Expression of α_2 -Receptor-Mediated Responses by Insulin in Primary Culture of Rat Hepatocytes

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ABSTRACT—The effects of the α_2 -adrenergic agonist, clonidine, on the glucagon-stimulated glucose output from serum-free cultures of adult rat hepatocytes were examined *in vitro*. When hepatocytes were cultured with 10 nM dexamethasone under the serum-free condition, 1 or 10 μ M clonidine did not inhibit the glucagon-induced glucose production. In contrast, clonidine dose-dependently inhibited the activity concomitantly with suppression of hepatocyte cAMP production by glucagon when they were cultured with 10 nM dexamethasone and 10 nM insulin. The inhibitory effects of clonidine were completely blocked by prior treatment of hepatocytes with pertussis toxin (100 ng/ml). In addition, forskolin-stimulated cAMP production was also inhibited by α_2 -adrenergic agonists (clonidine and oxymetazoline) in a dose-dependent manner when hepatocytes were cultured with 10 nM dexamethasone and 10 nM insulin. The inhibitory effects of α_2 -adrenergic agonists on forskolin-stimulated cAMP production were specifically blocked when they were combined with the α_2 -adrenergic antagonist yohimbine. Hepatocytes cultured with dexamethasone alone showed no response to the α_2 -adrenergic agonists. The α_2 -response was abolished when cycloheximide (0.5 μ M) was added to the cultures. These results suggest that insulin develops α_2 -adrenergic responsiveness through new protein synthesis during the primary culture of adult rat hepatocytes.

Keywords: Hepatic glucose output, cAMP production, Insulin, α_2 -Adrenergic agonist

Primary cultures of adult rat hepatocytes respond to various agents; e.g., hormones, neurotransmitters and growth factors. Among these circulating substances, catecholamines have been shown to be closely involved in the regulation of liver function (e.g., glycogenolysis, gluconeogenesis and cell growth) through adrenergic receptors (1, 2). It has been reported that during culture of adult rat hepatocytes, which show a very low β -adrenergic response *in vivo*, the responses increase rapidly (3). The acquisition of increased β -adrenergic response is associated with new synthesis of β -adrenergic receptors (4, 5). On the contrary, adult rat hepatocytes show almost no α_2 -adrenergic response, although they contain abundant G_i -protein. This is due to the presence of only very few post-junctional α_2 -adrenergic receptors in this preparation (2). However, it is unknown whether adult rat hepatocytes in primary culture can acquire a significant α_2 -adrenergic response.

In preliminary studies, I found that adult rat hepatocytes could respond to α_2 -adrenergic agonists when they are cultured with insulin. It is generally accepted that insulin is the principal hormone controlling blood glucose

levels by stimulating glucose metabolism in muscle and adipocytes and inhibiting gluconeogenesis by the liver (1, 6). In addition, higher concentrations of insulin (10 nM to 1000 nM) generally increase the rate of DNA synthesis by about twofold and act synergistically with epidermal growth factor in cultured hepatocytes (7, 8). In this report, therefore, I examined further the effect of insulin on the expression of the α_2 -adrenergic response in terms of inhibition of glucagon-stimulated gluconeogenesis and that of forskolin-stimulated cAMP production by α_2 -adrenergic agonists such as clonidine and oxymetazoline in cultured hepatocytes. The present results demonstrate that adult rat hepatocytes acquire an α_2 -adrenergic response during primary culture with higher concentrations of insulin.

MATERIALS AND METHODS

Hepatocyte isolation and culture

Parenchymal hepatocytes were isolated from adult male Wistar rats (200–250 g) by perfusion of the liver *in situ* with collagenase as described elsewhere (9). Routine-

ly, more than 85% of the cells were intact as assessed by the trypan blue exclusion test. The cells were suspended in Williams' medium E supplemented with 5% newborn calf serum, 0.1 $\mu\text{g}/\text{ml}$ aprotinin, and 10 nM dexamethasone. 1×10^6 cells were seeded to 35 mm diameter plastic dishes precoated with collagen type II. They were cultured first in a humidified incubator under an atmosphere of 5% CO_2 in air at 37°C. After the attachment period of 3 hr, the medium was replaced by a serum-free medium containing 10 nM dexamethasone with or without 10 nM insulin and cultured for a further 21 hr. Dexamethasone (10 nM) improved the plating efficiency and viability of the hepatocytes; Therefore, the glucocorticoid was routinely used in the subsequent experiments. Pertussis toxin (100 ng/ml) was added at the time of the medium exchange. The toxin is activated before use in a mixture containing 1 mM ATP, 20 mM dithiothreitol at 37°C for 10 min (10).

Hepatocyte incubation

Gluconeogenesis: After 21 hr of culture, hepatocytes were washed twice with Dulbecco's phosphate-buffered saline (pH 7.4); and then they were incubated for 1 hr at 37°C in 1.0 ml of Hanks–10 mM Hepes buffer (pH 7.2) containing 10 mM lactate as a gluconeogenic precursor. An aliquot (10 μl) of glucagon (0.1 nM to 1 μM) with or without clonidine (1 μM to 100 μM) was simultaneously added to the incubation medium. Gluconeogenesis is determined as the rate of formation of glucose from 10 mM lactate as described previously (11). The glucose in the medium was determined by the glucose oxidase-peroxidase method using a commercially available diagnostic kit.

cAMP production assay: After 21 hr of culture, hepatocytes were washed twice with Dulbecco's phosphate-buffered saline (pH 7.4). Then the cultured cells were preincubated with 1.0 ml of Hanks–10 mM Hepes buffer (pH 7.4) containing 0.2 mM 1-methyl-3-isobutylxanthine (IBMX) for 5 min at 37°C. After the preincubation, an aliquot (10 μl) of 10 μM forskolin (to stimulate cAMP production) and an α_2 -adrenergic agonist (to inhibit adenylate cyclase) with or without the α_2 -adrenergic antagonist yohimbine was simultaneously added to the incubation medium; The cells were then incubated for an additional 10 min at 37°C. The incubation was then stopped by the addition of 0.1 ml of 1.0 N HCl.

Intracellular cAMP levels were determined by the procedure of Honma et al. (12). Briefly, after incubation was terminated by 1.0 N HCl, the cells were harvested by scraping with a rubber policeman. Then the cells and medium were transferred to a small test tube and heated for 3 min in a boiling water bath to obtain a deproteinized extract. The suspension obtained was cooled and centrifuged at 3000 rpm for 5 min. A 0.5-ml aliquot of the

supernatant was stored at -70°C until assayed. cAMP in the supernatant was determined by the radioimmunoassay procedure using a commercially available assay kit (Yamasa Shoyu Co., Chiba). The cAMP content of the hepatocytes was expressed as pmol/mg cellular protein. The hepatocyte protein content was measured by the modified method of Lowry et al. with bovine serum albumin as a standard (13).

Materials

The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA): clonidine hydrochloride, oxymetazoline hydrochloride, yohimbine hydrochloride, 1-methyl-3-isobutylxanthine (IBMX), forskolin, aprotinin, insulin, glucagon, ATP and pertussis toxin. Williams' medium E and newborn bovine serum were purchased from Flow Laboratories (Irvine, Scotland). Dexamethasone, cycloheximide hydrochloride and glucose assay kit were obtained from Wako Pure Chemical Co. (Osaka). Bovine serum albumin (fraction v) and collagenase (Type II) were obtained from Worthington (Freehold, NJ, USA). All other reagents were of analytical grade. The stock solution of forskolin was 10 mM (in 50% dimethyl sulfoxide).

Statistical analyses

Results are presented as the mean $\pm$ S.E.M. for the indicated number of observations. The statistical significance of differences was analyzed by the unpaired Student's *t*-test. *P* values less than 0.05 were regarded as statistically significant.

RESULTS

Effect of clonidine on glucagon-stimulated glucose output

As shown in Fig. 1A, glucagon produced dose-dependent increases in the glucose output from 10 mM lactate in hepatocytes that were cultured in the presence of 10 nM dexamethasone alone, and the maximal stimulation was seen with 10 nM glucagon. In these hepatocytes, the α_2 -adrenergic receptor agonist clonidine (1 and 10 μM) did not alter the glucagon-stimulated glucose release. In contrast, the glucagon-stimulated glucose output was inhibited by 1 or 10 μM clonidine in hepatocytes that were cultured with 10 nM dexamethasone and 10 nM insulin (Fig. 1B). Thus, the dose-response curves to glucagon were shifted to the right and the maximal responses were significantly decreased (by 10%, lower dose of clonidine; by 16%, higher dose of clonidine).

Effect of clonidine on glucagon-stimulated cAMP levels

Glucagon produced dose-dependent increases in hepatocyte cAMP levels when the cells were cultured in

Fig. 1. Effects of clonidine on the glucagon-stimulated glucose output in hepatocytes cultured in Williams' medium E with or without insulin. Hepatocytes were cultured in serum-free Williams' medium E with 10 nM dexamethasone alone (A) or 10 nM dexamethasone and 10 nM insulin (B) for 21 hr at 37°C. Then they were washed twice with Dulbecco's phosphate-buffered saline and incubated for 1 hr at 37°C in Hanks-10 mM Hepes buffer (pH 7.2) containing 10 mM lactate with various concentrations of glucagon with or without clonidine. After incubation, the glucose in the medium was measured as described under experimental procedures. Results are reported as the mean $\pm$ S.E.M. of 3-4 independent preparations. * $P < 0.05$, compared with the respective control values (glucagon alone); glucagon ($\circ$), glucagon + 1 μ M clonidine (Δ), glucagon + 10 μ M clonidine ($\square$).

Fig. 2. Effects of clonidine on the glucagon-stimulated cAMP levels in hepatocytes cultured in Williams' medium E with (B) or without (A) insulin. After the incubation, cAMP levels in the hepatocytes were measured by radioimmunoassay as described under experimental procedures. Results are reported as the mean $\pm$ S.E.M. of 3-4 independent preparations. * $P < 0.05$, compared with the respective control values (glucagon alone); glucagon ($\circ$), glucagon + 1 μ M clonidine (Δ), glucagon + 10 μ M clonidine ($\square$).

the presence of 10 nM dexamethasone. α_2 -Adrenergic activation by clonidine did not change significantly glucagon-stimulated cAMP production in these hepatocytes (Fig. 2A). In contrast, clonidine did significantly reduce the glucagon-stimulated cAMP levels in hepatocytes that were cultured in a mixture of 10 nM dexamethasone and 10 nM insulin (Fig. 2B). The dose-response curves were consistently shifted to the right and the maximal responses in cAMP levels were significantly reduced in the presence of clonidine (by 21%, lower dose of clonidine; by 33%, higher dose of clonidine). Clonidine at 1 and 10 μ M had no significant effect on the basal cAMP levels in hepatocytes maintained in monolayer cultures. Pertussis toxin treatment of the hepatocytes abolished the inhibition by clonidine of the glucagon-stimulated glucose output (Fig. 3, inset) and cAMP levels (Fig. 3). The maximal

responses of glucose output and cAMP levels by glucagon tended to increase as compared to the control, when hepatocytes were cultured with pertussis toxin (100 ng/ml).

Effect of clonidine on forskolin-stimulated cAMP production

Figure 4 shows the effect of the α_2 -adrenergic receptor agonist clonidine on forskolin-stimulated cAMP production in hepatocytes cultured with 10 nM dexamethasone alone or in combination with 10 nM insulin. In both cultures, forskolin, a direct activator of adenylate cyclase (14, 15), caused a large increase in cAMP production. The α_2 -adrenergic agonist clonidine caused little change in the forskolin-stimulated cAMP production in hepatocytes cultured without insulin. In contrast, clonidine was

Fig. 3. The effects of pertussis toxin on the inhibition by clonidine of the glucagon-stimulated glucose output and cAMP levels in hepatocytes. After an attachment period of 3 hr, thiol-preactivated pertussis toxin (100 ng/ml) was added to the serum-free Williams' medium E supplemented with 10 nM dexamethasone and 10 nM insulin. The incubation was continued for a further 21 hr at 37°C. Then they were washed twice with Dulbecco's phosphate-buffered saline and incubated for 10 min at 37°C in Hanks-10 mM Hepes buffer (pH 7.2) containing 10 mM lactate with various concentrations of glucagon with or without clonidine as described in Fig. 1. cAMP levels of the cells were assayed as described under experimental procedures. Results are reported as the mean $\pm$ S.E.M. of 3-4 independent preparations. Glucagon ($\circ$), glucagon + 1 μ M clonidine ($\triangle$), glucagon + 10 μ M clonidine ($\square$); thin line: glucagon alone (without pertussis toxin treatment); Inset: the effects of pertussis toxin on the inhibition by clonidine of the glucagon-stimulated glucose production in hepatocytes. Glucose output was determined under the same conditions as described in Fig. 1. Glucagon ($\circ$), glucagon + 1 μ M clonidine ($\triangle$), glucagon + 10 μ M clonidine ($\square$).

Fig. 4. The effects of yohimbine on the inhibition by clonidine of the forskolin-stimulated cAMP production in hepatocytes cultured with 10 nM dexamethasone (Dex) and 10 nM insulin (Ins). Adult rat hepatocytes were cultured as those described in Materials and Methods. After the culture, the cells were washed twice with 2.0 ml of Dulbecco's phosphate-buffered saline. They were preincubated for 5 min at 37°C with Hanks-10 mM Hepes (pH 7.4) in the presence of 0.2 mM IBMX. Then 10 μ M forskolin and various concentrations of clonidine were added with or without 10 μ M yohimbine, and the incubation was continued for 10 min. Each column indicates the mean $\pm$ S.E.M. of 3-4 independent preparations. * $P < 0.05$, compared with forskolin alone. N: no addition, F: forskolin alone.

able to produce dose-dependent inhibition of forskolin-stimulated cAMP production in hepatocytes cultured with 10 nM dexamethasone and 10 nM insulin. Another α_2 -agonist, oxymetazoline, also showed the same effects as clonidine on forskolin-stimulated cAMP production (data not shown). The maximal inhibition obtained for each agonist (100 μ M) was about 25%. The similar effect of clonidine was observed in hepatocytes cultured with 100 nM insulin (data not shown). The α_2 -adrenergic receptor agonist-induced inhibition was almost completely blocked by combination with the α_2 -adrenoceptor-selective antagonist yohimbine (10 μ M).

Time course of appearance of α_2 -adrenergic response and inhibitory effect of cycloheximide

The time course of appearance of the α_2 -adrenergic response in adult rat hepatocytes was investigated in terms of the inhibition of forskolin-stimulated cAMP formation (Fig. 5). Suppressing effects of oxymetazoline on forskolin-stimulated cAMP formation was dose- and time-dependent. The maximal inhibition (22%) by 100

μ M oxymetazoline was seen in hepatocytes cultured for 7 hr with insulin. To determine whether the synthesis of new protein is involved in the α_2 -receptor-mediated response, the effect of cycloheximide (0.5 μ M) on the appearance of α_2 -response was examined. The α_2 -response was blocked almost completely by the addition of cycloheximide (0.5 μ M) to the culture medium, while cAMP production by 10 μ M forskolin was partially inhibited by the cycloheximide treatment (Fig. 6).

DISCUSSION

In this study, it was demonstrated that clonidine (1 and 10 μ M) dose-dependently inhibited the glucagon-stimulated glucose production from 10 mM lactate in hepatocytes that were cultured in the presence of 10 nM dexamethasone and 10 nM insulin, but this was not the case when the cells were cultured with 10 nM dexamethasone alone (Fig. 1). The effect of clonidine seemed to correlate well with its suppressive effect on glucagon-stimulated cAMP production (Fig. 2). These results indicate that hepato-

Fig. 5. Time course of the appearance of α_2 -adrenergic response in adult rat hepatocytes during primary culture. After the 3-hr attachment period, the culture medium was replaced by aspiration. Then the hepatocytes were cultured for a further 2, 4, 7 and 21 hr in the presence of dexamethasone (10 nM) with or without insulin (10 nM). The α_2 -response was determined as described in Fig. 4. Results are expressed as percent inhibition of forskolin-stimulated cAMP production and reported as the mean $\pm$ S.E.M. of 3 independent preparations. Oxymetazoline ($\circ$, 1 μ M; $\triangle$, 10 μ M; $\square$, 100 μ M). * $P < 0.05$, compared with the control ($\bullet$, 10 nM dexamethasone alone).

Fig. 6. Effect of cycloheximide on α_2 -adrenergic response in adult rat hepatocytes during primary culture. After the 3-hr attachment period, the hepatocytes were cultured for a further 4 or 21 hr in the presence of 10 nM insulin and 10 nM dexamethasone with or without cycloheximide (CHX, 0.5 μ M). The α_2 -response was determined in hepatocytes from the control and cycloheximide treatment groups. Each column indicates the mean $\pm$ S.E.M. of 3 independent preparations. F, 10 μ M forskolin alone. * $P < 0.05$, compared with forskolin alone.

cytes become responsive to α_2 -adrenergic receptor stimulation during culture with 10 nM insulin. In addition, the involvement of G_i -proteins in the regulation of adenylate cyclase activity in hepatocytes has been suggested, since the inhibitory effects of clonidine on the glucose production and cAMP levels could be reversed by pertussis toxin treatment (Fig. 3). These results indicate that the α_2 -adrenergic receptor couples functionally to adenylate cyclase through pertussis toxin-sensitive G_i -protein in hepatocytes cultured with 10 nM dexamethasone and 10 nM insulin.

The observation that forskolin, a direct stimulator of adenylate cyclase, activated cAMP production and its effects can be significantly inhibited by α_2 -adrenergic receptor activation are consistent with the appearance of α_2 -adrenergic receptor - G_i -protein - adenylate cyclase function in this cell preparation.

The findings that yohimbine completely reversed the α_2 -response produced by clonidine (Fig. 4) and oxymetazoline (data not shown) further support the possibility that the inhibition of forskolin-stimulated cAMP production is mediated by α_2 -adrenergic receptors. In these experiments, the α_2 -receptors mediated only a partial inhibition of forskolin-stimulated cAMP production. This is probably because clonidine is a partial agonist and oxymetazoline is a relatively weak activator of the hepatocyte α_{2B} -receptor subtype (16–18).

Although the definitive mechanism of action of insulin in stimulating the α_2 -adrenergic response is unknown, inhibitory effects of clonidine (or oxymetazoline) on the glucose output and cAMP production may be due to: 1) increase in the α_2 -adrenergic receptor number and/or 2) increase in functional coupling with α_2 -adrenergic receptor and G_i -protein that mediates suppression of adenylate cyclase. The present results suggest that the synthesis of new protein(s) is involved in increases in α_2 -adrenergic responsiveness acquired in adult male rat hepatocytes during primary culture with insulin. The target protein(s) may be α_2 -adrenergic receptors, since hepatocyte plasma membrane has been reported to contain abundant functional G_i -protein and adenylate cyclase (2, 19). The insulin concentration used in these experiments (10 nM) is about one order of magnitude higher than that reported for maximal plasma levels of insulin (~ 1 nM), but is comparable to the concentrations generally used to promote growth in hepatocyte culture (10 to 100 nM) (7, 8, 20). Therefore, it is tempting to speculate that the effect of insulin may be related to its growth-promoting activity. In addition, I also observed increased sensitivity to α_2 -adrenergic agonists in hepatocytes cultured with epidermal growth factor (20 ng/ml) in addition to dexamethasone where maximal inhibition by oxymetazoline of forskolin-stimulated cAMP production was about 30%

(unpublished data). However, it remains to be determined whether expression of α_2 -adrenergic receptor-specific mRNA and a change of α_2 -adrenergic receptor number are responsible for the present observations.

In conclusion, the present study provides the first experimental demonstration that insulin induces the appearance of α_2 -adrenergic receptor - G_i -protein - adenylate cyclase function in serum-free primary cultures of adult rat hepatocytes.

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