Role of Glutamine and Arginase in Protection against Ammonia-induced Cell Death in Rat Gastric Epithelial Cells

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ABSTRACT

Ammonia is a cytotoxic factor produced during *Helicobacter pylori* infection that may reduce the survival of surface epithelial cells. Here we examine whether ammonia kills cells and if L-glutamine (L-Gln) protects against cell death by stimulating ammonia detoxification pathways. Cell viability and vacuolation were quantified in rat gastric epithelial (RGM1) cells incubated with ammonium chloride (NH₄Cl) at pH 7.4 in the presence or absence of L-Gln. Incubation of RGM1 cells with NH₄Cl caused a dose-dependent increase in cell death and vacuolation, which were both inhibited by L-Gln. We show that RGM1 cells metabolize ammonia to urea via arginase, a process that is stimulated by L-Gln and results in reduced ammonia cytotoxicity. L-Gln also inhibits the uptake and facilitates the extrusion of ammonia from cells. Blockade of glutamine synthetase did not reduce the survival of RGM1 cells, demonstrating that the conversion of L-glutamate and ammonia to L-Gln is not involved in ammonia detoxification. Thus, our data support a role for L-Gln and arginase in protection against ammonia-induced cell death in gastric epithelial cells.

KEY WORDS: glutamine, ammonia, arginase, *Helicobacter pylori*
INTRODUCTION

*Helicobacter pylori (H. pylori)* infection of the stomach causes chronic-active gastritis and peptic/duodenal ulcer disease in humans and in many animal models. TUNEL staining of the mucosa has shown that apoptosis increases significantly during *H. pylori* infection and is most prevalent in surface epithelial cells (42, 51). Although apoptosis occurs, it is not clear what factors associated with *H. pylori* infection cause injury and death of surface epithelial cells. It has been reported that *H. pylori* produce many deleterious factors for gastric epithelial cells, such as vacuolating cytotoxin (VacA), gene products of the Cag pathogenicity island (PAI) and urease. Although VacA was initially thought to be cytotoxic to gastric epithelial cells, compelling evidence against this contention was recently shown in the gerbil model of infection by targeted deletion of the VacA gene in *H. pylori*, where severe gastritis and mucosal injury were present (36, 53). In addition, *H. felis*, a related gastric *Helicobacter* that does not possess VacA or the Cag PAI but has potent urease activity (33), induces severe gastritis and injury to gastric epithelial cells in several animal models (12, 30). These combined results suggest that injury to epithelial cells during *H. pylori* infection may occur from urease-derived ammonia rather than from VacA or gene products of the Cag PAI. In fact, the severity of gastric injury during *H. pylori* infection is correlated with the concentration of ammonia in the gastric juice (47) or the urease activity of *H. pylori* (26). Patients with *H. pylori* infection show a significant increase in gastric juice ammonia when compared to uninfected control patients (13, 14, 23, 24, 27, 32, 34, 35, 46, 50, 58).

A number of studies recently showed that ammonia affects the gastric mucosa *in vivo* and gastric epithelial cells *in vitro*. Ammonia, at a concentration below that detected in *H. pylori*-infected patients, inhibits oxygen consumption (48), cell proliferation (31) and acid secretion
In addition, ammonia kills parietal and chief cells in isolated gastric glands by necrosis and apoptosis, respectively (17). Ammonia, generated by using NH₄Cl or urea/urease, kills gastric MKN 45 cells alone and in combination with cytokines such as TNF-α or IFN-γ (21). Furthermore, ammonia retards restitution of the injured gastric mucosa (43), leading to impaired barrier function. Thus, ammonia may significantly impair mucosal homeostasis, resulting in injury and death of gastric epithelial cells during *H. pylori* infection.

Although gastric surface epithelial cells are exposed to high levels of ammonia during *H. pylori* infection, it is not established whether these cells are injured by ammonia or if they possess any mechanism(s) to protect against ammonia-induced injury. In the liver, systemic ammonia detoxification occurs in metabolic zones, where periportal and perivenous hepatocytes have unique enzymatic pathways for the production of non-toxic ammonia metabolites such as urea and glutamine, respectively (20, 59). Brain glial cells also produce glutamine from glutamate and ammonia to protect neurons from ammonia-induced cytotoxicity (9, 59). Thus, it is possible that gastric epithelial cells have the ability to process ammonia, either by facilitating the production of urea from ammonia or by converting glutamate and ammonia to glutamine. Either detoxification pathway would be beneficial to protect surface epithelial cells against the cytotoxic effects of ammonia in the gastric lumen, in general, and during *H. pylori* infection, in particular.

Thus, the purpose of this study was to determine if ammonia affects the survival of gastric surface epithelial cells and if so, to determine whether glutamine protects surface epithelial cells from injury by facilitating ammonia detoxification. To accomplish this, we measured cell viability and the degree of vacuolation in rat gastric epithelial (RGM1) cells that were exposed to NH₄Cl, producing ammonia and ammonium, with or without L-Gln. Our results
indicate that NH₄Cl significantly reduces the viability of RGM1 cells and that L-Gln and L-Glu both protect RGM1 cells against NH₄Cl-induced cell death. Our results establish that RGM1 cells metabolize ammonia to urea, that L-Gln protects by decreasing the intracellular accumulation of ammonia and increasing ammonia metabolism and that the conversion of ammonia and L-Glu to L-Gln via glutamine synthetase does not protect RGM1 cells. Since L-Gln completely reverses the cytotoxic effects of ammonia in our study, it is proposed that L-Gln supplementation may be beneficial to reduce mucosal injury during *H. pylori* infection.
MATERIALS AND METHODS

Preparation of RGM1 cell cultures. Rat gastric epithelial cell line, RGM1 cells, established by Dr. H. Matsui, Institute of Physical and Chemical Science (RIKEN) Cell Bank and Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan (29), are non-transformed gastric surface epithelial cells. RGM1 cells were cultured in DMEM/F12 (1:1) supplemented with heat-inactivated 10% FBS (Gibco/BRL, Gaithersburg, MA), 100 units/ml penicillin, 100 units/ml streptomycin and 0.25 µg/ml amphotericin B. Confluent monolayers of RGM1 cells were starved for 24 hr in culture medium without FBS (DMEM/F12 containing 15 mmol/L HEPES at pH 7.4) at 37 °C under 5% CO2 in air and then used for experiments. All experiments were performed in STD buffer at pH 7.4 in the presence and/or absence of reagent(s) without FBS.

Treatment of RGM1 cells with NH4Cl or methylamine with or without l-Gln or l-Glu. Starved RGM1 cells were transferred to standard (STD) buffer containing (in mM) 147 Na+, 5.0 K+, 131 Cl-, 1.3 Mg2+, 1.3 SO42-, 2 Ca2+, 25 HCO3-, 15 HEPES and 20 d-glucose at pH 7.4 and incubated with 0-100 mM NH4Cl or 0-30 mM methylamine hydrochloride (MeNH2) in the presence or absence of 0-20 mM l-Gln. NH4Cl was used as the source of ammonia (NH3), a primary amine weak base. Since NH3 is at equilibrium with its protonated form (NH4+) in a pH-dependent manner, 30 mM NH4Cl (pKa is 9.24) results in 0.44 mM NH3 (1.4%) and 29.56 mM NH4+ (98.6%) at pH 7.4. MeNH2 is also a primary amine weak base that is at equilibrium with its protonated form (MeNH2+) in a pH-dependent manner. MeNH2 at 3 mM (pKa is 10.66) results in 0.0017 mM MeNH2 (0.03%) and 2.9993 mM MeNH2+ (99.97%) at pH 7.4. Both NH3 and MeNH2 enter cells and result in intracellular and lysosomal alkalinization (10). However, NH3 is a substrate
in intracellular metabolic pathways and MeNH₂ is not. Thus, MeNH₂ was used as a control weak base for all experiments. To examine (indirectly) the role of urea production in protection of RGM1 cells against NH₃, RGM1 cells were incubated with 0.1-1 mM Nω-hydroxy-nor-L-arginine (nor-NOHA, Calbiochem, San Diego, CA) in the presence of 30 mM NH₄Cl or 3 mM MeNH₂ with or without L-Gln. nor-NOHA is a potent and specific competitive inhibitor of arginase (15, 45), a key enzyme in the production of urea (from arginine) in the urea cycle. To examine if the conversion of L-Glu and ammonia to L-Gln is involved in ammonia detoxification, RGM1 cells were incubated with 0.1-10 mM L-S-[3-amino-3-carboxypropyl]-S-methylsulfoximine (methionine sulfoximine: MS), a potent inhibitor of glutamine synthetase (52). Glutamine synthetase is responsible for the conversion of L-Glu plus ammonia to L-Gln. L-Gln is an amino acid with an uncharged R group. L-Glu is an amino acid with a charged polar group. NH₄Cl, MeNH₂, L-Gln, L-Glu, MS and all other buffer components were purchased from Sigma Chemical Company (St. Louis, MO).

Measurement of cell viability. The viability of RGM1 cells was evaluated by a colorimetric assay using crystal violet (25), a cytochemical stain that binds to chromatin. For this assay, RGM1 cells were washed once with PBS to remove dead cells, fixed with methanol for 15 minutes, and then air-dried. The dried cells were stained with 0.1% crystal violet for 5 minutes at room temperature, washed twice with PBS, and then air-dried. Stained cells were solubilized with 0.5% SDS for 30 minutes with slight agitation. Lysates were diluted with 0.5% SDS and the absorbance was measured at 590 nm using micro plate reader. Crystal violet stain was purchased from Sigma Chemical Company.
Measurement of cell vacuolation. Intracellular acidic vacuoles, containing H⁺ generated by the vacuolar ATPase, expand in the presence of a weak base (in a concentration-dependent manner) because the unprotonated weak base freely partitions into the acidic space, is protonated by H⁺ and cannot freely exit (10). The resulting loss of H⁺ alkalinizes the vacuole and initiates further H⁺ generation by the vacuolar ATPase, which is followed by water movement into the vacuole and vacuole expansion (10). Since vacuolation is an indicator of intracellular weak base concentration, we evaluated the intracellular concentration of NH₃ or MeNH₂ by quantifying vacuolation.

To quantify vacuolation in RGM1 cells, uptake of neural red into vacuoles was determined as described by Cover et al (7, 8), with slight modification. In brief, RGM1 cells were incubated for 10 minutes at 37 °C with 0.005% neutral red in STD buffer, and then washed twice with PBS containing 0.3% BSA. The dye was extracted with isopropyl alcohol containing 0.04 M HCl. The extract was diluted and the absorbance was measured at a test wavelength of 540 nm and reference wavelength of 650 nm using micro plate reader.

Assay for the Extrusion of NH₄Cl or MeNH₂ from Vacuoles. After the induction of vacuoles for 6 hr with 30 mM NH₄Cl or 3 mM MeNH₂, RGM1 cells were incubated for 1 hr in STD buffer with or without 0-20 mM L-Gln, in the absence of NH₄Cl or MeNH₂. Vacuolation was quantified as described above.

Measurement of MeNH₂ accumulation in RGM1 cells. Intracellular accumulation of [¹⁴C] MeNH₂ was measured in RGM1 cells that were incubated for 3 hr, at 37 °C, with 3 mM MeNH₂ containing 0.5 μCi of [¹⁴C]MeNH₂·HCl (NEN Life Science Products, Inc., Boston, MA) and 0-20 mM L-Gln. Washing the cells with ice-cold PBS terminated the
reaction. The cells were solubilized with 0.3 N NaOH and the radioactivity was measured by liquid scintillation (Packard Instruments Inc., Downers Grove, IL).

**Measurement of urea production in RGM1 cells.** Urea concentration in the culture supernatant was measured in two ways. First, by using a commercially available assay kit (Sigma), which follows the procedure of Ormsby (37) and second by measuring the conversion of \( L-[\text{guanido-}^{14}\text{C}] \)arginine to \([^{14}\text{C}]\)urea as described below for the measurement of arginase activity. For measurement of urea by the Sigma assay kit, RGM1 cells were cultured in 100 mm dishes to obtain \( 4 \times 10^6 \) cells/dish. After starvation for 24 hr, the cells were incubated with or without 20 mM \( L-\text{Gln} \) in the presence or absence of 30 mM \( \text{NH}_4\text{Cl} \) for 6 hr. The culture supernatant was collected from 4 dishes, combined into 1 sample and lyophilized. The lyophilized sample was solubilized in PBS and used for urea assay where the absorbance at 540 nm of hydroxylamine generated by the reaction of urea with diacetylmonoxime was measured. This assay is not affected by other nitrogen compounds such as ammonia or nitrogen oxides (37). The urea concentration was determined from a standard curve using urea purchased from Sigma.

**Measurement of arginase activity in RGM1 cells.** Starved RGM1 cells were incubated at 37 °C for 6 hr with STD buffer. The cells were solubilized and sonicated in lysis buffer containing 0.01% Triton X-100, 2 mg/ml BSA, 10 mM MnCl\(_2\) and 12 mM Na maleate (pH 7.5). After centrifugation at 1000 x g at 4 °C, arginase activity was determined in the supernatant by measuring the conversion of \( L-[\text{guanido-}^{14}\text{C}] \)arginine to \([^{14}\text{C}]\)urea (6, 39, 40). In brief, the supernatant was added to reaction buffer (100 mM glycine, pH 7.4) in the presence or absence of 30 mM \( \text{NH}_4\text{Cl} \), 20 mM \( L-\text{Gln} \) and 1 mM nor-NOHA and the reaction started by the addition of 250 mM \( L-\text{arginine} \) containing 0.05
µCi of l-[guanido-14C]arginine. After 90 min at 37 °C, the reaction was terminated by the addition of 0.8 ml of stop buffer containing 250 mM acetic acid, 100 mM urea, 10 mM l-arginine (pH 4.5) and a 50% suspension of Dowex 50-WX8 resin (H⁺ form). After centrifugation, the supernatant containing [14C]urea (500 µl) was measured by liquid scintillation. Under these conditions, the resin removed 99.8% of the arginine substrate and 99.0% of the converted ornithine. Arginase activity in the supernatant was extrapolated from a standard curve using purified arginase (Sigma Chemical Co.).

**Morphological analysis of cell cultures.** Cell morphology was evaluated in cultured RGM1 cells at 6 and 24 hr in STD buffer, or in STD buffer containing 30 mM NH₄Cl or 3 mM MeNH₂ with or without 20 mM l-Gln. Cells were photographed with a Nikon TE300 microscope (MicroVideo Instruments, Avon, MA) outfitted with an Orca CCD camera (Hamamatsu Photonics, Japan) and IP lab software (Scanalytics, Inc., Fairfax VA).

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**Statistical analysis.** The data represent means ± SE for 4 wells of RGM1 cells from 3 different experiments. Statistical differences were evaluated using the Dunnett's multiple comparison test and the Student's t test, with a value of $P < 0.05$ regarded as significant.
RESULTS

*NH₄Cl and MeNH₂ reduce the viability of cultured RGM1 cells.* Treatment of RGM1 cells with NH₄Cl or MeNH₂ at pH 7.4 reduced viability in a concentration-dependent manner (Fig. 1). The viability of RGM1 cells was significantly reduced with 3, 10, 30, and 100 mM NH₄Cl (Fig. 1A). Similarly, the viability of RGM1 cells was significantly reduced with 1, 3, 10, and 30 mM MeNH₂ (Fig. 1B). For all further experiments, we used 30 mM NH₄Cl, which reduced viability to 37.6 ± 1.1% of control, and 3 mM MeNH₂, which reduced viability to 54.6 ± 0.5% of control. It should be noted that a significantly greater concentration of NH₄Cl was required to produce the same reduction in viability when compared to MeNH₂.

Time course studies showed that the viability of RGM1 cells decreased over time in the presence of 30 mM NH₄Cl or 3 mM MeNH₂ (Fig. 2). The viability of RGM1 cells was significantly reduced at 12, 24 and 36 hr, when incubated with 30 mM NH₄Cl or 3 mM MeNH₂.

*l-Gln Improves the Viability of RGM1 Cells in the Presence of NH₄Cl and MeNH₂.* Treatment of RGM1 cells with l-Gln prevented the reduction in cell viability induced by 30 mM NH₄Cl or 3 mM MeNH₂ in a concentration-dependent manner (Fig. 3). In the presence of NH₄Cl, significant protection occurred with 0.2, 2 and 20 mM l-Gln (Fig. 3A). In fact, 20 mM l-Gln completely (100.3 ± 1.1% of the initial value) protected RGM1 cells that were incubated with 30 mM NH₄Cl (Fig. 3A). Similarly, significant protection occurred with 0.2, 2, and 20 mM l-Gln in RGM1 cells that were incubated with 3 mM MeNH₂. Like with NH₄Cl, 20 mM l-Gln completely (99.1 ± 1.1%)
protected RGM1 cells that were incubated with 3 mM MeNH2 (Fig. 3B).  l-Gln had no effect on the viability of RGM1 cells in the absence of NH4Cl or MeNH2 at 24 hr (104.2 ± 3.6% viability with l-Gln versus 100.0 ± 4.1% with STD buffer alone). In addition, treatment with 20 mM mannitol, used to control for osmotic changes produced by 20 mM l-Gln, had no effect on the viability of RGM-1 cells that were treated with 30 mM NH4Cl (42.2 ± 1.9% viability with mannitol and NH4Cl versus 45.0 ± 2.0% with NH4Cl alone).

NH4Cl or MeNH2 causes vacuolation of RGM1 cells that is reduced by l-Gln.

Incubation of RGM1 cells for 6 hr with NH4Cl or MeNH2 resulted in the vacuolation of RGM1 cells in a concentration-dependent manner (Table 1). Vacuolation increased significantly in the presence of 0.3-30 mM NH4Cl, resulting in a maximum increase of 86.4 ± 4.5% when compared to control cells treated with buffer alone (Table 1). Likewise, vacuolation increased significantly in the presence of 0.3-10 mM MeNH2, resulting in a maximum increase of 181.6 ± 9.1% when compared to control cells treated with buffer alone (Table 1). In all cases, the percentage of vacuolation induced by NH4Cl was significantly less than with an equal concentration of MeNH2 (Table 1).

When RGM1 cells were treated with l-Gln in the presence of 30 mM NH4Cl for 6 hr, vacuolation was reduced in a concentration-dependent manner (Fig. 4A). The vacuolation induced by NH4Cl decreased significantly with 0.02, 0.2, 2 and 20 mM l-Gln (Fig. 4A). Treatment with l-Gln also resulted in a decrease in vacuolation induced by 3 mM MeNH2 (Fig. 4B). l-Gln significantly reduced MeNH2-induced vacuolation at 0.2, 2 and 20 mM (Fig. 4B).

Time course experiments with 3 mM MeNH2 showed that vacuolation increased rapidly for the first hour and then increased slowly from 2-6 hr thereafter (Fig. 5A).
Treatment of RGM1 cells with l-Gln in the presence of MeNH₂ significantly reduced vacuolation (by more than 60%) in RGM1 cells (Fig. 5A). However, some vacuolation was always present with MeNH₂ and l-Gln, when compared to control cells incubated with buffer alone (Fig. 5A).

Time course experiments revealed that the formation of vacuoles induced by 30 mM NH₄Cl was slower than with MeNH₂, whereby vacuoles increased rapidly for the first 2 hr and then increased slowly for 2-6 hr thereafter (Fig. 5B). When RGM1 cells were treated with 30 mM NH₄Cl in the presence of 20 mM l-Gln, the response to l-Gln was significantly different than described in Fig. 5A for MeNH₂ and l-Gln (l-Gln-dependent response). For the first 2 hr, vacuole formation with 30 mM NH₄Cl was significantly reduced by 20 mM l-Gln (Fig. 5B) and would be a “predicted response” if the l-Gln-dependent response were to continue through the 6 hr experiment. However, there was an actual decline in vacuole formation 2-6 hr after the addition of 20 mM l-Gln to cells that were treated with 30 mM NH₄Cl. In fact, if the decline in vacuolation were to be extrapolated to the x axis in Fig. 5B, these data would suggest that after 10 hr of incubation with NH₄Cl and l-Gln, vacuolation would be the same as in control cells treated with buffer alone.

*Morphological studies show that l-Gln protects RGM1 cells against vacuolation, cell rounding, and detachment in the presence of NH₄Cl and MeNH₂.* RGM1 cells in culture formed a confluent monolayer that was unchanged by incubation with STD buffer for 6 hr (Fig. 6A). By 24 hr after the addition of STD buffer, some cell death occurred as demonstrated by cell rounding and loss of attachment to the culture dish (Fig. 6B). Cultures incubated for 6 and 24 hr in STD buffer containing 20 mM l-Gln were nearly
identical to cultures incubated in STD buffer alone (Fig. 6C and D). In contrast, RGM1 cells incubated with 30 mM NH₄Cl or 3 mM MeNH₂ showed significant vacuolation by 6 hr (Fig. 6E and G). By 24 hr in NH₄Cl or MeNH₂, more than 70% and 60%, respectively, of cells were rounded and/or detached from the culture plate (Fig. 6F and H). In cultures incubated with NH₄Cl or MeNH₂ containing l-Gln for 6 hr, vacuolation was significantly reduced (Fig. 6I and K). In cultures incubated with NH₄Cl or MeNH₂ containing l-Gln for 24 hr, cultures were confluent with cell rounding and detachment from the culture dish not significantly different from that of control cultures (Fig. 6J, L).

Identification of the Mechanism(s) by which l-Gln Protects RGM1 Cells in the Presence of NH₄Cl and MeNH₂. To determine the mechanism by which l-Gln improves viability and decreases the vacuolation of RGM1 cells, we studied two potential pathways. First, we investigated whether l-Gln inhibits the intracellular accumulation of MeNH₂ in RGM1 cells. The intracellular accumulation of MeNH₂, and not NH₃, was done because radiolabelled [¹⁵N]NH₃ is not commercially available. In addition, MeNH₂ is a weak base with no potential for entry into an intracellular metabolic pathway so that intracellular reduction in weak base concentration by metabolism is not a factor in the experiment. Second, we determined whether RGM1 cells, like liver or brain cells, utilize the urea cycle and/or glutamine synthetase, with or without l-Gln, as potential ammonia detoxification pathways. Ammonia detoxification would reduce the intracellular concentration of NH₃ in cells, resulting in less cell death and vacuolation.

l-Gln Reduces the Intracellular Accumulation of MeNH₂ in RGM1 Cells. When RGM1 cells were incubated with 3 mM MeNH₂, containing 0.5 μCi of [¹⁴C]MeNH₂, accumulation of MeNH₂ was 6.28 ± 0.13 μmol/5x10⁵ cells/well (Fig. 7).
cells were treated with 3 mM MeNH₂ containing l-Gln, [¹⁴C]MeNH₂ accumulation was reduced in a concentration-dependent manner (Fig. 7). Treatment with l-Gln resulted in a significant reduction in the accumulation of [¹⁴C]MeNH₂ by 9.8 ± 2.7%, 30.0 ± 3.6%, 45.8 ± 3.2% for 0.2, 2.0, and 20 mM l-Gln, respectively, when compared to 3 mM MeNH₂ alone. Incubation with STD buffer containing 0.5 µCi [¹⁴C]MeNH₂ and no additional l-Gln resulted in little accumulation of [¹⁴C]MeNH₂ (0.021 ± 0.001 µmol/5x10⁵ cells/well) or vacuolation (data not shown) in RGM1 cells.

The intracellular concentration of a weak base is regulated by the equilibrium between entry (uptake) and extrusion. We showed in Fig. 7 that l-Gln reduced the accumulation of MeNH₂ in RGM1 cells, however, these results cannot distinguish between inhibited uptake or facilitated extrusion. To examine the role of l-Gln in facilitated extrusion of weak bases, we used RGM1 cells pre-loaded with NH₄Cl or MeNH₂.

Pre-loading RGM1 cells for 6 hr with 30 mM NH₄Cl or 3 mM MeNH₂ caused a significant increase in vacuolation (195.6 ± 2.8% and 213.6 ± 2.2%, respectively) compared to control cells incubated with STD buffer alone (Figs. 8A and B). When NH₄Cl or MeNH₂ pre-loaded cells were incubated for 1 hr with STD buffer alone, the percent of vacuolation decreased by 64.5% in NH₄Cl-treated cells and 78.6% in MeNH₂-treated cells (Figs. 8A and B). This reduction in vacuolation reflects a decrease in the intracellular concentration of NH₄Cl or MeNH₂ due to diffusion into the culture medium (10), resulting in smaller vacuoles. When the preloaded RGM1 cells were incubated with STD buffer containing 0.2-20 mM l-Gln, vacuolation was significantly reduced in a concentration-dependent manner (Figs. 8A and B). In both NH₄Cl and MeNH₂ pre-
loaded cells, vacuolation was significantly reduced by 0.2, 2 and 20 mM \( \text{L-Gln} \) (Fig. 8A and B). Substitution of 20 mM mannitol for 20 mM \( \text{L-Gln} \), to control for the osmotic effects of \( \text{L-Gln} \), resulted in no reduction in the size of vacuoles compared with STD buffer alone (20 mM mannitol is \( 34.8 \pm 5.0 \% \) versus STD buffer of \( 35.0 \pm 1.0 \% \)). Thus, these results demonstrate that \( \text{L-Gln} \) stimulates the extrusion of both \( \text{NH}_4\text{Cl} \) and \( \text{MeNH}_2 \) from RGM1 cells.

*Production of urea contributes to \( \text{L-Gln-induced protection against NH}_4\text{Cl but not MeNH}_2 \) in RGM1 cells.* To determine whether gastric epithelial cells are protected from ammonia (but not methylamine) cytotoxicity by utilizing \( \text{NH}_3 \) to form urea, we blocked arginase activity, a key enzyme in the urea synthetic pathway, with \( \text{nor-NOHA} \). In RGM1 cells treated with 30 mM \( \text{NH}_4\text{Cl} \), cell viability was reduced significantly in the presence of 0.01-1 mM \( \text{nor-NOHA} \) (Fig. 9A). In fact, cell viability was reduced to \( 5.5 \pm 0.8 \% \) in the presence of 1 mM \( \text{nor-NOHA} \), a concentration that did not affect cell viability in the absence of \( \text{NH}_4\text{Cl} \) (Fig. 9A). When RGM1 cells were treated with \( \text{NH}_4\text{Cl} \) in the presence of 20 mM \( \text{L-Gln} \) and \( \text{nor-NOHA} \), protection induced by \( \text{L-Gln} \) was abolished (Fig. 9B). In contrast, \( \text{nor-NOHA} \) (at 1 mM) had no effect on viability in the presence of \( \text{MeNH}_2 \) (Fig. 9C) or on \( \text{L-Gln-induced protection against MeNH}_2 \) (Fig. 9D). RGM1 cells in STD buffer had arginase activity \( (87.2 \pm 3.1 \text{ mU/ml}) \) that increased significantly in the presence of \( \text{NH}_4\text{Cl} \) \( (112.9 \pm 4.9 \text{ mU/ml}) \). \( \text{nor-NOHA} \) blocked arginase activity in a dose dependent manner by 39.9%, 63.5% and 98.0% at 0.01, 0.1, and 1 mM, respectively, in the presence of \( \text{NH}_4\text{Cl} \), and by 38.3%, 71.6% and 100.3% at 0.01, 0.1 and 1 mM, respectively, in the presence of \( \text{NH}_4\text{Cl} \) and \( \text{L-Gln} \).
L-Glu improves the viability of RGM1 cells in the presence of NH4Cl but does not improve viability by the conversion of L-Glu and NH3 to L-Gln. Treatment of RGM1 cells with L-Glu prevented the reduction in cell viability induced by 30 mM NH4Cl in a concentration-dependent manner (Fig. 10A). In the presence of NH4Cl, significant protection occurred with 0.02, 0.2, 2 and 20 mM L-Glu (Fig. 10A). In fact, 20 mM L-Glu completely (100.3 ± 1.1% of the initial value) protected RGM1 cells that were incubated with 30 mM NH4Cl (Fig. 10A).

To determine whether the conversion of L-Glu and NH3 to L-Gln contributes to protection by L-Glu, we blocked this conversion with MS (Fig. 10B), a potent inhibitor of glutamine synthetase activity (52). If the conversion of L-Glu and NH3 to L-Gln is involved in ammonia detoxification, blockade of the pathway with MS would reduce viability in the presence of L-Glu. However, we found that there was no significant difference in survival with 30 mM NH4Cl and 20 mM L-Glu containing 0.1-10 mM MS when compared to RGM1 cells incubated with 30 mM NH4Cl and 20 mM L-Glu alone (Fig. 10B). These results demonstrate that RGM1 cells do not convert L-Glu and NH3 to L-Gln to protect against ammonia-induced cell death.
DISCUSSION

The present study shows that apical exposure of gastric surface epithelial (RGM1) cells to ammonia significantly reduces cell viability within 24 hr. The mean concentration of ammonia (measured as NH$_4^+$) in the gastric juice of *H. pylori*-infected patients is from 3.4 to 22.8 mM (13, 23, 24, 27, 32, 34, 35, 46, 50, 58), but may be much higher next to surface epithelial cells because most *H. pylori* colonize the adherent mucus layer, *in vivo*. Our study demonstrates that surface epithelial cells possess an active ammonia detoxification pathway that provides some level of protection against ammonia-induced cytotoxicity. When the luminal ammonia concentration increases, our results demonstrate that L-Gln facilitates ammonia detoxification and improves cell survival.

Although our work and other reports conclude that a high concentration of NH$_3$ is cytotoxic to cells, *in vitro*, several findings that address the role of NH$_3$ or NH$_4$Cl, *in vivo*, are not consistent with these results. For instance, intragastric administration of urea (6 %)/urease (100 units) or concentrations of NH$_4$Cl up to 3 % (560 mM, pH 4.8 or pH 8.0) for 1 hr in the rat, *in vivo*, caused no damage to epithelial or other cells in the stomach (44). Tsujii et al (49) showed that 187.5-250 mM of NH$_3$ decreased oxygen consumption, energy charge, and the survival of isolated mucosal cells but the same concentrations of NH$_4$Cl at pH 7.4, *in vivo*, did not (48). From these results it is tempting to conclude that ammonia plays no role in gastric epithelial injury under physiological conditions. However, we propose that the intact mucosa must be incubated with NH$_4$Cl for many hours before cell death is evident. To support this contention, the present study shows that RGM1 cells must be incubated with a high concentration of NH$_4$Cl (from the luminal surface) for at least 12 hr before ammonia initiates cell death. Although it is not known
why it takes NH₄Cl so long to kill cells from the luminal surface, parietal and chief cells in gastric glands have a permeability barrier to NH₃ (3), that may also occur in surface epithelial cells. Thus, with the slow paracellular flux of weak base that occurs in gastric tissues (18), ammonia may move from the lumen to the basolateral compartment and kill cells after entry from the basolateral surface. Alternatively, our results suggest that gastric surface cells are protected from NH₃ by the metabolic elimination of ammonia to urea, via arginase activity. The rate at which arginase metabolizes ammonia may determine ultimate cell fate.

That gastric surface epithelial cells can metabolize ammonia to urea is a concept demonstrated, for the first time, in the present study. The use of an intracellular detoxification pathway in RGM1 cells was suggested in our study because cell death occurs with approximately 10-fold higher concentrations of NH₄Cl than MeNH₂, a primary amine weak base with similar properties to NH₄Cl. In addition, NH₄Cl and MeNH₂ should cause the same degree of vacuolation due to similar properties as weak bases, but it requires 10-fold higher concentrations of NH₄Cl to cause the same degree of vacuolation as with MeNH₂. These results are even more significant if the weak base concentration is taken into consideration, where the NH₃ concentration in 30 mM NH₄Cl (pH 7.4) is 0.44 and the MeNH₂ (weak base) concentration in 3 mM MeNH₂ (pH 7.4) is 0.0017 mM. Since NH₃ can enter intracellular metabolic pathways and MeNH₂ cannot, we suggest that the metabolism of ammonia to urea must lower the effective concentration of ammonia in cells, causing less vacuolation and cytotoxicity.

The results presented here demonstrate that gastric RGM1 cells have arginase activity that is inhibitable by nor-NOHA, a selective arginase inhibitor (15, 45).
Arginase, an enzyme that catalyzes the hydrolysis of L-arginine to urea and L-ornithine, is a key enzyme in ammonia detoxification via the urea cycle (22). Arginase exists in two isoforms. Arginase I, a cytosolic enzyme, is expressed exclusively in liver as a component of the urea cycle (16, 22, 38). In contrast, arginase II is a mitochondrial enzyme that is expressed in many tissues including the stomach (16, 38). When compared to the intestine and liver, arginase activity is extremely low in the stomach and the glandular stomach (as a whole) produces very little urea (19). We also found this to be true in our study, because urea production by RGM1 cells, even in the presence of NH₄Cl, was below detectable levels using the commercial urea assay kit (Sigma). This finding was not surprising, because the urea kit measures between 1650-3300 μM of urea (37), a concentration that can easily be measured in blood, urine and liver, a tissue that produces urea at a rate of 158 μmol/min/g of tissue (19). Since the RGM1 cells in our study produced urea at a rate of 1 nmol/min/g of cells, it would take 27.5 hr to generate enough urea to measure using the commercial urea assay kit, which would not be possible in the presence of NH₄Cl. Thus, it was necessary to use a radioactive procedure, developed by Ruegg and Russel (39), to measure urea that is produced (by arginase activity) by the conversion of L-[guanido-14C]arginine to [14C]urea. Byrne et al (4) showed that arginase activity in the stomach is found predominately in a low-density fraction that contains 84 ± 2% parietal cells. Our study shows that arginase II activity must be present in surface epithelial cells and that arginase II activity may increase in the presence of L-Gln or other amino acids that regulate urea cycle activity. In the liver, there are five urea cycle enzymes that contribute to the synthesis of urea for ammonia detoxification (38). Since no other urea cycle intermediates have been described in
gastric tissues, further studies will be necessary to complete our understanding of the active components of the urea cycle in gastric mucosal cells.

In this study, we show that L-Gln protect RGM1 cells against NH₄Cl-induced cell death. It is noteworthy that protection was observed even at very low concentrations of L-Gln (0.2 mM), as shown in Fig. 3A. Because the plasma level of L-Gln is 0.5-0.8 mM (52), physiological concentrations of L-Gln may protect against the cytotoxic effects of ammonia in daily life. In general, L-Gln is involved in a wide variety of metabolic processes, such as the synthesis of proteins and nucleotides and in energy metabolism (41, 59). L-Gln plays an essential role in intestinal mucosal protection in many animal models of critical illness including burns, trauma, obstruction, radiation damage, cytotoxic chemotherapy, and sepsis (5, 11, 28). Cellular ATP levels are maintained in the presence of L-Gln, which protects mitochondria from damage and partially protects α-ketoglutarate dehydrogenase activity in the TCA cycle (1). L-Gln also induces heat shock protein expression to protect cells against injury (54, 56). Furthermore, L-Gln reduces the expression of proinflammatory cytokines (55), which may reduce inflammatory cell-induced mucosal damage, in vivo. Although it is not known how L-Gln protects against the cytotoxic effects of NH₄Cl, our study suggests that it inhibits the uptake and/or facilitates the extrusion of ammonia from cells and increases cellular metabolism of ammonia via arginase. Although L-Glu does not protect RGM1 by ammonia detoxification via glutamine synthetase activity, per se, it may act to increase cellular metabolism, ATP production, inhibit uptake/increase extrusion or facilitate some other process that facilitates protection against ammonia.
The results presented here clearly show that \(\text{L-Gln}\) protects cells differently in the presence of \(\text{NH}_4\text{Cl}\) than in the presence of \(\text{MeNH}_2\). Our data suggest that \(\text{L-Gln}\), in some way, accelerates cellular ammonia metabolism to reduce both the intracellular concentration of ammonia and ammonia cytotoxicity. Our data in Fig. 5B suggest that accelerated ammonia metabolism occurs 2 hr after the addition of \(\text{NH}_4\text{Cl}\) and \(\text{L-Gln}\) to RGM1 cells. In addition, blockade of urea production with nor-NOHA completely reversed the protective effect of \(\text{L-Gln}\) (against \(\text{NH}_4\text{Cl}\)-induced death), suggesting that ammonia detoxification via arginase activity is paramount in protecting RGM1 cells against \(\text{NH}_4\text{Cl}\)-induced cell death. In contrast, even though \(\text{L-Gln}\) completely protects against the cytotoxic effects of \(\text{MeNH}_2\), our data clearly show that urea cycle activity is not involved in \(\text{L-Gln}\) protection against \(\text{MeNH}_2\). This is because nor-NOHA did not reverse the protective effect of \(\text{L-Gln}\) against \(\text{MeNH}_2\)-induced cell death. If exposure to \(\text{L-Gln}\) protected RGM1 cells solely by extrusion of weak base, increased expression of heat shock proteins and/or by increased cellular ATP production, it is likely that protection would be similar with both \(\text{NH}_4\text{Cl}\) and \(\text{MeNH}_2\). Thus, the differential effect of weak bases on viability in RGM1 cells may lend important insights into the mechanism by which \(\text{L-Gln}\) protects against injury in gastric and other tissues.

In summary, we demonstrate that \(\text{L-Gln}\) and \(\text{L-Glu}\) protect gastric epithelial RGM1 cells against \(\text{NH}_4\text{Cl}\)-induced cell death. Since \(\text{L-Gln}\) alimentation is used routinely in human patients (2), it is possible that \(\text{L-Gln}\) alone or in combination with \(\text{L-Glu}\), would be effective as a therapeutic treatment for gastric epithelial damage induced by ammonia during \textit{H. pylori} infection.
ACKNOWLEDGEMENTS

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local nitric oxide production and parasite killing in experimental trypanosomiasis.


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55. **Wischmeyer PE, Khana M, Wolfson R, Ren H, Musch MM, and Chang EB.**

56. **Wischmeyer PE, Musch MM, Madonna MB, Thisted R, and Chang EB.**


FIGURE LEGENDS

Fig. 1. Effect of NH₄Cl or MeNH₂ on cell viability. RGM1 cells were incubated with 1-100 mM NH₄Cl (A) or 0.3-30 mM MeNH₂ (B) for 24 hr, and then the number of viable cells was quantified by the crystal violet assay. The data show that both NH₄Cl and MeNH₂ kill RGM1 cells in a dose-dependent manner. Values represent means ± SE of 4 wells from 3 different experiments and are expressed as the % of control cells that were incubated with standard buffer alone. * Denotes a significant decrease in viability (P < 0.05) when compared to control cells.

Fig. 2. Time course analysis of cell viability in the presence of NH₄Cl or MeNH₂. RGM1 cells were incubated for 36 hr in the presence or absence of 30 mM NH₄Cl (A) or 3 mM MeNH₂ (B) and the number of viable cells was quantified by the crystal violet assay. These data demonstrate that NH₄Cl and MeNH₂ significantly reduce the viability of RGM1 cells over time. Values represent means ± SE of 4 wells from 3 different experiments and are expressed as the % of initial viability (0 hr) in each group. * Denotes a significant decrease in viability (P < 0.05) when compared to time-matched control cells.

Fig. 3. Effect of L-Gln on the viability of RGM1 cells incubated with NH₄Cl or MeNH₂. RGM1 cells were incubated with 0.02-20 mM L-Gln, in the absence or presence of 30 mM NH₄Cl (A) or 3 mM MeNH₂ (B) for 24 hr, and then cell viability was quantified by the crystal violet assay. The data show that L-Gln significantly improves the viability of RGM1 cells in a dose-dependent manner. Values represent means ± SE of 4 wells from 3 different experiments and are expressed as % of control cells that were incubated with
standard buffer alone. * Denotes a significant decrease in viability ($P < 0.05$) when compared to control cells. * Denotes a significant increase in viability ($P < 0.05$) when compared to cells treated with NH$_4$Cl ($A$) or MeNH$_2$ ($B$).

Fig. 4. Effect of l-Gln on NH$_4$Cl- and MeNH$_2$-induced vacuolation. RGM1 cells were incubated for 6 hr with 0.02-20 mM l-Gln in the absence or presence of 30 mM NH$_4$Cl ($A$) or 3 mM MeNH$_2$ ($B$), and then vacuolation was quantified by the neutral red uptake assay. These data show that both NH$_4$Cl and MeNH$_2$ significantly increase the number and size of vacuoles in RGM1 cells and that l-Gln significantly decreases the degree to which RGM1 cell vacuolation occurs in the presence of NH$_4$Cl ($A$) or MeNH$_2$ ($B$). Values represent means ± SE of 4 wells from 3 different experiments and are expressed as % of control cells incubated with standard buffer alone. * Denotes a significant increase in vacuolation ($P < 0.05$) when compared to control cells. * Denotes a significant ($P < 0.05$) reduction in vacuolation when compared to cells incubated with NH$_4$Cl ($A$) or MeNH$_2$ ($B$) alone.

Fig. 5. Time course analysis of MeNH$_2$- and NH$_4$Cl-induced vacuolation of RGM1 cells in the presence or absence of l-Gln. RGM1 cells were incubated with 20 mM l-Gln in the presence of 3 mM MeNH$_2$ ($A$), 30 mM NH$_4$Cl ($B$), or standard buffer alone (control in $A$ and $B$) for 6 hr, and the rate of vacuolation was quantified by the neutral red uptake assay. Values represent means ± SE of 4 wells from 3 different experiments and the data are expressed as the change in optical density (OD) compared to the initial value at 0 hr. The results demonstrate that l-Gln significantly decreases the rate of vacuolation in RGM1 cells. In addition, the data in Fig. 5B show that when RGM1 cells are incubated
with NH₄Cl and l-Gln, the l-Gln-dependent response, identified in Fig. 5A, is greater than the “predicted response” to l-Gln. Thus, there is a significant difference in the response of MeNH₂ and NH₄Cl to l-Gln. *Denotes a significant (P<0.05) decrease in vacuolation when compared to cells treated with (A) MeNH₂ or (B) NH₄Cl alone.

Fig. 6. Phase-contrast images of RGM1 cells that were incubated with or without l-Gln in the absence or presence of NH₄Cl or MeNH₂. (A and B) RGM1 cells maintained a confluent monolayer in STD buffer for 6 and 24 hr, respectively. At 24 hr, some dying cells (arrows) were present in the monolayer from control cells. (C and D) l-Gln alone resulted in no change in the monolayer of cells for 6 and 24 hr, respectively. Like in control cells, some dying cells (arrows) were present in the monolayer at 24 hr. (E and G) Addition of 30 mM NH₄Cl or 3 mM MeNH₂ for 6 hr resulted in the vacuolation of RGM1 cells (arrowheads). (F and H) By 24 hr, the number of adherent cells decreased significantly in the presence of 30 mM NH₄Cl or 3 mM MeNH₂, respectively. Many cells were rounded (arrows) and the few remaining attached cells (arrowheads) had large vacuoles. (I and K) l-Gln at 20 mM protected RGM1 cells against NH₄Cl- or MeNH₂-induced vacuolation at 6 hr. The number of rounded and detached cells (arrows) with l-Gln was not different from that of control cells in (B). (J and L) l-Gln at 20 mM protected RGM1 cells against NH₄Cl- or MeNH₂-induced vacuolation and cell death at 24 hr. The number of rounded and detached cells (arrows) was not different from that of control cells in (B). Bar = 20 μm.

Fig. 7. Effect of l-Gln on the intracellular accumulation of radiolabeled MeNH₂ ([¹⁴C]MeNH₂) in RGM1 cells. RGM1 cells were incubated for 3 hr with 0.02-20 mM l-
Gln in the presence of 3 mM MeNH₂ containing 0.5 μCi of [¹⁴C]MeNH₂, and then the intracellular accumulation of [¹⁴C]MeNH₂ was measured. The data show that l-Gln significantly decreased the intracellular concentration of [¹⁴C]MeNH₂ in a concentration-dependent manner. * Denotes a significant increase ($P < 0.05$) of intracellular [¹⁴C]MeNH₂ when compared to control cells. * Denotes a significant decrease ($P < 0.05$) of intracellular [¹⁴C]MeNH₂ when compared to cells treated with 3 mM MeNH₂ and no l-Gln.

Fig. 8. Effect of l-Gln on vacuolation in the NH₄Cl- or MeNH₂-preloaded cells. RGM1 cells were incubated for 6 hr with 30 mM NH₄Cl (A) or 3 mM MeNH₂ (B) to create large vacuoles (preloaded condition). Next, the preloaded cells were incubated for 1 hr with standard (STD) buffer containing 0-20 mM l-Gln and no NH₄Cl or MeNH₂. Vacuolation was quantified by the neutral red uptake assay and expressed as means ± SE of 4 wells from 3 different experiments. The results show that vacuolation decreased faster in the presence of l-Gln suggesting that l-Gln accelerates the extrusion of both NH₄Cl and MeNH₂. * Denotes a significant ($P < 0.05$) increase in vacuolation when compared to control cells that were not exposed to NH₄Cl or MeNH₂. * Denotes a significant ($P < 0.05$) reduction in vacuolation when compared to cells in STD buffer alone.

Fig. 9. Viability of RGM1 cells in the presence of Nω-hydroxy-nor-l-arginine, nor-NOHA, a potent arginase inhibitor that blocks urea formation from ammonia. (A and B) RGM1 cells were incubated with 30 mM NH₄Cl (A) or 30 mM NH₄Cl and 20 mM l-Gln (B) in the presence of 0.01-1 mM nor-NOHA and cell viability was determined by the
crystal violet assay. *(A)* NH$_4$Cl decreased viability that was further reduced when urea production was blocked by nor-NOHA. *(B)* Blockade of urea production with nor-NOHA completely reversed the protection in viability provided by 20 mM l-Gln. In contrast, either alone *(C)* or in combination with l-Gln *(D)*, nor-NOHA had no effect on viability in the presence of 3 mM MeNH$_2$. * Denotes a significant decrease in viability ($P < 0.05$) when compared to control cells. * Denotes a significant increase in viability ($P < 0.05$) when compared to cells treated with NH$_4$Cl *(B)* or MeNH$_2$ *(D)* alone. # Denotes a significant ($P < 0.05$) reduction in viability when compared to cells treated with NH$_4$Cl alone. § Denotes a significant ($P < 0.05$) reduction in viability when compared to cells treated with NH$_4$Cl and l-Gln alone. Note that 1 mM nor-NOHA has no effect on the viability of untreated RGM1 cells *(A and C)*.

Fig. 10. Effect of l-Glu with or without methionine sulfoximine (MS) on cell viability in the presence of NH$_4$Cl. *(A)* RGM1 cells were incubated with 30 mM NH$_4$Cl with or without 0.02-20 mM l-Glu for 24 hr and the number of viable cells was quantified by the crystal violet assay. The data show that l-Glu protects RGM1 cell viability in the presence of NH$_4$Cl in a dose-dependent manner. *(B)* RGM1 cells were incubated with 30 mM NH$_4$Cl and 20 mM l-Glu in the presence of 0.1-10 mM MS for 24 hr and the number of viable cells was quantified by the crystal violet assay. The data show that there was no difference in viability with NH$_4$Cl, l-Glu and MS when compared to RGM1 cells incubated in NH$_4$Cl and l-Glu alone. Values represent means ± SE of 4 wells from 3 different experiments and are expressed as the % of control cells that were incubated with standard buffer alone. * Denotes a significant decrease in viability ($P < 0.05$) when
compared to control cells. * Denotes a significant increase in viability ($P < 0.05$) when compared to cells incubated with 30 mM NH$_4$Cl without l-Glu.
Table 1. *Vacuolation of RGM1 Cells by NH₄Cl or MeNH₂*

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<th>Concentration (mM)</th>
<th>NH₄Cl (%)</th>
<th>MeNH₂ (%)</th>
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<tr>
<td>Control (0)</td>
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<td>0.0 ± 2.7</td>
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<td>0.3</td>
<td>4.3 ± 2.4</td>
<td>11.5 ± 1.0*</td>
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<tr>
<td>1</td>
<td>06.6 ± 3.7</td>
<td>19.9 ± 2.0*</td>
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<tr>
<td>3</td>
<td>14.3 ± 4.4*</td>
<td>102.6 ± 2.7*</td>
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<tr>
<td>10</td>
<td>42.8 ± 5.6*</td>
<td>181.6 ± 9.1*§</td>
</tr>
<tr>
<td>30</td>
<td>86.4 ± 4.5*</td>
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Data are expressed as mean ± SE. * Denotes a significant ($P<0.05$) increase in vacuolation when compared to control cells incubated with buffer alone. Viability of RGM1 cells was not significantly different in the presence of 0-30 mM NH₄Cl or 0-3 mM MeNH₂ at 6 hr. § Denotes that 10 mM MeNH₂ significantly decreased the viability of RGM1 cells at 6 hr.
Figure 1

**A**

Viability (% of Control)

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<th>NH₄Cl (mM)</th>
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**B**

Viability (% of Control)

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Nakamura and Hagen
Viability (% of the initial value) vs. Time (hr)

A: Control vs. 30mM NH₄Cl

B: Control vs. 3mM MeNH₂

Nakamura and Hagen
Figure 2
A

Viability (% of Control)

Control 0.02 0.2 2 20

L-Gln (mM)

30mM NH₄Cl

B

Viability (% of Control)

Control 0.02 0.2 2 20

L-Gln (mM)

3mM MeNH₂

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Figure 3
Figure 4

Nakamura and Hagen

Figure 4
Figure 5

Nakamura and Hagen
Control 0.02 0.2 2 20

L-Gln (mM)

3mM MeNH₂

[¹⁴C]-MeNH₂ 0.5µCi
A

B

Nakamura and Hagen
Figure 8
Figure 9

Nakamura and Hagen
Figure 9

Nakamura and Hagen
Nakamura and Hagen
Figure 10