Insulin Facilitates Formation of Taurine-Conjugated Bile Acids — Analysis Using Primary Cultured Hepatocytes —

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It is well known that various hormones such as insulin, glucagon, catecholamines and glucocorticoids modulate not only metabolisms but also membranous transport of various biological substances in the liver. For investigating the effect of hormones on the membranous transport and metabolism of amino acids, freshly isolated hepatocytes and hepatocytes in primary culture have been found to be suitable (1). Most of the previous studies investigating hormone effects on amino acid transport by hepatocytes, however, used α -aminoisobutyric acid (AIB), an artificially synthesized amino acid and non metabolizable in hepatocytes, as a test substance (1, 2); and little information is available with regard to the modulating action of hormones on the transport of amino acids which are biosynthesized and metabolized in mammalian organs.

Taurine is known as one of the amino acids biosynthesized and metabolized in hepatocytes (3), and it is used for the formation of taurine-conjugated bile acids in the liver (4–6). The taurine transport in freshly isolated hepatocytes has been reported to be a saturable, sodium- and energy-dependent, and carrier-mediated process (7). The presence of a similar transport system for taurine has been reported in hepatocytes maintained in primary culture (8, 9).

In this study, we have investigated the possible modulatory effect of hormones such as insulin and glucagon on the transport of taurine as well as on the formation of taurine-conjugated bile acids in freshly isolated hepatocytes and hepatocytes maintained in primary culture.

Adult rat liver parenchymal cells (hepa-

tocytes) were isolated from Wistar male rats (weighing 200–300 g), which were maintained under free access to standard laboratory chow and drinking water. Freshly isolated hepatocytes were prepared by a collagenase perfusion technique according to the method of Berry and Friend (10) with a minor modification (7). The hepatocytes obtained were washed three times with ice-cold Ca^{2+} -free Krebs-Henseleit buffer (pH 7.4) by the repetition of the centrifugation at $50\times g$ for 3 min, and then resuspended in ice-cold Krebs-Henseleit buffer containing 3% bovine serum albumin, penicillin-G (K-salt, 0.2 mg/ml) and streptomycin (0.2 mg/ml). These hepatocytes had a viability of more than 90% as estimated by the trypan blue exclusion test. For maintaining hepatocytes as a monolayer in primary culture, the procedure described by Bonney et al. (11) was used. The freshly isolated and washed hepatocytes were suspended in Eagle's minimal essential medium [MEM, pH 7.4; supplemented with 10% fetal calf serum, 10 mM N-Tris (hydroxymethyl)-methyl-2-amino ethanesulfonic acid (TES), 10 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), 10 μM dexamethasone, insulin (10 mU/ml), carbenicillin (0.1 mg/ml)], and the cell number was adjusted to 1.0×10^6 cells/ml of suspension. Three ml of the suspension were added into each Corning plastic culture dish (60 mm in diameter) and placed in an incubator for maintaining at 37°C under humidified 5% CO_2 in air for 24 hr. The planting efficiency 24 hr after the initiation of incubation was examined by the method of Morse and Potter (12) and was found to be approximately 60 percent.

For investigating the effect of insulin,

glucagon and isoproterenol on the uptakes of taurine and AIB by hepatocytes in primary culture, the experiments were carried out as follows: MEM in a culture dish to which hepatocytes were attached was discarded by aspiration, and the culture dish was rinsed three times with 5 ml of warm (37°C) Krebs-Henseleit buffer (pH 7.4). This procedure was followed by the addition of 2 ml of Krebs-Henseleit buffer (37°C, pH 7.4) containing penicillin G (0.2 mg/ml) and streptomycin (0.2 mg/ml) into the culture dish. The incubation was started following the simultaneous addition of insulin, glucagon or isoproterenol, and 100 μ M [3 H]taurine or 1 mM [14 C]AIB, respectively. The incubation was carried out at 37°C in humidified 5% CO₂ in air, and it was terminated by rapid aspiration of the Krebs-Henseleit buffer containing radio-labeled amino acids, followed by rinsing the culture dish five times with 3 ml of ice-cold Krebs-Henseleit buffer within 45 sec. Two ml of 0.1 N NaOH were added to the culture dish for the digestion of hepatocytes, and an aliquot of digested hepatocytes was transferred to a scintillation counting vial with 10 ml of Triton toluene scintillator [Triton:toluene (containing 5 g PPO and 0.3 g POPOP per liter)=1:2] for the measurement of radio-activity accumulated in hepatocytes. An aliquot of hepatocytes dissolved in 0.1 N NaOH was also used for the measurement of protein content.

The amount of [3 H]taurine-conjugated bile acids formed by primary cultured hepatocytes was determined 5 hr after the incubation with [3 H]taurine. The incubation was terminated by withdrawing the medium. The incubation medium was aspirated, and the aliquots of medium used for washings were combined and transferred to another vessel, followed by the addition of methanol-acetone (1:1, v/v). Hepatocytes were then scraped off by a rubber policeman and also mixed with methanol-acetone. The methanol-acetone mixtures with hepatocytes and incubation medium were centrifuged respectively at 800 $\times$ g for 10 min. After decanting the supernatant, each sediment was mixed with methanol-acetone and recentrifuged. The supernatants obtained by the first and

second centrifugations were mixed and lyophilized at 80°C (13). The lyophilized samples prepared from hepatocytes and incubation medium were separately dissolved into methanol. After separating bile acids in each fraction from hepatocytes and incubation medium by thin layer chromatography (14), spots corresponding with authentic taurocholic acid and taurodihydroxybile acids were scraped off and transferred to vials containing 500 μ l methanol to extract the bile acids from the silica gel G. Finally, the radioactivities of [3 H]taurine-conjugated bile acids in hepatocytes and incubation medium were measured respectively in a liquid scintillation spectrometer after the addition of Triton-toluene scintillator. The net amount of [3 H]taurine-conjugated bile acids formed by primary cultured hepatocytes was calculated by summing the amount of [3 H]-taurine-conjugated bile acids detected in hepatocytes and that detected in the incubation medium. The recoveries of standard conjugated bile acids during thin layer chromatographic procedures were more than 85%.

Protein contents in freshly isolated and primary cultured hepatocytes were determined by the method of Lowry et al. (15) using bovine serum albumin as a standard.

Insulin (10^{-6} M), glucagon (10^{-6} M) and isoproterenol (10^{-4} M) had no effect on the transport of taurine as well as that of AIB in freshly isolated hepatocytes during 1 hr incubation. Similarly, no effect of these drugs was observed when the time of incubation was extended to 2 hr. Based on these results, it is suspected that freshly isolated hepatocytes used in this study may lose the responsiveness to hormones including insulin and glucagon, which agrees well with a previous report (16).

On the other hand, the transport of AIB in primary cultured hepatocytes was obviously facilitated by glucagon following 3 hr incubation (Fig. 1A). The increase in the amount of AIB accumulated in hepatocytes was also observed 5 hr after the initiation of incubation in the presence of insulin, glucagon and isoproterenol, although no effect of these drugs was noticed following 1 hr incubation. These results clearly indicate

Fig. 1. Effect of isoproterenol, insulin and glucagon on uptakes of α -aminoisobutyric acid (AIB: A) and taurine (B) by hepatocytes in primary culture. Hepatocytes were incubated with Krebs-Henseleit buffer containing 1 mM [14 C]AIB or 100 μ M [3 H]taurine up to 5 hr in the presence and absence of various drugs. Each value represents the mean $\pm$ S.E.M. obtained from four to eight dishes. * $P < 0.001$, ** $P < 0.01$, *** $P < 0.02$, **** $P < 0.05$, compared with each control value.

that freshly isolated hepatocytes have no responsiveness to various hormones, but these hormonal sensitivities are recovered, at least in part, following the primary culture of these cells for 24 hr. These findings essentially agree with previous reports (17–19).

In contrast to a remarkable increase in the accumulation of AIB in primary cultured hepatocytes by these hormones, no induction of the increase in taurine transport was noted under the same experimental conditions (Fig. 1B). The results clearly indicate that hepatocellular transport of taurine is not modulated by these hormones and is distinct from that of AIB in terms of hormonal sensitivity.

The amount of [3 H]taurine-conjugated bile acids formed in primary cultured hepatocytes in the absence or presence of insulin, glucagon and isoproterenol is shown in Table 1. One-tenth μ M of insulin, which induced a significant increase in AIB accumulation, significantly facilitated the formation of [3 H]taurine-conjugated bile acids by primary cultured hepatocytes. In contrast, 10^{-5} – 10^{-7} M glucagon and isoproterenol, which exhibited a significant accentuating effect on AIB transport, had no effect on the formation of [3 H]taurine-conjugated bile acids in these cells. These results indicate that insulin, but not glucagon and isoproterenol, facilitates the formation of

Table 1. Effect of isoproterenol, insulin and glucagon on the formation of [^3H]taurine-conjugated bile acids by hepatocytes in primary culture

	[^3H]taurine-conjugated bile acids formed (nmoles/mg prot. $\pm$ S.E.M.)
Control	12.85 $\pm$ 0.73
Isoproterenol (10^{-5} M)	13.28 $\pm$ 1.01
Insulin (10^{-7} M)	17.97 $\pm$ 1.24*
Glucagon (10^{-7} M)	13.28 $\pm$ 0.72

Hepatocytes were incubated with Krebs-Henseleit buffer containing 100 μM [^3H]taurine in the presence of isoproterenol, insulin and glucagon for 5 hr. Each value represents the sum of taurine-conjugated bile acids detected in both hepatocytes and incubation medium. The mean $\pm$ S.E.M. obtained from four separate experiments is presented. * $P < 0.05$, compared with the control value.

taurine-conjugated bile acids.

It has been previously reported that AIB transport in primary cultured hepatocytes is facilitated by insulin, glucagon and isoproterenol (17–19). Similar results in regard to the hormonal sensitivity of AIB transport in hepatocytes maintained in primary culture have been observed in this study. These results indicate that primary cultured hepatocytes undoubtedly possess the capacity to respond to hormones, although these cells have no sensitivity to hormones immediately after the isolation. This rapid restoration of hepatocellular hormonal sensitivity due to primary culture for 24 hr is in sharp contrast to the fact that primary culture induces the deterioration of metabolic activities such as the metabolism of carbohydrate (20) and various drugs (21) in hepatocytes.

With regard to the effect of insulin, glucagon and isoproterenol on the taurine transport in primary cultured hepatocytes, no apparent alteration in taurine transport was detected as shown in Fig. 1. Since it has been well established that taurine transported into hepatocytes is conjugated with bile acids (22, 23), and taurocholic acid, one of the major taurine-conjugated bile acids in rats, is rapidly secreted into medium by isolated hepatocytes (24–26), we have examined the formation of [^3H]taurine-conjugated bile acids in primary cultured hepatocytes in the presence and absence of insulin, glucagon and isoproterenol. The experimental results clearly demonstrated that insulin increased the formation of [^3H]taurine-conjugated bile acids by primary cultured hepatocytes, but

glucagon and isoproterenol had no such stimulatory effects on the formation of [^3H]taurine-conjugated bile acids.

The metabolic steps by which taurine is conjugated with bile acids consist of two enzymatic reactions (4, 27). The first reaction is the formation of bile acid-Co A complex mediated by cholesteryl-Co A synthetase (EC 6:2.1.7). The reaction of bile acid-Co A complex with taurine is successively catalyzed by bile acid-Co A: amino acid N-acyl-transferase (EC 2.3.1) and forms taurine-conjugated bile acid. Since it is well known that insulin facilitates not only protein synthesis but also the synthesis of various enzymes, it is probable that this hormone accelerates the biosynthesis of these enzymes involved in the biosynthetic pathway of taurine-conjugated bile acids. Although exact molecular mechanisms underlying the facilitating effect of insulin on the hepatocellular formation of taurine-conjugated bile acids remain to be elucidated, present results clearly indicate that this facilitation is not due to the increased transport of taurine in these cells.

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