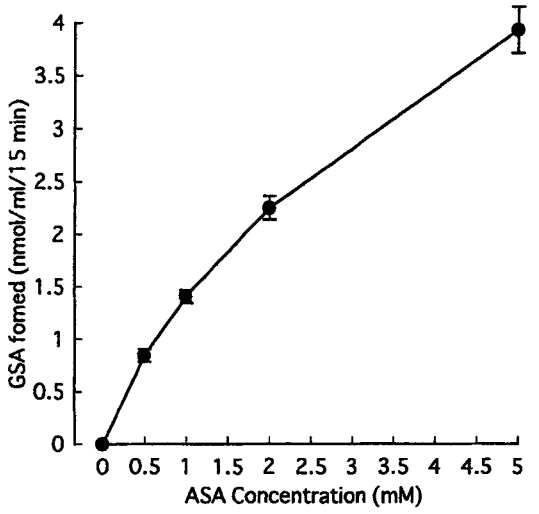

FIGURE 3 GSA synthesis by SIN-1 from various concentrations of ASA. ASA from 0 to 5 mM was incubated with 1 mM SIN-1 for 30 min as described in Materials and Methods. Each point represents the mean of duplicate incubations. Bars indicate the range of each determination.

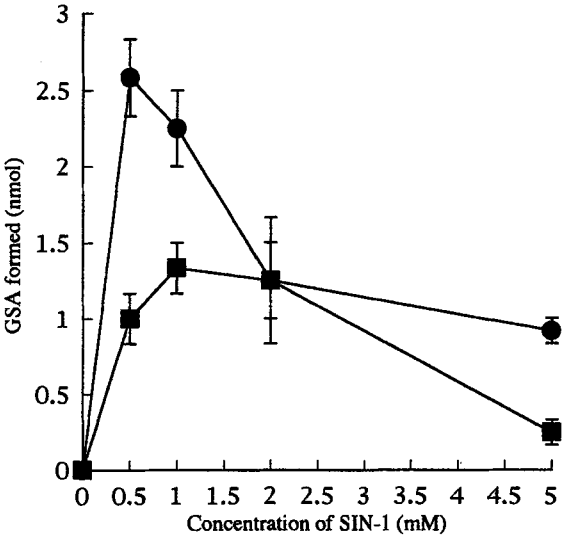

FIGURE 4 GSA synthesis from ASA by various concentrations of SIN-1. SIN-1 from 0 to 5 mM was incubated with 1 mM ASA for 15 or 30 min as described in Materials and Methods. Each point represents the mean of duplicate incubations. Bars indicate the range of each determination. GSA amounts produced at 15 or 30 min incubation are expressed as ■ and •, respectively.

generates GSA may decrease. Because, SIN-1 is degraded to the superoxide anion and, subsequently, to NO through the action of O_2, the amount of O_2 in the reaction mixture may be

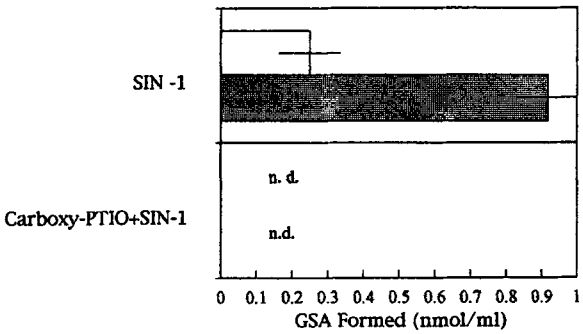

FIGURE 5 Effect of Carboxy-PTIO on GSA synthesis from ASA and SIN-1. ASA (1 mM) was incubated with 1 mM SIN-1 for 15 or 30 min as described in Materials and Methods. The open column represents GSA at 15 min and the dotted column represents GSA at 30 min. Each column represents the mean of duplicate incubations. Vertical bars depict the range of each determination

the limiting factor. At these high concentrations of SIN-1, limited O_2 in the reaction mixture coupled with the fact that the half life of the superoxide anion is shorter than that of NO, might result in an excess amount of NO, which in turn, scavenges the hydroxyl radical, which is needed to generate GSA.

Inhibition of GSA Synthesis from ASA and SIN-1 by Carboxy-PTIO

Carboxy-PTIO,[28] a specific scavenger of NO, completely inhibited GSA synthesis from ASA in the presence of 1 mM SIN-1 as shown in Figure 5. These data suggest that NO is essential for GSA synthesis under these conditions.

Effect of SOD, Catalase, SOD + Catalase and DMSO on GSA Synthesis by SIN-1

The effect of SOD and/or catalase on the synthesis of GSA from ASA and SIN-1 was studied. SOD (42 U/ml), an enzyme that converts the superoxide anion to hydrogen peroxide, inhibited GSA synthesis by 20% as shown in Figure 6. Catalase inhibited GSA synthesis only 12%. Combining catalase and SOD inhibited GSA synthesis by 40%. These data confirm the report that SOD partly inhibits generation of the hydroxyl radical

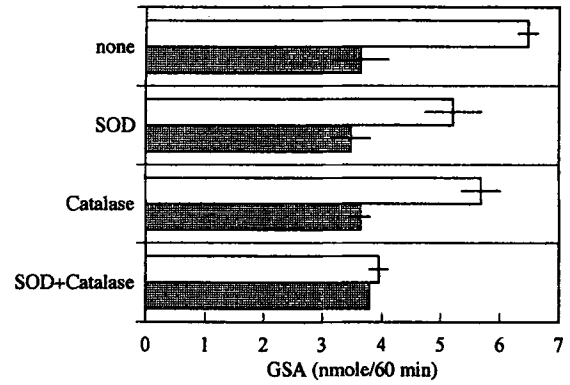

FIGURE 6 Effect of SOD and/or catalase on GSA synthesis from ASA and SIN-1. ASA (1 mM) was incubated with 1 or 4 mM SIN-1 for 60 min with or without SOD and/or catalase for 60 min as described in Materials and Methods. The open columns represent GSA incubated with 1 mM SIN-1 and the dotted columns represent GSA incubated with 4 mM SIN-1. Each column represents the mean of duplicate incubations. Vertical bars depict the range of each determination.

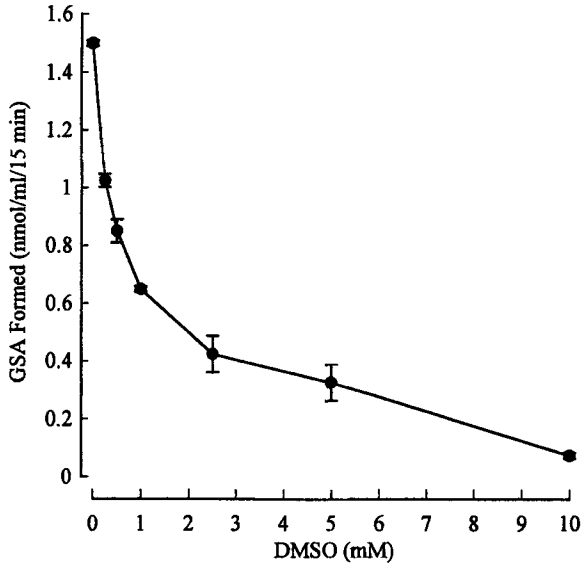

FIGURE 7 Effect of DMSO on GSA synthesis by SIN-1. ASA (1 mM) was incubated with 1 mM SIN-1 and various concentrations of DMSO for 15 min as described in Materials and Methods. Each point represents the mean of duplicate incubations. Vertical bars depict the range of each determination.

from SIN-1, and catalase did not.[24] However, neither SOD nor catalase affected GSA synthesis from ASA when incubated with 4 mM SIN-1, an amount that formed less GSA than 1 mM SIN-1. The reason for this is not clear although the rapid consumption of O_2 in the reaction mixture at these high concentrations of SIN-1 and excess amount of NO that can scavenge the hydroxyl radical may also be operative in this instance.

DMSO, a hydroxyl radical scavenger, inhibited GSA synthesis by 56% at the concentration of 1 mM and the inhibition depended on the concentrations of DMSO as shown in Figure 7.

DISCUSSION

In this study, we demonstrate that NO derived reactive oxygen species such as the hydroxyl radical react with ASA to form GSA in *vitro*. The serum GSA concentration in patients with acute renal failure caused by endotoxcemia was higher than that of caused by other mechanisms.[29,30] These reports suggested that NO is the source of reactive oxygen that generates GSA *in vivo*. In addition to the reported dilatation of the aorta by GSA accompanied by an increase in cGMP, activation by GSA of NMDA receptor, which causes convulsions, is another NO mimicking action of GSA recently reported.[8] Levels of GSA in uremic brain were comparable to those previously observed in brains of experimental animals displaying convulsions following intraperitoneal injection of GSA.[31] Activation of the NMDA receptor has been known to activate NO synthetase.[32] Moreover, the existence of ASA in the nervous system has been reported.[22] These data imply a mechanism that amplifies NO generation in the nervous system especially under hypoxia. Hypoxia, then, causes activation of the NMDA receptor which generates NO, as well as causing generation of the superoxide anion.[33] The simultaneous generation of NO and superoxide could generate GSA, a stable NO mimic, that activates, in turn, the NMDA receptor as illustrated in Figure 8. Thus, the conditions that generate both NO and superoxide facilitate the production of GSA from ASA. This amplification

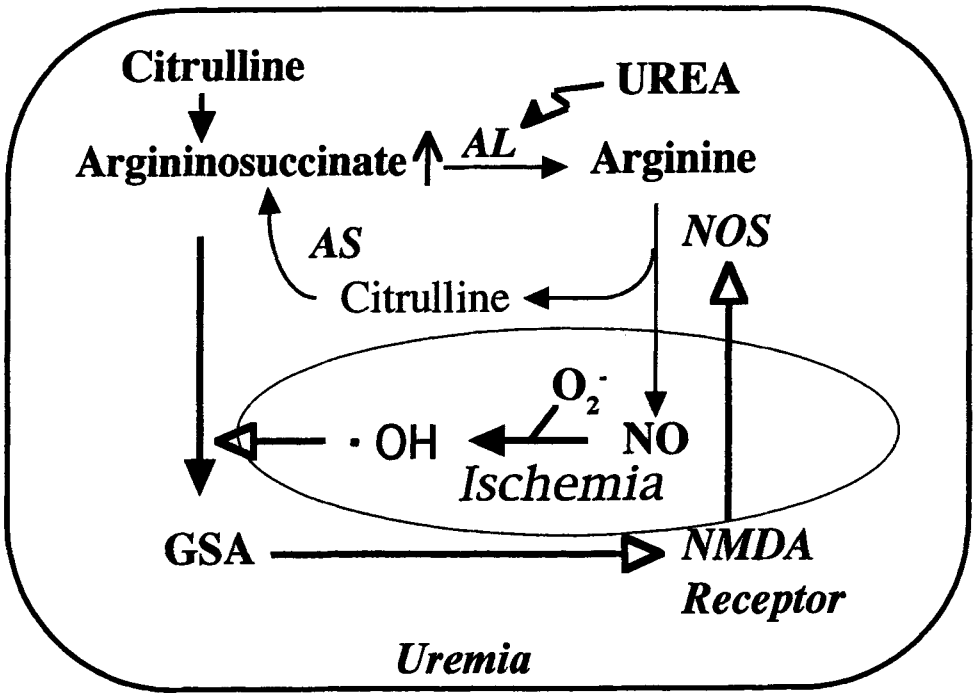

FIGURE 8 A proposed mechanism for NO amplification by GSA, the NMDA receptor and NO derived reactive oxygen under hypoxic conditions. GSA activates the NMDA receptor that generates NO. Superoxide generation increases under hypoxic conditions. Simultaneous production of NO and superoxide generates the hydroxyl radical, which in turn generates GSA from ASA. Thus NO may be amplified by this cycle. NOS: NO synthetase, AL: argininosuccinic acid lyase.

of NO by GSA will occur more easily in uremic patients who have high concentrations of GSA.

Acknowledgments

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