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Density-dependent proliferation of adult rat hepatocytes in primary culture induced by epidermal growth factor is potentiated by cAMP-elevating agents

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Abstract

We investigated whether or not epidermal growth factor (EGF) and cAMP-elevating agents induce the proliferation of adult rat hepatocytes during the early (4 h after adding EGF) and late phases (21 h after adding EGF) of primary cultures. Adult rat hepatocytes did not significantly proliferate after culture with 20 ng/ml EGF for 4 h at a density of 1×10^5 cells/cm^2. In contrast, when the density was decreased by about one-third to 3.3×10^4 cells/cm^2, the number of nuclei increased about 1.2-fold after culture with 10–20 ng/ml EGF for 4 h. Under these culture conditions, DNA synthesis began within 2–4 h of exposure to 20 ng/ml of EGF, although at the high cell density, DNA was not synthesized during this period. The β-adrenoceptor agonists, metaproterenol and isoproterenol, and other cAMP-elevating agents, such as glucagon, forskolin, and dibutyryl cAMP, potentiated both hepatocyte DNA synthesis and proliferation about 1.4-fold when cultured in combination with 20 ng/ml EGF. The stimulatory effects of metaproterenol and other cAMP-elevating agents were specifically blocked by the cAMP-dependent protein kinase inhibitor, H-89 (10^{-7} M). The effect of EGF was almost completely suppressed by genistein (5×10^{-6} M) and rapamycin (10 ng/ml), but it was unaffected by wortmannin (10^{-7} M). These results demonstrate that mature rat hepatocytes can proliferate very rapidly in low-density cultures with EGF, the effects of which were potentiated by β-adrenoceptor agonists and cAMP-elevating agents. In addition, the activation of receptor tyrosine kinase and p70 ribosomal protein S6 kinase may be involved in EGF-induced hepatocyte DNA synthesis and proliferation. © 1997 Elsevier Science B.V.

Keywords: EGF (epidermal growth factor); β-Adrenoceptor agonist; Forskolin; DNA synthesis; Hepatocyte proliferation

1. Introduction

Hepatocyte proliferation is controlled by a series of highly integrated events (Michalopoulos, 1990). When two-thirds of the rat liver is removed, the remaining cells undergo replication. The original mass of tissue is restored within one week. The most remarkable feature of liver regeneration is not only the fact that it proceeds at all, but that it stops at the point of the original hepatic mass. Therefore, most investigators studying hepatocyte proliferation in vivo and in vitro have focused upon proliferation-initiating and terminating factors and their mechanisms of action. Several of these factors have been defined.

Some aspects of liver cell proliferation in vivo should be recognized as they are relevant to primary cultures of hepatocytes in vitro. Early studies on hepatocytes in primary culture indicated little mitogenic activity at confluence, although the cells entered S phase, when insulin and epidermal growth factor (EGF) were added at 2 days in vitro (Richman et al., 1976; McGowan et al., 1981). In contrast, adult rat hepatocytes can initiate DNA synthesis and replication in response to EGF and insulin with a shorter lag time (20 h) when cultured at a low cell density (Nakamura et al., 1983a; Takai et al., 1988). Therefore, the cell division appeared to be dependent on the mitogens in the culture medium as well as the plating density.

On the other hand, the role of the second messenger, cAMP, in the control of hepatocyte proliferation is controversial. Cyclic AMP can either stimulate or inhibit DNA synthesis depending on the experimental conditions (Bronstad et al., 1983; Vintermyr et al., 1989; Refsnes et al., 1992). For example, elevated hepatocyte cAMP levels inhibited hepatocyte growth factor-stimulated DNA synthesis (Marker et al., 1992). In contrast, cAMP and EGF may potently stimulate the growth of hepatocytes (Fried-

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man et al., 1981) and other cell types (Rosengurt, 1982) and mediate liver regeneration following injury (Bronstad and Christoffersen, 1980). Furthermore, adrenaline responsiveness increases in the liver after partial hepatectomy and in other situations that increase hepatocyte proliferation in vivo (Refsnes et al., 1983; Sandnes et al., 1986; Mahler and Wilce, 1988). Under these conditions, elevated cAMP levels precede and may be required for the onset of DNA replication. Therefore, it is of interest to determine how β-adrenoceptor agonists and cAMP are involved in transduction of the proliferative response in primary cultures of hepatocytes.

There are some indications that a high β-adrenoceptor-mediated response and cell growth are related, since hepatocytes from young rats (Christoffersen et al., 1973), regenerating liver (Bronstad and Christoffersen, 1980) and rats exposed to carcinogens (Christoffersen et al., 1972) show a high β-adrenoceptor-mediated response. In a previous report, we showed that fresh hepatocytes, which show a very low β-adrenoceptor-mediated response, become responsive to the β_2-adrenoceptor agonist, metaproterenol, when cultured with 20 ng/ml EGF for 3–7 h (Ogihara, 1996b). Therefore, whereas EGF alone can stimulate cell proliferation in adult rat hepatocytes, it is likely that expression of the hepatocyte β-adrenoceptor-mediated response elicited by EGF also plays a positive role in this process. Furthermore, the transient effects of cAMP on the EGF-stimulated induction of hepatocyte proliferation have not been studied. We therefore investigated whether or not hepatocytes can respond to EGF, metaproterenol and other agents that increase cAMP to produce cell division. The results showed that the proliferation of adult rat hepatocytes induced by EGF proceeds rapidly (4 h after culture) and is significantly potentiated by β-adrenoceptor agonists and other cAMP-elevating agents in primary cultures at low density.

2. Materials and methods

2.1. Animals

Male rats (weighing 200–250 g) of the Wistar strain were obtained from Saitama Experimental (Saitama, Japan). The rats were housed in animal rooms at 21 ± 2°C with a relative humidity of 55 ± 10% and 12 h day/night cycle (lights on, 6 a.m.–6 a.m.). They had free access to commercial food pellets (MF, Oriental Yeast, Japan) and water.

2.2. Procedure

2.2.1. Hepatocyte isolation and culture

Parenchymal cells were isolated from male Wistar rats by perfusing the liver in situ with collagenase, essentially as described by Seglen (1975). Routinely, more than 93% of the cells were intact as monitored by trypan blue dye exclusion. Hepatocytes were suspended in Williams' medium E containing 10^{-9} M dexamethasone, 5% newborn calf serum, 0.1 μg/ml aprotinin, 100 μg/ml streptomycin and 100 U/ml penicillin. Unless otherwise indicated, hepatocytes at a density of 3.3×10^4 cells/cm^2 (low density) were plated in collagen-coated six-well plastic plates (35-mm diameter; Sumitomo Bakelite, Tokyo, Japan), then incubated at 37°C under an atmosphere of 5% CO_2 in humidified air. After 3 h, non-attached cells were removed by aspiration, and attached cells were washed twice with serum-free Williams' medium E containing dexamethasone, aprotinin, and the antibiotics as described above. The medium was finally replaced by serum-free Williams' medium E containing 20 ng/ml EGF. The cAMP- elevating agents and other factors were added directly to the culture medium and the cells were incubated for a further 4 and 21 h, respectively.

2.2.2. Counting nuclei

To precisely measure hepatocyte proliferation, we counted the nuclei instead of the cells according to the procedure of Nakamura et al. (1983a) with minor modifications. Briefly, cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4), then lysed by a 30 min incubation with 0.25 ml of 0.1 M citric acid containing 0.1% Triton X-100 at 37°C. This procedure was performed because the hepatocytes firmly attached to the collagen-coated plates and were not dispersed by EDTA-trypsin. An equal volume of the nucleus suspension was mixed with 0.3% Trypan blue in phosphate-buffered saline and the nuclei were counted in a hemocytometer. The plating efficiency of hepatocytes as determined by the number of nuclei was consistently over 96% in 3 h at 37°C under 5% CO_2 in humidified air.

2.2.3. Measurement of DNA synthesis

DNA synthesis was assessed by measuring the amount of [^{3}H]thymidine incorporated into acid-precipitable materials (Morley and Kingdon, 1972). Briefly, after an attachment period of 3 h, cultured hepatocytes were washed twice with serum-free Williams' medium E. Cells were pulsed at 2 and 19 h post-EGF stimulation for 2 h with 0.5 μCi/ml [^{3}H]thymidine and incorporation into DNA was determined as described below. After 2 h, the incorporation was stopped by removing the medium and immediately adding 2 ml of ice-cold phosphate-buffered saline (pH 7.4). Cells were washed twice with ice-cold phosphate-buffered saline and incubated in 5% trichloroacetic acid for 30 min at 4°C. The 5% trichloroacetic acid was then aspired and the cells were solubilized with 1 ml of 20% sodium carbonate containing 0.1 M sodium hydroxide, 1% sodium dodecyl sulfate. Thereafter, 0.4 ml of this solution was neutralized by 40% trichloroacetic acid and the amount of [^{3}H]thymidine incorporation was measured using a liquid scintillation counter (Aloka, model 5100).

Specific [^{3}H]thymidine incorporation was determined by subtracting the value obtained in the presence of the DNA polymerase α inhibitor, aphidicolin (10 μg/ml), since the values of the incorporation of [^{3}H]thymidine in the presence of aphidicolin (or hydroxyurea), subtracted from that in the absence, yields the true replicative incorporation rate. DNA synthesis is described as dpm/h/mg protein. Cellular protein was determined by modification of the Lowry procedure using bovine serum albumin as the standard (Lee and Paxman, 1972).

2.3. Materials

The following reagents were obtained from Sigma (St. Louis, MO, USA): EGF (human recombinant), forskolin, dibutyryl cAMP (db-cAMP), aphidicolin, genistein, metaproterenol hemisulfate, butoxamine hydrochloride, metoprolol tartrate, dobutamine hydrochloride, 1-methyl-3-isobutylxanthine (IBMX), insulin (porcine), glucagon (porcine), wortmannin, rapamycin and dexamethasone. H-8 · 2HCl (*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). UK14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline; Cambridge, 1981) was generously provided by Pfizer Central Research (Sandwich, UK). Williams' medium E and newborn bovine serum albumin were purchased from Flow Laboratories (Irvine, UK). Collagenase (type II) was purchased from Worthington (Freehold, NJ, USA). [*methyl*-^{3}H]Thymidine (20 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, USA). All other reagents were of analytical grade.

2.4. Statistics

Values reported are expressed as mean ± S.E.M. Data were analyzed by the unpaired Student's *t*-test. *P* values less than 0.05 were regarded as statistically significant.

3. Results

3.1. Time-course of the stimulation of hepatocyte DNA synthesis and proliferation by EGF, with or without metaproterenol

Fig. 1 shows significant [^{3}H]thymidine incorporation into DNA during culture with EGF (20 ng/ml) for 3.0 h (6.0 h after plating), and that the level remained constant for the following 1 h. The number of nuclei significantly increased at about 4 h after adding EGF (Fig. 1). The very rapid stimulation of DNA synthesis by EGF in cultured hepatocytes was preceded by an increase in the number of nuclei. When EGF (20 ng/ml) and metaproterenol (10^{-6} M) were simultaneously added, hepatocyte DNA synthesis was potentiated within 2.5 h after and the number of nuclei was significantly increased (Fig. 1). To determine whether or not the metaproterenol effect was mediated through cAMP, metaproterenol was replaced with the cell-permeable cAMP analogue, db-cAMP. As shown in Fig. 2, db-cAMP (10^{-7} M) also potentiated the EGF-induced increase in the number of nuclei within a similar time frame.

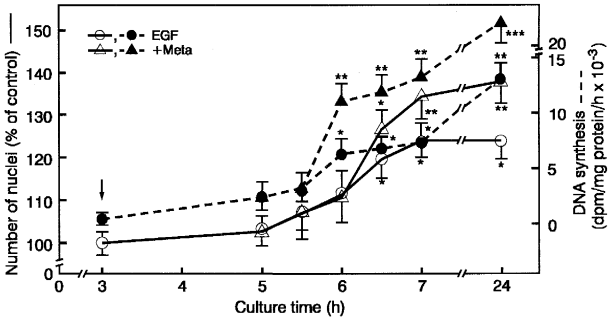

Fig. 1. Time-course of hepatocyte DNA synthesis and number of nuclei (proliferation) stimulated by EGF, with or without metaproterenol. Fresh hepatocytes were prepared by collagenase perfusion of a normal rat liver. Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured in Williams' medium E containing 5% newborn calf serum and dexamethasone (10^{-9} M) for 3 h. Medium was then replaced with fresh serum-free Williams' medium E containing EGF (20 ng/ml) alone and with metaproterenol (10^{-6} M) as indicated by the arrow. [^{3}H]Thymidine (1.0 μCi/well) was added at 2 or 19 h after EGF stimulation. DNA synthesis was measured by incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable materials as described in Section 2. EGF (●, dotted lines); EGF with metaproterenol (▲, dotted lines). Nuclei of hepatic parenchymal cells were processed by exposing hepatocyte cultures to 0.1 M citric acid containing 1% Triton X-100, then counted using a hemocytometer as described in Section 2. EGF (○, solid lines); EGF and metaproterenol (△, solid lines). Results are expressed as mean ± S.E.M. of 3 independent preparations. Values significantly different from respective controls are indicated by * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

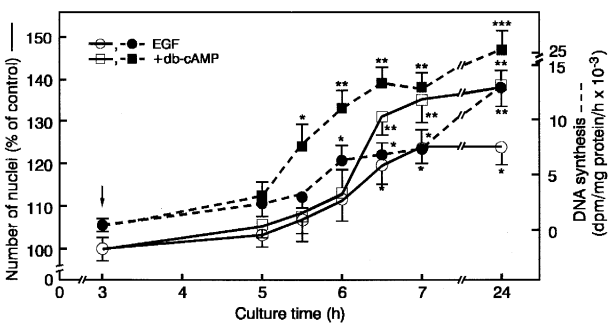

Fig. 2. Time-course of hepatocyte DNA synthesis and number of nuclei (proliferation) stimulated by EGF with or without db-cAMP in cultured hepatocytes. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured as described in the legend to Fig. 1, except metaproterenol was replaced by db-cAMP (10^{-7} M). EGF (●, dotted lines); EGF with db-cAMP (■, dotted lines). Nuclei of hepatic parenchymal cells were processed and counted as described in the legend to Fig. 1. EGF (○, solid lines); EGF and db-cAMP (□, solid lines). Results are expressed as mean ± S.E.M. of 3 independent preparations. Values significantly different from respective controls are indicated by * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

3.2. Effect of EGF concentration on hepatocyte DNA synthesis and proliferation

Since EGF very rapidly stimulated hepatocyte DNA synthesis and proliferation when cultured at a density of 3.3×10^4 cells/cm^2 for 4 h, we examined the dose-response effects of EGF on hepatocyte DNA synthesis and proliferation in low-density culture. The stimulatory effect of EGF on hepatocyte DNA synthesis was dose-dependent with the maximal concentration being about 10 ng/ml. EGF alone, dose-dependently increased the number of nuclei by about 1.2-fold with the maximal concentration being 10 ng/ml (Fig. 3).

3.3. Influence of cell density on the effects of metaproterenol and db-cAMP on the EGF-stimulated DNA synthesis and proliferation

We investigated the density dependence of hepatocyte DNA synthesis and proliferation induced by EGF and its potentiation by the β_2-adrenoceptor agonist, metaproterenol (10^{-6} M), in cells cultured for 4 h at various densities. Fig. 4 shows that EGF stimulated DNA synthesis in primary culture of adult rat hepatocytes at a low cell density. In contrast, when the hepatocytes were cultured at a high density, the stimulatory effects of EGF on hepatocyte DNA synthesis were reduced or absent. Metaproterenol markedly potentiated the EGF-stimulated hepatocyte DNA synthesis at a lower cell density. Fig. 5 shows the influence of cell density on the effects of EGF with and without metaproterenol on the number of nuclei in hepatocytes cultured for 4 and 21 h. The levels of EGF- and metaproterenol-stimulated hepatocyte proliferation (at 4 and 21 h) were increased at a lower cell density and absent at near-confluence (1×10^5 cells/cm^2). The effects of metaproterenol were replaced by glucagon (10^{-7} M), which is also associated with the adenylate cyclase system (data not shown).

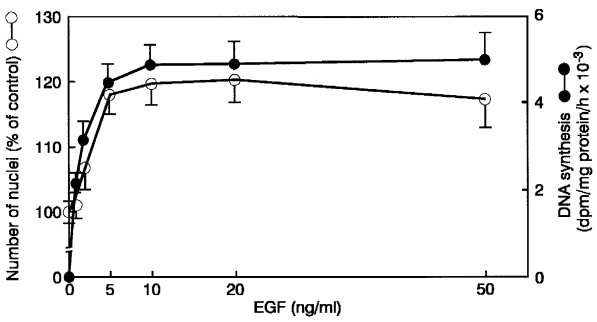

Fig. 3. Effect of EGF concentration on hepatocyte DNA synthesis and number of nuclei (proliferation). For details of hepatocyte DNA synthesis and nucleus counting see legend to Fig. 1. Number of nuclei (○); DNA synthesis (●). Each value is expressed as mean ± S.E.M. from 3 independent preparations.

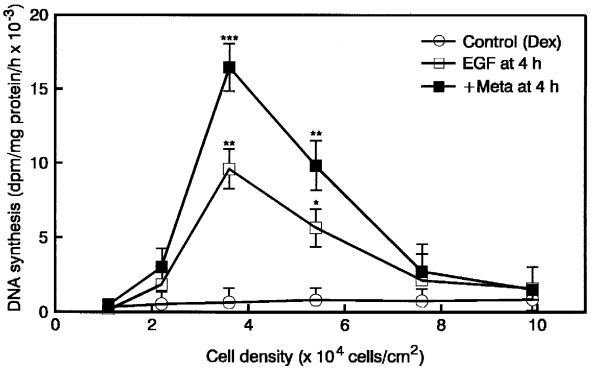

Fig. 4. Influence of cell density on the effect of metaproterenol on EGF-stimulated DNA synthesis at an early phase (4 h after EGF addition) of culture. Hepatocytes were plated at various densities and cultured as described in the legend to Fig. 1. Dexamethasone alone (○); EGF alone (□); EGF with metaproterenol (■). Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from respective controls are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.4. Dose-dependent effects of metaproterenol and db-cAMP on EGF-stimulated hepatocyte DNA synthesis and increase in number of nuclei at early and late phases of primary culture

We then examined the dose-dependent effects of metaproterenol and db-cAMP on EGF-stimulated hepatocyte DNA synthesis and proliferation in hepatocytes cultured at the density of 3.3×10^4 cells/cm^2 (low density). Table 1 shows dose-response effects of metaproterenol and db-cAMP on EGF-stimulated hepatocyte DNA synthesis during 4 (early phase) and 21 h (late phase) of culture. EGF-stimulated hepatocyte DNA synthesis was potentiated by the β_2-adrenoceptor agonist metaproterenol at peak concentrations of 10^{-6} and 3×10^{-6} M, but only slightly inhibited at 10 μM. Similar results were obtained using

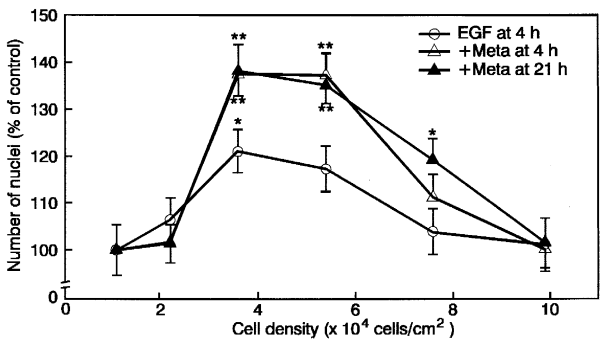

Fig. 5. Influence of cell density on the effect of metaproterenol on EGF-stimulated increase in number of nuclei. Hepatocytes were cultured at various densities for 4 or 21 h after 20 ng/ml EGF addition. Density-dependent changes in number of nuclei were measured in the absence and presence of 1×10^{-6} M metaproterenol (Meta). EGF alone (○); EGF with metaproterenol at 4 h (△); EGF with metaproterenol at 21 h (▲). Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from respective controls are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1
Dose-dependent effects of metaproterenol and dibutyryl cAMP on EGF-stimulated hepatocyte DNA synthesis and increase in number of nuclei at early (4 h) and late phases (21 h) of culture

Treatment		DNA synthesis (dpm/mg protein per h $\times 10^{-3}$)		Number of nuclei (% of control)	
		Culture time (h)		Culture time (h)	
		4	21	4	21
Control (dexamethasone 1 nM)		0.732 ± 0.430	0.743 ± 0.130	100.0 ± 4.0	100.2 ± 3.3
EGF		6.863 ± 1.530	13.011 ± 1.413	120.4 ± 4.0	119.8 ± 3.2
+ metaproterenol	0.1 μM	7.571 ± 1.463	16.190 ± 1.642	123.3 ± 4.0	123.9 ± 3.2
	0.3 μM	9.072 ± 2.229	17.963 ± 1.084 [a]	128.1 ± 3.6	130.1 ± 4.2
	1 μM	14.610 ± 1.452 [a]	22.983 ± 2.542 [a]	142.4 ± 5.0 [a]	137.3 ± 3.3 [a]
	3 μM	12.212 ± 1.130 [a]	22.300 ± 2.382 [a]	141.0 ± 4.9 [a]	136.9 ± 3.6 [a]
	10 μM	10.191 ± 1.943	20.651 ± 1.700 [a]	139.3 ± 8.5	134.8 ± 6.5
+ dibutyryl cAMP	0.01 μM	7.860 ± 1.750	16.911 ± 1.362	136.5 ± 4.0 [a]	124.5 ± 4.9
	0.03 μM	10.482 ± 2.286	23.081 ± 2.082 [a]	139.2 ± 4.5 [a]	132.3 ± 3.9
	0.1 μM	13.833 ± 1.462 [a]	24.293 ± 2.323 [a]	140.8 ± 5.2 [a]	142.8 ± 5.5 [a]
	0.3 μM	9.180 ± 2.032	21.673 ± 2.565 [a]	124.1 ± 4.8	123.8 ± 4.1
	1 μM	8.546 ± 0.833	17.503 ± 3.833	119.9 ± 4.9	119.9 ± 3.7
	10 μM	7.992 ± 1.663	15.736 ± 2.346	107.4 ± 3.4	106.9 ± 3.6

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2. After an attachment period of 3 h, they were cultured for a further 4 and 21 h with 20 ng/ml EGF alone or EGF with various concentrations of metaproterenol or dibutyryl cAMP. For details of hepatocyte DNA synthesis and nucleus counting see legend to Fig. 1. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from EGF alone are indicated by [a] $P < 0.05$.

the non-specific β-adrenoceptor agonist, isoproterenol (10^{-7} and 10^{-6} M; data not shown). In addition, db-cAMP in combination with EGF also had biphasic effects on hepatocyte DNA synthesis. Dibutyryl cAMP at lower concentrations (3×10^{-8} and 10^{-7} M) stimulated hepatocyte DNA synthesis, whereas at higher concentrations (10^{-6} M and 10^{-5} M) it was markedly reduced. Table 1 also shows that the stimulatory effects of metaproterenol and db-cAMP on EGF-stimulated [^{3}H]thymidine incorporation were simply related to the increase in the number of nuclei (proliferation).

3.5. Effect of adrenoceptor agonists and cAMP-elevating agents on EGF-stimulated hepatocyte DNA synthesis and proliferation

Since cAMP mediates the effects of β_2-adrenoceptor agonists, we examined the potential of glucagon, forskolin, and db-cAMP that promote intracellular cAMP levels by several mechanisms. As shown in Fig. 6, the EGF-stimulated hepatocyte proliferation was significantly potentiated by the β_2-adrenoceptor agonist metaproterenol (10^{-6} M), as well as glucagon (10^{-7} M), forskolin (10^{-7} M) and db-cAMP (10^{-7} M) at early and late phases of culture.

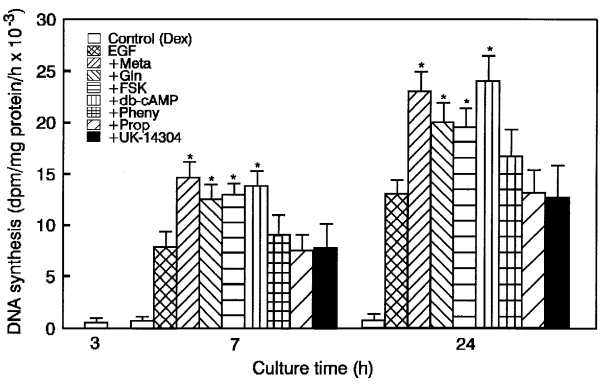

Fig. 6. Effect of adrenoceptor agents and cAMP-elevating agents on EGF-stimulated DNA synthesis. Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured for 4 and 21 h with EGF (20 ng/ml) alone or EGF with various cAMP-elevating agents. Meta, metaproterenol (10^{-6} M); Gln, glucagon (10^{-7} M); FSK, forskolin (10^{-7} M); db-cAMP, dibutyryl cAMP (10^{-7} M); Pheny, phenylephrine (10^{-4} M); Prop, propranolol (10^{-5} M); Aphi, aphidicolin (10 μg/ml). Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from EGF alone are indicated by * $P < 0.05$.

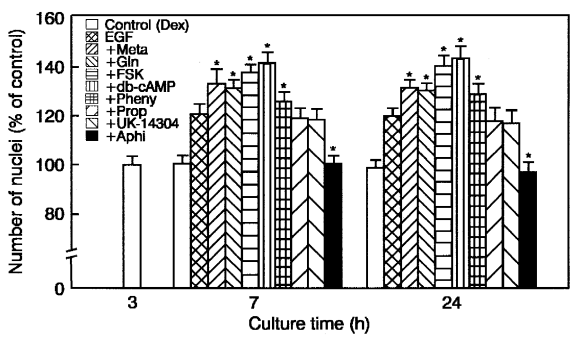

Fig. 7. Effect of adrenoceptor agonists and cAMP-elevating agents on EGF-stimulated increase in number of nuclei. Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and cultured for 4 and 21 h with EGF (20 ng/ml) alone or EGF with various cAMP-elevating agents. Meta, metaproterenol (10^{-6} M); Gln, glucagon (10^{-7} M); FSK, forskolin (10^{-7} M); db-cAMP, dibutyryl cAMP (10^{-7} M); Pheny, phenylephrine (10^{-4} M); Prop, propranolol (10^{-5} M); Aphi, aphidicolin (10 μg/ml). Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from EGF alone are indicated by * $P < 0.05$.

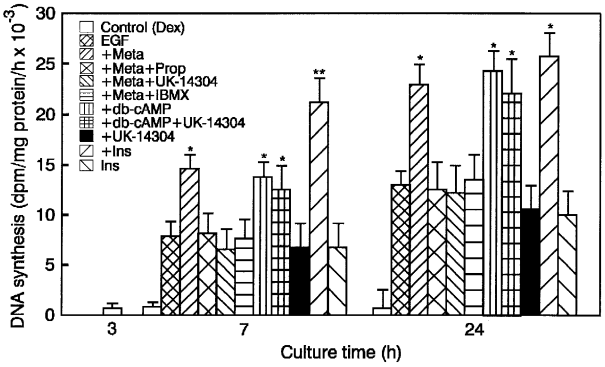

Fig. 8. Interaction between adrenoceptor agents on metaproterenol- and db-cAMP-stimulated DNA synthesis. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured for 4 and 21 h. Adrenoceptor agonists or antagonists with metaproterenol or db-cAMP were added immediately after medium change. Meta, metaproterenol (10^{-6} M); UK14304 (10^{-6} M); IBMX, 1-methyl-3-isobutylxanthine (2×10^{-4} M); db-cAMP, dibutyryl cAMP (10^{-7} M); Prop, propranolol (10^{-5} M); Ins, insulin (10^{-7} M). Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from EGF alone are indicated by * $P < 0.05$.

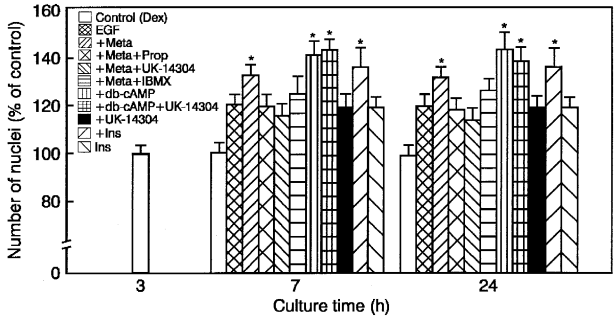

Fig. 9. Interaction between adrenoceptor agents on metaproterenol- and db-cAMP-stimulated increases in number of nuclei. Experimental details are the same as those described in the legend to Fig. 8. Meta, metaproterenol (10^{-6} M); db-cAMP, dibutyryl cAMP (10^{-7} M); Prop, propranolol (10^{-5} M); UK-14304 (10^{-6} M); IBMX, 1-methyl-3-isobutylxanthine (2×10^{-4} M); Ins, insulin (10^{-7} M). Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from EGF alone are indicated by * $P < 0.05$.

Metaproterenol, glucagon, forskolin, and db-cAMP alone had no direct effects on hepatocyte DNA synthesis and proliferation during these periods (data not shown). The α_1-adrenoceptor agonist, phenylephrine (10^{-6} M–10^{-4} M), the α_2-adrenoceptor agonist, UK14304, and the β-adrenoceptor antagonist, propranolol, did not affect EGF-induced hepatocyte DNA synthesis at the early and late phases of culture. As shown in Fig. 7, the stimulatory effects of these cAMP-elevating agents on EGF-induced [^{3}H]thymidine incorporation are simply related to the increases in the number of nuclei. Aphidicolin (10 μg/ml) completely inhibited the EGF-induced increase in the number of nuclei.

3.6. Interaction between adrenergic agents on the metaproterenol- and db-cAMP-stimulated hepatocyte DNA synthesis and proliferation

Fig. 8 shows that the metaproterenol-stimulated hepatocyte DNA synthesis was blocked by propranolol (10^{-5} M) without affecting the EGF response. UK14304 inhibited hepatocyte DNA synthesis caused by 1.0 μM metaproterenol in the presence of 20 ng/ml EGF.

Table 2
Effects of specific β_1- and β_2-adrenoceptor antagonists and H-89 on potentiation of hepatocyte DNA synthesis and number of nuclei increased by cAMP-elevating agents

Treatment	DNA synthesis (dpm/mg protein per h × 10^{-3})		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control (dexamethasone)	0.487 ± 0.044	0.477 ± 0.044	100.0 ± 4.0	100.2 ± 3.3
Dexamethasone + H-89	0.487 ± 0.034	0.475 ± 0.037	100.1 ± 2.8	103.8 ± 4.1
EGF	6.769 ± 1.243	14.385 ± 1.075	123.4 ± 4.0	119.8 ± 3.2
+ metaproterenol	15.227 ± 1.438 [a]	25.039 ± 2.614 [a]	138.4 ± 3.3 [a]	137.9 ± 3.4 [a]
+ metoprolol	6.518 ± 1.341	14.222 ± 1.633	118.9 ± 3.6	121.1 ± 4.2
+ metaproterenol + metoprolol	14.833 ± 1.443 [a]	24.745 ± 2.286 [a]	136.7 ± 2.6 [a]	135.3 ± 2.8 [a]
+ butoxamine	6.663 ± 1.243	13.885 ± 1.275	122.0 ± 4.9	121.9 ± 3.6
+ metaproterenol + butoxamine	6.539 ± 1.366	13.385 ± 1.325	119.3 ± 4.8	118.8 ± 5.5
+ H-89	6.387 ± 1.201	14.353 ± 1.550	118.2 ± 3.5	117.7 ± 3.9
+ metaproterenol + H-89	6.211 ± 1.318	14.130 ± 1.805	116.8 ± 4.2	118.8 ± 4.5
+ dibutyryl cAMP	13.908 ± 1.482 [a]	24.353 ± 2.422 [a]	140.5 ± 4.5 [a]	142.5 ± 4.9 [a]
+ dibutyryl cAMP + H-89	7.373 ± 1.331	14.288 ± 1.172	119.9 ± 3.9	120.9 ± 2.7
+ dobutamine	6.896 ± 1.462	13.966 ± 2.123	121.9 ± 4.6	122.1 ± 3.3

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. Specific β_1- and β_2-adrenoceptor antagonists, H-89, metaproterenol and dibutyryl cAMP were added with 20 ng/ml EGF immediately after medium change and cells were cultured for a further 4 and 21 h. Dexamethasone, 10^{-9} M; metaproterenol, 10^{-6} M; metoprolol, 10^{-6} M; butoxamine, 10^{-6} M; H-89, 10^{-7} M; dibutyryl cAMP, 10^{-7} M; dobutamine, 10^{-6} M. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from EGF alone are indicated by [a] $P < 0.05$.

Table 3
Effect of specific inhibitors of signal-transducing elements on hepatocyte DNA synthesis and number of nuclei induced by EGF with and without metaproterenol

Treatment	DNA synthesis (dpm/mg protein per h $\times 10^{-3}$)		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control (dexamethasone)	0.885 ± 0.435	0.755 ± 0.044	101.5 ± 4.1	100.2 ± 3.3
EGF	7.234 ± 1.459	14.090 ± 1.366	120.6 ± 4.4	120.0 ± 3.5
+ metaproterenol	15.027 ± 0.938 [a]	24.039 ± 1.614 [a]	135.9 ± 3.1 [a]	136.9 ± 4.8 [a]
+ genistein	0.839 ± 0.337 [a]	0.755 ± 0.767 [b]	101.7 ± 5.1 [a]	101.6 ± 5.6 [a]
+ metaproterenol + genistein	0.864 ± 0.430 [a]	0.877 ± 0.788 [b]	104.6 ± 3.5 [a]	106.2 ± 3.4 [a]
+ metaproterenol + aphidicolin	0.434 ± 0.404 [a]	0.468 ± 0.332 [b]	100.7 ± 4.9 [a]	95.3 ± 6.4 [a]
+ wortmannin	6.539 ± 1.366	13.785 ± 1.075	123.3 ± 6.4	119.2 ± 4.2
+ rapamycin	0.462 ± 0.412 [a]	0.885 ± 1.505 [b]	100.6 ± 5.7 [a]	100.8 ± 5.3 [a]

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. Specific inhibitors of signal-transducing elements were added with 20 ng/ml EGF immediately after medium change and cells were cultured for a further 4 and 21 h. Dexamethasone, 10^{-9} M; metaproterenol, 10^{-6} M; genistein, 5×10^{-6} M; wortmannin, 10^{-7} M; rapamycin, 10 ng/ml; aphidicolin, 10 μg/ml. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from EGF alone are indicated by [a] $P < 0.05$, [b] $P < 0.01$.

Metaproterenol-stimulated hepatocyte DNA synthesis was reproduced by db-cAMP (10^{-7} M); however, the db-cAMP effect on the EGF-stimulated hepatocyte DNA synthesis was not influenced by UK14304 (10^{-6} M). The ability of UK14304 to inhibit hepatocyte DNA synthesis was reversed by yohimbine (10^{-5} M, data not shown). In contrast to earlier reports, insulin (10^{-7} M) alone significantly increased the rate of DNA synthesis and acted additively with EGF. As shown in Fig. 9, the stimulatory effects of metaproterenol and db-cAMP on EGF-induced [^{3}H]thymidine incorporation were simply related to the increases in the number of nuclei (proliferation). In addition, the metaproterenol-stimulated hepatocyte proliferation was blocked by propranolol (10^{-5} M) without affecting the EGF response. The db-cAMP effect on the EGF-stimulated hepatocyte proliferation was not affected by UK14304 (10^{-6} M). Insulin (10^{-7} M) alone significantly increased hepatocyte proliferation and acted additively with EGF.

3.7. Effect of specific β_1- and β_2-adrenoceptor antagonists and H-89 on potentiation of hepatocyte DNA synthesis and number of nuclei increased by cAMP-elevating agents

Table 2 shows that metoprolol (10^{-7}–10^{-5} M), a specific β_1-adrenoceptor antagonist, had no effect on the metaproterenol-stimulated DNA synthesis in the presence of EGF. In contrast, the metaproterenol effect was dose-dependently blocked by a specific β_2-adrenoceptor antagonist, butoxamine, in the range of 10^{-6} to 10^{-5} M at the early and late phases of culture. Metoprolol and butoxamine had no effect on the EGF-stimulated hepatocyte DNA synthesis. A specific β_1-adrenoceptor agonist, dobutamine (10^{-8}–10^{-5} M), did not stimulate the hepatocyte DNA synthesis induced by EGF. Table 2 shows that metoprolol (10^{-7}–10^{-5} M) alone had no effect on the metaproterenol-stimulated number of nuclei in the presence of EGF. In contrast, the metaproterenol effects were dose-dependently blocked by butoxamine in the range of 10^{-6} to 10^{-5} M. Metoprolol, butoxamine and dobutamine (10^{-8}–10^{-5} M) had no effect on EGF-stimulated hepatocyte proliferation.

The role of cAMP-dependent protein kinase in the transduction of various extracellular signals has been investigated using H-89, a selective inhibitor of the cAMP-dependent protein kinase activity in several cell systems (cf., Section 4). Therefore, we investigated the effects of H-89 on the rate of [^{3}H]thymidine incorporation stimulated by metaproterenol and cAMP-elevating agents in the presence of EGF. The results indicated that H-89 (10^{-7}, 10^{-6} M) specifically inhibits the hepatocyte DNA synthesis and the number of nuclei (Table 2) increased by metaproterenol (10^{-6} M) and db-cAMP (10^{-7} M), but not those induced by EGF (Table 2).

3.8. Effect of specific inhibitors of signal-transducing elements on hepatocyte DNA synthesis and number of nuclei induced by EGF with and without metaproterenol

We investigated whether or not the rapid mitogenic responses of hepatocytes to EGF and EGF with metaproterenol are mediated by the signal transducers, receptor tyrosine kinase, phosphoinositide 3-kinase and p70 ribosomal protein S6 kinase. The results showed that genistein, a specific receptor tyrosine kinase inhibitor, greatly diminished hepatocyte DNA synthesis and the increased nuclear number (Table 3) caused by 20 ng/ml EGF alone or with metaproterenol. To examine the involvement of phosphoinositide 3-kinase in hepatocyte mitogenesis, we investigated the effects of wortmannin, a potent inhibitor of phosphoinositide 3-kinase, on the DNA synthesis and increase in the nucleus number by EGF with or without metaproterenol. The results showed that hepatocyte DNA synthesis induced by EGF alone was unaffected by 10^{-8} or 10^{-7} M wortmannin. By contrast, wortmannin (10^{-7} M) almost completely inhibited hepatocyte DNA synthesis

and increased the nuclear number induced by metaproterenol. The results also showed that the immunosuppressant, rapamycin (10 ng/ml), markedly attenuated both the mitogenic effects of EGF and co-mitogenic effects of metaproterenol on both hepatocyte DNA synthesis and proliferation (Table 3). Aphidicolin (10 μg/ml) completely inhibited the hepatocyte DNA synthesis induced by EGF with metaproterenol, as well as the increase in the number of nuclei (Table 3).

4. Discussion

EGF requires other growth-promoting hormones to maximally stimulate DNA synthesis in hepatocytes in primary culture. Early studies on hepatocyte proliferation using a mixture of EGF and insulin stimulation were performed only during the relatively late phase of culture (Richman et al., 1976; McGowan et al., 1981). However, our findings indicated that the stimulation of DNA synthesis and proliferation by EGF alone proceed rapidly (within 4 h of adding EGF) in adult rat hepatocytes. Human recombinant EGF rapidly caused a dose-dependent increase in hepatocyte DNA synthesis and proliferation with the maximal effects at 10–20 ng/ml EGF in short-term cultures. The differences in time course cannot be explained, although it might reflect repair after collagenase perfusion and adaptation of hepatocytes to their experimental conditions. In accordance with earlier reports, we also found that the rapid proliferative effects of EGF were density-dependent. The density-dependent mechanisms of hepatocyte growth and proliferation are considered to be related to cell-to-cell contact (Nakamura et al., 1983a,b, 1984; Kajiyama and Ui, 1994) and the production of inhibitory autocrine factors (Nakamura et al., 1983a).

We showed that hepatocytes cultured with 20 ng/ml EGF at a low cell density acquire a rapid β-adrenergic response (Ogihara, 1996b). Since there is some indication that the β-adrenergic response and cell growth are closely related (cf., Section 1), we investigated the combined effect of EGF and β-adrenoceptor agonists on hepatocyte proliferation in primary culture. The results showed that the β_2-adrenoceptor agonists, metaproterenol, isoproterenol and glucagon (10^{-7} M, not shown) in combination with EGF, potentiated EGF-stimulated hepatocyte proliferation in a density-dependent manner (Fig. 4). Unlike EGF, metaproterenol and other cAMP-elevating agents alone essentially did not stimulate hepatocyte DNA synthesis and proliferation during these culture periods. Therefore, these agents may be considered co-mitogenic rather than as a primary growth factor.

Because cAMP mediates the effects of β-adrenoceptor agonists and glucagon, we explored the potential of other agents to elevate intracellular cAMP. Forskolin, IBMX, and db-cAMP, as distinct from that triggered by plasma membrane receptor, also rapidly and significantly potentiated the hepatocyte proliferation induced by EGF. This finding suggests that metaproterenol and other cAMP-elevating agents act via the same mechanism. The EGF-stimulated hepatocyte proliferation potentiated by metaproterenol and other cAMP-elevating agents was inhibited by the α_2-adrenoceptor agonist, UK14304 (10^{-6} M). In contrast, potentiation of EGF-stimulated hepatocyte proliferation by db-cAMP was resistant to UK14304 (10^{-6} M), suggesting that UK14304 acts through the inhibition of adenylate cyclase. The α_2-adrenoceptor-mediated response also increases rapidly in hepatocytes cultured with EGF (Ogihara, 1996a) or insulin (Ogihara, 1995).

The β-adrenoceptor consists of β_1 and β_2 subtypes, both of which activate adenylate cyclase. Therefore, we investigated which of the subtypes is mainly involved in the potentiation of EGF-stimulated hepatocyte DNA synthesis and proliferation. As shown in Table 2, the metaproterenol effect was inhibited by butoxamine, a β_2-adrenoceptor antagonist, and the non-specific β-adrenoceptor antagonist, propranolol, but not by metoprolol, a β_1-adrenoceptor antagonist, indicating that the potentiation of the EGF effects by metaproterenol is predominantly mediated through its β_2-adrenoceptor. This was further confirmed by the finding that dobutamine (10^{-6}–10^{-4} M), a β_1-adrenoceptor agonist, did not potentiate the EGF-induced hepatocyte DNA synthesis and proliferation.

We then examined whether the proliferative effect of metaproterenol, forskolin, and glucagon in synergy with EGF is dependent on cAMP-dependent protein kinase action. H-89 (10^{-7} M), a specific cAMP-dependent protein kinase inhibitor (Zuscik et al., 1994), alone did not affect EGF-stimulated hepatocyte proliferation. Inhibiting cAMP-dependent protein kinase activity with H-89 (10^{-7} M) in adult rat hepatocyte cultures significantly reduced the ability of metaproterenol and other cAMP-elevating agents to potentiate EGF-stimulated hepatocyte proliferation without affecting EGF action (Table 2). Therefore, we confirmed that the co-mitogenic effect of cAMP-elevating agents is mediated via cAMP-dependent protein kinase in primary cultures of adult rat hepatocytes. All these results support our hypothesis that EGF directly stimulates the proliferation of mature rat hepatocytes and the hormone also indirectly stimulates the hepatocyte proliferation through the density-dependent induction of the β_2-adrenoceptor-mediated responses (Ogihara, 1996b). Both mechanisms may be involved in an early phase of liver regeneration in vivo. In addition, it is likely that α_2-adrenoceptor-mediated responses are involved in the termination of liver regeneration in vivo.

The binding of EGF stimulates phosphorylation of its own receptor by receptor-associated tyrosine kinase. This process is an essential step in the propagation of the proliferative signals into the nucleus (Cantley et al., 1991). Therefore, a specific tyrosine kinase inhibitor, such as genistein (Akiyama et al., 1987), is useful as a tool for elucidating the role of this enzyme in EGF action. Table 3

show that tyrosine kinase activation plays an important role in EGF-induced hepatocyte proliferation. Wortmannin is a fungal metabolite which is a specific and potent inhibitor of phosphoinositide 3-kinase (Baggiolini et al., 1987; Dewald et al., 1988; Ui et al., 1995). Therefore, we investigated whether or not phosphoinositide 3-kinase is involved in hepatocyte proliferation induced by EGF with or without metaproterenol. Wortmannin (10^{-7} M) interferes with the signaling pathway used by metaproterenol, but not by EGF (Table 3). These findings suggest a requirement for phosphoinositide 3-kinase in cAMP-stimulated hepatocyte DNA synthesis and proliferation in the presence of EGF.

The immunosuppressant rapamycin inhibits proliferation of the H4 hepatoma cell line (Price et al., 1992) and other cell types (Chung et al., 1992). Therefore, we examined whether or not this agent also inhibits adult rat hepatocyte proliferation in primary culture. Rapamycin almost completely inhibited the hepatocyte mitogenic responses to EGF with or without cAMP-elevating agents. Rapamycin inhibits a signal transduction element that is necessary for the activation of p70 ribosomal protein S6 kinase. The target for rapamycin is thought to lie downstream of phosphoinositide 3-kinase and mitogen-activated protein kinase in some cell types (Downward, 1994). If the receptor tyrosine kinase-related upstream elements act directly on phosphoinositide 3-kinase or through further intermediates such as mitogen-activated protein kinase in adult rat hepatocyte in primary culture (Gines et al., 1995), the results of our study using rapamycin support this notion. However, the precise mechanisms by which metaproterenol and other cAMP-elevating agents potentiate the EGF-induced hepatocyte proliferation are not known. Furthermore, the relationship between these pathways is not clear.

In conclusion, we found that the activation of DNA synthesis and proliferation proceeds rapidly (within 4 h of adding EGF) in low-density cultures of adult rat hepatocytes. The rapid, proliferative effects of EGF are density-dependent, and may be mediated by the activation of receptor tyrosine kinase and p70 ribosomal protein S6 kinase. In addition, we showed that incubating hepatocytes with agents that stimulate cAMP production potentiates the EGF-stimulated hepatocyte DNA synthesis and proliferation in a density-dependent manner. The transmission of proliferative signals by cAMP-elevating agents may be mediated through phosphoinositide 3-kinase and cAMP-dependent protein kinase. These two signaling systems (i.e., EGF and cAMP pathways) may synergistically act in the control of hepatocyte growth in vivo.

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