Role of nitric oxide in the synthesis of guanidinosuccinic acid, an activator of the N-methyl-D-aspartate receptor

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Background. We propose that reactive oxygen and argininosuccinic acid (ASA) form guanidinosuccinic acid (GSA). An alternative to this hypothesis is the so-called guanidine cycle, which consists of a series of hydroxyurea derivatives that serve as intermediates in a pathway leading from urea to GSA. We compare the role of the guanidine cycle to that of nitric oxide (NO) in the synthesis of GSA.

Methods. The members of the guanidine cycle (hydroxyurea, hydroxylamine plus homoserine, L-canaline, and L-canavanine) were incubated with isolated rat hepatocytes. The known NO donors, NOR-2, NOC-7, and SIN-1, were incubated with ASA in vitro. Ornithine, arginine, or citrulline, which increase arginine, a precursor of NO, were incubated with isolated rat hepatocytes. GSA was determined by high-performance liquid chromatography.

Results. None of guanidine cycle members except for urea formed GSA. SIN-1, which generates superoxide and NO formed GSA, but other simple NO donors, did not. Both carboxy-PTIO, a scavenger of NO, and dimethyl sulfoxide, a hydroxyl radical scavenger, completely inhibited GSA synthesis by SIN-1. GSA formation by SIN-1 reached a maximum at 0.5 mmol/L and decreased at higher concentrations. GSA synthesis, stimulated by urea in isolated hepatocytes, was inhibited by ornithine, arginine, or citrulline with ammonia, but not by ornithine without ammonia, where arginine production is limited.

Conclusion. GSA is formed from ASA and the hydroxyl radical. When arginine increased in hepatocytes, GSA synthesis decreased. These data suggest that increased NO, which results from high concentrations of arginine, or SIN-1 scavenges the hydroxyl radical. This may explain the decreased GSA synthesis in inborn errors of the urea cycle where ASA is decreased, and also the diminished GSA excretion in argininemia.

Guanidinosuccinic acid (GSA) is a recognized uremic toxin because of its many biological activities [1], such as inhibition of platelet aggregation [2], induction of hemolysis [3], and production of generalized clonic and tonic convulsions [4]. Recently, GSA became noteworthy for its actions mimicking nitric oxide (NO) [5] as well as activating the N-methyl-D-aspartate (NMDA) receptor, which generates NO in the nervous system [6].

With regard to the mechanism of GSA synthesis, it is reported to increase in patients with renal failure depending on both the level [7] and the synthetic rate [8] of urea. We have also reported that GSA synthesis in isolated rat hepatocytes is dependent on the concentration of urea in the preparation [9]. Two routes have been proposed for this urea-stimulated GSA synthesis. Natelson and Sherwin suggested that urea is oxidized to hydroxyurea, which forms, in turn, ureidohomoserine via carbamoyl phosphate and that urea forms canaline via hydroxylamine [10]. They called it the "guanidine cycle," the components of which are ureidohomoserine, canavaninosuccinic acid, canavanine, and canaline. GSA is formed from the canavaninosuccinic acid produced in this cycle [10].

We propose an alternate route, calling attention to the inhibition of argininosuccinase by urea, which leads to an increase of argininosuccinic acid (ASA) [11]. We show that GSA is formed from ASA and a reactive oxygen species, specifically the hydroxyl radical [12, 13]. ASA is an intermediate in the urea cycle and is also a precursor of arginine. This mechanism explains the inhibition of GSA synthesis by D,L-norvaline, which inhibits ASA synthesis [9]. However, it does not explain the fact that arginine, which increases urea synthesis, inhibits the urea-stimulated synthesis of GSA [9].

In this study, isolated rat hepatocytes were used to examine whether members of the guanidine cycle form GSA. In addition, we explore the role of NO on GSA synthesis in an effort to determine the reason for the contradictory effect of arginine.

METHODS

Isolated rat hepatocytes prepared from male Wistar rats were incubated in Krebs-Henseleit bicarbonate buffer as described previously [9]. Cells were incubated in 6 mL of Krebs-Henseleit bicarbonate buffer containing 3% bovine serum albumin (BSA), 10 mmol/L sodium lactate, and 36 mmol/L of urea and indicated substances at 37°C for four hours. Incubation was arrested by the addition of 0.6 mL of 100% (wt/vol) trichloroacetic acid. After sonication, the supernatant of cells and medium was obtained by centrifugation at 1700 × g for 15 minutes at 0°C, and 0.2 mL of the extract was used for GSA measurement.

Argininosuccinic acid was incubated at 37°C in 1 mL of 50 mmol/L potassium phosphate buffer (pH 7.4) with 3-morpholinosydomine hydrochloride (SIN-1) (±)-(E)-4-methyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR-2) or 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propamine (NOC-7), all of which are NO donors. SIN-1 (10 mmol/L) was dissolved in 20 mmol/L hydrochloric acid immediately before the experiments. At the end of incubation, 1 mL of 20% (wt/vol) 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, which reacts specifically with mono-substituted guanidino compounds, for postlabeling after separation on a cation exchanging column as described previously [9].

RESULTS

Evaluation of the guanidine cycle intermediates in GSA synthesis

Isolated hepatocytes (0.18 g wet liver) were incubated with 5 or 40 mmol/L hydroxyurea, 1 mmol/L hydroxylamine, homoserine, canavanine, or canaline with or without 36 mmol/L urea. All reagents related to the guanidine cycle except for urea did not form GSA in isolated rat hepatocytes. However, canavanine gave a peak (peak C) that had almost the same retention time of GSA after incubation (Fig. 1). We would have to use a different cation exchange column for HPLC to separate these peaks; however, peak C appeared promptly following the addition of canaline without incubation and decreased after incubation with the hepatocytes. Therefore, we conclude that peak C is canaline.

GSA synthesis from ASA with various NO donors

Among the tested NO donors, NOR-2 and NOC-7, which generate NO alone, did not form GSA, but SIN-1 formed GSA from ASA. SIN-1 is an agent that generates NO and the superoxide anion simultaneously; together, both radicals form peroxynitrite, which in turn generates

the hydroxyl radical or a hydroxyl radical-like reactive oxygen species [14]. GSA synthesis increased depending on the concentration of ASA when incubated with 1 mmol/L SIN-1. This result is compatible our observation that the hydroxyl radical is much more effective than the superoxide anion in the synthesis of GSA from ASA [12]. When 1 mmol/L ASA was incubated with 1 mmol/L SIN-1 at 37°C, GSA increased almost linearly depending on the incubation period for up to 60 minutes 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide. Sodium salt (carboxy-PTIO), a specific scavenger of NO, completely inhibited GSA synthesis from ASA in the presence of 1 mmol/L SIN-1. These data suggest that NO is essential for GSA synthesis under these conditions. Dimethyl sulfoxide (DMSO), a hydroxyl radical scavenger, inhibited GSA synthesis by 56% at a concentration of 1 mmol/L, and the amount of inhibition depended on the concentration of DMSO. These data indicate that the hydroxyl radical derived from NO and superoxide forms GSA from ASA. Among the concentrations of SIN-1 tested, ranging from 0.5 to 5 mmol/L, GSA synthesis from 1 mmol/L ASA was maximum at 0.5 mmol/L and decreased with increasing concentrations of SIN-1, as shown in Figure 2, despite the fact that the amount of GSA synthesized increased depending on the incubation time. The reason for less GSA synthesis at higher concentrations of SIN-1 is not clear. A possible reason is that at high concentrations of SIN-1, the hydroxyl radical that generates GSA, may decrease because the superoxide radical, having a shorter life span than that of NO, might increase the relative concentration of NO enough to scavenge the hydroxyl radical. NO and the hydroxyl radical form nitrous acid.

Effect of urea cycle members on urea stimulated GSA synthesis

We have reported previously that D,L-norvaline, which inhibits ornithine transcarbamylase leading to decreased ASA, also inhibited GSA synthesis despite high concentrations of urea [9]. In accordance with the hypothesis that ASA is the precursor of GSA, ornithine, which increases urea synthesis in the presence of ammonium chloride, should increase GSA synthesis. We observed, however, that ornithine inhibited urea-stimulated GSA synthesis in isolated hepatocytes [9]. A possible explanation is that there is a threshold amount of arginine that increases NO, and it then scavenges the hydroxyl radical. We therefore set out to learn whether arginine regulates GSA synthesis or whether ornithine alone regulates GSA synthesis by another mechanism.

Ornithine inhibited GSA synthesis in the presence of 10 mmol/L ammonium chloride, but did not inhibit it without ammonium chloride (Fig. 3). However, citrulline, which does not need ammonium chloride to form arginine, inhibited GSA synthesis with or without ammo-
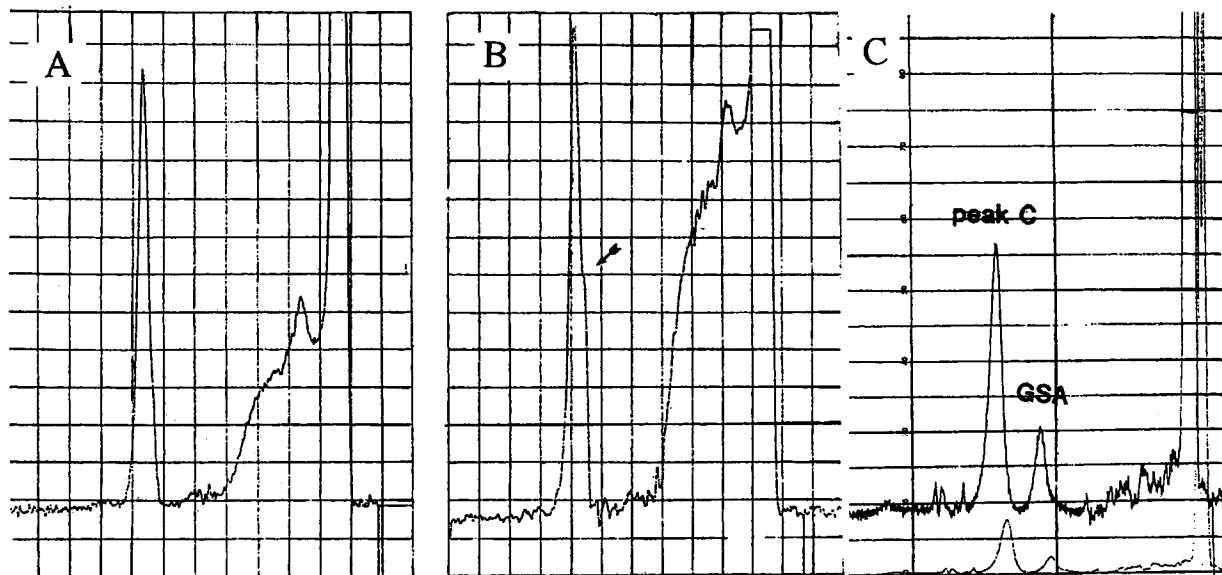
Fig. 1. Chromatograms obtained from the trichloric acid (TCA) extract of isolated rat hepatocytes incubated with 1 mmol/L canavanine (*A*) or 36 mmol/L urea plus 1 mmol/L canavanine (*B*). Notching, which appeared on peak C, is indicated by an arrow. The peak of GSA was separated from the peak of GSA (*C*) using a different column.
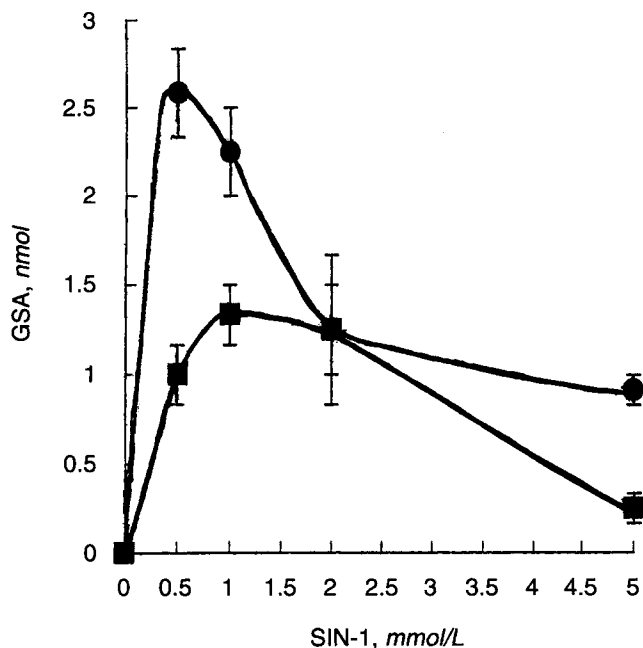

Fig. 2. Guanidinosuccinic acid (GSA) synthesis from argininosuccinic acid (ASA) in the presence of various concentrations of 3-morpholino-sydomine hydrochloride (SIN-1). SIN-1 from 0 to 5 mmol/L was incubated with 1 mmol/L ASA for 15 or 30 minutes at 37°C. Each point represents the mean of duplicate incubations. Whiskers indicate the range of each determination. GSA amounts produced at 15- or 30-minute incubations are expressed as n and l, respectively. Symbols are (●) ???; (■) ???.

nium chloride (Fig. 3). Inhibition by citrulline was dose dependent up to 5 mmol/L (data are not shown). The addition of arginine also inhibited GSA synthesis with or without ammonium chloride (Fig. 3). These data show

that when arginine increases, GSA synthesis is inhibited. Recently, it was reported that NO synthetase competes with arginase for arginine [15], and arginine levels in hepatocytes are very low because of the very high arginase activity. From these data, it can be postulated that increased arginine forms sufficient NO to scavenge the hydroxyl radical.

DISCUSSION

In this study, intermediates of the proposed guanidine cycle did not form GSA in isolated rat hepatocytes. We show that GSA is formed from ASA and the hydroxyl radical derived from NO and superoxide. Both the lack of NO, which is associated with a reduced generation of the hydroxyl radial, and an excess of NO, which scavenges the hydroxyl radical, may lead to decreased GSA synthesis. Among the inborn errors of the urea cycle, those in which there is a decreased ASA synthesis such as citrullinemia, ornithine transcarbamylase deficiency, and hyperornithinemia show an undetectable amount of GSA excretion in the urine [16]. It is not surprising, based on our hypothesis, that patients who have a low synthetic activity of ASA will excrete less than normal GSA in urine. However, in one of two patients with argininosuccinic aciduria, GSA output, while it did not increase, was found to be comparable to that of normal subjects [7]. Considering the high ASA level in the patients with a deficiency of argininosuccinase, the hydroxyl radical generation may be limited by the very low arginine synthetic activity. Inhibition of GSA synthesis by arginine, ornithine plus ammonia, and citrulline, all
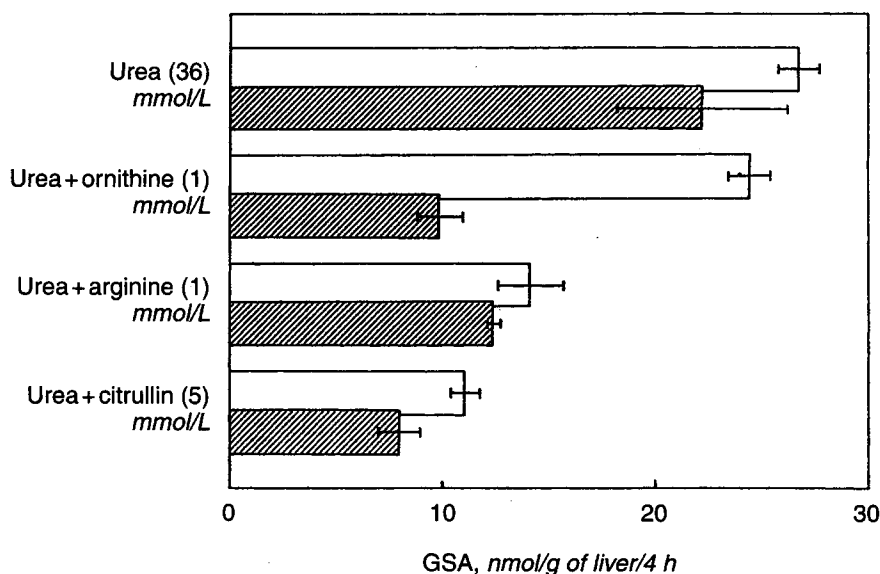
Fig. 3. Effect of urea cycle members that affect arginine synthesis in isolated rat hepatocytes. Hepatocytes were incubated with indicated substances and 36 mmol/L urea with or without 10 mmol/L ammonium chloride for four hours as described in the **Methods** section. Symbols are: (■) with 10 mmol/L ammonium chloride; (□) without ammonium chloride.

of which increase both ASA and arginine levels in isolated rat hepatocytes, may be explained by increased NO synthesis, which in turn scavenges the hydroxyl radical. This hypothesis is also supported by the report that in arginemia, where arginine is very high, very small amounts of GSA are excreted [16].

Argininosuccinic acid synthesis from citrulline, formed from arginine by NO synthetase, is known as the citrulline-arginine-NO cycle. This cycle is active in a variety of tissues, including endothelial cells, smooth muscle cells, macrophages, and nerve cells [17]. In the nervous system especially, GSA activates the NMDA receptor, which increases NO synthesis. Therefore, this GSA-NMDA receptor-NO pathway might amplify NO synthesis whenever superoxide is generated. The NMDA receptor has been implicated in various pathologic processes such as memory loss, nervous system maldevelopment, seizure disorders, and ischemic and excitotoxic brain damage [4]. Our study suggests that a sufficient supply of arginine as a source of NO could inhibit this damage.

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