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Proliferation of adult rat hepatocytes in primary culture induced by insulin is potentiated by cAMP-elevating agents

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Abstract

We investigated whether or not insulin and cAMP-elevating agents induce the proliferation of adult rat hepatocytes during the early and late phases of primary culture. Adult rat hepatocytes synthesized a significant amount of DNA when cultured in the presence of 10^{-7} M insulin for 3 h. Under these conditions, the number of nuclei increased within 4 h. Hepatocyte DNA synthesis and proliferation were not essentially affected by the initial plating densities. Other cAMP-elevating agents, such as glucagon, forskolin and dibutyryl cAMP, as well as β-adrenoceptor agonists (i.e., metaproterenol and isoproterenol) alone had no effect on either hepatocyte DNA synthesis or proliferation in primary culture. In contrast, these agents potentiated both processes at concentrations as low as 10^{-7} M when cultured in combination with 10^{-7} M insulin. The stimulatory effects of β-adrenoceptor agonists and other cAMP-elevating agents were significantly blocked by the cAMP-dependent protein kinase inhibitor, H-89 (*N*-[2-(*p*-(bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; 10^{-7} M). The mitogenic effect of insulin upon hepatocytes was almost completely suppressed by genistein (5×10^{-6} M), wortmannin (10^{-7} M) and by rapamycin (10 ng/ml). These results show that insulin rapidly induced the proliferation of adult rat hepatocytes in primary culture. The mitogenic effects of insulin were potentiated by β-adrenoceptor agonists and cAMP-elevating agents. The effects of β-adrenoceptor agonists and cAMP-elevating agents may be mediated through cAMP-dependent protein kinase. In addition, the activation of receptor tyrosine kinase, phosphoinositide 3-kinase and p70 ribosomal protein S6 kinase may be involved in the insulin signal transduction pathway.

Keywords: Insulin; Hepatocyte DNA synthesis; Hepatocyte proliferation; β-Adrenoceptor agonist; cAMP-elevating agent

1. Introduction

In mammals, insulin is an anabolic hormone that has many effects on metabolism in vivo, especially processes that control blood glucose. In addition, insulin stimulates cellular growth in vitro (Straus, 1981), as well as DNA synthesis in some cell types, particularly in the presence of other growth factors such as epidermal growth factor (EGF) (Richman et al., 1976; McGowan et al., 1981; Nakamura et al., 1983a; Reid and Reid, 1987). However, insulin has no apparent mitogenic effect upon adult rat hepatocytes unless added with EGF for at least 20 h, although the cells can enter S phase (Nakamura et al., 1983a). Therefore, insulin is thought to function as a key part of the background hormonal activity under which normal cells proliferate.

The signal transduction pathway that is activated in response to insulin in hepatocytes has been partially elucidated (Cantley et al., 1991; Anderson and Olefsky, 1994; White and Kahn, 1994). The biological effects of insulin are initiated through the activation of tyrosine kinase-linked receptors, thereby enabling insulin to modulate the proliferation of adult rat hepatocytes. However, little is known at the molecular level about the mechanism of insulin action on cellular activities such as the post-receptor transfer of signals and the involvement of a second messenger.

We reported that EGF rapidly stimulates DNA synthesis and proliferation during short-term culture, and that the proliferative effect is strictly density-dependent (Kimura and Ogihara, 1997). Furthermore, the proliferative effect appears to be potentiated by β-adrenoceptor agonists and other cAMP-elevating agents. In the present study, we investigated whether or not insulin participates in the intracellular events involved in the proliferation of adult rat hepatocytes. In addition, we examined the effects of β-adrenoceptor agonists and cAMP-elevating agents on insulin-stimulated DNA synthesis in adult rat hepatocytes

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in order to clarify the relationship between insulin action and β-adrenoceptor-mediated responses. Finally, we investigated the cell signalling systems involved in insulin responsiveness using a pharmacological approach.

The results show that insulin stimulated hepatocyte DNA synthesis and proliferation after a lag of about 4 h in primary culture, and that these effects were apparently independent of the initial plating density. The mitogenic effects of insulin were potentiated by β-adrenoceptor agonists and cAMP-elevating agents mediated through cAMP-dependent protein kinase. Since the hepatocyte DNA synthesis and proliferation induced by insulin was almost completely blocked by specific inhibitors of signal transducers such as the specific tyrosine kinase inhibitor, genistein, the phosphoinositide 3-kinase inhibitor, wortmannin, and the p70 ribosomal protein S6 kinase inhibitor, rapamycin, these signal transducers may play a role in the mitogenic activity induced by insulin. To our knowledge, this report is the first direct demonstration of a rapid and synergistic hepatocyte proliferation induced by insulin and β-adrenoceptor agonists (cAMP-elevating agents).

2. Materials and methods

2.1. Hepatocyte isolation and culture

Rats (weighing 200–250 g) of Wistar strain were obtained from Saitama Experimental (Saitama, Japan). They were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). The liver was disaggregated by two-step in situ collagenase perfusion based on the method of Seglen (1975). In brief, the liver was first washed via the portal vein with a calcium-free Hanks–10 mM HEPES buffer (pH 7.4) at 37°C at a flow rate of 30 ml/min, then with the same buffer containing 0.025% collagenase and 0.075% $CaCl_2$ at the same flow rate for 10 min. The cells were dispersed in Ca^{2+}-free Hanks' solution. They were then washed 3 times by slow centrifugation ($120 \times g$) for 1 min to remove cell debris, damaged cells and non-parenchymal cells. The viability of hepatocytes was monitored by trypan blue dye exclusion. Routinely, over 94% of the cells remained intact. Unless otherwise indicated, isolated hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 in Williams' medium E containing 5% bovine calf serum and 10^{-9} M dexamethasone for 3 h in 5% CO_2 in air. Thereafter the cells were cultured in serum-free Williams' medium E containing various concentrations of insulin without or with β-adrenoceptor agonists and cAMP-elevating agents.

2.2. Measurement of DNA synthesis

Hepatocyte 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, hepatocytes were washed twice with serum-free Williams' medium E and cultured in medium containing 10^{-7} M insulin for a further 4 h and 21 h. They were pulsed at 2 h and 19 h post-insulin stimulation for 2 h with [^{3}H]thymidine (1.0 μCi/well) and incorporation into DNA was determined as described previously (Kimura and Ogihara, 1997). 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 value 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. The cellular protein was determined by the modification of the Lowry procedure with bovine serum albumin as the standard (Lee and Paxman, 1972).

2.3. Counting nuclei

Nuclei instead of the cells were counted according to the procedure of Nakamura et al. (1983a), with minor modifications, because the hepatocytes attached firmly to the collagen-coated plates (Sumitomo Bakelite, Tokyo, Japan) and failed to form single cells after exposure to trypsin. Briefly, cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4). Then, the cells were lysed by incubation with 0.25 ml of 0.1 M citric acid containing 0.1% Triton X-100 for 30 min at 37°C. An equal volume of the nuclear suspension was mixed with 0.3% Trypan blue in Dulbecco's phosphate-buffered saline and the nuclei were counted using a hemocytometer.

2.4. Materials

The following reagents were obtained from Sigma (St. Louis, MO, USA): insulin (porcine), EGF (human recombinant), forskolin, dibutyryl cAMP (db-cAMP), genistein, aphidicolin, metaproterenol hemisulfate, butoxamine hydrochloride, metoprolol tartrate, butoxamine hydrochloride, UK14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline), 1-methyl-3-isobutylxanthine (IBMX), glucagon (porcine), wortmannin, rapamycin and dexamethasone. H-89 · 2HCl (*N*-[2-(*p*-(bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). 8-Bromo cAMP (8-bromoadenosine-3′-5′-cyclophosphate sodium) was obtained from Research Biochemicals International (Natick, MA, USA). Williams' medium E and new-born calf serum were purchased from Flow Laboratories (Irvine, UK). Collagenase (type II) was obtained 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.

2.5. Statistics

Values are reported as means ± S.E.M. Data were analyzed using the unpaired Student's *t*-test. Significance was established at the $P < 0.05$ level.

3. Results

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

Growth-arrested hepatocytes were incubated in the presence of insulin (10^{-7} M) with or without metaproterenol (10^{-8} M) for various periods, then DNA synthesis was determined according to [^{3}H]thymidine incorporation at a low cell density (3.3×10^4 cells/cm^2). DNA synthesis was induced in hepatocytes at about 3 h in the presence of 10^{-7} M insulin (Fig. 1). The number of nuclei increased in response to insulin from about 3.5 h, reaching a peak at about 4 h. Thereafter, DNA synthesis was rapidly stimulated by insulin. Hepatocyte DNA synthesis and proliferation induced by insulin (10^{-7} M) were potentiated by the β_2-adrenoceptor agonist, metaproterenol (10^{-8} M) and the non-specific β-adrenoceptor agonist, isoproterenol (10^{-7} M, data not shown).

3.2. Time-course of stimulation of hepatocyte DNA synthesis and proliferation by insulin with or without dibutyryl cAMP

To determine whether or not the metaproterenol effect was mediated through cAMP, metaproterenol was replaced with the cell-permeable cAMP analogue, dibutyryl cAMP (db-cAMP). Fig. 2 shows that the hepatocyte DNA synthesis and proliferation induced by insulin (10^{-7} M) was also potentiated by db-cAMP (10^{-8} M) added at various culture times. The effect of metaproterenol was therefore mimicked by db-cAMP.

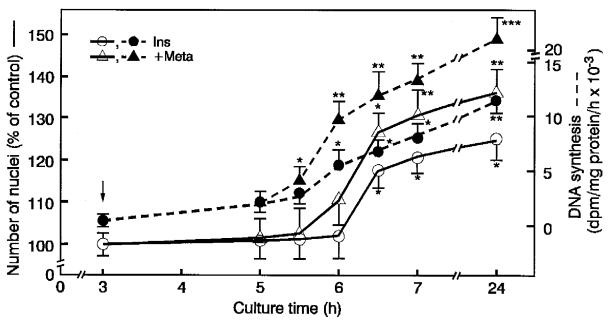

Fig. 1. Time-course of DNA synthesis and number of nuclei (proliferation) stimulated by insulin, with or without metaproterenol in cultured hepatocytes. Hepatocytes were prepared and incubated as described in Section 2. Insulin and /or metaproterenol were added at the time of medium change as indicated by the arrow. Results are expressed as means ± S.E.M. of 3 independent preparations. Values significantly different from respective controls are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Insulin, 10^{-7} M (●, dotted lines); insulin with metaproterenol, 10^{-7} M (▲, dotted lines). Insulin (○, solid lines); insulin and metaproterenol (△, solid lines).

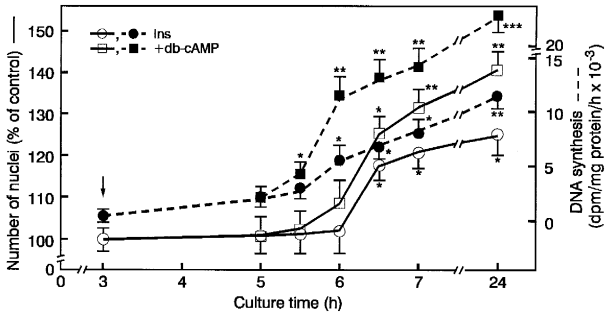

Fig. 2. Time-course of stimulation of hepatocyte DNA synthesis and number of nuclei (proliferation) by insulin with or without dibutyryl cAMP (db-cAMP) in cultured hepatocytes. Insulin and/or db-cAMP were added at the time of medium change as indicated by the arrow. Results are expressed as means ± S.E.M. of 3 independent preparations. Values significantly different from respective controls are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Insulin, 10^{-7} (●, dotted lines); insulin with db-cAMP, 10^{-7} M (■, dotted lines). Insulin (○, solid lines); insulin and db-cAMP (□, solid lines).

3.3. Dose-dependent effect of insulin on hepatocyte DNA synthesis and proliferation in the presence or absence of EGF

We examined the dose–response effects of insulin on hepatocyte DNA synthesis and proliferation in the presence or absence of 20 ng/ml EGF in cells cultured at low density (3.3×10^4 cells/cm^2) for 4 h. A bell-shaped dose–response curve showed that the effect of insulin on hepatocyte DNA synthesis was dose-dependent (Fig. 3), with a peak at 10^{-7} M insulin and inhibition at 10^{-6} M. The maximum response induced by insulin was similar to that seen in response to EGF (20 ng/ml) alone. We determined the range of concentrations at which insulin

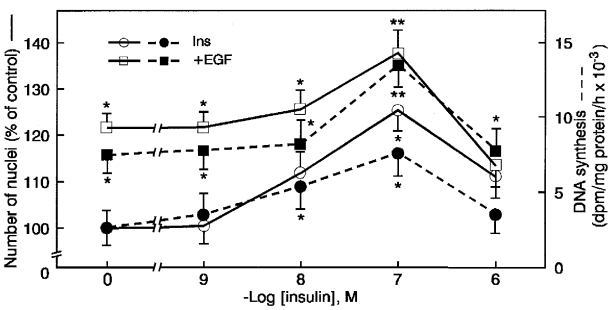

Fig. 3. Dose-dependent effects of insulin on hepatocyte DNA synthesis and proliferation in the presence or absence of EGF. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from respective controls are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$. Insulin, 10^{-7} M (●, dotted lines); 10^{-7} M insulin + 20 ng/ml EGF (■, dotted lines); insulin alone, 10^{-7} M (○, solid lines); 10^{-7} M insulin + 20 ng/ml EGF (□, solid lines).

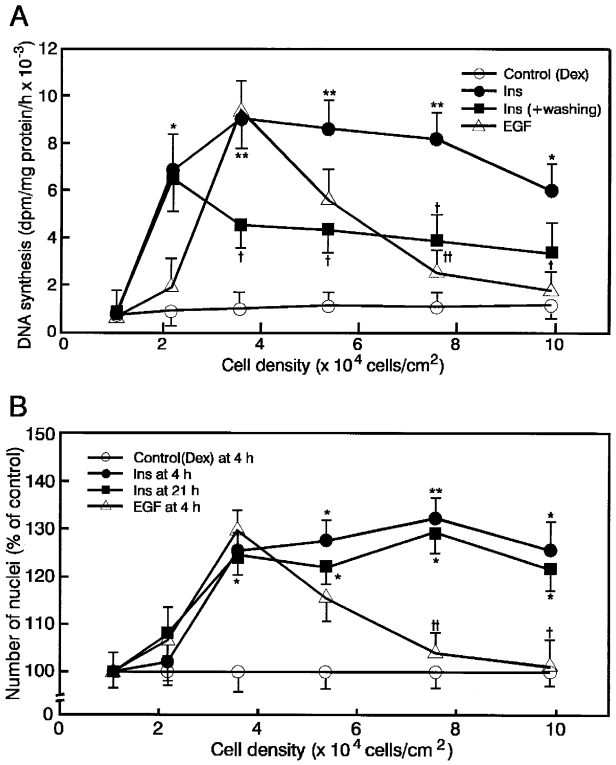

Fig. 4. (A) Influence of cell density on insulin- and EGF-stimulated DNA synthesis at early phase (4 h after hormone addition) of culture. For washing experiments, hepatocytes were cultured for 1 h with 10^{-7} M insulin and then the medium was replaced by a fresh amount of the same medium. Hepatocyte DNA synthesis was determined after culture with 10^{-7} M insulin for a further 3 h. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from respective controls are indicated by * $P < 0.05$. Dexamethasone, 10^{-9} M (○); EGF, 20 ng/ml (△); insulin, 10^{-7} M (●); insulin, 10^{-7} M (washing, ■). (B) Influence of cell density on insulin-stimulated increase in the number of nuclei at early (4 h) and late phases (21 h) of cultures. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from respective controls are indicated by * $P < 0.05$, ** $P < 0.01$. Insulin, 10^{-7} M at 4 h (○); insulin, 10^{-7} M at 21 h (●); EGF, 20 ng/ml at 4 h.

stimulated proliferation under serum-free conditions. Insulin dose-dependently increased the number of hepatocyte nuclei by about 1.2-fold with the maximal concentration being 10^{-7} M (Fig. 3). Various concentrations of insulin and EGF (20 ng/ml) synergistically stimulated both hepatocyte DNA synthesis and proliferation.

3.4. Influence of cell density on the insulin-stimulated hepatocyte DNA synthesis and proliferation

We examined the density dependence of hepatocyte DNA synthesis and proliferation induced by insulin. Fig. 4A shows that an important step leading to hepatocyte DNA synthesis was sensitive to the initial cell density and proceeded only 4 h after 20 ng/ml EGF addition. In contrast, insulin initiated hepatocyte DNA synthesis at a low and even at a relatively high cell density. Therefore, it appeared that the stimulation of hepatocyte proliferation by insulin was not essentially density-dependent, unlike that of EGF alone. Renewal of the medium at 1 h after culture with insulin (10^{-7} M) significantly reduced the insulin-induced hepatocyte DNA synthesis. Hepatocytes cultured with dexamethasone (10^{-9} M) did not significantly synthesize DNA at any of the densities tested. As shown in Fig. 4B, the number of nuclei induced by insulin reached a plateau at densities over 4×10^4 cells/cm^2, at the early (4 h) and late (21 h) phases of primary culture.

3.5. Dose-dependent effects of metaproterenol with or without IBMX on insulin-stimulated hepatocyte DNA synthesis and proliferation at the early and late phases of primary culture

To understand the role of metaproterenol in insulin action, we examined insulin (10^{-7} M)-stimulated DNA synthesis and proliferation in low density cultures of adult rat hepatocytes in the presence of varying concentrations of metaproterenol. Metaproterenol alone had little or no effect on hepatocyte DNA synthesis and proliferation in the range of 10^{-8} M–10^{-6} M. Therefore, activation of the cAMP pathway is not involved per se in the induction of hepatocyte proliferation. However, as shown in Figs. 5 and 6, the ability of insulin to induce hepatocyte DNA synthesis and proliferation was potentiated by metaproterenol up to a concentration of 10^{-8} M. Metaproterenol potentiation was dose-dependent and became inhibitory at 10^{-7} M. 1-Methyl-3-isobutylxanthine (IBMX), which is known to inhibit cAMP phosphodiesterase, potentiated the effect of metaproterenol on insulin-induced hepatocyte DNA synthesis and proliferation in a dose-dependent manner. In addition, the inhibitory phase induced by metaproterenol (10^{-8} M–10^{-6} M) was not observed in the presence of IBMX (2×10^{-4} M). The potentiative effect of metapro-

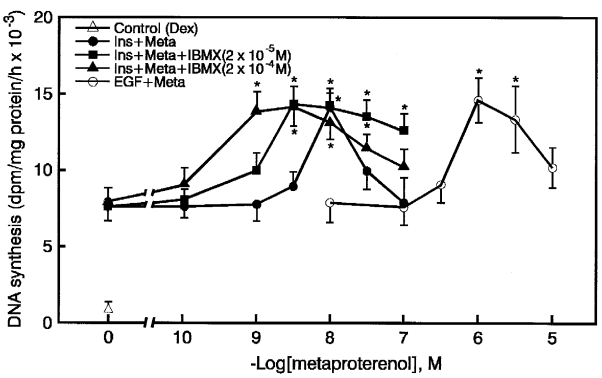

Fig. 5. Dose-dependent effects of metaproterenol with or without IBMX on insulin- and EGF-stimulated hepatocyte DNA synthesis at early phase (4 h) of culture. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from respective controls are indicated by * $P < 0.05$. Dex, dexamethasone (10^{-9} M); Ins, insulin (10^{-7} M); IBMX, 1-methyl-3-isobutylxanthine (2×10^{-5} M or 2×10^{-4} M); Meta, metaproterenol (10^{-10} M–10^{-5} M); EGF, epidermal growth factor (20 ng/ml).

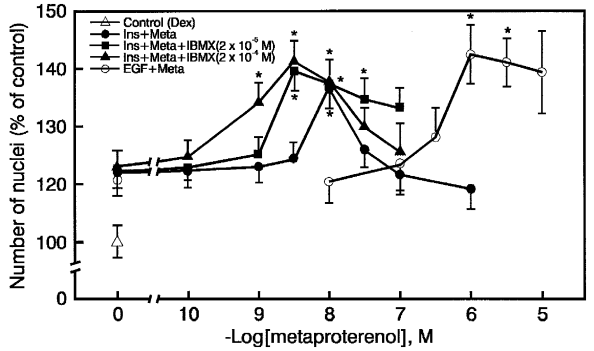

Fig. 6. Dose-dependent effects of metaproterenol with or without IBMX on insulin- and EGF-stimulated increases in the number of nuclei at the early phase (4 h) of hepatocyte culture. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from respective controls are indicated by * $P < 0.05$. Dex, dexamethasone (10^{-9} M); Ins, insulin (10^{-7} M); IBMX, 1-methyl-3-isobutylxanthine (2×10^{-5} M or 2×10^{-4} M); Meta, metaproterenol (10^{-10} M–10^{-5} M); EGF, epidermal growth factor (20 ng/ml).

terenol on insulin-induced hepatocyte DNA synthesis and proliferation was far more sensitive than that on hepatocyte proliferation induced by 20 ng/ml EGF. Similar results were obtained using dibutyryl cAMP (10^{-8} M and 10^{-7} M, peak concentrations for insulin and EGF action, respectively) instead of metaproterenol (results not shown).

3.6. Effect of adrenoceptor agents and cAMP-elevating agents on insulin-stimulated hepatocyte DNA synthesis and proliferation

Since cAMP mediates the effects of β-adrenoceptor agonists, we examined the potential of glucagon, forskolin and db-cAMP, which promote intracellular cAMP levels by several mechanisms, as well as that of other adrenoceptor agents. The potentiative effect of metaproterenol (10^{-8} M) on insulin-stimulated hepatocyte DNA synthesis and proliferation (Table 1) was mimicked by forskolin (10^{-7} M), db-cAMP (10^{-8} M) and glucagon (10^{-7} M) in the early (4 h) phase of primary cultures. In the presence of insulin (10^{-7} M), the stimulatory effects of 8-bromo cAMP (8-bromoadenosine-3′-5′-cyclophosphate sodium; 10^{-8} M) was comparable to that achieved with db-cAMP (10^{-8} M). Unlike insulin, metaproterenol and other cAMP-elevating agents (i.e., db-cAMP, forskolin and glucagon) alone 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 (data not shown). The β-adrenoceptor antagonist, propranolol (10^{-6} M), the α_1-adrenoceptor agonist, phenylephrine (10^{-4} M), and the specific α_2-adrenoceptor agonist (Cambridge, 1981), UK14304 (10^{-7} M), did not potentiate insulin-stimulated hepatocyte DNA synthesis and proliferation. None of these agents alone had any direct effects on hepatocyte DNA synthesis and proliferation during these periods (data not shown). Although hepatocyte DNA synthetic activity was highly induced by insulin in the late (21 h) phase of cultures, the profiles of these adrenoceptor agents were similar.

3.7. Interaction between α_2-adrenoceptor agonists on the metaproterenol- and db-cAMP-stimulated hepatocyte DNA synthesis and proliferation

We showed that adult rat hepatocytes in vivo normally have very low α_2- and β-adrenoceptor-mediated re-

Table 1
Effects of adrenoceptor agents and cAMP-elevating agents on insulin-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 × 10^{-3})		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control (dexamethasone)	0.465 ± 0.211	0.490 ± 0.438	100.6 ± 2.4	99.8 ± 3.7
Insulin	7.706 ± 1.413 [b]	13.090 ± 1.320 [b]	120.1 ± 3.6 [a]	122.6 ± 3.4 [a]
+ propranolol	7.884 ± 1.501 [b]	13.185 ± 2.138 [b]	122.4 ± 4.2 [a]	120.8 ± 5.6 [a]
+ UK-14304	7.572 ± 2.323 [b]	12.745 ± 3.079 [b]	122.9 ± 4.1 [a]	120.0 ± 5.4 [a]
+ metaproterenol	13.530 ± 1.382 [b,c]	23.044 ± 1.883 [b,c]	134.8 ± 3.1 [b,c]	139.4 ± 3.9 [b,c]
+ metaproterenol + propranolol	7.965 ± 1.813 [b]	12.668 ± 2.604 [b]	121.3 ± 4.3 [a]	119.5 ± 5.7 [a]
+ metaproterenol + UK-14304	8.034 ± 2.016 [b]	12.867 ± 2.018 [b]	120.8 ± 3.1 [a]	120.0 ± 3.9 [a]
+ dibutyryl cAMP	14.187 ± 1.318 [b,c]	25.807 ± 1.344 [b,d]	139.1 ± 4.4 [b,c]	141.5 ± 5.8 [b,c]
+ dibutyryl cAMP + propranolol	14.032 ± 1.323 [b,c]	24.993 ± 1.423 [b,d]	138.9 ± 3.4 [b,c]	140.8 ± 4.3 [b,c]
+ dibutyryl cAMP + UK-44304	13.887 ± 2.018 [b,c]	23.976 ± 1.306 [b,d]	139.2 ± 4.9 [b,c]	143.6 ± 6.0 [b,c]
+ 8-bromo cAMP	14.262 ± 1.433 [b,c]	26.645 ± 1.333 [b,d]	139.7 ± 4.8 [b,c]	142.5 ± 5.3 [b,c]
+ glucagon	12.927 ± 1.203 [b,c]	20.030 ± 1.820 [b,c]	131.9 ± 2.2 [b,c]	137.9 ± 4.3 [b,c]
+ forskolin	13.469 ± 1.089 [b,c]	18.839 ± 1.574 [b,c]	135.6 ± 4.2 [b,c]	140.6 ± 5.5 [b,c]
+ phenylephrine	11.977 ± 1.653 [b]	18.222 ± 2.952 [b]	131.3 ± 4.1 [b]	131.7 ± 4.2 [b]

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured for 4 h and 21 h with 10^{-7} M insulin alone or insulin with various cAMP-elevating agents immediately after medium change. Dexamethasone, 10^{-9} M; propranolol, 10^{-5} M; UK-14304, 10^{-7} M; metaproterenol, 10^{-8} M; dibutyryl cAMP, 10^{-8} M; 8-bromo cAMP, 10^{-8} M; glucagon, 10^{-7} M; forskolin, 10^{-7} M; phenylephrine, 10^{-4} M. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from control are indicated by [a] $P < 0.05$, [b] $P < 0.01$. Values significantly different from insulin alone are indicated by [c] $P < 0.05$, [d] $P < 0.01$.

sponses; these responses of adult rat hepatocytes in vitro were rapidly increased by insulin (10^{-7} M) (Ogihara, 1995) or EGF (20 ng/ml) (Ogihara, 1996a,b). Therefore, we examined the effect of an α_2-adrenoceptor agonist, UK14304, on metaproterenol- and db-cAMP-stimulated hepatocyte DNA synthesis and proliferation (Table 1) in the presence of insulin (10^{-7} M). The metaproterenol-stimulated hepatocyte DNA synthesis was blocked by propranolol (10^{-6} M) without affecting the insulin response. UK14304 (10^{-7} M) inhibited hepatocyte DNA synthesis caused by 10^{-8} M metaproterenol in the presence of 10^{-7} M insulin. The UK14304 inhibition of hepatocyte DNA synthesis was reversed by yohimbine (10^{-5} M; data not shown). In contrast, UK14304 did not affect db-cAMP-stimulated hepatocyte DNA synthesis and proliferation in the presence of insulin. Each agent alone had no direct effect on either hepatocyte DNA synthesis or proliferation in primary cultures.

3.8. Effects of selective β_1- and β_2-adrenoceptor antagonists and H-89 on metaproterenol-stimulated DNA synthesis and proliferation in the presence of insulin

The β-adrenoceptor consists of β_1 and β_2 subtypes, both of which activate adenylate cyclase to increase intracellular cAMP levels. Therefore, to further confirm β_2-adrenoceptor mediation of metaproterenol-stimulated hepatocyte DNA synthesis and proliferation in the presence of insulin (Table 2), we examined the effects of the specific β_1-adrenoceptor antagonist, metoprolol, and the specific β_2-adrenoceptor antagonist, butoxamine. These effects were mediated via a β_2-adrenoceptor, because the β_2-selective antagonist, butoxamine, inhibited and the β_1-selective antagonist, metoprolol, did not affect the responses at the early and late phases of primary culture. Metoprolol (10^{-6} M) and butoxamine (10^{-7} M) alone had no direct effect upon insulin-stimulated hepatocyte DNA synthesis. Notably, DNA synthesis was not stimulated with the β_1-selective agonist, dobutamine (10^{-7} M–10^{-5} M), indicating that the insulin effect is potentiated by metaproterenol mainly through β_2-adrenoceptors.

The second messenger cAMP acts via cAMP-dependent protein kinase. The isoquinoline sulfonamide, H-89 (*N*-[2-(*p*-(bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), is a specific inhibitor of cAMP-dependent protein kinase in some cell types (Zuscik et al., 1994). Therefore, it is a useful tool with which to investigate the involvement of cAMP-dependent protein kinase in signal transduction pathways. H-89 (10^{-7} M and 10^{-6} M) alone had no significant effect on the hepatocyte DNA synthesis and proliferation induced by insulin (Table 2). On the other hand, H-89 completely blocked db-cAMP-, forskolin- and metaproterenol-stimulated hepatocyte DNA synthesis and proliferation in the presence of insulin.

3.9. Effect of specific inhibitors of signal-transducing elements on hepatocyte DNA synthesis and proliferation induced by insulin with or without agents that elevate cAMP

Specific inhibitors of the intracellular signaling cascade are useful probes with which to characterize target proteins. Therefore, we investigated whether or not the rapid

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 $\times 10^{-3}$)		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control (dexamethasone)	0.465 ± 0.211	0.490 ± 0.438	100.6 ± 2.4	99.8 ± 3.7
Dexamethasone + H-89	0.523 ± 0.306	0.490 ± 0.333	100.8 ± 5.2	100.1 ± 6.2
Insulin	7.708 ± 1.394 [b]	13.029 ± 1.919 [b]	119.3 ± 3.4 [a]	121.6 ± 3.0 [a]
+ metaproterenol	13.804 ± 1.611 [b,c]	23.029 ± 2.288 [b,c]	133.4 ± 3.3 [b,c]	137.9 ± 5.0 [b,c]
+ metoprolol	7.752 ± 1.127 [b]	12.107 ± 2.435 [b]	119.0 ± 6.0 [a]	121.3 ± 5.8 [a]
+ metaproterenol + metoprolol	13.854 ± 1.707 [b,c]	22.218 ± 2.329 [b,c]	133.1 ± 3.1 [b,c]	138.5 ± 5.2 [b,c]
+ butoxamine	7.428 ± 1.181 [b]	11.293 ± 2.658 [b]	120.6 ± 5.1 [a]	119.0 ± 4.4 [a]
+ metaproterenol + butoxamine	7.212 ± 1.882 [b]	11.884 ± 2.434 [b]	118.6 ± 3.8 [a]	119.8 ± 5.4 [a]
+ H-89	7.655 ± 1.200 [b,f]	14.402 ± 1.590 [b,f]	122.9 ± 5.4 [a,e]	117.7 ± 3.9 [a,e]
+ metaproterenol + H-89	7.294 ± 1.978 [b,f]	14.586 ± 1.957 [b,f]	117.1 ± 4.2 [a,e]	121.3 ± 4.7 [a,e]
+ dibutyryl cAMP	14.187 ± 1.318 [b,c,f]	25.807 ± 1.341 [b,d,f]	140.8 ± 4.4 [b,c,f]	141.5 ± 6.7 [b,c,f]
+ dibutyryl cAMP + H-89	7.373 ± 1.331 [b,f]	14.288 ± 1.172 [b,f]	115.6 ± 4.8 [a,e]	119.9 ± 4.0 [a,e]
+ dobutamine	7.588 ± 1.396 [b]	13.350 ± 1.254 [b]	120.3 ± 4.4 [a]	120.0 ± 3.4 [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 β_1- and β_2-adrenoceptor antagonists, H-89, metaproterenol and db-cAMP, were added with 10^{-7} M insulin immediately after medium change and cells were cultured for a further 4 h and 21 h. Dexamethasone, 10^{-9} M; metaproterenol, 10^{-8} M; metoprolol, 10^{-6} M; butoxamine, 10^{-6} M; H-89, 10^{-7} M; dibutyryl cAMP, 10^{-8} M; dobutamine, 10^{-6} M. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from control are indicated by [a] $P < 0.05$, [b] $P < 0.01$. Values significantly different from insulin alone are indicated by [c] $P < 0.05$, [d] $P < 0.01$. Values significantly different from dexamethasone with H-89 are indicated by [e] $P < 0.05$, [f] $P < 0.01$.

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

Treatment	DNA synthesis (dpm/mg protein/h $\times 10^{-3}$)		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control (dexamethasone)	0.603 ± 0.459	0.729 ± 0.691	100.5 ± 3.4	100.3 ± 6.1
Insulin	7.824 ± 1.487 [b]	13.039 ± 1.390 [b]	120.4 ± 3.5 [a]	123.2 ± 3.0 [a]
+ metaproterenol	14.598 ± 1.558 [b,c]	21.279 ± 1.820 [b,c]	135.8 ± 3.2 [b,c]	136.7 ± 3.1 [b,c]
+ genistein	0.801 ± 0.741 [c]	0.729 ± 0.630 [d]	105.3 ± 4.1 [c]	106.9 ± 3.6 [c]
+ metaproterenol + genistein	0.856 ± 0.459 [c]	0.902 ± 0.779 [d]	104.2 ± 3.5 [c]	108.2 ± 4.4 [c]
+ metaproterenol + aphidicolin	0.342 ± 0.317 [d]	0.456 ± 0.341 [d]	100.3 ± 5.1 [c]	95.2 ± 3.4 [d]
+ wortmannin	0.742 ± 0.687 [d]	0.802 ± 0.607 [d]	102.2 ± 5.5 [c]	100.3 ± 5.0 [c]
+ EGF	18.225 ± 1.574 [b,d]	26.434 ± 2.956 [b,c]	141.4 ± 6.4 [b,c]	147.2 ± 4.2 [b,d]
+ EGF + wortmannin	8.472 ± 1.256 [b]	13.907 ± 1.301 [b]	122.5 ± 3.6 [a]	124.7 ± 4.7 [a]
EGF + wortmannin	8.755 ± 1.343 [b]	13.650 ± 1.297 [b]	123.2 ± 3.8 [a]	123.4 ± 5.7 [a]
Insulin + rapamycin	0.515 ± 0.454 [d]	0.641 ± 0.695 [d]	100.3 ± 6.1 [c]	100.7 ± 4.7 [c]

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 10^{-7} M insulin immediately after medium change and cells were cultured for a further 4 h and 21 h. Dexamethasone, 10^{-9} M; metaproterenol, 10^{-8} M; genistein, 5×10^{-6} M; aphidicolin, 10 μg/ml; wortmannin, 10^{-7} M; EGF, 20 ng/ml; rapamycin, 10 ng/ml. Each value is expressed as mean ± S.E.M. from 3 independent preparations. Values significantly different from control are indicated by [a] $P < 0.05$, [b] $P < 0.01$. Values significantly different from insulin alone are indicated by [c] $P < 0.05$, [d] $P < 0.01$.

mitogenic response of hepatocytes to insulin and insulin with metaproterenol is mediated by signal transducers, such as receptor tyrosine kinase, phosphoinositide 3-kinase and p70 ribosomal protein S6 kinase. To determine whether insulin-stimulated DNA synthesis requires protein tyrosine kinase activity, hepatocytes were incubated with insulin in the presence or absence of the specific tyrosine kinase inhibitor, genistein (5×10^{-6} M). As shown in Table 3, genistein almost completely blocked the DNA synthesis stimulated by insulin and the proliferative effects of insulin with or without metaproterenol. Incubating hepatocytes with the specific phosphoinositide 3-kinase inhibitor, wortmannin (10^{-7} M), almost completely inhibited the insulin-induced stimulation of DNA synthesis and proliferation without altering the effects of EGF. Table 3 also show that the immunosuppressant rapamycin (10 ng/ml) almost completely attenuated the mitogenic effects of insulin and the co-mitogenic effect of metaproterenol on both hepatocyte DNA synthesis and proliferation. The mitogenic effects of insulin with metaproterenol were completely blocked by aphidicolin (10 μg/ml).

4. Discussion

As shown in Figs. 1 and 2, hepatocyte DNA synthesis and proliferation in primary culture of adult rat hepatocytes is activated about 3.5 h and 4.0 h, respectively, by 10^{-7} M insulin. The differences between our results and others (Nakamura et al., 1983a) in time course cannot be explained, although it might reflect repair after collagenase digestion and adaptation of hepatocytes to their culture conditions. The maximal response induced by insulin was similar to that seen in response to 20 ng/ml EGF and both factors acted synergistically to stimulate hepatocyte DNA synthesis and proliferation (Fig. 3). To our knowledge, there have been no studies on the short-term effects of insulin on hepatocyte DNA synthesis and the number of nuclei (proliferation).

Several studies have suggested that plating density determines hepatocellular responsiveness to growth factor stimulation (Nakamura et al., 1983a,b, 1984; Kajiyama and Ui, 1994). The density-dependent mechanisms of hepatocyte growth and proliferation might be related to cell-cell contact and the production of inhibitory (or stimulatory) autocrine factor(s). In accordance with earlier reports, we showed that EGF-induced hepatocyte proliferation was strictly density-dependent. However, as shown in Fig. 4A and B, we found that insulin initiates hepatocyte DNA synthesis and proliferation even at relatively high cell densities. In contrast to EGF, hepatocyte proliferation stimulated by insulin, therefore, was not entirely density-dependent. The reason for this remains unknown; however, since renewal of medium (washing) at 1 h after culture with insulin (10^{-7} M) significantly depressed the hepatocyte DNA synthesis, growth-stimulatory substances that stimulate these processes may be generated in culture.

As shown in Table 1, β-adrenoceptor agonists and other cAMP-elevating agents stimulated hepatocyte DNA synthesis and proliferation in an insulin-dependent manner. The stimulation of hepatocyte DNA synthesis and proliferation was completely inhibited by the β_2-selective adrenoceptor antagonist, butoxamine, but not by β_1-selective adrenoceptor antagonist, metoprolol (10^{-7} M–10^{-5} M), indicating that the effects were mediated via β_2-adrenoceptors. This was further supported by the findings that β_1-selective agonist, dobutamine (10^{-7} M–10^{-5} M), did not potentiate the insulin-induced hepatocyte DNA synthesis and proliferation (Table 2). The metaproterenol-stimulated

hepatocyte DNA synthesis and proliferation was inhibited by the specific α_2-adrenoceptor agonist, UK14304 (Table 1), suggesting that it acts through the inhibition of adenylate cyclase. Since phenylephrine had little or no action upon insulin-induced hepatocyte DNA synthesis and proliferation, α_1-adrenoceptor mechanisms are not involved in the modulation of the insulin effects. However, we should not exclude that a possible stimulation of Ca^{2+} influx through the R-type Ca^{2+} channel and increased intracellular Ca^{2+} by insulin as reported in heart and vascular smooth muscle cells (Bkaily et al., 1992) may contribute to the observed insulin-mediated proliferation in cultured hepatocytes. In contrast to insulin, relatively high concentrations of metaproterenol (and db-cAMP) are required for maximal hepatocyte DNA synthesis and proliferation induced by EGF (Figs. 5 and 6). This difference in sensitivity may be explained by isoforms of cAMP-dependent protein kinase (i.e., type I and type II) (Ogreid et al., 1985; Rothermel et al., 1984), that might couple to insulin or EGF signalling pathway.

Hepatocyte proliferation induced by both metaproterenol and db-cAMP was inhibited by a submaximal concentration of H-89, confirming the involvement of cAMP-dependent protein kinase (Table 2). This finding suggests that metaproterenol and other cAMP-elevating agents act via the same mechanism. The livers of young rats (Christoffersen et al., 1973), regenerating livers (Bronstad and Christoffersen, 1980; Sandnes et al., 1986; Mahler and Wilce, 1988; Michalopoulos, 1990) and those treated with carcinogens (Christoffersen et al., 1972) are highly sensitive to β-adrenoceptor stimulation. The notion that some growth factors can stimulate hepatocyte DNA synthesis and replication and act indirectly via stimulating a β-adrenoceptor mechanism (Ogihara, 1996a,b) is supported by this observation. It is generally accepted that cAMP inhibits cell proliferation (Bronstad et al., 1983; Vintermyr et al., 1989; Marker et al., 1992; Refsnes et al., 1992). However, our results showed that the potentiation of hepatocyte DNA synthesis and proliferation is mediated via an increase in the activity of the β_2-adrenoceptor-adenylate cyclase system, especially in the presence of IBMX (Figs. 5 and 6).

Although the signal transduction pathway activated in response to insulin in hepatocytes is not completely understood, insulin initiates its biological effects by activating receptor tyrosine kinase in the β-subunit and phosphorylates several intracellular proteins (Anderson and Olefsky, 1994; White and Kahn, 1994). To determine whether insulin-stimulated DNA synthesis and proliferation requires receptor tyrosine kinase activity, hepatocytes were incubated with insulin in the presence or absence of the specific tyrosine kinase inhibitor, genistein (Akiyama et al., 1987). Since the mitogenic effect of insulin was almost completely blocked by 5×10^{-5} M genistein, receptor tyrosine kinase activity is required for insulin-induced hepatocyte DNA synthesis and proliferation (Table 3).

To examine the involvement of phosphoinositide 3-kinase in hepatocyte mitogenic responses to insulin, we investigated the effect of a specific phosphoinositide 3-kinase inhibitor, wortmannin (Baggiolini et al., 1987; Dewald et al., 1988; Ui et al., 1995), on DNA synthesis and the increase in the number of nuclei induced by the hormone with or without metaproterenol. Since the specific phosphoinositide 3-kinase inhibitor, wortmannin, completely inhibited insulin-induced stimulation of DNA synthesis and proliferation in hepatocytes, we suggest that these responses are phosphoinositide 3-kinase-dependent. Similar results have been reported in other cell type (Sanchez-Margalet et al., 1994). Rapamycin is a potent inhibitor of the hepatocyte proliferation induced by insulin. As shown in Table 3, these findings suggest that hepatocyte DNA synthesis is regulated by a cascade involving p70 ribosomal protein S6 kinase. In liver cells, insulin stimulates the activity of several protein kinases, including p42 mitogen-activated protein kinase and p70 ribosomal protein S6 kinase (Downward, 1994; Gines et al., 1995). Therefore, the rapamycin target may also be a candidate for the transmission of proliferative signals (Chung et al., 1992; Price et al., 1992).

In conclusion, we found that the activation of DNA synthesis and proliferation proceeds rapidly (within 4 h of adding insulin) in primary cultures of adult hepatocytes. The rapid, proliferative effects of insulin are not density-dependent, and may be accomplished through receptor tyrosine kinase, phosphoinositide 3-kinase and p70 ribosomal protein S6 kinase. In addition, we demonstrated that cAMP-elevating agents can potentiate insulin-induced hepatocyte DNA synthesis and proliferation, especially in the presence of phosphodiesterase inhibitors. These two signalling systems (i.e. insulin and cAMP pathways) may synergistically act in the control of hepatocyte growth in vivo.

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