Regulation of urea synthesis by agmatine in the perfused liver: studies with $^{15}\text{N}$

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Nissim, Itzhak, Oksana Horyn, Yevgeny Daikhin, Ilana Nissim, Adam Lazarow, and Marc Yudkoff. Regulation of urea synthesis by agmatine in the perfused liver: studies with $^{15}\text{N}$. Am J Physiol Endocrinol Metab 283: E1123–E1134, 2002. First published August 13, 2002; 10.1152/ajpendo.00246.2002.—Administration of arginine or a high-protein diet increases the hepatic content of $N$-acetylglutamate (NAG) and the synthesis of urea. However, the underlying mechanism is unknown. We have explored the hypothesis that agmatine, a metabolite of arginine, may stimulate NAG synthesis and, thereby, urea synthesis. We tested this hypothesis in a liver perfusion system to determine 1) the metabolism of $L$-[guanidino-$^{15}\text{N}_2$]arginine to either agmatine, nitric oxide (NO), and/or urea; 2) hepatic uptake of perfusate agmatine and its action on hepatic N metabolism; and 3) the role of arginine, agmatine, or NO in regulating NAG synthesis and ureagenesis in livers perfused with $^{15}\text{N}$-labeled glutamine and unlabeled ammonia or $^{15}\text{NH}_4\text{Cl}$ and unlabeled glutamine. Our principal findings are 1) $L$-[guanidino-$^{15}\text{N}_2$]arginine is formed in the liver from perfusate $L$-[guanidino-$^{15}\text{N}_2$]arginine ($\sim 90\%$ of hepatic agmatine is derived from perfusate arginine); 2) perfusions with agmatine significantly stimulated the synthesis of $^{15}\text{N}$-labeled NAG and $^{[15]}\text{N}$urea from $^{15}\text{N}$-labeled ammonia or glutamine; and 3) the increased levels of hepatic agmatine are strongly correlated with increased levels and synthesis of $^{15}\text{N}$-labeled NAG and $^{[15]}\text{N}$urea. These data suggest a possible therapeutic strategy encompassing the use of agmatine for the treatment of disturbed ureagenesis, whether secondary to inborn errors of metabolism or to liver disease.

arginine; $N$-acetylglutamate; carbamoyl phosphate synthetase I; hyperammonemia; nitric oxide

THE REGULATION OF UREAGENESIS has posed a complex problem since the first description of the urea cycle by Krebs and Henseleit in 1932 (20). The first and most critical step in urea synthesis is the conversion of $\text{NH}_4^+$ and $\text{HCO}_3^-$ to carbamoyl phosphate by mitochondrial carbamoyl-phosphate synthetase I (CPS-I; EC 6.3.5.5; see Refs. 9, 13, 16, 22), which requires $N$-acylglutamate (NAG) as an obligatory effector for its activation (9, 13, 14). Brosnan (3) has suggested that acute increases in ureagenesis are initiated by greater availability of NAG. Increased NAG synthesis and concomitant ureagenesis is observed after intake of arginine or a high-protein diet (7, 19, 26, 44). However, it is not clear how and why arginine or a high-protein diet increases the hepatic NAG level and, thereby, urea synthesis. It is unknown whether arginine directly or one of its metabolites stimulates the synthesis of NAG and, thereby, ureagenesis.

As shown in Fig. 1, $L$-arginine can be metabolized in the liver via several pathways. The first is hydrolysis via arginase to form urea, the end product of the urea cycle. There are two isoenzymes of mammalian arginase (9, 17, 38, 47). Arginase I is mainly present in the cytosol of periportal hepatocytes and is linked to urea cycle activity (2, 22, 38, 47). Arginase II is found primarily in mitochondria (7, 17, 38, 47). The second pathway is decarboxylation via mitochondrial arginine decarboxylase (ADC) to produce agmatine and $\text{CO}_2$ (14, 39, 47). The third pathway is metabolism via nitric oxide (NO) synthase to form NO and citrulline (21, 47). The ADC and NO synthase reactions are quantitatively minor routes of overall arginine metabolism, but the products of these pathways (NO and agmatine) have significant roles as signaling molecules that regulate many metabolic and physiological functions (21, 40, 42, 47). However, their role in the regulation of ureagenesis is uncertain. Understanding this relationship may provide a new therapeutic strategy for treatment of urea cycle disorders and hyperammonemia.

It has been demonstrated that the ADC pathway is present in rat kidney, brain, liver, and gut (40, 42). Recent studies have highlighted the importance of agmatine for a number of physiological processes, including dose-dependent release of insulin from islet cells exposed to glucose, proliferation of vascular smooth muscle, regulation of intracellular polyamine levels and cellular proliferation, regulation of neurotransmitter receptors, modulation of opioid analgesics, and inhibition of NO synthesis (2, 40, 42). Agmatine may stimulate the release of catecholamines from adrenal...
chromaffin cells by binding to α2-adrenergic receptors (14). Thus agmatine, which is widely distributed in mammalian tissue (37, 39), may have a role as a hormone and/or polyamine precursor for multiple metabolic functions. However, the significance of agmatine as a signaling molecule in regulating the urea cycle remains to be defined.

In the current investigation, we have explored the hypothesis that dietary arginine (whether free or as a part of a high-protein diet) is decarboxylated to agmatine in hepatic mitochondria and that agmatine, not arginine, is the regulator of NAG synthesis and the resulting activation of mitochondrial CPS-I. As shown in Fig. 1, both arginate and NAG are synthesized in mitochondria, the latter from acetyl-CoA and glutamate by NAG synthetase (22). The mitochondrial location of both ADC and NAG synthetase makes agmatine a reasonable candidate for regulation of NAG synthesis and, thereby, activation of CPS-I.

We have tested the above hypothesis in the following three steps: 1) initial studies that involve perfusions with L-[guanidino-15N2]arginine to determine the hepatic uptake of arginine and its relative metabolism to either agmatine, NO, and/or urea, as indicated in Fig. 1; 2) perfusions with arginine to determine its hepatic uptake and the dose dependence of agmatine action on urea synthesis; and 3) studies to elucidate the role of arginine, agmatine, or NO in the regulation of NAG synthesis and ureagenesis in liver perfused with either [2-15N]- or [5-15N]glutamine and unlabeled ammonia or 15 NH4Cl and unlabeled glutamine, as we have described previously (4, 5, 31, 28). The results obtained substantiate the hypothesis that agmatine regulates NAG and urea synthesis.

MATERIALS AND METHODS

Liver Perfusions

Livers from fed, male Sprague-Dawley rats were perfused “in situ” in the nonrecirculating mode as described by Pastor and Billiar (35). We employed the single-pass perfusion with antegrade flow because this model preserves the normal lobular microcirculation of the liver and physiological flow direction. The model also avoids the problem of substrate recycling from perivenous to periportal hepatocytes. Oxygen consumption was measured continuously using a Clark electrode and the Oxygen Measuring System (Instech SYS203). One electrode was attached to the inflow cannula, and one was attached to the outflow cannula. This system was linked to a computer equipped with the WinDaq/Lite/Pro/Pro Data System (DATAQ Instrument, Akron, OH) for on-line acquisition and recording of signals corresponding to inflow and outflow levels of PO2. The basic perfusion medium was Krebs saline continuously gassed with 95% O2-5% CO2 and containing lactate (2.1 mM) and pyruvate (0.3 mM) as metabolic fuels. Perfusion flow rate (3–3.5 ml/min) g–1), hepatic temperature, pH, and PO2 (in influent and effluent media) were monitored throughout, and oxygen consumption was calculated. After 15 min of preperfusion with the basic perfusate, we changed to a medium that contained, in addition to the lactate and pyruvate, precursors for urea N or modulators of urea synthesis as indicated below.

Experimental Design

To establish the role of arginine or its metabolites, NO or agmatine, in the regulation of NAG and urea synthesis, we first performed experiments to determine the rate of hepatic uptake of arginine and its relative metabolism to agmatine (ADC reaction), urea (arginase reaction), or nitric oxide (NO synthase reaction). To this end, after 15 min of preperfusion with a basic perfusate as indicated above, we changed to a perfusate that contained 0.3 mM NH4Cl, 1 mM unlabeled glutamine, and 0.5 mM L-[guanidino-15N2]arginine [99 mole % excess (MPE)]. Separate perfusate reservoirs, each containing different media, were used to facilitate changes in perfusate medium. Perfusion was continued for a total of 70 min. Samples were taken from the influent and effluent media for chemical and gas chromatography-mass spectrometry (GC-MS) analyses. At the end of the perfusions, livers were freeze-clamped and used for measurements of metabolite concentrations and 15N enrichment in arginine and agmatine.

The next series of experiments was designed to examine the effect of exogenous agmatine or nitroprusside (SNP), a donor of NO, on total urea synthesis. We also evaluated a possible dose-dependent action of arginine, agmatine, or NO on urea synthesis from ammonia and glutamine. Perfusions were performed with 1) arginine at increasing concentrations (μM) of 25, 50, 100, 250, 500, or 1,000; 2) SNP at increasing concentrations (μM) of 25, 50, or 100; 3) arginine at increasing concentrations (μM) of 250, 500, and 1,000. The
dose dependence was studied in the same liver perfusion. At the indicated times, a separate perfusate reservoir containing different media was used to facilitate changes in the concentration of each modulator.

To examine the role of arginine or its metabolites in the regulation of NAG synthesis and ureagenesis under conditions and concentrations that approximate those that exist in vivo, determinations of NAG levels were performed without (control) or with addition of 100 μM arginine, 25 μM SNP, or 500 μM arginine. The 15N-labeled precursor for urea N was either glutamine (1 mM), [5-15N]glutamate or [2-15N]glutamine (99 MPE) and NH4Cl (0.3 mM) or 2 unlabeled glutamine (1 mM) and 15NH4Cl (0.3 mM; 99 MPE). Preperusions with basic medium were performed for 15 min and continued for 70 min with the indicated 15N precursor or modulator.

Chemical Analyses

Samples were taken at the indicated times from influent and effluent media for chemical determination of metabolites. At the end of the perfusion (70 min), livers were freeze-clamped. The frozen livers were extracted in perchloric acid, and the neutralized extracts were used for chemical analysis. Adenine nucleotides (ATP, ADP, and AMP) were determined using 31P-NMR analysis as described (30). Amino acids and arginine were measured by HPLC using precolumn derivatization with o-phthalaldehyde (18). Measurements also were made of glucose (1), urea and ammonia (29), lactate (15), and pyruvate (12).

GC-MS Methodology

GC-MS measurements of hepatic NAG levels and 15N isotopic enrichment in various metabolites were performed on a Hewlett-Packard 5970 MSD and/or 5971 MSD coupled with a 5890 HP-GC, as described previously (4, 5, 31, 28). The following measurements were performed.

Amino acids, ammonia, and urea. For measurement of 15N enrichment in urea and amino acids, samples were prepared as we have described previously (4, 5, 31, 28). Briefly, a 500-μl aliquot of effluent or liver extract was purified via an AG-50 column (H+; 100–200 mesh; 0.5 × 2.5 cm) and then converted to the t-butylmethylsilyl (t-BDMS) derivatives. The mass-to-charge ratios (m/z) 231, 232, 233, and 234 of the urea t-BDMS derivative (46) were monitored for singly (Um−1) and doubly (Um−2) labeled urea determination (4, 31, 28). Isotopic enrichment in glutamate, glutamine, aspartate, and alanine was monitored using ratios of ions at m/z of 433/432, 432/431, 419/418, and 261/260, respectively. 15N enrichment was measured after conversion of ammonia to norvaline as described (5). 15N enrichment in L-[guanidino-15N2]arginine was determined after derivatization with 100 μl of trifluoroacetic anhydride (TFAA) and monitoring the ratio of ions at m/z 377/375 as we have described previously (32).

Measurement of NAG concentration. The level of NAG in freeze-clamped livers was determined using GC-MS and a modification of the conventional isotope dilution technique, as described (31). First 15N(NAG) was synthesized (99 MPE) by reacting [15N]glutamate (99 MPE) with acetic anhydride. The [15N]NAG was used to prepare standard dilution curves by mixing labeled and unlabeled NAG and spiking samples for determination of NAG levels by isotope dilution.

NAG in standard solutions or samples was converted into the methyl esters as follows. Samples were dried under N2 and azeotroped with methylene chloride. Next, 100 μl of methanolic hydrochloride (3 N; Supelco) were added. Capped vials were heated at 60°C for 10 min, cooled, and dried under a gentle stream of N2. The residue was extracted in 1 ml ethyl acetate and 300 μl H2O after vortexing for 30 s, and the organic layer was removed, dried, and reconstituted in 75 μl ethyl acetate. Usually 1–2 μl were injected in the GC-MS, and isotopic enrichment in NAG was determined using m/z 159/158.

To determine the concentration of NAG in freeze-clamped liver extracts, an aliquot (500 μl) was assayed as indicated above for 15N enrichment (I1), after 15N-labeled precursor was used in the perfusion, e.g., in experiments with 15NH4Cl or [2-15N]glutamine (in this case, the value of I1 was between 20 and 30 MPE). A second aliquot (500 μl) was spiked with 5 nmol unlabeled NAG, and the second isotopic enrichment (I2) was determined. In experiments with [5-15N]glutamine, I1 usually was <3 MPE. Therefore, the second aliquot of 500 μl was spiked with 5 nmol [15N]NAG, and I2 was determined. NAG concentrations were calculated using the isotope dilution technique (31, 48). With each series of measurements, a calibration curve of NAG with a known isotopic enrichment (1–50 MPE) was prepared and analyzed by GC-MS. In nearly every preparation, we achieved an excellent agreement between the observed and the expected 15N enrichment in NAG with r values around 0.9.

Determination of [12N]agmatine. We first synthesized [guanidino–15N2]agmatine as described (6). Briefly, 18 mM L-[guanidino–15N2]arginine in 0.2 M sodium acetate, pH 5.2, containing 5 mM pyridoxal phosphate (Sigma), 0.1% BSA, and 1 IU bacterial ADC (no. A8134; Sigma) was incubated at 37°C for 2 h. Next, an additional 1 IU ADC was added, and incubation was continued for another 3 h. The reaction was stopped by addition of 5 M KOH. Agmatine was extracted with n-butanol and dried under vacuum. Purity was determined by HPLC, and yield was ∼100%. The [15N]agmatine was used to develop the GC-MS methodology for measurement of [15N]agmatine in biological samples and for preparation of standard isotope dilution curves by mixing labeled and unlabeled agmatine.

For GC-MS analysis of 15N-labeled agmatine, we applied a previously described method for determination of 15N-labeled arginine in biological samples (32). To examine the accuracy of this method for measurement of 15N enrichment in agmatine, a standard dilution curve of N-labeled agmatine, with a known isotopic enrichment, e.g., (1–50 MPE) was prepared. Samples were alkalinated with 1 ml of 3 M NH4OH and then loaded in a column containing the acetate form of Dowex 1-X8 (100–200 mesh; 0.5 × 2.5 cm) and temperature program as follows: injector temperature 250°C isothermal for 2 min and then 10°C/min. We have found that ∼90% of agmatine was converted to N-tri-TFA-agmatine with a major ion at m/z 349, and ∼10% was converted into N-tetra-TFA with a major ion at m/z 445. We used the N-tri-TFA-agmatine and m/z 351/349 to determine isotopic enrichment in [guanidino–15N]agmatine. When the standard isotope dilution curve was analyzed, we achieved excellent agreement between the observed and the expected 15N enrichment in agmatine with r values of 0.9 or better. For determination of [15N]agmatine after liver perfusion with L-[guanidino–15N2]arginine, 1 ml of perfusate or liver extract was alkalinized with 1 ml of 3 M NH4OH and then loaded on
Materials and Animals

Male Sprague-Dawley rats (Charles River) were fed ad libitum on a standard rat chow diet. Chemicals were of analytical grade and were obtained from Sigma-Aldrich. Enzymes and cofactors for the analysis of urea, lactate, pyruvate, glucose, and ammonia were obtained from Sigma. Labelled arginine, glutamine, and ammonia (99 MPE) were obtained from Isotec.

RESULTS

Hepatic Uptake and Metabolism of Arginine: Production of Agmatine

The initial series of experiments was designed to determine the extent to which perfusate arginine is taken up and metabolized to urea, agmatine, or NO. Figure 2 demonstrates the formation of these metabolites and oxygen consumption during perfusion with L-[guanidino-15N2]arginine. The constancy of oxygen consumption is an indication of the viability and stability of the perfused livers. The rate of L-[guanidino-15N2]arginine uptake is ~120–140 nmol·min⁻¹·g liver⁻¹ (Fig. 2B). At the end of 70-min perfusion, the isotopic enrichment of hepatic arginine was 46.3 ± 9.3 MPE (Fig. 2C), indicating that ~50% of hepatic arginine was derived from arginine entering the liver via the portal vein.

Arginine in the effluent was ~0.45 mM and that in the perfusate 0.5 mM, a finding in agreement with a previous estimate that ~15% of perfusate arginine is metabolized in the liver (33, 34). The major metabolites of L-[guanidino-15N2]arginine are [15N2]urea and [guanidino-15N2]agmatine (Fig. 2A). The 15N enrichment in doubly labeled urea was ~10–13 MPE between 20 and 40 min, and labeling decreased to 5–6 MPE by 70 min. The decreased enrichment in [15N2]urea after 40 min of perfusion reflects increased formation of unlabeled urea from ammonia and glutamine via the urea cycle. The total (labeled and unlabeled) urea output between 20 and 70 min was ~1,000 nmol·min⁻¹·g liver⁻¹. Total [15N2]urea output was about 70–90 nmol·min⁻¹·g⁻¹ (Fig. 2A), which is about 80% of arginine uptake.

Therefore, when physiological concentrations of glutamine and ammonia are provided, ~7–9% of total urea in the effluent was formed from external arginine entering the liver via the portal vein.

Agmatine is the second major metabolite of arginine. The output of [guanidino-15N2]agmatine in the effluent was ~14–20 nmol·min⁻¹·g⁻¹ (Fig. 2A), indicating that ~10–15% of arginine uptake was recovered as agmatine in the effluent. The concentration of agmatine in the liver extract was ~130 nmol/g, and 41.1 ± 11.2% of this was in the form of [guanidino-15N2]agmatine (Fig. 2C). The ratio between [guanidino-15N2]agmatine and L-[guanidino-15N2]arginine was 0.87, indicating that ~87% of hepatic agmatine was derived from external arginine.

An additional pathway of arginine metabolism is via NO synthetase (21, 46). The output of NO, measured as NO2 plus NO3, was 1.5–2 nmol·min⁻¹·g⁻¹, which is ~1% of arginine uptake (Fig. 2A). The current value of NO output is in good agreement with a previous study.
showing that, in livers perfused with 1 mM arginine, the sum of $\text{NO}_2^- + \text{NO}_3^-$ output was $\sim 4 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g liver dry wt}^{-1}$ (35).

**Dose Dependence of Agmatine Action on Urea Synthesis**

After the demonstration that agmatine is formed in the liver from external arginine, we next examined the hepatic uptake of perfusate agmatine and its action on hepatic $\text{N}$ metabolism and ureagenesis. We also evaluated the effect of perfusate agmatine on these parameters. Figure 3 depicts the results of perfusions with unlabeled glutamine (1 mM), ammonia (0.3 mM), and 25, 50, or 100 $\mu$M agmatine. We found that the maximum effect of agmatine on urea synthesis occurred at a concentration of 100 $\mu$M in the perfusate. Higher levels (data not shown) had little incremental effect on urea synthesis or oxygen consumption. Similarly, no significant differences were found with an arginine concentration $> 500$ $\mu$M or SNP $> 25$ $\mu$M (data not shown). These data indicate that agmatine is taken up by the liver, where it stimulates oxygen consumption and urea synthesis from glutamine and ammonia. This action is dose dependent, with a maximum effect at perfusate agmatine of 100 $\mu$M.

It has been shown that agmatine is taken up by system y $^+$, the same system used for arginine and polyamine transport (6, 41). In cultured hepatocytes, $\sim 10\%$ of agmatine was converted to putrescine via the agmatinase pathway (6). In the current study, if we assume that 10% of agmatine uptake was metabolized to putrescine and urea, then the expected amount of urea formed from agmatine will be $\sim 10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$, which is $< 0.1\%$ of total urea output (Fig. 3C). Thus the amount of urea that may be derived from agmatine is negligible. This conclusion is further supported by the formation of $[^{15}\text{N}]$urea from $^{15}\text{N}$-labeled precursors, as indicated below.

**Effect of Arginine, Agmatine, or NO on Hepatic Metabolic Activity and $\text{N}$ Balance**

Viability of the livers is documented by the constancy of oxygen consumption during the course of perfusions (Fig. 4). The addition of arginine or agmatine to the perfusate significantly ($P < 0.05$) increased $\text{O}_2$ consumption between 40 and 70 min. The increased $\text{O}_2$ consumption was more significant with agmatine ($P = 0.006$) than with arginine (Fig. 4B).

The increased oxygen consumption in perfusions with agmatine was associated with stimulated glutamine-$\text{N}$ uptake (Table 1 and Fig. 4A). Between 40 and 70 min of perfusion, glutamine-$\text{N}$ uptake (nmol $\text{N} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$) was increased by approximately fourfold in perfusions with agmatine and by twofold in perfusion with arginine or SNP (Table 1). There were only minor differences in ammonia-$\text{N}$ uptake between the various experimental groups (Table 1 and Fig. 4C). The appearance of $\text{N}$ in urea, alanine, and glutamate represents the major nitrogenous output (the release of other amino acids was minor). Therefore, we calculated the extent to which these three compounds could account for $\text{N}$ balance across the liver. In control experiments, the combined nitrogenous uptake of glutamine and ammonia $\text{N}$ was 1,127 nmol $\text{N} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$. The output of $\text{N}$ in urea, alanine, and glutamate was 1,031 nmol $\text{N} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$. In perfusions with agmatine, arginine, or SNP, the uptake of $\text{N}$ was 2,652, 1,610, or 1,652 nmol $\text{N} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$, respectively (Table 1). The output of $\text{N}$ was 1,890, 1,332, or 1,225 nmol $\text{N} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ in perfusion with agmatine, arginine, or SNP, respectively (Table 1). Thus, in control perfusions and with the addition of arginine or SNP, there was almost complete recovery of $\text{N}$ uptake in the release of urea, alanine, and glutamate. In perfusions with agmatine, the uptake of $\text{N}$ exceeded the output by $\sim 30\%$.

Table 2 presents the level of hepatic metabolites at the end of the 70-min perfusion. We present only those metabolites that are directly related to the urea cycle. The agmatine level in control perfusions was 29.6 $\pm$ 4.2 nmol/g and increased by $\sim 10\text{-fold}$ after perfusion with agmatine, 5-fold after perfusion with arginine, but relatively little with SNP. Agmatine significantly increased the level of hepatic ornithine, and most notably...
Fig. 4. Total N balance and O2 consumption in perfused liver. Livers were perfused without (control) or with agmatine (100 μM), sodium nitroprusside (SNP; 25 μM), or arginine (500 μM) and 1 mM [2-15N]- or [5-15N]glutamine and 0.3 mM unlabeled ammonia or 15NH4Cl and unlabeled glutamine as 15N precursors. Because all perfusions were performed with the same concentrations of substrates (i.e., 1 mM glutamine, 0.3 mM NH4Cl, 2.1 mM lactate, and 0.3 mM pyruvate), it was possible to combine those with 15N-labeled glutamine or ammonia.

Table 1. Effect of agmatine, arginine, or SNP on nitrogen balance

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Control</th>
<th>+Agmatine</th>
<th>+Arginine</th>
<th>+SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen uptake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From Glutamine-N</td>
<td>466 ± 64</td>
<td>1,877 ± 150</td>
<td>812 ± 50</td>
<td>1,020 ± 218</td>
</tr>
<tr>
<td>From Ammonia-N</td>
<td>661 ± 24</td>
<td>775 ± 29</td>
<td>798 ± 20</td>
<td>632 ± 19</td>
</tr>
<tr>
<td>Nitrogen output</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Urea-N</td>
<td>961 ± 87</td>
<td>1,745 ± 99</td>
<td>1,229 ± 120</td>
<td>1,146 ± 24</td>
</tr>
<tr>
<td>As Amino-N</td>
<td>70 ± 13</td>
<td>145 ± 24</td>
<td>104 ± 15</td>
<td>79 ± 18</td>
</tr>
</tbody>
</table>

Values are means ± SD of data points presented in Fig. 4 between 40 and 70 min of perfusion; units are nmol·g−1·min−1. SNP, sodium nitroprusside. Values of total glutamine and urea N are nmol·min−1·g−1 × 2. Amino-N represents the sum of glutamate and alanine nitrogen.

Effect of Arginine, Agmatine, or NO on 15N-Labeled Glutamine Metabolism and Synthesis of [15N]Urea

Hepatic glutamine metabolism and ammonia formation are mediated via flux through the phosphate-dependent glutaminase (PDG) reaction (22, 29, 31, 28). In the current study, we estimated flux through PDG as the sum of 15N-labeled ammonia, urea, alanine, and glutamate output in the effluent when [5-15N]glutamine was used as labeled precursor. We have found that the formation of other amino acids accounted for <5% of [5-15N]glutamine consumption (28). The calculated flux through the PDG reaction based upon values at 60 min of perfusion (Fig. 5) shows rates (nmol·min−1·g−1) of 426 ± 113 (control), 691 ± 190 (plus arginine, P = 0.027), 584 ± 118 (plus arginine, P = 0.062), and 433 ± 121 (plus SNP, P > 0.05). Therefore, the addition of arginine to the perfusate significantly stimulated flux through PDG, whereas the addition of arginine is less significant.

The PDG reaction converts [5-15N]glutamine to 15NH3, which either can be released in the effluent as 15NH3 or incorporated into glutamate by glutamate dehydrogenase (GDH) or into urea via the CPS-I reaction (29, 31, 28). Figure 6A shows the production of...
Table 2. Hepatic content of agmatine, N-acetylglutamate, and amino acids after 70 min of perfusion with agmatine, arginine, or SNP

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control (15)</th>
<th>+Agmatine (18)</th>
<th>+Arginine (9)</th>
<th>+SNP (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agmatine, nmol/g wet wt</td>
<td>29 ± 4</td>
<td>311 ± 61.8*</td>
<td>129 ± 40*</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Ornithine, nmol/g wet wt</td>
<td>28.9 ± 8.6</td>
<td>47.6 ± 16.6*</td>
<td>33.6 ± 8.5</td>
<td>26.8 ± 6.7</td>
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<tr>
<td>Citrulline, nmol/g wet wt</td>
<td>46.2 ± 15.7</td>
<td>71.9 ± 33.7*</td>
<td>134 ± 37*</td>
<td>74 ± 8*</td>
</tr>
<tr>
<td>Arginine, nmol/g wet wt</td>
<td>66 ± 25</td>
<td>57 ± 26</td>
<td>96 ± 34*</td>
<td>52 ± 15</td>
</tr>
<tr>
<td>Glutamine, µmol/g wet wt</td>
<td>51 ± 21</td>
<td>69 ± 17</td>
<td>148 ± 37*</td>
<td>75 ± 22</td>
</tr>
<tr>
<td>Glutamate, µmol/g wet wt</td>
<td>2.4 ± 0.7</td>
<td>2.7 ± 0.8</td>
<td>1.8 ± 0.3</td>
<td>1.6 ± 0.4*</td>
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<tr>
<td>Alanine, µmol/g wet wt</td>
<td>1.31 ± 0.4</td>
<td>1.6 ± 0.7</td>
<td>1.5 ± 0.5</td>
<td>0.8 ± 0.2*</td>
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<tr>
<td>Aspartate, µmol/g wet wt</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>Ornithine, nmol/g wet wt</td>
<td>0.29 ± 0.1</td>
<td>0.36 ± 0.1</td>
<td>0.28 ± 0.1</td>
<td>0.19 ± 0.1*</td>
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</tbody>
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Values are means ± SD; no. of experimental determinations is given in parentheses for each experimental group. Perfusions performed as described in MATERIALS AND METHODS. Livers were perfused with either agmatine (100 µM), arginine (500 µM), or SNP (25 µM). Perfusions were terminated as described and processed for quantitation of the indicated amino acid and agmatine. Perfusate also included glutamine (1 mM), NH₄Cl (0.3 mM), lactate (2.1 mM), and pyruvate (0.3 mM). *P < 0.05, significantly different from control perfusions.

[¹⁵N]urea (sum of Uₘ₋₁ + Uₘ₋₂). Between 20 and 40 min of perfusion, [¹⁵N]urea production from [⁵-¹⁵N]glutamine amounted to 150–190 nmol N·min⁻¹·g⁻¹ and continued to increase to 250–280 nmol N·min⁻¹·g⁻¹ at 60 min in control perfusions or perfusions with SNP. In perfusions with agmatine, however, [¹⁵N]urea production increased to ~431 nmol N·min⁻¹·g⁻¹ (P = 0.02) at 60 min. In perfusions with arginine, [¹⁵N]urea production increased by ~25%, to ~310 nmol N·min⁻¹·g⁻¹, which is not significant (P = 0.1).

To distinguish between a possible effect of agmatine on glutamine metabolism via the mitochondrial PDG reaction or via the deamination of glutamate in the GDH reaction (29, 31, 28), perfusions were performed with [⁵-¹⁵N]glutamine. As with [⁵-¹⁵N]glutamine, perfusions with [⁵-¹⁵N]glutamine demonstrate a control rate of [¹⁵N]urea production of 100–150 nmol·min⁻¹·g⁻¹ between 20 and 40 min and 260 nmol·min⁻¹·g⁻¹ from 40 to 60 min. In perfusions with arginine, [¹⁵N]urea output was 320 nmol N·min⁻¹·g⁻¹ at 60 min, but these differences are not significant. However, in perfusions with agmatine, [¹⁵N]urea production increased to ~600 nmol N·min⁻¹·g⁻¹ from 40 to 60 min (P = 0.001; Fig. 6B).

Similarly, livers infused with SNP increased [¹⁵N]urea production from [⁵-¹⁵N]glutamine to 385 nmol N·min⁻¹·g⁻¹ from 40 to 60 min (P = 0.026).

Incorporation of [¹⁵N]NH₄Cl into Urea: Regulation by Agmatine

To determine whether the stimulatory action of agmatine on [¹⁵N]urea production from [¹⁵N]-labeled glutamine is directly linked to glutamine metabolism, or is subsequent to a stimulation of carbamoyl phosphate synthesis, perfusions were carried out with agmatine, [¹⁵N]NH₄Cl, and unlabeled glutamine. Figure 6C demonstrates that the presence of [¹⁵N]NH₄Cl resulted in an immediate and massive production of [¹⁵N]urea (between 20 and 60 min) of ~1,000 nmol N·min⁻¹·g⁻¹ (control) and 1,700 nmol N·min⁻¹·g⁻¹ (plus agmatine, P < 0.05). Agmatine, independent of an effect on glutamine metabolism, increased [¹⁵N]urea production from [¹⁵N]NH₄Cl by ~40% (P < 0.05), even with 1 mM unlabeled glutamine in the perfusate. Thus it is reasonable to propose that agmatine stimulates the formation of [¹⁵N]carbamoyl phosphate from [¹⁵N]NH₄Cl secondary to its action on mitochondrial NAG synthesis.

Synthesis of NAG: Regulation by Agmatine

Figure 7 shows the relationship between hepatic agmatine and NAG levels under all experimental conditions. Data points from perfusions with [⁵-¹⁵N]glutamine, [⁵-¹⁵N]glutamine, and [¹⁵N]NH₄Cl are included in these correlation analyses. It is evident that there was a highly significant relationship between hepatic agmatine and NAG concentrations (P = 0.0003), between agmatine and [¹⁵N]urea synthesis (P = 0.0002), and between NAG and [¹⁵N]urea synthesis (P = 0.0002). This observation indicates that the level of NAG in the liver is strongly associated with levels of agmatine and that agmatine may regulate the synthesis of NAG in the mitochondria. This observation suggests that agmatine regulates NAG synthesis independently of an
The data suggest that increased hepatic agmatine can induce NAG and urea synthesis. A related question is: does this change reflect increased synthesis or diminished translocation of NAG from mitochondrion to cytosol, where it is degraded? To answer this question, we have measured the production of $^{15}$N-NAG in liver perfused with $^{15}$NH$_4$Cl and unlabeled glutamine or [2-15N]glutamine and unlabeled ammonia (see MATERIALS AND METHODS). With $^{15}$NH$_4$Cl, $^{15}$N-glutamate will be formed in the mitochondria via the reductiveamination of α-ketoglutarate catalyzed by GDH. In addition, some mitochondrial glutamate will be derived from unlabeled glutamine via the PDG pathway. In the case of [2-15N]glutamine, $^{15}$N-glutamate will be formed via the PDG pathway (28, 29, 31). Therefore, the $^{15}$N-NAG will be derived from the overall 15N-labeled mitochondrial glutamate pool regardless of the respective fluxes through PDG or GDH. Figure 8 shows the production of $^{15}$N-NAG from [2-15N]glutamine. Similar observations were obtained with $^{15}$NH$_4$Cl.

**Fig. 6.** Total $^{15}$N urea production (sum of $U_{m+1}$ and $U_{m+2}$), during the course of liver perfusion with [5-15N]glutamine (A), [2-15N]glutamine (B), and $^{15}$NH$_4$Cl (C). Perfusion conditions are as indicated in Fig. 4. The output of $^{15}$N urea is the product of $^{15}$N enrichment (mole %excess/100) times one-half of total urea N (nmol N·min$^{-1}$·g$^{-1}$) for $U_{m+1}$ and times total urea N for $U_{m+2}$. Data are means ± SD for control perfusions (○, n = 3–5), plus agmatine (●, n = 4–6), plus arginine (◆, n = 3–5), and plus SNP (▲, n = 3–4).

**Fig. 7.** Dependence of N-acetylglutamate (NAG) levels and urea synthesis on agmatine concentrations in freeze-clamped liver at the end of perfusions (A). Data points included in these correlations are from perfusions with [2-15N]glutamine, [5-15N]glutamine, and $^{15}$NH$_4$Cl of control, plus agmatine, arginine, and SNP perfusions. B, C: relationship between hepatic agmatine and NAG in freeze-clamped liver extracts and $^{15}$N urea in the effluent at the end of perfusions.
naturally present in the intact liver, significantly increases in concentration after addition of arginine to the perfusate. As much as 90% of hepatic agmatine can be derived from arginine entering the liver via the portal vein. 4) The increase of hepatic agmatine strongly correlates with an increase of the newly synthesized [15N]NAG and [15N]urea production from either [2-15N]- or [5-15N]glutamine and unlabeled ammonia or 15NH4Cl and unlabeled glutamine as 15N precursors. 5) Perfusions with agmatine significantly stimulated (P < 0.0001) the synthesis of [15N]NAG and [15N]urea from 15N-labeled ammonia or glutamine. However, perfusion with arginine marginally increased (P = 0.08) NAG and urea synthesis.

The current observations suggest that the arginine entering the liver via the portal vein is mainly metabolized through the mitochondrial ADC and arginase II reactions, and there is minimal or no equilibrium with the arginase pool that is linked with the urea cycle. Evidence of this is the observation that label in [15N]-urea was ~5–10% of total urea output with 0.5 mM L-[guanidino-15N2]arginine, unlabeled ammonia, and glutamine in the perfusate. If the perfusate arginine is in equilibrium with the cytosolic pool of arginine formed via the urea cycle, then the isotopic enrichment in hepatic arginine at the end of perfusion should be similar to the isotopic enrichment of [15N2]urea in the effluent. However, the observed enrichment in hepatic [guanidino-15N2]arginine was ~50 MPE, and enrichment in effluent urea was ~5–10 MPE at the end of perfusion. This is expected, since the urea cycle does not allow net production of intermediates, and arginine formed is hydrolyzed to ornithine and urea (22, 47).

In addition, the percentage of [15N2]urea of the total urea in the effluent indicates that the relative activity of arginase II is ~5–10% of the total (sum of arginase I and II reactions) hepatic arginase. This estimate is in agreement with previous observations indicating that, in human liver, arginase II is ~2% of liver arginase (17) and in rat ~10% of total arginase activity (9).

In addition to mitochondrial formation of [15N2]urea from external L-[guanidino-15N2]arginine, the current study demonstrates that ~15–20% of L-[guanidino-15N2]arginine uptake was catabolized via the mitochondrial ADC reaction to form agmatine (Fig. 2A). O’Sullivan et al. (33, 34) suggested that [U-14C]arginine is catabolized in the liver by ornithine aminotransferase after its degradation via arginase I. Their conclusion is based on the release of 14CO2 in the effluent, which was ~13 nmol·min−1·g−1 in liver obtained from rats fed a normal protein diet (34), a regimen similar to that of this study. If we assume that 1 mol agmatine formed via the ADC reaction will be accompanied by the release of 1 mol CO2, then the rate of CO2 formation would be 14–20 nmol·min−1·g−1, similar to the values obtained by O’Sullivan et al.

Agmatine is widely distributed in mammalian tissues (37, 40, 42) and may have a role in multiple metabolic functions. The current observations demonstrate that agmatine entering the liver via the portal vein stimulates the uptake of glutamine (Table 1),

Fig. 8. Appearance of [15N]NAG in extracts of livers that were freeze-clamped at the end of perfusions with [2-15N]glutamine. Amount of [15N]NAG is the product of isotopic enrichment (mole % excess/100) times total concentration (nmol/g). Data are means±SD for control perfusions (n = 4), plus agmatine (n = 4), plus arginine (n = 3), and plus SNP (n = 3). P values indicate the degree of significance compared with the control level.

DISCUSSION

The liver regulates whole body N metabolism and detoxifies ammonia via conversion of excess amino N to urea (22, 49). Although ureagenesis is a major hepatic function, the factors that regulate this process have been the subject of conflicting views and controversies (3). Understanding these mechanism(s) is important not only in terms of understanding ammonia detoxification but also because ureagenesis has provided insight into whole body N balance and metabolism (49).

The initial step in urea synthesis is the conversion of NH4+ and HCO3− to carbamoyl phosphate by mitochondrial CPS-I (32), which requires NAG as an obligatory effector (8, 13, 16, 22). Increased NAG synthesis and concomitant ureagenesis have been observed after the intake of arginine or a high-protein diet (9, 19, 26, 44). However, it is unknown whether arginine itself or a metabolite of arginine stimulates the synthesis of NAG and, thereby, ureagenesis. In the current investigation, we have explored the hypothesis that agmatine, not arginine, is responsible for increased NAG synthesis and concomitant ureagenesis.

The principal observations supporting our hypothesis are as follows. 1) L-[guanidino-15N2]arginine enters the liver via the portal vein and is catabolized to [guanidino-15N2]agmatine and [15N2]urea. About 90% of arginine uptake was recovered in urea and agmatine output in the effluent. 2) Metabolism of perfusate arginine is primarily mediated via the mitochondrial ADC and arginase II reactions. 3) Agmatine, which is
synthesis of NAG, and urea (Figs. 4–8). This possibility is consistent with an increased rate of oxygen consumption in perfusions with agmatine and to a lesser extent with arginine (Fig. 4B). The increased oxygen consumption in perfusions with agmatine may indicate 1) higher energy requirements; 2) stimulation of substrate supply to the electron transport chain; and/or 2) increased respiratory chain activity (45). Agmatine may increase the activity of respiratory enzymes secondary to stimulated mitochondrial energy-consuming functions such as the synthesis of NAG, carbamoyl phosphate, and urea itself.

In line with the above suggestion are data (Fig. 8) demonstrating that agmatine significantly (P < 0.0001) increased the formation of newly synthesized [15N]NAG from [2-15N]glutamine. However, arginine marginally increased (P = 0.08) and SNP significantly (P = 0.002) decreased the production of NAG compared with control. The linear correlation between agmatine and NAG levels (Fig. 7) demonstrates that synthesis of NAG and, thereby, activity of CPS-I would depend on the concentration of agmatine. Therefore, the current data strongly support the hypothesis that agmatine is a positive effector for mitochondrial synthesis of NAG. In addition, the data in Fig. 7 suggest that the concentration of intrahepatic agmatine is an important feature in the regulation of urea synthesis and ammonia detoxification. Nevertheless, it is uncertain whether increased NAG levels are secondary to increased synthesis or diminished translocation of NAG from mitochondrion into cytosol, where it is degraded. It has been shown that efflux of NAG out of mitochondria and its subsequent degradation are diminished in rats fed a high-protein diet compared with rats on a protein-free diet (23, 24, 27). The current data clearly demonstrate that agmatine stimulates the synthesis of NAG (Fig. 8). The diminished rate of NAG translocation in response to a high-protein diet (27) may reflect the presence in this diet of arginine, which is converted to agmatine.

It has been suggested that hepatic glutaminase and concomitant glutamate production are key factors in facilitating NAG synthesis (3). Consistent with this interpretation are the data demonstrating that agmatine stimulates glutamine uptake (Fig. 4A and Table 1) and flux through glutaminase (Fig. 5). However, the positive association between NAG synthesis, [15N]urea synthesis, and agmatine concentration is valid whether [2-15N]glutamine, [5-15N]glutamine, or 15NH4Cl was used to monitor these metabolic processes (Fig. 7). Thus agmatine may regulate NAG synthesis independently of an effect on flux through the PDG reaction. The observed increased flux through PDG in perfusions with agmatine may be subsequent to elevated NAG levels in the mitochondrial matrix, as previously indicated (25). In addition, agmatine-stimulated glutamine uptake (Table 1) may provide an additional fuel that supports NAG and/or carbamoyl phosphate synthesis, both of which require ATP. This possibility would be in line with the increased oxygen consumption associated with agmatine-induced glutamine uptake (Fig. 4, A and B, and Table 1).

A significant decrease in hepatic glutamate level was observed after perfusions with SNP (Table 2). This finding may explain the decreased NAG synthesis in perfusions with SNP (Fig. 8). The uptake of glutamine is increased with SNP (Table 1 and Fig. 4), but this is not reflected in augmented output of [15N]urea and 15NH3 from [5-15N]glutamine (i.e., flux through the PDG pathway), which show minor differences compared with control (Figs. 5 and 6). Measurements of 15NH3 enrichment in perfusions with [2-15N]glutamine show that, in control, [15N]ammonia enrichment in the effluent was ~3 atom percent excess, with little differences in perfusions with agmatine or arginine. In perfusions with SNP, 15NH3 enrichment was ~11 atom percent excess, indicating a stimulation of glutamate catabolism mediated via the GDH reaction. Therefore, stimulated mitochondrial glutamate catabolism in perfusions with SNP may account for the reduction of the newly synthesized NAG, as shown in Fig. 8.

An alternative mechanism for increased NAG synthesis by agmatine is stimulation of β-oxidation and/or the pyruvate dehydrogenase and pyruvate carboxylase reactions. This possibility is supported by the significant (P < 0.05) increase in oxygen consumption in perfusions with agmatine (Figs. 3B and 4B) and increased pyruvate and lactate uptake (μmol·min⁻¹·g⁻¹) from 1.7 (control) to ~3 (plus agmatine). Stimulation of the pyruvate dehydrogenase reaction would increase the availability of acetyl-CoA for NAG synthesis, and an increase in the pyruvate carboxylase activity would provide more oxaloacetate and, thereby, aspartate for synthesis of argininosuccinate, as we have indicated previously in studies with [3-13C]pyruvate (31). These possibilities are currently under investigation using 13C-labeled precursors and NMR as an analytical tool.

In conclusion, the current observations provide evidence to support the hypothesis that availability of agmatine rather than arginine may have a major regulatory role in hepatic ammonia detoxification and urea synthesis. The current findings may have clinical implications for the treatment of disturbed urea synthesis and toxic hyperammonemia. Effective drugs have been introduced for removal of waste toxic N from the body (10), but agmatine might prove a valuable therapeutic adjunct. This agent has been proposed as a treatment for other disorders (40, 42). Agmatine can be useful, especially in cases of hyperactivity of the hepatic GDH reaction, which is associated with the hyperinsulinism/hyperammonemia syndrome (43). In this case, hyperammonemia is secondary to diminished synthesis of NAG (43). The stimulation of NAG synthesis by agmatine (Fig. 8) lends support to the importance of agmatine as a potential candidate for the treatment of the hyperinsulinism/hyperammonemia syndrome in infants (43).

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