Analysis of the ammonia metabolism of rat primary hepatocytes and a human hepatocyte cell line Huh 7

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Abstract

Ammonia metabolism of rat primary hepatocytes and a human hepatocyte cell line, Huh 7, at different concentrations of glutamine, glucose and ammonia was examined. During the incubation of the primary hepatocyte cells, glutamine and ammonia concentrations decreased, that of urea increased, and that of glucose remained the same. In the case of Huh 7 cells, glucose was consumed rapidly, the concentration of ammonia increased and that of urea remained the same. The major energy sources among medium components were glutamine for the primary cells and glucose for Huh 7 cells, although the primary hepatocytes may utilize intracellular glycogen as energy source. As the glutamine concentration in the incubation medium increased, the specific rates of not only glutamine consumption, but also ammonia production by the primary cells and Huh 7 cells increased. Besides, specific urea production rate by the primary cells increased then. Increase of glucose concentration had no effect on glutamine and ammonia metabolism by both cells, although it increased glucose consumption by Huh 7 cells. The incubation of the primary cells with higher ammonia concentration increased all specific rates of glutamine consumption, ammonia consumption and urea production. An increase in the ammonia concentration to 5 mM changed the ammonia metabolism from production to consumption and increased the specific glucose consumption rate. Consequently, increases in the glutamine and ammonia concentrations were revealed to have negative and positive effects, respectively, on decreasing ammonia concentration by both of rat primary hepatocytes and Huh 7 cells.

Introduction

Hybrid artificial liver employing hepatocyte cells is being explored as a treatment of acute liver failure patients (Kamlot et al., 1996). One of the most important functions of artificial liver is to decrease ammonia concentrations in the blood. Several kinds of reactors such as hollow fibers and non-woven fabrics have performed high hepatocyte density with high activity (Kamlot et al., 1996; Miwa et al., 1996; Wu et al., 1996; Naruse et al., 1996). Clinical trials are underway in a few group, in order to confirm the advantages of hybrid artificial liver employing each type reactor (Hewitt et al., 1997; Miwa et al., 1996). Kinetic information such as the effect of metabolite concentrations on hepatocyte activity should be necessary for the control of the artificial liver reactor, because

there might be variety in serum conditions and necessary treatment among patients. However, there is little information with respect to the effect of metabolite concentration on hepatocyte metabolism.

Ammonia concentration in blood is normally less than 0.1 mM and the concentration may rise higher than 0.2 mM in liver failure patient. Liver cells convert the toxic ammonia in blood stream to nontoxic urea through the urea cycle. However, ammonia is also produced mainly through glutamine and other amino acids. Before entering TCA cycle, the amino group in glutamine is removed by two reaction steps in mammalian cell. (Ljunggren and Haggstron, 1994). The first step is the glutaminase reaction converting glutamine to glutamate and producing 1 mol ammonia for each mol of glutamine. The second is the glutamate dehydrogenase reaction converting glutamate to

α -ketoglutarate which also produces 1 mol ammonia per mol glutamine. There is another reaction catalyzed by glutamate pyruvate transaminase which converts glutamate to α -ketoglutarate and produces alanine. One mol of glutamine can produce 1 to 2 mol of ammonia through these pathways. In general, glutamine and glucose are the major energy sources for mammalian cells and it has been reported that glucose and glutamine concentrations affect the consumption rates of each other in a culture of hybridoma cells (Oh et al., 1996). Consequently, ammonia metabolism is affected not only its own concentration but also by glutamine and glucose.

Almost all the hybrid artificial liver models have utilized rat or porcine primary hepatocyte cells because human and other animal primary hepatocyte cells growth *in vitro* is too slow; while attempts to successfully culture human primary hepatocytes *in vitro* are ongoing (Tateno et al., 1996). Although there is a virtually inexhaustible supply of porcine hepatocytes, each lot of harvested cells is from different animals and ensuring consistent quality from different harvest is a daunting task. Furthermore, potential incompatibility problems exist regarding animal cell antigens in humans. An alternative approach involves the use of continuous cell lines that retain key differentiated properties of normal hepatocytes. These cells can be grown to give rise to a large, homogeneous population. Huh 7 (Nakabayashi et al., 1982) was selected as a cell line for the construction of a hybrid artificial liver model among cell lines established from hepatocellular carcinoma which had been reported to maintain certain biochemical functions of hepatocytes to a high degree and were available from public cell banks, e.g. ATCC, JCRB (Takagi et al., 1997). The ammonia metabolism in both rat primary hepatocytes and a human hepatocyte cancer-derived cell line, Huh 7, were analyzed in this study by varying the concentrations of glutamine, glucose and ammonia concentration in the incubation medium.

Materials and Methods

Cells and medium

Primary hepatocytes were harvested from 4 to 6-week-old male Wistar rats, weighing 200–250 g, using a modified two-step *in situ* collagenase perfusion technique (Seglen et al., 1976). Postharvest hepatocyte viability was higher than 90%, based on trypan blue

exclusion analysis. Freshly isolated hepatocyte cells were plated at 5×10^4 cells/cm² onto tissue culture dishes (21 cm², Corning Co. Ltd.) containing 5 ml culture medium and incubated for 24 h before assay of metabolic rates at 37 °C in a CO₂ incubator (5% CO₂). The hepatocyte cell line Huh 7 (JCRB403) cells were grown to confluency (13×10^4 cells/cm²) on the culture dishes in 5 ml culture medium before the assay. The basal medium used for both rat hepatocytes and Huh 7 cells was DMEM (Gibco Co., NY, U.S.A.) with glucose and glutamine concentrations of 25 and 4 mM, respectively, containing 10% FCS (Gibco), 5000 U/l penicillin and 5 mg/l streptomycin (Sigma, MO, U.S.A.).

Assays of cell metabolic rates

For the study of the effects of glutamine, glucose and ammonia concentrations, three modified media were formulated based on the basal medium mentioned above referring the concentration of glucose and glutamine in normal human serum (approx. 2.8 and 0.5 mM) as follows: Medium 1 was basal medium containing 1 mM NH₄Cl, 2.8 mM glucose and 0–4 mM glutamine, medium 2 was basal medium containing 1 mM NH₄Cl, 1.1–25 mM glucose and 0.5 mM glutamine, and medium 3 was modified basal medium (glutamine 4 mM, glucose 25 mM) supplemented with 0–5 mM NH₄Cl, respectively. After washing with PBS(–), primary hepatocytes and Huh 7 cells adhered on dish were incubated with the medium for 3–24 h at 37 °C in a CO₂ incubator (5% CO₂). The metabolite concentrations were assayed from samples of each culture and the reaction rates were calculated. Control without cells was performed each time because glutamine and ammonia concentrations change slightly during incubation even without cells.

Analysis

Initial cell concentration in the incubation media was determined using the nuclei staining method (Sanford et al., 1950). Glucose and lactate concentrations in the culture medium were analyzed using the glucose oxidase-peroxidase and lactic acid oxidase-peroxidase methods, respectively, in an auto-analyzer (Biochemistry Analyzer 2700; YSI Inc., Ohio, U.S.A.). Glutamine and glutamate concentrations were analyzed using the glutaminase-glutamate oxidase-peroxidase method in an auto-analyzer (BF-4, Oji Scientific Instruments Co. Ltd., Osaka). The ammonia and urea concentrations were determined

Figure 1. Change of metabolite concentrations during the incubation of rat primary hepatocytes. The medium 3 supplemented with 1 mM ammonia was employed. Symbols: ○ glutamine; ●, glutamate; □, Urea; ■, ammonia; Δ, glucose; ▲, lactate.

using the indophenol (Ammonia-test Wako) and diacetyl monoxime (Ureic nitrogen-test Wako) methods, respectively.

Results and Discussion

Change of metabolite concentrations during the incubation

To study the ammonia and related energy metabolism in rat primary hepatocytes and Huh 7 cells, cells were incubated with the medium 3 (glutamine; 4.0 mM, glucose; 25 mM) supplemented with 1 mM ammonium chloride. As shown in Figure 1, ammonia concentration decreased from 0.90 to 0.04 mM over 3 h and urea concentration increased from 0.60

Figure 2. Change of metabolite concentrations during the incubation of Huh 7 cells. The medium 3 supplemented with 1 mM ammonia was employed. The symbols are the same as those in Figure 1.

to 1.30 mM in the culture of primary hepatocytes. Glutamine may be metabolized in the TCA cycle because glutamine concentration decreased from 0.65 to 0.54 mM and there was almost no accumulation of glutamate (from 0.095 to 0.100 mM). The rate of conversion from ammonia to urea by the primary hepatocytes was 81.5% on a molar basis. Although the primary hepatocytes could utilize intracellular glycogen as energy source, there was no consumption of extracellular glucose. In contrast, the Huh 7 cells rapidly consumed glucose and converted it into lactate at a rate of 85% (Figure 2). The amounts of accumulated glutamate (0.27 mM) and consumed glutamine (0.030 mM) were almost the same each other over 24 h. This indicated that little glutamine was metabolized into the TCA cycle. The ammonia concentration

Figure 3. Effect of glutamine concentration on specific rates of ammonia and related energy metabolism of rat primary hepatocytes. Cells were incubated using medium 1 containing glucose (2.8 mM), ammonium chloride (1 mM) and various concentrations of glutamine. Specific change rates of metabolite concentrations were calculated from the metabolite concentrations during the incubation. The symbols are the same as those in Figure 1.

increased and urea was not produced. Consequently, the major energy source in culture medium (or plasma in the case of clinical application of the artificial liver containing hepatocytes) may be glutamine for the primary hepatocytes and glucose for Huh 7 cells.

Effect of glutamine and glucose concentration on ammonia and related energy metabolisms

To investigate the effect of glutamine and glucose concentration on the rate of ammonia metabolism by primary hepatocytes and Huh 7 cells, both cell were incubated in medium 1 and 2 which were defined in the Materials and Methods section.

As glutamine concentration increased, specific consumption rates of glutamine and ammonia increased and decreased, respectively, with the increase in glutamine concentration while the specific urea pro-

Figure 4. Effect of glutamine concentration on specific rates of ammonia and related energy metabolism of Huh 7 cells. Cells were incubated using medium 1 containing glucose (2.8 mM), ammonium chloride (1 mM) and various concentrations of glutamine. Specific change rates of metabolite concentrations were calculated from the metabolite concentrations during the incubation. The symbols are the same as those in Figure 1.

duction rate increased slightly (Figure 3). The large increase in ammonia production derived from the increase in glutamine consumption was larger than the maximum activity of the urea cycle. This tendency was more markedly for Huh 7 cells. Specific rates of glutamine consumption and ammonia production by Huh 7 cells increased steadily with the increase of glutamine concentration (Figure 4). On the other hand, glucose concentration had almost no effect on the specific metabolic rates in either the primary hepatocytes or the Huh 7 cells (Figures 5 and 6), except for glycolysis (the major energy metabolism) of Huh 7 cells, which increased with an increase in glucose concentration from 2.8 to 11 mM (Figures 4 and 6). Increase of specific glucose production rate due to the increase of glutamine (Figure 3) and glucose concen-

Figure 5. Effect of glucose concentration on specific rates of ammonia and related energy metabolism of rat primary hepatocytes. Cells were incubated using medium 2 containing glutamine (0.5 mM), ammonium chloride (1 mM) and various concentrations of glucose. Specific change rates of metabolite concentrations were calculated from the metabolite concentrations during the incubation. The symbols are the same as those in Figure 1.

trations (Figure 5) by the primary hepatocytes may due to the change of glycogen decomposition rate.

Consequently, the higher concentration of glutamine and glucose had a negative and no effect, respectively, on the change of ammonia concentration in primary hepatocytes and Huh 7 cells.

Effect of ammonia concentration on ammonia and related energy metabolisms

To investigate the effect of ammonia concentration on ammonia and related metabolic rates of primary hepatocytes and Huh 7 cells, both cells were incubated in medium 3.

The specific rates of ammonia consumption and urea production by primary hepatocytes increased with the increase in ammonia concentration (Fig-

Figure 6. Effect of glucose concentration on specific rates of ammonia and related energy metabolism of Huh 7 cells. Cells were incubated using medium 2 containing glutamine (0.5 mM), ammonium chloride (1 mM) and various concentrations of glucose. Specific change rates of metabolite concentrations were calculated from the metabolite concentrations during the incubation. The symbols are the same as those in Figure 1.

ure 7). Ammonia metabolism by Huh 7 cells turned from production to consumption of ammonia with the higher ammonia concentration than 3 mM. Specific urea production rate of Huh 7 cells increased slightly at high ammonia concentration (Figure 8). However, ammonia detoxification by Huh 7 cells at ammonia concentration higher than 3 mM has no meaning clinically, because ammonia concentration in plasma is less than 0.5 mM even if in the case of liver failure patient. These results may due to the activation of carbamoyl phosphate synthetase at high substrate (ammonia) concentration. Consequently, the increase in ammonia concentration had the positive effect on decreasing ammonia concentration in both of rat primary hepatocytes and Huh 7 cells.

The specific glutamine consumption rate in primary hepatocytes increased markedly with higher

Figure 7. Effect of ammonia concentration on specific rates of ammonia and related energy metabolism of rat primary hepatocytes. Cells were incubated using medium 3 containing various concentrations of ammonium chloride (0.1–0.5 mM). Specific change rates of metabolite concentrations were calculated from the metabolite concentrations during the incubation. The symbols are the same as those in Figure 1.

ammonia concentrations (Figure 7). In the case of Huh 7 cells, the specific glucose consumption rate at 3 mM of ammonia was slightly higher than that at 1 mM (Figure 8). In another culture of Huh 7 cells with a lower range of ammonia concentration (Figure 9), there was marked increase in the specific glucose consumption rate with an increase in ammonia concentration from 0.1 to 2 mM. Glutamine and glucose were the major energy sources in the medium for the primary hepatocytes and Huh 7 cells, respectively, as mentioned above. These results indicate that higher ammonia concentrations accelerated the major energy metabolism. There may be some regulation mechanism in which the rate of energy metabolism is increased in order to obtain more energy to compensate for the damage caused by a high ammonia concentration.

Figure 8. Effect of ammonia concentration on specific rates of ammonia and related energy metabolism of Huh 7 cells. Cells were incubated using medium 3 containing various concentrations of ammonium chloride (1–5 mM). Specific change rates of metabolite concentrations were calculated from the metabolite concentrations during the incubation. The symbols are the same as those in Figure 1.

There were some fluctuation of cell activities between batches of culture; e.g. the metabolic rates in Figure 9 were almost twice that shown in Figure 8. While the reason was not clear, there might be difference in primary hepatocyte activity due to harvest conditions such as required time and cell yield at collagenase perfusion.

Conclusion

The specific ammonia consumption rate of rat primary hepatocytes was markedly higher than that of Huh 7 cells. The major energy sources were glutamine for primary hepatocytes and glucose for Huh 7 cells. The presence of a higher glutamine concentration decreased the specific ammonia consumption rates of

Figure 9. Effect of ammonia concentration on specific rates of glycolysis of Huh 7 cells. A lower range of ammonia concentration (0.1–3 mM) was employed compared with that in Figure 8. The symbols are the same as those in Figure 1.

primary hepatocytes and Huh 7, and glucose concentration had no effect. A higher ammonia concentration increased the rate in both cells. Consequently, glutamine and ammonia were considered to be important variables for the operation of hybrid artificial liver reactors.

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