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CYTOTOXICITY OF D-GALACTOSAMINE ON PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

K. H. Yang, Kwan Ha Park and Byung Sam Kim
Department of Biological Science and Engineering
Korea Advanced Institute of Science and Technology
P.O. Box 150, Chongyang, Seoul, Korea

ABSTRACT: *Primary cultures of adult rat hepatocytes were used to study the cytotoxicity of D-galactosamine. Hepatocytes were isolated by a collagenase perfusion technique and maintained as monolayers in serum-free medium on collagen-coated culture dishes. Treatment of galactosamine to the culture markedly inhibited the uptake of α-aminoisobutyric acid (AIB) inducible with glucagon and dexamethasone. At 0.1 mM of galactosamine, AIB uptake was inhibited significantly when treated for 12 hr. At higher doses (0.25, 0.5 and 1.0mM), a significant inhibition was noticed after 1 hr exposure. Generally the magnitude of the inhibition was related to the dose and treatment time of galactosamine. Treatment of galactosamine also produced a dose- and treatment time-related suppression of the tyrosine aminotransferase (TAT) induction caused by dexamethasone. Meanwhile, uptake of ouabain was not affected by the treatment of galactosamine. The viability of the hepatocytes was decreased only slightly by the treatment of galactosamine; more than 87% of the cells excluded tryphane blue when treated 1 mM galactosamine for 12 hr. Galactosamine induced depressions of AIB uptake and TAT activity were prevented by the simultaneous addition of uridine to the culture.*

Key words: *D-Galactosamine, cytotoxicity, hepatocytes culture, α-aminoisobutyric acid uptake, tyrosine aminotransferase.*

INTRODUCTION

D-Galactosamine in the N-acetylated form is found together with D-glucosamine as integral units of glycoprotein and mucopolysaccharides throughout the body. In 1968, Keppler *et al.* reported the induction of severe hepatic cell damage in laboratory animals after repeated administration of large doses of galactosamine. The lesions consisted of spotty necrosis of liver cells and periportal infiltration of inflammatory cells,

features, closely resembling human viral hepatitis. The observation was soon confirmed by other investigators and formed the basis of a new experimental model for possibly another type of liver cell injury.

For the fast several years our laboratory has extensively used primary hepatocyte cultures as an experimental model to study chemical toxicity, and as metabolizing components in the *in vitro* toxicity studies (Yang *et al.* 1983, 1984, 1986; Kim *et al.*, 1987). Because the *in vivo,* hepatotoxicity of galactosamine has been investigated extensively we choose it as a model hepatotoxin to evaluate the specificity and sensitivity of our *in vitro* system for hepatotoxicity studies. Uptake of ouabain and hormonal induction of amino acid transport and tyrosine aminotransferase activity were examined for the cytotoxic effects of galactosamine along with trypan blue viability test.

MATERIALS AND METHODS

Primary Hepatocyte Cultures and Treatment

Male Sprague-Dawley rats (200 — 250g) were used. Hepatocytes were isolated by a collagenase perfusion technique and maintained as monolayers on 60 mm -diamater collagen-coated plates as reported previously (Yang *et al.*, 1983). Cells were initially cultured in a modified serum-free Waymouth's MB 752/1 medium at 37°C in a humidified 5% CO_2/95% air icubator. After 24 hr, the medium was changed to Swim's S-77 medium containing 4 mM glutamine and gentamycin (50ug/m*l*). D-galactosamine dissolved in saline was added directly to the culture 30, 36, 39 and 41 hr after initial plating for 12, 6, 3 and 1 hr exposure, respectively.

AIB and Quabain Uptake Determination

At 42 hr, culture medium was changed with fresh Swim's S-77 medium containing 1 uM dexamethasone and 0.2 uM glucagon to induce AIB transport in the hepatocytes culture. At 48 hr, the culture medium was aspirated off and the plate was rinsed with warm (37°C) Hank's-Hepes salt solution. AIB uptake was measured by incubating the cells in 2 to 3 m*l* of Hank's-Hepes salt solution containing 1 mM α-aminoisobutyric acid and α-amino [^{14}-C] isobutyric acid (0.2 uCi/m*l* medium) for four min at 37°C. Incubation was terminated by aspirating off the medium and rinsing the cell several times with a total of 15 to 20 m*l* of cold Hank's-Hepes buffer. Cells were digested in 0.2 N NaOH. Protein concentration in the aliquot was determined by the method of Lowry *et al.* (1951) and radioactivity was estimated in a liquid scintillation counter (Medel LS-2133T, Beckman Instrument, Irvine, CA).

Uptake of ouabain was determined similarly to the AIB experiment except no hormonal induction was made and the assay was done at 42 hr.

TAT Activity Determination

At 42 hr, culture medium was changed with fresh Swim S-77 medium containing 10^{-5} M dexamethasone. At 48 hr, the medium was aspirated off and the plate was rinsed with 3 m*l* of cold saline. One m*l* of homogenizing buffer containing 0.2 M potassium

phosphate, 0.04 mM pyridoxal phosphate, and 1.0 mM EDTA, pH 7.3, was added and the plate was frozen on dry ice. They were subsequently thawed, and the cells were scraped into a test tube and frozen in a dry ice-acetone bath and thawed twice. The resulting suspension was centrifuged at 48,000 × g for 30 min. Enzyme activity in the supernatant was assayed by the method of Diamondstone (1966) and protein concentrations in the supernatant and the precipitate were determined. The enzyme activity was expressed as 1 unit being equal to 1 umole of *p*-hydroxyphenylpyruvate formed in 1 min.

Viability Test

The viability of hepatocytes was assayed by trypan blue exclusion. Trypan blue (0.4% in 0.95% NaCl) was added directly to the cultures and allowed for 5 min at room temperature. The number of viable and nonviable cell was counted under microscope.

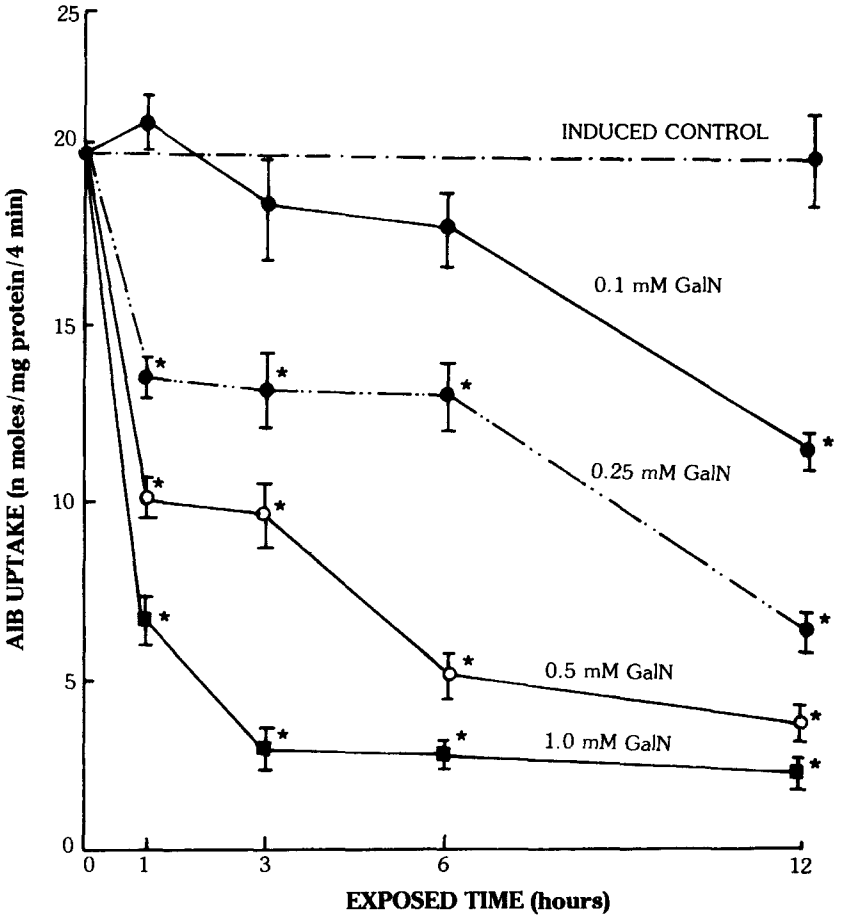

Fig. 1. Effects of D-galactosamine on hormonal induction of AIB uptake. All values represent the mean ± SE of triplicate determinatins. An asterisk indicates values significantly different from control.

Statistical Analysis

Means of control and galactosamine-treated groups were compared by Student t-test. Significance was set at $p < 0.05$.

RESULTS

Fig. 1 shows the effects of galactosamine on hormonal induction of AIB uptake. At 0.1 mM of galactosamine, AIB uptake was significantly inhibited after 12 hr treatment. At higher doses (0.25, 0.5 and 1.0 mM) of galactosamine, a significant inhibition was noticed after 1 hr treatment. Generally the magnitude of inhibition was related to the dose and treatment time of galactosamine.

Fig. 2 shows the effects of galactosamine on the induction of TAT by dexametha-

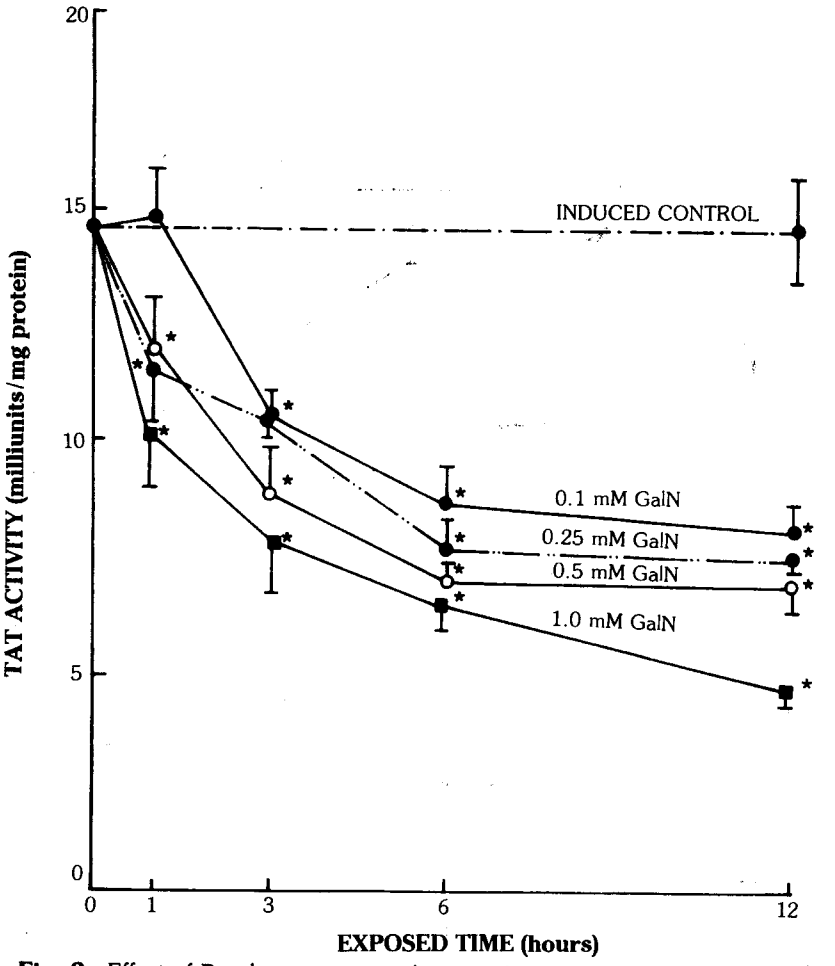

Fig. 2. Effect of D-galactosamine on hormonal induction of TAT activity. All values represent the mean ± SE of triplicate determinations. An asterisk indicates values significantly different from control.

Table 1. Effect of D-galactosamine on ouabain uptake in primary rat hepatocyte cultures.

Galactosamine (mM)	Exposed time (hr) 1	3	6	12
0		2.58 ± 0.42		
0.1	2.57 ± 0.13	2.50 ± 0.28	2.54 ± 0.17	2.53 ± 0.13
0.25	2.50 ± 0.23	2.50 ± 0.22	2.45 ± 0.22	2.26 ± 0.16
0.5	2.57 ± 0.13	2.69 ± 0.47	2.66 ± 0.21	2.33 ± 0.17
1.0	2.33 ± 0.36	2.50 ± 0.13	2.4 ± 0.32	2.16 ± 0.17

Table 2. Effect of D-galactosamine (1 mM) on the viability of primary cultures of rat hepatocytes.

Exposed time (hr)	Viability (%)
0	100.0 ± 4.2
1	93.3 ± 3.4
3	87.7 ± 5.4
6	88.4 ± 6.8
12	88.6 ± 7.2

sone. Similar to AIB transport, treatment of galactosamine caused a significant inhibition of TAT induction in hepatocytes culture. At 0.1 mM of galactosamine, TAT activity was significantly lower than the induced control when treated for 3 hr. At higher dose (0.25, 0.5 and 1.0 mM), TAT activity was significantly lower than induced control at 1 hr treatment.

The cardiac glycoside, ouabain is known to be transported by an active transport system specific for organic neutral compounds, and is not metabolized by hepatocytes. It has been widely used to test the damage of the plasma membrane associated active transport system. As shown in Table 1, ouabain uptake was not significantly affected by the treatment of galactosamine; only 12 hr treatment of 1.0 mM galactosamine decreased the uptake slightly but it was still not statistically significant.

The viability of cultured hepatocytes exposed to 1mM of galactosamine is shown in Table 2. A slight decrease of viability (less than 13%) was observed when treated with galactosamine for 3 hr. No gradual increase of cell death was observed in longer treatment periods (6 and 12 hr). The results suggested that the depression of AIB uptake and TAT activity by galactosamine was not directly related to the cell death.

Since uridine is known to protect against galactosamineinduced liver damage (Keppler *et al.*, 1974, Holstege *et al.*, 1981), we studied whether uridine could protect from the galactosamine-induced depression of AIB uptake and TAT activity. As showon in Fig. 3. AIB uptake was partially protected by the addition of 1 mM uridine and

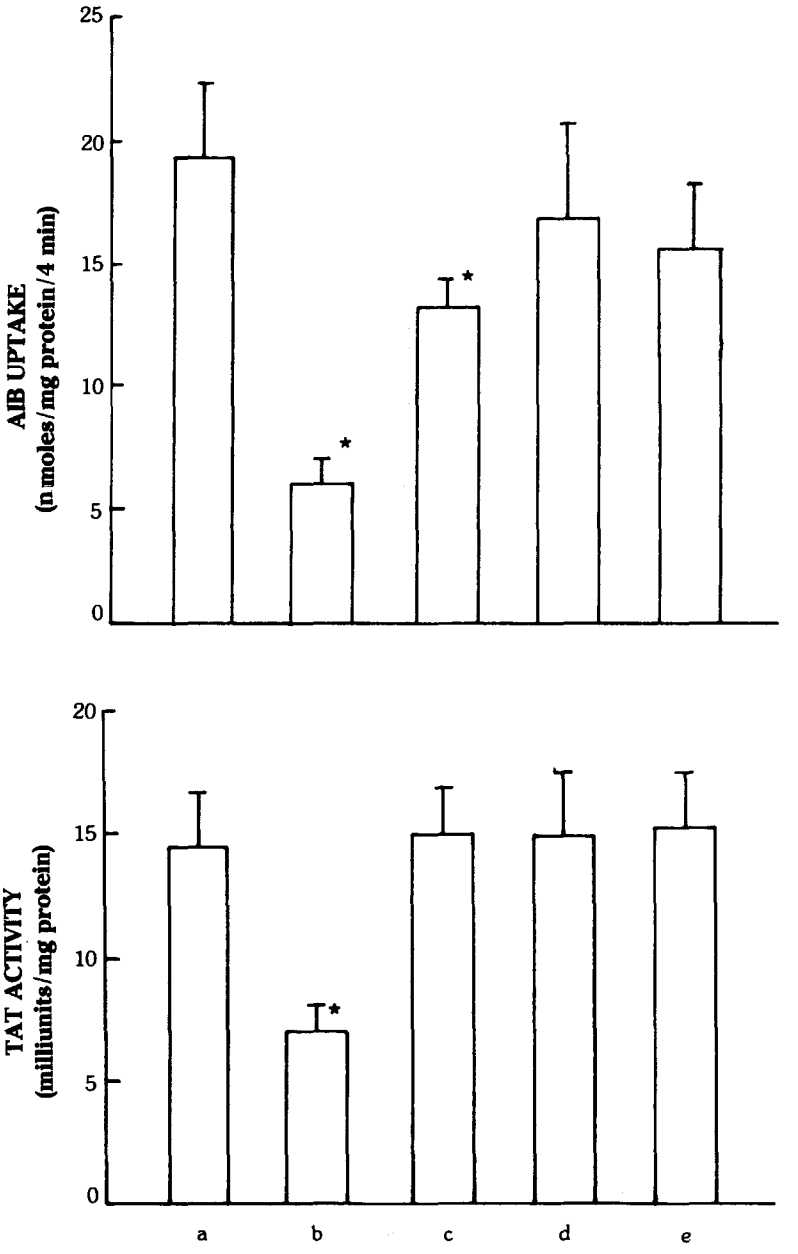

Fig 3. Reversal of D-galactosamine-induced depression of the hormonal induction of AIB uptake and TAT activity by uridine. Cultures were treated with uridine and D-glactosamine (0.5 mM) for 6 hr.

a: Control,
b: Galactosamine only,
c: Galactosamine + uridine (1 mM)
d: Galactosamine + uridine (2 mM)
e: Galactosamine + uridine (3 mM)

a complete protection was observed at 2 mM uridine. Meanwhile TAT activity was completely protected by 1 mM uridine.

DISCUSSION

The results of the present study demonstrated that treatment of galactosamine to hepatocytes culture caused a dose- and treatment-time related inhibition of the hormonal induction of AIB uptake and TAT activity. These effects of galactosamine were not related to the cell death and were protected by simultaneous addition of uridine to the culture. Meanwhile, uptake of cardiac glycoside, ouabain was not affected by galactosamine. According to Keppler and Decker (1969), galactosamine is first phosphorylated to form galactosamine-1-phosphate that inhibits UDPG pyrophosphorylase reaction with subsequent decrease of UDP-glucose. Disturbance of UDPG-linked biosynthesis of glycogen, heteropolysaccharides, and glucuronides will then take place. Furthermore, trapping of uridine phosphate by the formation of large quantities of UDP-hexosamines induces severe uridinetriphosphate (UTP) deficiency. Because UTP is one of the substrate of RNA synthesis, rapid and marked UTP deficiency results in inhibition of RNA synthesis. They explained that this inhibition of RNA synthesis and subsequent inhibition of protein synthesis led to liver cell necrosis. Since the induction of AIB uptake and TAT by glucocorticoides reported to require the mRNA synthesis (Butcher *et al.*, 1972; Pariza *et al.*, 1976), the effects of galactosamine on AIB uptake and TAT might be possibly due to the inhibition of RNA synthesis. Meanwhile, membrane ouabain transport system, known to depend on metabolic energy, may not require mRNA synthesis. The overall significance of this study is that it demonstrated that primary cultures of rat hepatocyte could be used as an *in vitro* system to study the mechanism of toxic liver cell injury by galactosamine.

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REFERENCES

Butcher, F.R., Bushnell, D.E., Becker, J.E. and Potter, V.R. (1972): Effect of cordycepin on induction of tyrosine aminotransferase employing hepatoma cells in tissue culture. *Exp. Cell Res.* 74: 115—123.

Diamondstone, T.I. (1966): Assay of tyrosine transaminase activity by conversion of p-hydroxy-phenylpyruvate to phydroxybenzeldehyde. *Anal. Biochem.* 16: 395-401.

Holstege, A., Anukarahanonta, T., Yamamoto, M., Koch, H. K., Kattermann, R., Lesch, R. and Keppler, D. (1981): Preventive effects of 5-fluorouridine and uridine on D-galactosamineinduced liver injury. *Exp. Mol. Pathol.* 34: 170—182.

Keppler, D. and Decker, K. (1969): Studies on the mechanism of galactosamine hepatitis: Accumulation of galactosamine-1-phosphate and its inhibition of UDP-

glucose pyrophosphorylase. *Eur. J. Biochem.* 10: 219—225.

Keppler, D., Lesch, R., Reutter, W. and Decker, K. (1968): Experimental hepatitis induced by D-glactosamine. *Exp. Mol. Pathol.* 9: 279—290.

Keppler, D., Pausch, J. and Decker, K. (1974): Selective uridine triphosphate deficiency induced by D-galactosamine in liver and reversed by pyridine nucleotide precursors. *J. Biol. Chem.* 249: 211—216.

Kim, D.H., Yang, K.H., Johnson, K.W. and Holsapple, M.P. (1987): Suppression of *in vitro* antibody production by dimethylnitrosamine in mixed cultures of mouse primary hepatocytes and mouse splenocytes. *Toxicol. Appl. Pharmacol.* 87: 32—42.

Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951): Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265—275.

Pariza, M.W., Kletzien, R.F. Butcher, F.R. and Potter, R. (1976): Induction by hormones added signly, simultaneously or sequentially: What cultured hepatocytes can tell us about metabolic regulation in the whole animal. *Adv. Enz. Reg.* 14: 103—115.

Yang, K.H., Choi, E.J. and Choe, S.Y. (1983): Cytotoxicity of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin on primary cultures of adult rat hepatocytes. *Arch. Environ. Contam. Toxicol.* 12: 183—188.

Yang, K.H., B.S., Munson, A.E. and Holsapple, M.P. (1986): Immunosuppresison induced by chemicals requiring metabolic activation in mixed cultures of rat hepatocytes and murine splenocytes. *Toxicol. Appl. Pharmacol.* 83: 420—429.

Yang, K.H., Shin, C.S., Choe, S.Y. and Kim, D.H. (1984): Detection of DNA singlestrand breaks induced by procarcinogens in Chinese hamster ovary cells coculatured with rat hepatocytes. *J. Toxicol. Environ. Health* 13: 133-144.