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Agmatine Stimulates Hepatic Fatty Acid Oxidation

*A POSSIBLE MECHANISM FOR UP-REGULATION OF UREAGENESIS**

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We demonstrated previously in a liver perfusion system that agmatine increases oxygen consumption as well as the synthesis of *N*-acetylglutamate and urea by an undefined mechanism. In this study our aim was to identify the mechanism(s) by which agmatine up-regulates ureagenesis. We hypothesized that increased oxygen consumption and *N*-acetylglutamate and urea synthesis are coupled to agmatine-induced stimulation of mitochondrial fatty acid oxidation. We used ^{13}C-labeled fatty acid as a tracer in either a liver perfusion system or isolated mitochondria to monitor fatty acid oxidation and the incorporation of ^{13}C-labeled acetyl-CoA into ketone bodies, tricarboxylic acid cycle intermediates, amino acids, and *N*-acetylglutamate. With [U-$^{13}C_{16}$] palmitate in the perfusate, agmatine significantly increased the output of ^{13}C-labeled β-hydroxybutyrate, acetoacetate, and CO_2, indicating stimulated fatty acid oxidation. The stimulation of [U-$^{13}C_{16}$]palmitate oxidation was accompanied by greater production of urea and a higher ^{13}C enrichment in glutamate, *N*-acetylglutamate, and aspartate. These observations suggest that agmatine leads to increased incorporation and flux of ^{13}C-labeled acetyl-CoA in the tricarboxylic acid cycle and to increased utilization of ^{13}C-labeled acetyl-CoA for synthesis of *N*-acetylglutamate. Experiments with isolated mitochondria and ^{13}C-labeled octanoic acid also demonstrated that agmatine increased synthesis of ^{13}C-labeled β-hydroxybutyrate, acetoacetate, and *N*-acetylglutamate. The current data document that agmatine stimulates mitochondrial β-oxidation and suggest a coupling between the stimulation of hepatic β-oxidation and up-regulation of ureagenesis. This action of agmatine may be mediated via a second messenger such as cAMP, and the effects on ureagenesis and fatty acid oxidation may occur simultaneously and/or independently.

Agmatine (Agm)[2] is widely distributed in mammalian tissue (1, 2) and may act as a hormone affecting multiple metabolic functions (3–8). We demonstrated previously that addition of Agm to a liver perfusion system significantly stimulated oxygen consumption and synthesis of ^{15}N-labeled NAG and urea from ^{15}N-labeled glutamine (9). However, the nature and/or the mechanism(s) underlying these actions of Agm are unknown. One possibility is that Agm stimulates fatty acid oxidation (FAO), thereby providing more reducing equivalents (NADH and FADH) to the respiratory chain and increasing availability of substrates and/or ATP for NAG and urea synthesis. This possibility is in line with the increased oxygen consumption associated with agmatine up-regulation of urea synthesis (9).

As illustrated in Fig. 1, Agm may stimulate FAO, thereby increasing the availability of reducing substrates and acetyl-CoA. The latter may be converted to β-HB and AcAc (ketone bodies), utilized for NAG synthesis, and/or incorporated into the tricarboxylic acid cycle. The increased [acetyl-CoA] and/or [NADH] is expected to up-regulate the production of oxaloacetate (OXA) via the pyruvate carboxylase (PC) reaction (10–13), thus increasing the anaplerotic incorporation of pyruvate carbon into glucose and/or the tricarboxylic acid cycle (10). Simultaneously, increased [NADH] can promote reductive amination of α-ketoglutarate and production of glutamate, *i.e.* cataplerosis (10). Alternatively, agmatine may stimulate deamidation of glutamine, thereby elevating the glutamate concentration, as indicated previously (9). Glutamate plus acetyl-CoA provide more substrates for the synthesis of NAG, an obligatory activator of carbamoylphosphate synthetase I (CPS-I) (14). In addition, some mitochondrial OXA will be transaminated to aspartate, which then is transported into the cytosol to support the synthesis of argininosuccinate (15, 16). In addition, an elevation of mitochondrial [glutamate] supports production of ornithine, and then citrulline, in the mitochondrial matrix (16). Hence, the proposed metabolic cascade may lead to the following: (i) increased reducing substrates such as NADH and FADH, thus furnishing more ATP (for ureagenesis) via oxidative phosphorylation; (ii) increased mitochondrial synthesis of NAG and citrulline; and (iii) increased availability of aspartate for cytosolic synthesis of argininosuccinate. This sequence of events leads to up-regulation of urea synthesis. This study also examined an alternative possibility, *i.e.* the action of Agm may be mediated through a second messenger such as cAMP. Agm and/or its second messenger may act simultaneously and independently on ureagenesis and FAO, with the increase of urea synthesis and FAO being mediated by independent events.

Hepatic oxidation of fatty acids begins in cytosol and finishes in the mitochondrion (17, 18). The transport of long chain fatty acids into mitochondria involves their conversion into acylcarnitine esters at the outer mitochondrial membrane (17, 18). Acylcarnitine is transported into the mitochondrial matrix and metabolized via the β-oxidation pathway (17, 18). Agm may act either at the site of long chain fatty acid activation in the mitochondrial outer membrane or on the β-oxidation chain shortening in the mitochondrial matrix. Therefore, in this study we investigated the action of Agm in a liver perfusion system with [U-$^{13}C_{16}$]palmitate as tracer and in isolated mitochondria incubated with ^{13}C-labeled octanoic acid, a medium chain fatty acid, that does not require activation by the carnitine transport system at the mitochondrial outer membrane (18). Experiments with isolated mitochondria or a liver perfusion system allow differentiation between a possible action of Agm on carnitine acyltransferase and the β-oxidation pathway. In addition, perfusion of the structurally intact liver with physiological concentrations of [U-$^{13}C_{16}$]palmitate and other metabolites would

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[2] The abbreviations used are: Agm, agmatine; AcAc, acetoacetate; FAO, fatty acid oxidation; GC-MS, gas chromatography-mass spectrometry; β-HB, β-hydroxybutyrate; MPE, mol % excess; NAG, *N*-acetylglutamate; OXA, oxaloacetate; PC, pyruvate carboxylase; HPLC, high pressure liquid chromatography.

reveal a possible coupling between Agm action on β-oxidation and the up-regulation of urea synthesis, as illustrated in Fig. 1. Furthermore, a comparison between experiments with isolated mitochondria and a liver perfusion system should differentiate between Agm actions on mitochondrial *versus* peroxisomal β-oxidation (19, 20).

The results demonstrate that, in isolated mitochondria and liver perfusions, Agm stimulated the production of ^{13}C-labeled ketone bodies and CO_2, indicating a stimulation of β-oxidation in the mitochondrial matrix. The stimulation of FAO was accompanied by increased [NAG] and urea output, suggesting a coupling between urea synthesis and the stimulation of fatty acid oxidation by agmatine.

EXPERIMENTAL PROCEDURES

Materials and Animals—Male Sprague-Dawley rats (Charles River Breeding Laboratories) were fed a standard rat chow diet *ad libitum*. Chemicals were of analytical grade and obtained from Sigma. Enzymes and cofactors for the analysis of adenine nucleotides, β-HB, AcAc, NADH, NAD, urea, lactate, pyruvate, and ammonia were obtained from Sigma. [U-$^{13}C_{16}$]Palmitate and [1,2,3,4-$^{13}C_4$]octanoic acid, $^{15}NH_4Cl$, or [^{15}N]glutamate, 99 mol % excess (MPE), were from Isotec.

Experiments with Liver Perfusions—Livers from overnight fasted male rats were perfused in the non-recirculating mode and antegrade flow at the rate of 3–3.5 ml·g^{-1}·min^{-1} as described previously (9, 16). The basic perfusion medium was Krebs saline, pH 7.4, continuously gassed with 95% O_2, 5% CO_2 and containing lactate (2.1 mM) and pyruvate (0.3 mM) as metabolic fuels. pO_2 (in influent and effluent media) was monitored throughout, and oxygen consumption was calculated. After 10 min of conditioning with a basic perfusion medium (Medium A), perfusate was replaced with one containing lactate, pyruvate (as in Medium A), plus 0.3 mM NH_4Cl, and 1 mM glutamine with or without 0.1 mM agmatine (Medium B). After a 10-min perfusion with Medium B, the perfusate was replaced with one that contained 0.5 mM [U-$^{13}C_{16}$]palmitate (as potassium salt bound to bovine serum albumin in a 5:1 molar ratio), NH_4Cl, glutamine, lactate, and pyruvate (as in Medium A and B), with or without 0.1 mM agmatine. The perfusion was continued for an additional 5, 10, 15, 20, 25, and 30 min. Samples were taken from the influent and effluent media for chemical and GC-MS analyses. At the indicated times, perfusion was stopped, and the liver was freeze-clamped with aluminum tongs pre-cooled in liquid N_2. The frozen liver was ground into a fine powder, extracted into perchloric acid, and used for metabolite determination and ^{13}C enrichment.

Metabolic Studies with Isolated Mitochondria—Mitochondria were isolated from the liver of overnight fasted rats by differential centrifugation as described previously (21). Respiratory control and oxygen consumption were determined in each mitochondrial preparation (16, 22). Metabolic studies were carried out with mitochondria having a state 3/state 2 respiratory ratio greater than 3.

The mitochondrial suspension (3–4 mg of protein/ml) was incubated in Erlenmeyer flasks (2 ml final volume) at 30 °C in a shaking water bath for 10 min and with the addition of substrates as indicated below. The basic incubation medium consisted of the following (mM): Tris (50), EDTA (2), KCl (5), $MgCl_2$ (5), $KHCO_3$ (15), KH_2PO_4 (5), $^{15}NH_4Cl$ (2), α-ketoglutarate (5), and ornithine (5), pH 7.4.

In the first series of experiments, mitochondria were incubated for 10 min at 30 °C with basic medium plus 2 mM [1,2,3,4-$^{13}C_4$]octanoic acid and an increasing concentration (0–1 mM) of Agm. The second series of experiments was carried out with broken mitochondria (22), basic medium, increasing concentrations of Agm, 5 mM ATP, 2 mM acetyl-CoA, 5 mM succinate, and 5 mM [^{15}N]glutamate without octanoic acid.

TABLE 1

Metabolic state of the liver during and at the end of liver perfusions with [U-$^{13}C_{16}$]palmitate and with or without agmatine

Livers from overnight fasted male rats were perfused in the non-recirculating mode with lactate, pyruvate, glutamine, and ammonia plus 0.5 mM [U-$^{13}C_{16}$]palmitate as outlined under "Experimental Procedures." Samples were taken from the influent and effluent media for determination of metabolite levels. At the end of the perfusion, the liver was freeze-clamped, extracted into perchloric acid, and used for metabolite determination.

	Control	(+) Agmatine
Oxygen consumption and metabolite output during liver perfusions[a]		
Oxygen consumption (μmol·g−1·min−1)[b]	3.53 ± 0.05	3.71 ± 0.07
Metabolite output (nmol·g−1·min−1)[c]		
Urea	955 ± 65	1229 ± 68[d]
β-Hydroxybutyrate	197 ± 22	313 ± 11[d]
Acetoacetate	70 ± 12	119 ± 15[d]
Glucose	937 ± 174	1172 ± 62
Metabolites in liver extract at the end of perfusions[e]		
ATP[b]	2.3 ± 0.7	2.4 ± 0.3
ADP[b]	1.3 ± 0.5	1.3 ± 0.4
AMP[b]	0.8 ± 0.2	0.7 ± 0.1
cAMP[c]	1.4 ± 0.2	1.7 ± 0.2
Acetoacetate[c]	165 ± 26	204 ± 22[d]
β-Hydroxybutyrate[c]	407 ± 71	556 ± 89[d]
Lactate[c]	1912 ± 978	2441 ± 481
Pyruvate[c]	313 ± 82	378 ± 143
Ornithine[c]	94 ± 17	136 ± 31[d]
Citrulline[c]	51 ± 6	91 ± 37[d]

[a] The rates of oxygen consumption or urea, ketone body, and glucose output are the steady-state values obtained between 15 and 30 min after the start of [U-$^{13}C_{16}$]palmitate infusion. Values are mean ± S.D.
[b] Values are μmol/g wet weight of livers.
[c] Values are nmol/g wet weight of livers.
[d] Value indicates a significant difference ($p < 0.05$) compared with perfusions without agmatine.
[e] Metabolite levels in liver extract are mean ± S.D. of separate livers (3–5) obtained at 15, 20, 25, and 30 min after the start of [U-$^{13}C_{16}$]palmitate infusion.

At the end of the incubation, an aliquot (100 μl) was taken for protein determination, and the incubation was stopped with 100–150 μl of $HClO_4$ (60%). Metabolite measurements were done in neutralized extracts. Three to five independent experiments were carried out for each series.

GC-MS and NMR Methodology; Determination of ^{13}C-Labeled Metabolites—GC-MS measurements of ^{13}C isotopic enrichment were performed on either a Hewlett-Packard 5970 MSD and/or 5971 Mass Selective Detector (MSD), coupled with a 5890 HP-GC, GC-MS Agilent System (6890 GC-5973 MSD) or Hewlett-Packard (HP-5970 MSD), using electron impact ionization with an ionizing voltage of −70 eV and an electron multiplier set to 2000 V.

For measurement of the ^{13}C enrichment in amino acids, organic acids (α-ketoglutarate or citrate), or ketone bodies, samples were prepared as described previously (9, 16, 22). Briefly, an aliquot of effluent, liver, or mitochondrial extract was purified by passage on an AG-1 (Cl^-, 100–200 mesh; 0.5 × 2.5 cm) or AG-50 (H^+, 100–200 mesh) column, and then converted into the *t*-butyldimethylsilyl derivatives. Isotopic enrichment in glutamate isotopomers was monitored using ions at *m*/*z* 432, 433, 434, 435, 436, and 437 for M, M + 1, M + 2, M + 3, M + 4 and M + 5 (containing 1–5 ^{13}C atoms). Isotopic enrichment in aspartate isotopomers was monitored using ions at *m*/*z* 418, 419, 420, 421, and 422 for M + 1, M + 2, M + 3 and M + 4 (containing 1–4 ^{13}C atoms). Isotopic enrichment in β-HB was monitored using ions at *m*/*z* 275, 276, 277, 278, and 279 for M + 1, M +2, M + 3, and M + 4 (containing 1–4 ^{13}C atoms), respectively, and ^{13}C enrichment in AcAc was monitored using ions at *m*/*z* 273, 274, 275, 276, and 277 for M + 1, M + 2, M + 3, and M + 4 (containing 1–4 ^{13}C atoms), respectively.

The concentration and ^{13}C enrichment in *N*-acetylglutamate in liver or mitochondrial extracts were determined using GC-MS and an isotope dilution approach (9, 16). The formation of ^{13}C-labeled NAG iso-

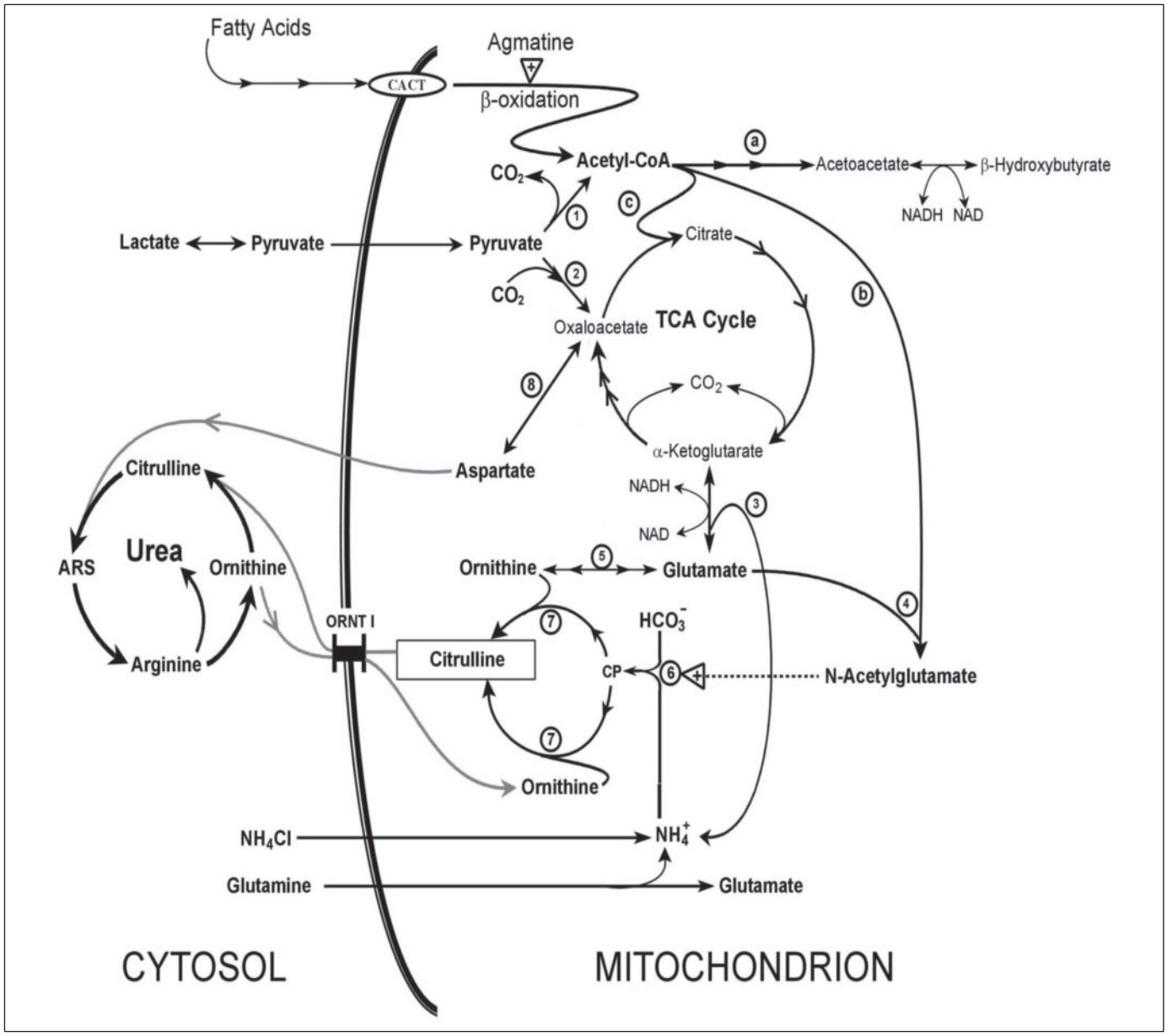

FIGURE 1. **Hypothetical schematic of agmatine effects on hepatic metabolism.** We propose that agmatine stimulates mitochondrial β-oxidation, thereby increasing the production of reducing equivalents and acetyl-CoA. The latter may be converted to acetoacetate and β-hydroxybutyrate (ketone bodies) (*a*), utilized for *N*-acetylglutamate synthesis (*b*), and/or metabolized in the tricarboxylic acid cycle (*TCA*) (*c*). Increased mitochondrial [acetyl-CoA] might down-regulate flux through pyruvate dehydrogenase and up-regulate flux through pyruvate carboxylase and oxaloacetate production (anaplerosis). Oxaloacetate may be used to form aspartate or may react with acetyl-CoA to form citrate, thereby favoring formation of α-ketoglutarate and glutamate (cataplerosis). In addition, glutamate is generated via deamidation of perfusate glutamine. The production of glutamate and acetyl-CoA provides more substrates for *N*-acetylglutamate synthesis, thus stimulating the urea cycle. Metabolic reactions are as follows: *1*, pyruvate dehydrogenase; *2*, pyruvate carboxylase; *3*, glutamate dehydrogenase; *4*, *N*-acetylglutamate synthetase; *5*, ornithine aminotransferase; *6*, carbamoyl-phosphate synthetase I; *7*, ornithine transcarbamylase; *8*, glutamate-aspartate aminotransferase. *ARS*, argininosuccinate; *CACT*, carnitine:acylcarnitine translocase; *CP*, carbamoyl phosphate.

topomers was monitored using ions at *m*/*z* 158, 159, 160, 161, 162, and 163, for M + 1, M + 2, M + 3, M + 4, and M + 5 (containing 1–5 ^{13}C atoms). In experiments with isolated mitochondria, the production of ^{15}N-labeled citrulline from $^{15}NH_4Cl$ was determined as described (16).

The production of $^{13}CO_2$ during liver perfusion was monitored in the effluent as follows: 1 ml of effluent was added to a sealed tube free of CO_2 and containing 1 ml of 1 mM NaOH; 0.5 ml of 40% phosphoric acid was then added. Tubes were left for about 30 min to liberate $^{13}CO_2$. The latter was removed with a sealed syringe and transferred to auto-sampler tubes for analysis. Isotopic enrichment in $^{13}CO_2$ was determined by an isotope ratio-mass spectrometer (Thermoquest Finnigan Delta Plus) using the *m*/*z* 45/44 ratio.

To evaluate further the effect of Agm on hepatic catabolism of [U-$^{13}C_{16}$]palmitate, a portion (about 2 g wet weight) of the neutralized liver extracts was analyzed by ^{13}C NMR methodology using a Bruker DMX 400 wide bore equipped with a Silicon Graphic O2 computer. The chemical shifts of analyte containing ^{13}C atoms were measured relative to the resonance of trimethylsilylpropionic acid at −2.7 ppm. Data acquisition and calculation of % ^{13}C enrichment in various glutamate carbons were done as described (23).

Analytical Measurements—The concentration of amino acids was determined by HPLC, utilizing pre-column derivatization with *o*-phthalaldehyde (24). The levels of ammonia and urea were measured (9, 16) as well as ATP (25), ADP, and AMP as in Ref. 26. cAMP was meas-

ured as described (27) using a cAMP EIA kit (Cayman Chemical Co.). We also measured NAD and NADH (28), AcAc and β-HB (29), lactate (30), pyruvate (31), and glucose (32).

Calculations and Statistical Analyses—During liver perfusions, the rate of uptake or the output of metabolites was determined by the measurement of metabolite concentration in the influent and effluent (nmol/ml), normalized to the flow rate (ml/min) and liver wet weight (9, 15, 16). ^{13}C enrichment in a given mass isotopomer is expressed by molar percent enrichment (MPE), which is the mol fraction (%) of analyte containing ^{13}C atoms above natural abundance. The MPE was calculated using the peak area from GC-MS ions corrected for natural abundance as described (33, 34). The appearance of ^{13}C-labeled glutamate, aspartate, or NAG isotopomers was calculated by the product of ^{13}C enrichment (MPE) in a given isotopomer/100 times concentration (nmol/g wet wt) and is expressed as nanomoles of ^{13}C-labeled metabolite/g wet wt. The rate of appearance of ^{13}C-labeled isotopomers was calculated by fitting the time course appearance of ^{13}C-labeled glutamate, aspartate, or NAG isotopomers to a one-phase exponential association or to a linear regression analysis using GraphPad Prism-4 software for linear and nonlinear curve fitting as indicated (16). The output of ^{13}C-labeled ketone bodies was calculated by the product of MPE/100 times rate of output (nmol·g^{-1}·min^{-1} wet wt) and is expressed as nanomoles of ^{13}C-labeled metabolite·g^{-1}·min^{-1}. The output of $^{13}CO_2$ (nmol·g^{-1}·min^{-1}) was calculated by the product of $^{13}CO_2$ enrichment MPE/100 times 25 mM, the concentration of $NaHCO_3$ in the perfusate. Data obtained from mitochondrial incubations were analyzed with GraphPad Prism-4 software for linear and nonlinear curve fitting.

Each series of experiments was repeated 3–4 times with different mitochondrial preparations or with individual liver perfusion systems as outlined above. Statistical analysis was carried out using In-STAT 1.14 software for the Macintosh. The Student's *t* test or analysis of variance test was employed to compare two groups or differences among groups as needed. A *p* value less than 0.05 was taken as indicating a statistically significant difference.

RESULTS

The initial series of experiments was designed to determine the relationship between the products of FAO, *i.e.* β-HB, AcAc (ketone bodies), CO_2, and urea output in the effluent. Fig. 2 illustrates the effect of Agm on the output of ^{13}C-labeled ketone bodies and CO_2 in the effluent during perfusion with 0.5 mM [U-$^{13}C_{16}$]palmitate, glutamine, and ammonia, with or without 0.1 mM Agm. This dose of Agm was found to exert the maximum effect on the up-regulation of urea synthesis (9). During perfusions with Agm and [U-$^{13}C_{16}$]palmitate, the output of both ^{13}C-labeled ketone bodies and CO_2 was significantly higher compared with perfusions without Agm. The elevated output of ^{13}C-labeled ketone bodies (Fig. 2*A*) and CO_2 (Fig. 2*B*) was accompanied by a significant increase in urea output (Table 1), indicating a possible coupling between the oxidation of [U-$^{13}C_{16}$]palmitate and ureagenesis. In perfusion without 0.5 mM [U-$^{13}C_{16}$]palmitate (Medium B, see under "Experimental Procedures"), the release of ketone bodies in the effluent was about 54 ± 9 nmol·g^{-1}·min^{-1}, and the β-HB/AcAc ratio was about 1. When 0.5 mM [U-$^{13}C_{16}$]palmitate was added to the perfusate, the release of ketone bodies was increased by about 6-fold (Table 1), and the β-HB/AcAc ratio was increased to 2.7–2.9. These values are similar to those reported previously during liver perfusions with octanoate (35, 36) or palmitate (37).

[1,2-^{13}C]Acetyl-CoA generated from the oxidation of [U-$^{13}C_{16}$]palmitate may be converted to ketone bodies, utilized for NAG synthesis, and/or metabolized in the tricarboxylic acid cycle (Fig. 1). The increased

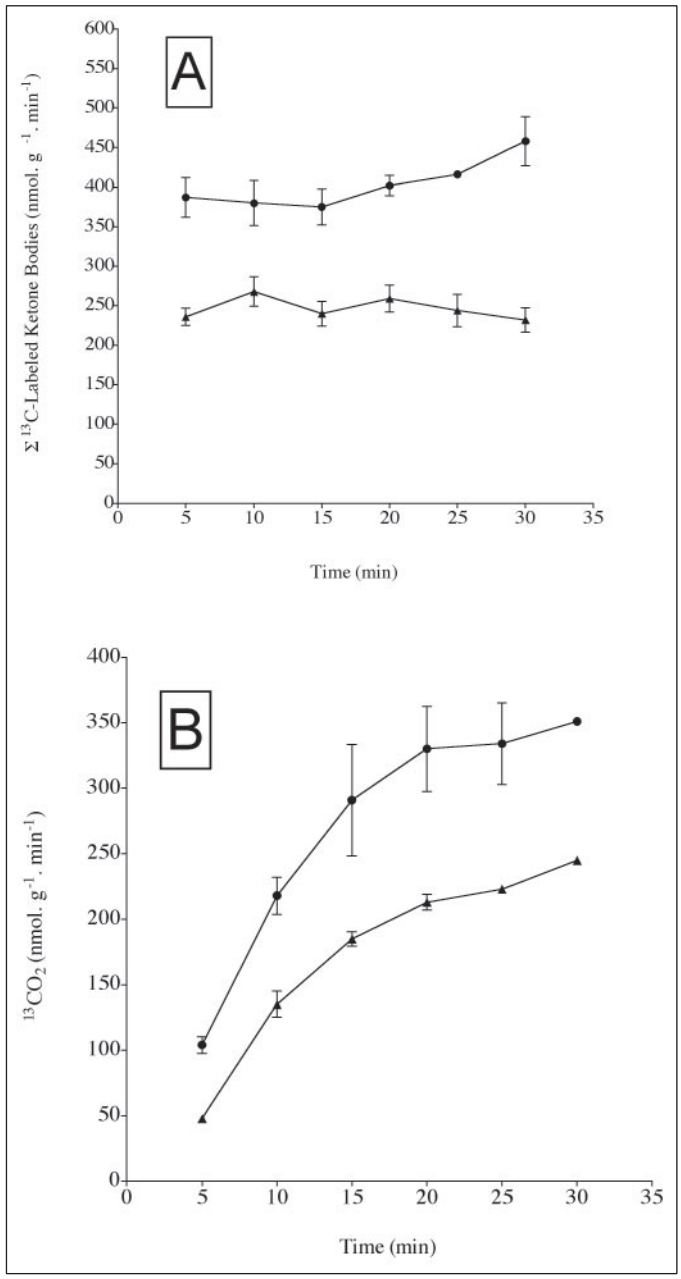

FIGURE 2. **The effect of agmatine on the output of ^{13}C-labeled ketone bodies (*A*) or CO_2 (*B*) during the course of perfusions with [U-$^{13}C_{16}$]palmitate.** Preperfusions (10 min) were carried out with basic perfusate containing 2.1 mM lactate and 0.3 mM pyruvate. Liver was then perfused for an additional 10 min with perfusate supplemented with 1 mM glutamine and 0.3 mM NH_4Cl, lactate, and pyruvate with or without 0.1 mM agmatine. Finally, liver was perfused for the times indicated with lactate, pyruvate, glutamine and ammonia (as indicated above) plus 0.5 mM [U-$^{13}C_{16}$]palmitate, with (●) or without (▲) 0.1 mM agmatine. The indicated time represents time of collection of effluent samples following the start of [U-$^{13}C_{16}$]palmitate infusion. *Bars* are mean ± S.D. of 3–4 independent liver perfusions.

output of $^{13}CO_2$ in perfusion with Agm indicates increased incorporation of ^{13}C-labeled acetyl-CoA in the tricarboxylic acid cycle. At steady state, which is 15–30 min after the start of the [U-$^{13}C_{16}$]palmitate infusion, the rates of $^{13}CO_2$ output were 326 ± 25 or 216 ± 28 nmol·g^{-1}·min^{-1} in perfusions with or without Agm, respectively (Fig. 2*B*), indicating a significantly higher flux of ^{13}C-labeled acetyl-CoA through the tricarboxylic acid cycle with Agm.

To evaluate further the flux of ^{13}C-labeled acetyl-CoA through the tricarboxylic acid cycle, we utilized the transfer of ^{13}C from palmitate to glu-

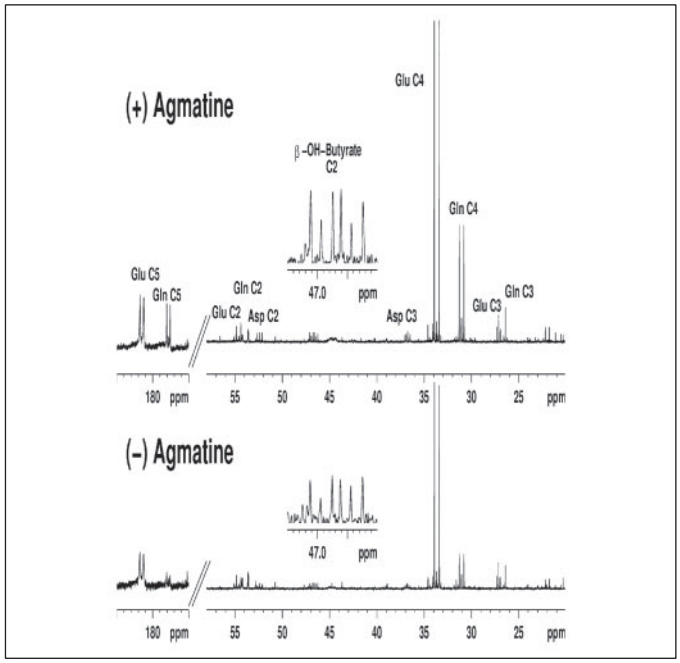

FIGURE 3. **A representative ^{13}C NMR spectrum of freeze-clamped liver extracts following perfusion with [U-$^{13}C_{16}$]palmitate with or without agmatine.** Experimental details are as in the legend to Fig. 2. This spectrum was obtained from neutralized liver extracts (≈2 g wet weight) following 25 min of perfusion with [U-$^{13}C_{16}$]palmitate. Analysis was performed with a Bruker DMX 400 wide-bore spectrometer equipped with a Silicon Graphic O2 computer. *Insets* demonstrate the production of ^{13}C-labeled β-OH-butyrate, which was remarkably higher with agmatine compared with perfusion without agmatine (The release in the effluent is demonstrated in Fig. 2.)

tamate as a marker for flow of [1,2-^{13}C]acetyl-CoA through the tricarboxylic acid cycle. To this end, the neutralized liver extracts were analyzed by GC-MS and ^{13}C NMR methodologies. This analytical approach is the most precise to follow the metabolism of ^{13}C-labeled precursor through the tricarboxylic acid cycle and to determine a possible precursor-product relationship. ^{13}C NMR spectra (Fig. 3) demonstrate that glutamate is mainly labeled at carbons 4 and 5, with insignificant ^{13}C enrichment in carbons 1–3. Because of the randomization of [1,2-^{13}C]acetyl-CoA in the tricarboxylic acid cycle (12, 33), we used the M + 2 glutamate isotopomer (Fig. 4) and the time course of [glutamate] in liver extract (Table 2), to estimate the rate of ^{13}C-labeled glutamate appearance. This rate was about 20 and 38 nmol·g^{-1}·min^{-1} in the absence or presence of Agm, respectively (Fig. 4, *C* and *D*). However, the rate of ^{13}C-labeled ketone bodies production and their output was about 400 and 250 nmol·g^{-1}·min^{-1} in experiments with and without Agm, respectively (Fig. 2*A*), indicating 11–13-fold higher incorporation of [1,2-^{13}C]acetyl-CoA into ketone bodies than into glutamate formation. This is in keeping with previous studies, which proposed that acetyl-CoA generated from FAO is primarily used for ketogenesis (35, 38).

Calculations based on the ^{13}C NMR analysis indicate that ~58 and 40% of the glutamate pool is labeled at carbons 4 and 5 in experiments with or without Agm, respectively, in keeping with the GC-MS measurements of the MPE in the M + 2 isotopomer of glutamate (Fig. 4). The remaining unlabeled glutamate pool (40–60%) probably was derived from the perfusate glutamine and proteolysis (39, 40). Nonetheless, despite the addition of 1 mM unlabeled glutamine in the perfusate, there was a robust formation of ^{13}C-labeled glutamine at carbons 4 and 5 (Fig. 3), suggesting glutamine recycling, *i.e.* part of glutamine that was hydrolyzed to glutamate and ammonia via the glutaminase reaction in the periportal system is resynthesized from [4,5-^{13}C]glutamate in the perivenous hepatocytes.

Fig. 5 depicts the production of ^{13}C-labeled NAG isotopomers. The time course of the hepatic [NAG] is presented in Table 2. The M + 2 isotopomer of NAG probably results from the reaction of [1,2-^{13}C]acetyl-CoA with unlabeled glutamate via mitochondrial *N*-acetylglutamate synthase (EC 2.3.1.1) (41). The M + 4 isotopomer forms following the reaction of [4,5-^{13}C]glutamate with [1,2-^{13}C]acetyl-CoA. Other isotopomers of NAG (Fig. 5, *A* and *B*) are the result of the reaction of [1,2-^{13}C]acetyl-CoA with various isotopomers of glutamate (Fig. 4). Therefore, the production of NAG is represented by the formation of M + 2 plus M + 4 isotopomers (Fig. 5, *C* and *D*). In experiments with Agm, the rate of M + 2 and M + 4 isotopomers production was 2.02 ± 0.37 nmol·g^{-1}·min^{-1}, whereas without Agm the rate of M + 2 and M + 4 isotopomers production was 0.78 ± 0.17 nmol·g^{-1}·min^{-1} (Fig. 5, *C* and *D*). Furthermore, total hepatic [NAG] was significantly higher at 20, 25, and 30 min after the start of infusions with Agm and [U-^{13}C]palmitate compared with infusions without Agm (Table 2). Therefore, there was approximately a 2-fold higher [NAG] and a 3-fold increase in ^{13}C-labeled NAG production ($p < 0.05$) from perfusate [U-$^{13}C_{16}$]palmitate with the addition of Agm to the perfusate.

As shown in Fig. 6, ^{13}C-labeled aspartate also was formed, reflecting the appearance of ^{13}C-labeled oxaloacetate and subsequent transamination with glutamate N. This observation is in agreement with the suggestion that mitochondrial glutamate-oxaloacetate transaminase favors aspartate synthesis (15, 38). However, the appearance of ^{13}C-labeled aspartate may not represent net production of aspartate, rather an isotopic equilibrium between ^{13}C-labeled OXA and the mitochondrial aspartate pool. Nonetheless, Agm significantly increased hepatic aspartate levels (Table 2) and the appearance ^{13}C-labeled aspartate (Fig. 6). The increased mitochondrial [aspartate] may promote synthesis of argininosuccinate, as suggested previously (15).

The stimulation of FAO may also increase hepatic gluconeogenesis secondary to decreased flux through pyruvate dehydrogenase and increased flux through pyruvate carboxylase (11, 13, 37, 38, 42). The current observations indicate about a 20% increase in glucose output in perfusions with Agm (Table 1). This increment was likely generated from perfusate lactate and pyruvate and mediated via increased flux through the PC reaction as well as elevated [NADH] secondary to stimulation of FAO by Agm. This observation agrees with previous reports, which showed that hepatic gluconeogenesis from lactate and pyruvate was greater with increased FAO (13, 37, 38, 42).

Concentrations of hepatic metabolites at the end of perfusion with or without 0.1 mM Agm and 0.5 mM [U-$^{13}C_{16}$]palmitate are presented in Table 1. We present data only for those metabolites or co-factors that are directly related to FAO and the urea cycle. There were no significant differences in the adenine nucleotide concentrations (Table 1). Calculation of the NAD^+/NADH ratio in the cytoplasmic and mitochondrial compartments as described previously (43, 44), using the lactate/pyruvate and β-HB/AcAc ratios (Table 1), shows that mitochondrial NAD^+/NADH is about 5 without Agm and 5.8 with Agm. For cytosolic NAD^+/NADH, the ratios are about 1500 without and 1400 with Agm. The current calculated values of mitochondrial and cytosolic redox state are in the range of those reported previously (28, 43, 44), indicating that Agm has little effect on the hepatic redox state. An important observation is that [cAMP] was increased by ~20–25% after perfusions with Agm (Table 1). Although this was not of statistical significance ($p = 0.08$), this increase of [cAMP] may have a key role in the control of FAO and ureagenesis, as discussed below.

Hepatic concentrations of glutamate and NAG and aspartate were increased after perfusions with Agm (Table 2). In addition, we found

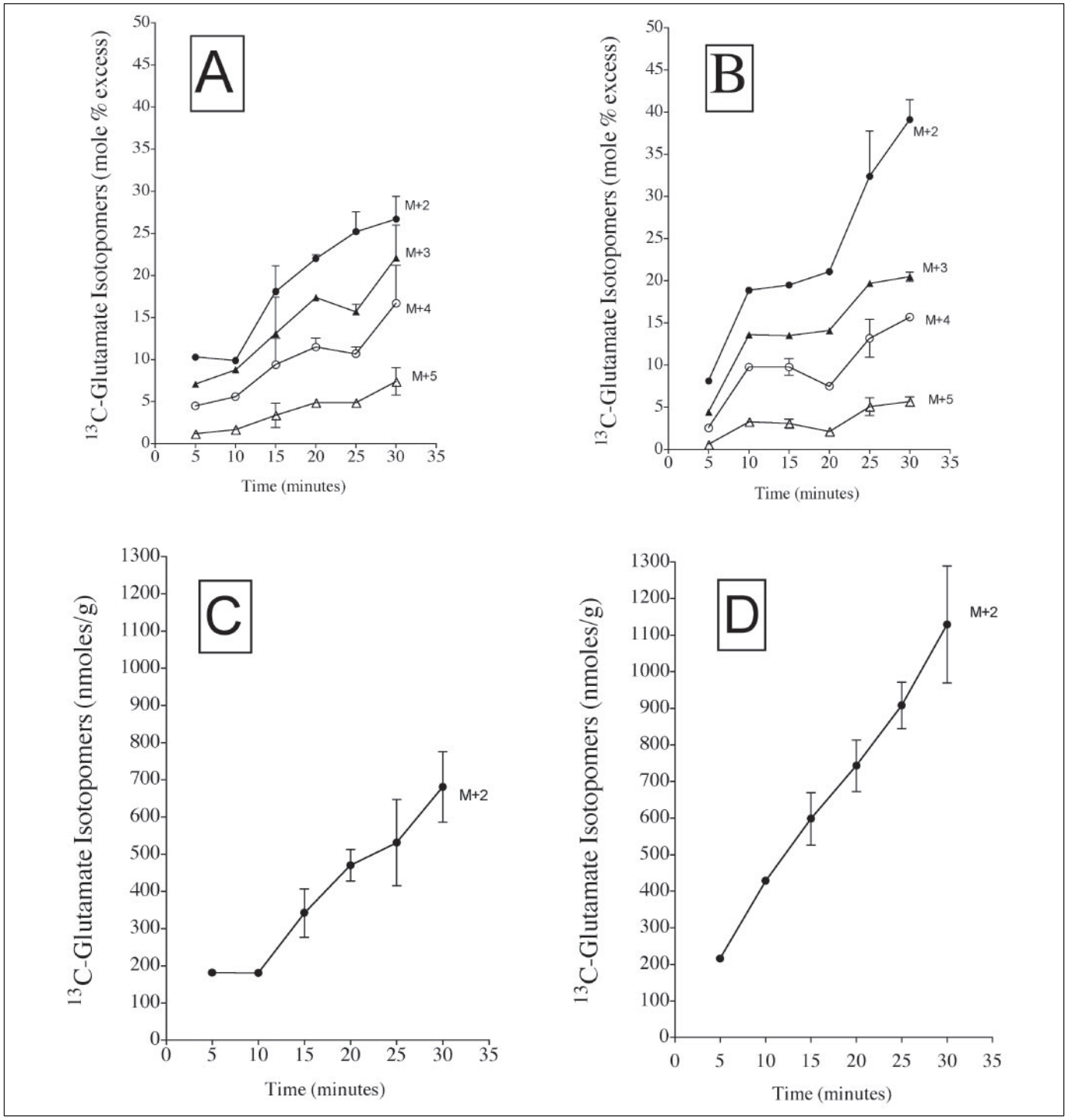

FIGURE 4. **The effect of agmatine on the appearance of ^{13}C-labeled glutamate isotopomers.** Perfusions were carried out as indicated in the legend to Fig. 2. At the indicated times, perfusion was stopped, and liver was freeze-clamped, extracted with perchloric acid, and analyzed with GC-MS and HPLC for determination of ^{13}C enrichment (MPE) and amino acid concentrations (nmol/g), respectively, as detailed under "Experimental Procedures." *A* and *C,* perfusions without agmatine. *B* and *D,* perfusions with 0.1 mM agmatine. The appearance of ^{13}C-labeled isotopomers is the product of glutamate concentration (nmol/g) at the indicated time point × ^{13}C enrichment (MPE/100) in the M + 2 isotopomer. Because of the randomization of [1,2,^{13}C]acetyl-CoA in the tricarboxylic acid cycle (12, 33), we used the M + 2 isotopomer to estimate the rate of ^{13}C-labeled glutamate appearance. Mass isotopomers of glutamate containing 1–5 ^{13}C atoms are identified as M + 1, M + 2, etc. *Bars* are mean ± S.D. of 3–4 individual livers obtained at the indicated time point.

significantly increased ornithine and citrulline levels following perfusion with Agm (Table 1). The increase in glutamate concentration may reflect the following: (i) higher flux through phosphate-dependent glutaminase and deamidation of perfusate glutamine, as indicated (9); (ii) transamination with α-ketoglutarate; and/or (iii) reductive amination of α-ketoglutarate. The elevated aspartate and ornithine concentration was most likely derived from glutamate metabolism and was mediated via aminotransferase reactions (16).

It has been shown that a portion of long chain fatty acid oxidation occurs in peroxisomes with formation of acetate (19, 20, 45). The current GC-MS analysis did not detect ^{13}C-labeled acetate in the effluent, but we did note increased ^{13}C-labeled ketone bodies, gluta-

TABLE 2

Time course of glutamate, aspartate, and N-acetylglutamate concentration in liver extract at the end of liver perfusions with [U-$^{13}C_{16}$] palmitate and with or without agmatine

Livers from overnight fasted male rats were perfused in the non-recirculating mode with lactate, pyruvate, glutamine, and ammonia plus 0.5 mM [U-$^{13}C_{16}$]palmitate as outlined under "Experimental Procedures." At the end of the perfusion, the liver was freeze-clamped, extracted into perchloric acid, and used for metabolite determination. The metabolite levels are means ($n = 2$) or means ± S.D. ($n = 3–4$) of separate livers obtained at each time point.

Time[a]	Control			+ Agmatine		
	Glu	Asp	NAG	Glu	Asp	NAG
min	*nmol/g wet weight of liver*					
5	1756	689	32	2653	1197	49
10	1821	515	49	2262	732	65
15	1868 ± 573	522 ± 93	41 ± 5	3101 ± 344[b]	1060 ± 486	42 ± 12
20	2180 ± 252	426 ± 98	42 ± 9.6	4046 ± 1040[b]	1094 ± 266[b]	90 ± 11[b]
25	2054 ± 802	445 ± 96	34 ± 6	3050 ± 437	836 ± 374	83 ± 9[b]
30	2596 ± 784	597 ± 69	39 ± 16	2874 ± 532	912 ± 358	63 ± 7[b]

[a] The indicated time represents the time of obtaining the liver extract after the start of [U-$^{13}C_{16}$]palmitate infusion with or without agmatine.
[b] Value indicates significant difference ($p < 0.05$) compared with perfusions without agmatine.

mate, and CO_2, which are formed in mitochondria. Thus, Agm enhanced FAO primarily in mitochondria and not peroxisomes. This conclusion is in agreement with a prior study indicating that the contribution of peroxisomes to palmitate oxidation is about 5% of the overall FAO (45).

To determine whether the stimulation of [U-$^{13}C_{16}$]palmitate oxidation by Agm occurs at the outer mitochondrial membrane or the intramitochondrial β-oxidation pathway, incubations were carried out with isolated mitochondria and ^{13}C-labeled octanoate. Experiments were designed to examine a possible dose effect of Agm on octanoate oxidation and the relationship between the products of β-oxidation, NAG, and citrulline synthesis. Fig. 7 demonstrates that Agm significantly increased the production of ^{13}C-labeled ketone bodies following oxidation of ^{13}C-labeled octanoate. The increased mitochondrial β-oxidation by Agm was coupled with increased production of ^{13}C-labeled *N*-acetylglutamate and ^{15}N-labeled citrulline from $^{15}NH_4Cl$ added to the incubation medium. This action of Agm was dose-dependent with a maximal effect occurring at a concentration of 0.05–0.1 mM and a slight decline noted at higher concentrations (Fig. 7). Experiments with broken mitochondria, 5 mM [^{15}N]glutamate, 2 mM acetyl-CoA, and increasing Agm concentrations without ^{13}C-labeled octanoate indicated that Agm does not act allosterically on *N*-acetylglutamate synthetase (data not shown). In addition, experiments with exogenous acetyl-CoA did not show a significant increase in the production of ketone bodies with the addition of Agm, suggesting that Agm has no effect on the mitochondrial acetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-coenzyme A synthase, and/or lyase or β-hydroxybutyrate dehydrogenase pathways (13).

DISCUSSION

We recently found that Agm augmented oxygen consumption and urea synthesis during liver perfusion (9) through an incompletely defined mechanism. In this study we hypothesized that Agm stimulates mitochondrial FAO, thereby increasing the availability of substrates and ATP required for urea synthesis. This mechanism is consistent with prior studies both *in vivo* (46–48) and *in vitro* (16, 39, 46), indicating that the availability of substrates (*i.e.* glutamate and acetyl-CoA) may regulate the rate of NAG synthesis and thus ureagenesis. However, our previous study (9) demonstrated that Agm increased O_2 uptake and urea synthesis even without addition of exogenous fatty acid. Therefore, an alternative but not mutually exclusive possibility is that Agm may favor release of second messenger(s) that promote ureagenesis and FAO by independent events.

To explore the current hypothesis, we investigated the action of Agm in a liver perfusion system with [U-$^{13}C_{16}$]palmitate as tracer and in isolated mitochondria incubated with ^{13}C-labeled octanoic acid. Experiments with liver perfusion demonstrate that Agm increased hepatic production of ^{13}C-labeled ketone bodies and CO_2 from perfusate [U-^{13}C]palmitate (Fig. 2), indicating stimulated β-oxidation. The stimulation of [U-^{13}C]palmitate oxidation was accompanied by a significantly higher urea output (Table 1), suggesting a coupling between β-oxidation and ureagenesis. In addition, experiments with isolated mitochondria (Fig. 7) indicate the following. (i) Agm stimulates intramitochondrial β-oxidation, and this action is independent of the carnitine acyltransferase reaction. (ii) The stimulation of FAO and the increased ketogenesis and NAG synthesis are similar to those observed in a liver perfusion system (Figs. 2, 4, and 5). (iii) Agm does not act allosterically on *N*-acetylglutamate synthetase. Therefore, the combined observations obtained in experiments with isolated mitochondria (Fig. 7) and liver perfusion (Figs. 2–6 and Tables 1 and 2) are consistent with the hypothesis that Agm stimulation of NAG and urea synthesis is coupled with mitochondrial stimulation of β-oxidation.

The stimulation of [U-^{13}C]palmitate oxidation leads to a greater production of [1,2-^{13}C]acetyl-CoA. The current observations indicate that Agm increased the conversion of [1,2-^{13}C]acetyl-CoA to ketone bodies (Fig. 2*A*) and NAG (Fig. 5). Simultaneously, Agm increased the incorporation and flux of [1,2-^{13}C]acetyl-CoA in the tricarboxylic acid cycle as indicated by increased release of $^{13}CO_2$ (Fig. 2*B*) and increased ^{13}C-labeled glutamate (Figs. 3 and 4). The formation of ^{13}C-labeled glutamate isotopomers (Fig. 3) reflects hepatic metabolism of the tricarboxylic acid cycle and FAO, because glutamate is in isotopic equilibrium with α-ketoglutarate, and formation of ^{13}C-labeled α-ketoglutarate reflects conversion of ^{13}C-labeled fatty acid to ^{13}C-labeled acetyl-CoA (49). The data suggest that, in the course of enhancing the oxidation of [U-$^{13}C_{16}$]palmitate (Fig. 2*B*), agmatine also may have favored an accelerated isotopic equilibrium between glutamate and α-[4,5-^{13}C]ketoglutarate, as evidenced by the higher enrichment in glutamate isotopomers and the more active appearance of ^{13}C in this amino acid (Figs. 3 and 4). The isotopic equilibrium between α-[4,5-^{13}C]ketoglutarate and [4,5-^{13}C]glutamate is further supported by the GC-MS analyses that indicate similar isotopic enrichment in α-ketoglutarate and glutamate isotopomers (data not shown). However, the amination of α-ketoglutarate and/or transamination reactions are not the sole routes of glutamate formation, the phosphate-dependent glutaminase reaction being yet another source. Indeed, the enhanced production of urea (Table 1) points to augmented flux through glutaminase, a conclusion supported by our prior research (9). Thus, an agmatine-induced increase in the concentration of glutamate (Table 2) could result from a direct effect on several reactions, including glutaminase, glutamate dehydrogenase, and/or transaminases.

Numerous *in vivo* (46–48) and *in vitro* (16, 31, 47, 50, 51) studies have indicated that NAG is an important regulator of ureagenesis. Agm may

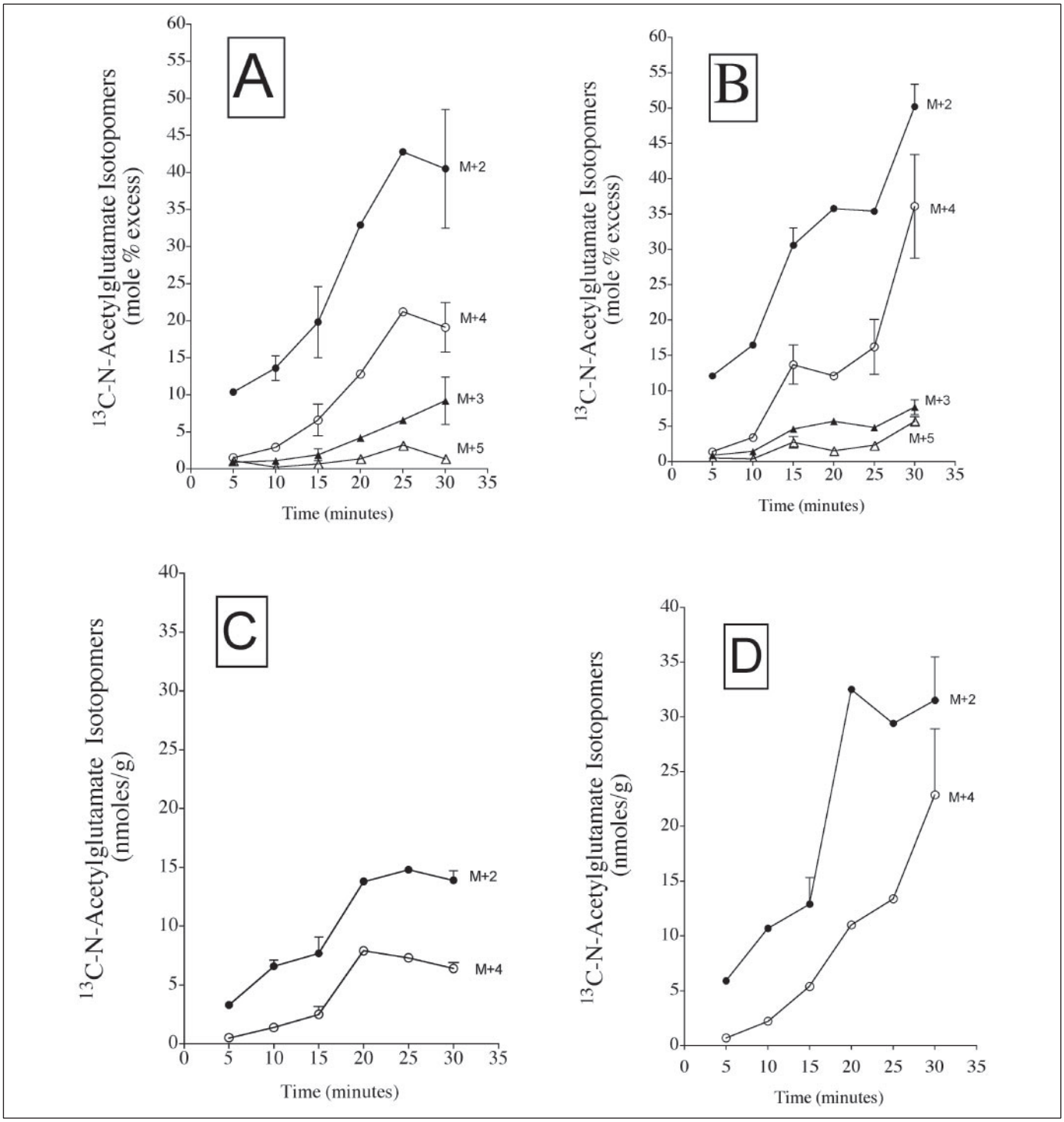

FIGURE 5. **The effect of agmatine on the production of ^{13}C-labeled *N*-acetylglutamate isotopomers.** Perfusions were carried out as indicated in the legend to Figs. 2 and 3. At the indicated times, perfusion was stopped, and liver was freeze-clamped, extracted with perchloric acid, and analyzed with GC-MS for determination of ^{13}C enrichment (MPE) and NAG concentrations (nmol/g) as detailed under "Experimental Procedures." *A* and *C*, perfusions without agmatine. *B* and *D*, perfusions with 0.1 mM agmatine. ^{13}C-Labeled isotopomer production (nmol/g) is the product of NAG concentration at the indicated time point (nmol/g) × ^{13}C enrichment (MPE/100) in the corresponding isotopomer. Mass isotopomers of NAG containing 1–5 ^{13}C atoms are identified as M + 1, M + 2, etc. *Bars* are mean ± S.D. of 3–4 individual livers obtained at the indicated time point.

up-regulate production of carbamoyl phosphate and urea by increasing the concentration of NAG. The data in Figs. 2, 4, and 5 indicate that the output in the effluent of $^{13}CO_2$ was accompanied by increased concentration of ^{13}C-labeled glutamate and NAG. Because the effluent $^{13}CO_2$ represents the end product of [U-$^{13}C_{16}$]palmitate oxidation, this observation suggests a coupling between hepatic FAO and NAG and urea synthesis. Furthermore, the highly significant relationship between the concentration of ^{13}C-labeled glutamate and NAG (Figs. 4 and 5) demonstrates the dependence of mitochondrial NAG synthesis on [glutamate] as indicated previously (39, 46, 50). Therefore, an important aspect of Agm action is that the stimulation of [U-$^{13}C_{16}$]palmitate oxidation was coupled with augmented production of NAG. These observations are in line with the possibility that Agm stimulates mitochondrial β-oxidation and, therefore, up-regulates NAG and carbamoyl phosphate synthesis.

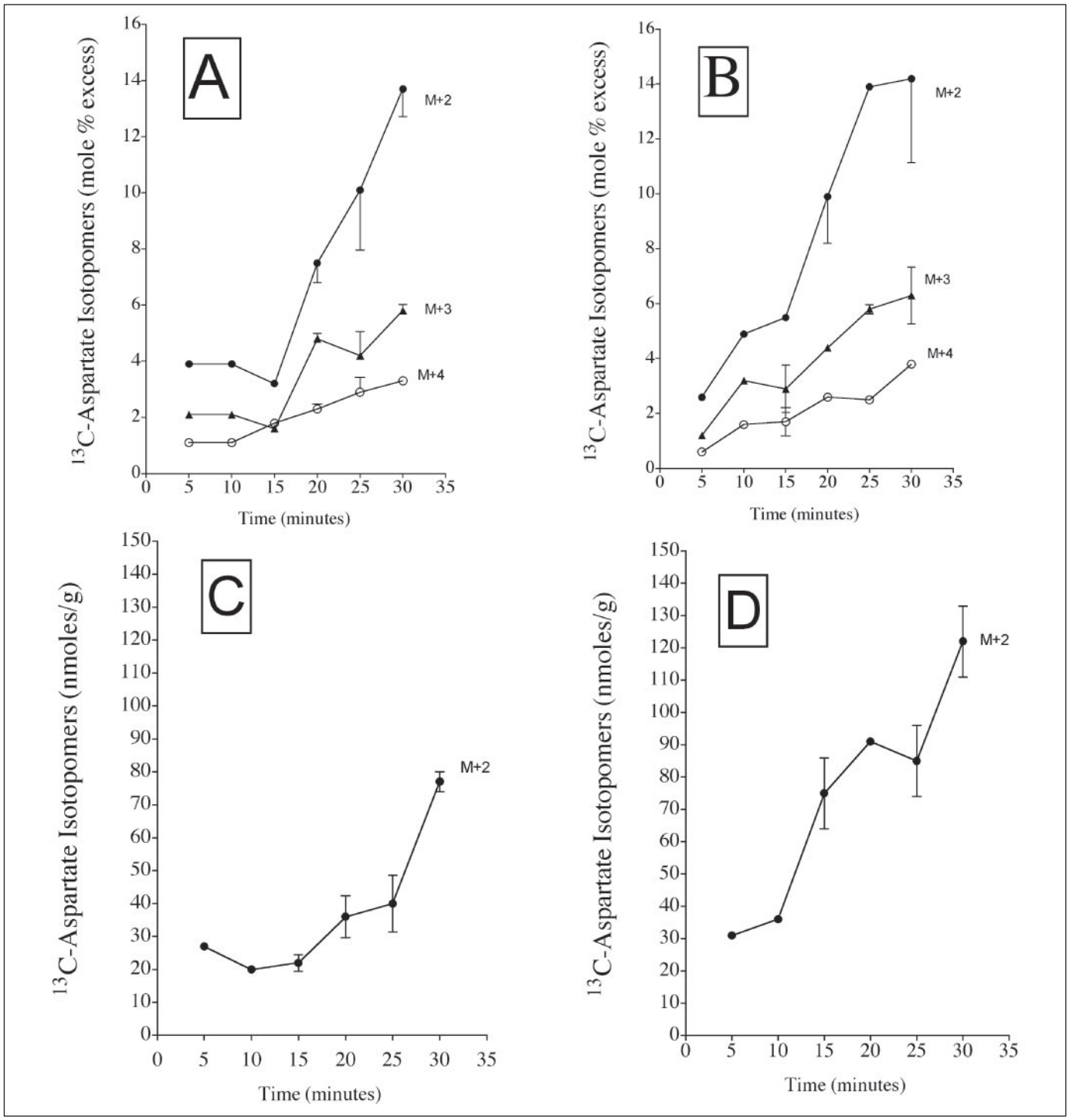

FIGURE 6. **The effect of agmatine on the appearance of ^{13}C-labeled aspartate isotopomers.** Perfusions were carried out as indicated in the legend to Figs. 2 and 3. At the indicated times, perfusion was stopped, and liver was freeze-clamped, extracted with perchloric acid, and analyzed with GC-MS and HPLC for determination of ^{13}C enrichment (MPE) and amino acid concentrations (nmol/g), respectively, as detailed under "Experimental Procedures." *A* and *C,* perfusions without agmatine. *B* and *D,* perfusions with agmatine. The appearance of ^{13}C-labeled isotopomers is the product of aspartate concentration (nmol/g) at the indicated time point × ^{13}C enrichment (MPE/100) in the corresponding M + 2 isotopomer. Mass isotopomers of aspartate containing 1 to 4 ^{13}C atoms are identified as M + 1, M + 2, etc. *Bars* are mean ± S.D. of 3–4 independent livers obtained at the indicated time point.

An important feature of Agm action is the observation that the stimulation of palmitate oxidation was accompanied by about a 25% increase in urea output ($p < 0.05$) but an insignificant increase in oxygen consumption (Table 1). However, Agm significantly increased oxygen consumption and urea synthesis by about 40–50% ($p < 0.05$), during liver perfusions without palmitate (this study and see Ref. 9). These findings suggest that the effects of Agm and palmitate on O_2 consumption are not additive. This may be explained by the formulation that, in perfusions with lactate and pyruvate without exogenous addition of fatty acid (this study and see Ref. 9), NADH is generated mainly via pyruvate dehydrogenase and is oxidized by complex I of the respiratory chain. In this case, the demand for ATP to support greater ureagenesis (9) leads to augmented oxidative phosphorylation and oxygen consumption mediated by increased activity of complex I. With the addition of palmitate,

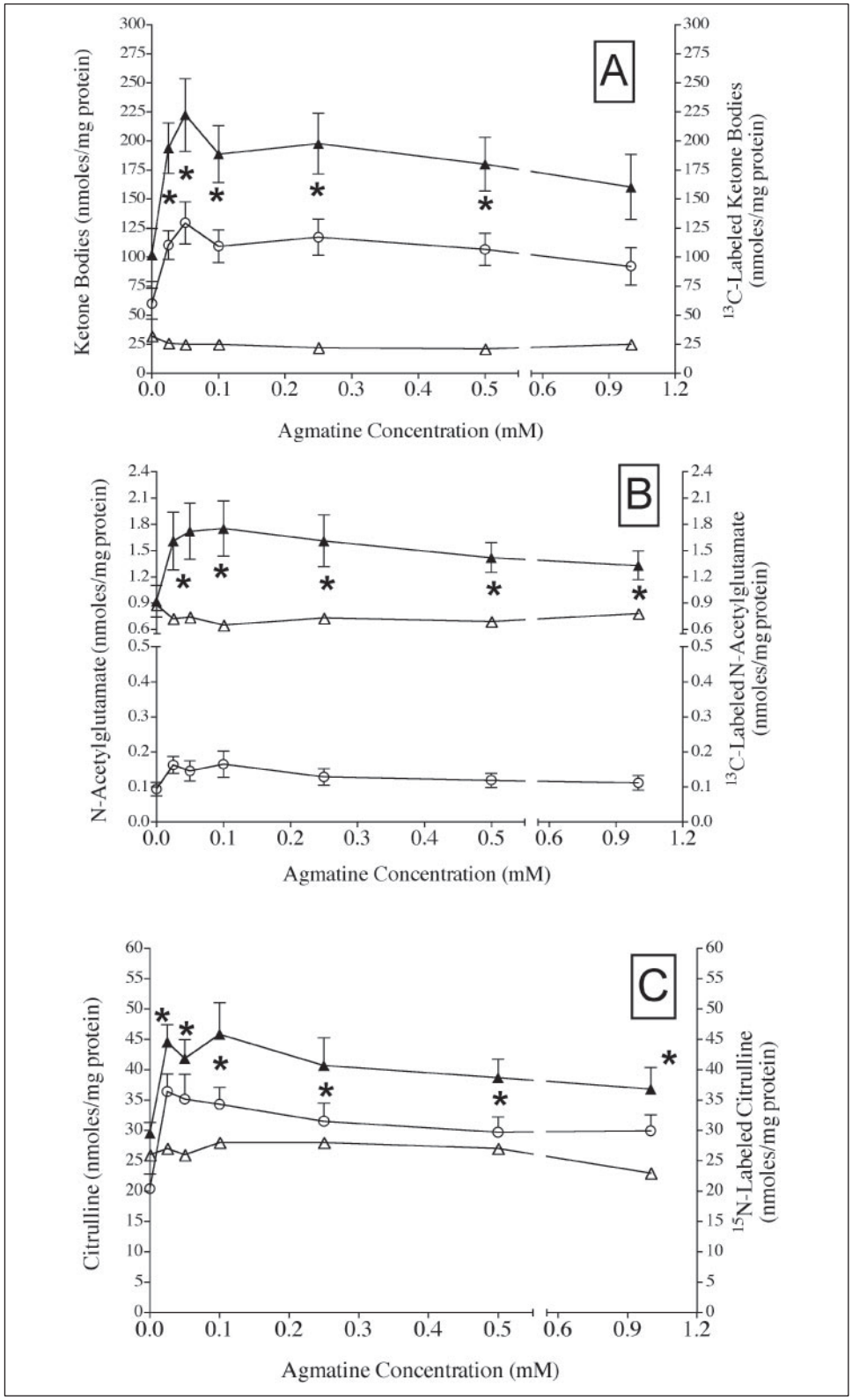

FIGURE 7. **Dependence of ketone bodies, *N*-acetylglutamate, and citrulline production on the concentration of agmatine.** Experiments were carried out with isolated mitochondria incubated with an increasing concentrations (0–1 mM) of agmatine and basic incubation medium as outlined under "Experimental Procedures" with or without 2 mM [1,2,3,4-$^{13}C_4$]octanoic acid. Data were obtained following a 10-min incubation. Total metabolite concentration is presented by ▲ in experiments with [1,2,3,4-$^{13}C_4$]octanoic acid or △ without [1,2,3,4-$^{13}C_4$]octanoic acid. ^{13}C-Labeled metabolites (*A* and *B*, in experiments with [1,2,3,4-$^{13}C_4$]octanoic acid) and ^{15}N-labeled citrulline production, in experiments with $^{15}NH_4Cl$ (*C*), are presented by ○. *Bars* are mean ± S.D. of 5–6 independent experiments. * indicates significant difference compared with incubations without agmatine.

the ATP required for increased urea synthesis was likely furnished by the already elevated activity of complex I and the greater production of FADH, which is oxidized via complex II. Thus, the increment in O_2 consumption with palmitate alone when compared with palmitate plus agmatine was insignificant (Table 1). This possibility is further supported by the observations that without addition of palmitate, the reducing equivalents generated from endogenous fatty acids were oxidized, and thus the release in the effluent of ketone bodies was minimal, *i.e.* about 54 nmol·g^{-1}·min^{-1}, and the β-HB/AcAc ratio was about 1. In experiments with palmitate plus Agm, the production of reducing equivalents was much higher than the increment in ureagenesis. The more intense production of reducing equivalents is reflected in

increased release of β-HB in the effluent, as well as a higher β-HB/AcAc ratio, which was about 3-fold greater than in perfusions without palmitate (Table 1).

The current findings raise two important questions. (i) How does Agm stimulate FAO? (ii) Is the stimulation by Agm of ureagenesis coupled to FAO or is the up-regulation of ureagenesis and FAO mediated by independent events? The current data indicate that the increased FAO is not related to peroxisomal β-oxidation or cytosolic activation of long chain fatty acids, but to intramitochondrial effects. The process of mitochondrial β-oxidation is subject to acute regulation by cAMP and/or hormones such as glucagon (13, 17, 42, 52, 53). The current results suggest that Agm mimics the action of glucagon on FAO, which involves cAMP as second messenger (17, 42, 52, 53). A marginal (20%, $p = 0.08$) rise of mitochondrial cAMP was observed in liver extracts following perfusion with agmatine (Table 1). This measurement was done in whole liver and may have underestimated mitochondrial [cAMP]. Furthermore, it has been indicated that small changes in [cAMP] may have relatively large effects on metabolic processes (53), suggesting that the ≈20% increase in the cAMP level may be sufficient to mediate the up-regulation of FAO and ureagenesis. Agm may elicit effects on multiple metabolic and physiologic events that are independent of one another (2–8). Two such independent events may be FAO and ureagenesis, both of which are stimulated by cAMP (53). An increase in the level of cAMP in the liver extract following perfusion with Agm may independently stimulate ureagenesis and FAO. This may be the most plausible mechanism by which Agm stimulates FAO and urea synthesis, because in our previous study (9) Agm stimulated NAG and urea synthesis without the addition of fatty acid.

Hepatic FAO is an essential process that furnishes substrate to the respiratory chain and energy for multiple functions (54). The current finding that Agm stimulated mitochondrial β-oxidation is of great relevance to human health, especially in cases such as obesity, abnormal energy metabolism in heart tissue (55), or type II diabetes associated with disturbed fatty acid metabolism (56). In the current study, stimulation of FAO occurred at a perfusate Agm concentration (0.1 mM) that exceeds reported plasma levels (57, 58), but the concentration of Agm in plasma and liver may increase severalfold under certain conditions such as a diet supplemented with high arginine and, perhaps more importantly, after increased release of Agm by intestinal bacteria. Regulation of hepatic FAO and ketogenesis by Agm may occur naturally in normal and disease states, and may have significant implications for whole body energy metabolism.

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