Proliferation of Adult Rat Hepatocytes in Primary Cultures Induced by Platelet-Derived Growth Factor Is Potentiated by Phenylephrine

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ABSTRACT--We investigated whether or not proliferation of adult rat hepatocytes induced by platelet-derived growth factor (PDGF) is affected by α_1-adrenoceptor agonists such as phenylephrine during the early and late phases of primary culture. Adult rat hepatocytes underwent significant DNA synthesis after culture with 10 ng/ml PDGF for 2 hr at a low cell density (3.3 x 10^4 cells/cm^2). Under these culture conditions, the number of nuclei increased significantly during the 3.5 hr culture period. Hepatocyte DNA synthesis and proliferation induced by 10 ng/ml PDGF decreased slightly as a result of increasing the initial plating density. An α_1-adrenoceptor agonist, phenylephrine (10^{-6} - 10^{-5} M), alone did not affect hepatocyte DNA synthesis and proliferation, but the markedly potentiated PDGF-induced hepatocyte DNA synthesis and proliferation. The phenylephrine effect was mimicked by phorbol myristate acetate (10^{-7} M), but not by ionomycin (10^{-5} M). The mitogenic effects of PDGF were almost completely blocked by treating hepatocytes with genistein (5 x 10^{-6} M), U-73122 (3 x 10^{-6} M), sphingosine (10^{-5} M), wortmannin (10^{-7} M), and rapamycin (10 ng/ml). These results demonstrate that PDGF can induce the proliferation of adult rat hepatocytes rapidly in primary culture, regardless of the initial plating density. The present results also suggest that following stimulation with PDGF, activation of tyrosine kinase, phospholipase C, phosphatidylinositol 3-kinase, protein kinase C (PKC) and p70 ribosomal protein S6 kinase is essential for proliferation of adult rat hepatocytes. The co-mitogenic effects of phenylephrine may involve PKC activation.

INTRODUCTION

The majority of research on the mechanisms associated with hepatic growth has used partially hepatectomized animals *in vivo* (1). Studying hepatocytes in primary culture in conjunction with current *in vivo* methods would provide a valuable tool for increasing our understanding of hepatocyte proliferation, because the influences of stimulatory and inhibitory factors *in vivo* can be isolated in the culture model. Thus, the growth conditions that may play a role in hepatocyte replication *in vivo* may be clarified in vitro. For example, hepatic parenchymal cells that are cultured in a defined medium for 24 hr and 48 hr undergo DNA synthesis and proliferation in the presence of a variety of growth factors, including epidermal growth factor (EGF), insulin and hepatocyte growth factor (HGF) (2-4).

In contrast, we have reported previously that EGF and insulin alone can rapidly stimulate hepatocyte DNA synthesis and proliferation during short-term cultures (i.e. approx. 3-4 hr) (5, 6). The density dependency of hepatocyte proliferation is regulated differently between these growth factors. Hepatocyte DNA synthesis and proliferation induced by EGF are strictly dependent on the initial plating density, unlike insulin induction, which appears to be independent of the initial plating density. Furthermore, EGF- or insulin-stimulated hepatocyte DNA synthesis and proliferation appear to be potentiated by β-adrenoceptor agonists and other cAMP-elevating agents.

Similarly, platelet-derived growth factor (PDGF) is a potent stimulator of the proliferation of connective-tissue cell such as fibroblasts and smooth muscle cells (7, 8), and may play a role in the liver regeneration process (1). Recent studies have begun to demonstrate the signal transduction pathway activated in response to PDGF in some cell types (9,10). The receptor for PDGF, like the receptor for EGF, insulin and HGF, contains intrinsic tyrosine kinase activity. The mitogenic effects of PDGF are reportedly mediated through the activation of tyrosine kinase-linked receptors. However, little is known about which of the signals originating in the plasma membrane are involved in the induction of hepatocyte DNA synthesis and proliferation by PDGF. Thus, the present study investigated the direct action of PDGF on DNA synthesis and replication of adult rat hepatocytes in primary culture *in vitro*. It also investigated the effects of α_1-adrenoceptor agonist on PDGF-induced DNA synthesis and proliferation in adult rat hepatocytes to clarify the relationship between PDGF action and α_1-adrenoceptor responses, since there are indications that hepatocyte DNA synthesis and proliferation are modified by α_1-adrenoceptor receptor-mediated responses (11-15) in the presence of HGF. Finally, the cell signaling systems involved in PDGF responsiveness in primary cultures of adult rat hepatocytes were investigated by pharmacological approaches.

MATERIALS AND METHODS

Hepatocyte isolation and culture

Male Wistar rats (weight 200-250 g) were obtained from Saitama Experimental Co. (Saitama). The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). Two-step *in situ* collagenase perfusion was performed to facilitate disaggregation of the adult rat liver as described previously (16, 17). Briefly, the liver was first washed via the portal vein with a calcium-free 10 mM HEPES buffer (pH 7.4) at 37℃ and a flow rate of 30 ml/min for 10 min. The second step was performed with the same buffer containing 0.025% collagenase and 0.075% $CaCl_2$ at flow rate of 30 ml/min for 10 min. The cells were dispersed in Ca^{2+}-free Hanks' solution containing kanamycin (48 μg/ml). The cells were then washed three times by slow centrifugation (120g) for 1 min in order to remove cell debris, damaged cells and non-parenchymal cells. The viability of hepatocytes always exceeded 94%, as determined by the trypan blue exclusion test. Unless otherwise indicated, isolated hepatocytes were plated onto collagen-coated plastic culture dishes (Sumitomo Bakelite Co., Tokyo) at a density of 3.3 x 10^4 cells/cm^2 in Williams' medium E containing 5% bovine calf serum, 10^{-10} M dexamethasone for 3 hr in 5% CO_2 in air. The medium was then changed, and the cells were cultured in serum-free Williams' medium E containing various concentrations of PDGF with or without a β-adrenoceptor agonist, cAMP-elevating agents, an α_1-adrenoceptor agonist, and/or specific inhibitors of signal transducers.

Measurement of DNA synthesis

Hepatocyte DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into acid-precipitable materials (18). Briefly, after an initial attachment period of 3 hr, hepatocytes were washed twice with serum-free Williams' medium E and cultured in a medium containing 10 ng/ml PDGF for a further 4 hr and 21 hr. The cells were pulsed at 2 hr and 19 hr post-PDGF stimulation for 2 hr with [^{3}H]thymidine (1.0 μCi/well). Incorporation into DNA was then determined as described previously (5). Hepatocyte protein content was measured by a modified Lowry procedure with bovine serum albumin as a standard (19).

Counting nuclei

The number of nuclei was counted instead of the cell number according to the previously described procedure of Nakamura et al. with minor modifications (4). Briefly, the primary cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4). The cells were then lysed by incubation with 0.25 ml of 0.1 M citric acid containing 0.1 % Triton x-100 for 30 min at 37℃. An equal volume of the nucleus suspension was mixed with 0.3% trypan blue in Dulbecco's phosphate-buffered saline (pH 7.4) and the number of nuclei was counted in a hemocytometer (5). This procedure was performed because the hepatocytes had firmly attached to the collagen-coated plates and were not dispersed by EDTA-trypsin treatment.

Materials

The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA): forskolin, dibutyryl cAMP (db-cAMP), genistein, aphidicolin, metaproterenol hemisulfate, phenylephrine hydrochloride, prazosin hydrochloride, yohimbine hydrochloride, D-sphingosine, ionomycin calcium salt, UK14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline), wortmannin, rapamycin, angiotensin II, arginine vasopressin, and dexamethasone. Platelet-derived growth factor-BB (human recombinant) was obtained from R & D Systems (Mineapolis, MN, USA). Phorbol 12-myristate 13-acetate (PMA) and suramin hexasodium salt was purchased from Research Biochemicals International (Natick, MA, USA). U-73122 (1-[6-[[17β-3-methoxyestra-1, 3, 5 (10)-triene-17-yl] amino] hexyl]-1H pyrrol-2, 5-dione) and U-73343 (1- [6-[[17β-3-methoxyestra-1, 3, 5 (10)-triene-17-yl] amino] hexyl]-2, 5-pyrrolidine-dione) were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Williams' medium E and new born calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical Co., (Freehold, NJ, USA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, USA). All reagents were of analytical grade.

Statistical analysis

Data 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.

RESULTS

Time course of stimulation of hepatocyte DNA synthesis and proliferation by PDGF with or without phenylephrine

Isolated adult rat hepatocytes were treated using PDGF with or without phenylephrine for various culture periods, and DNA synthesis was measured by [^{3}H]thymidine incorporation at a low cell density (3.3 x 10^4 cells/cm^2). DNA synthesis was induced in hepatocytes after only 2 h and reached a maximum 6.5 - 7.0 hr after the addition of PDGF (10 ng/ml). DNA synthesis then became reduced by 21 hr after addition (Fig. 1). A significant increase in the number of nuclei (proliferation) induced by PDGF (10 ng/ml) was observed approximately 3.5 hr after PDGF addition, reached a peak at 4 hr and was sustained for a further 21 hr. Therefore, the detected increase in the number of nuclei could be due to an increase in [^{3}H]thymidine incorporation. Thus, both hepatocyte DNA synthesis and proliferation induced by PDGF (10 ng/ml) were found to be potentiated in the presence of an α_1-adrenoceptor agonist, phenylephrine (10^{-6} M).

Dose-dependent effects of PDGF on hepatocyte DNA synthesis and proliferation

Figure 2 shows the dose-dependent effect of PDGF on DNA synthesis in hepatocytes cultured for 4 hr at a low density (3.3 x 10^4 cells/cm^2). Peak stimulation of hepatocyte DNA synthesis occurred at 10 ng/ml and showed an EC_{50} of 2.5 ± 0.3 ng/ml (n=3). PDGF increased the number of nuclei by approximately 1.2fold with a maximal concentration of 10 ng/ml (EC_{50}:3.5 ± 0.3 ng/ml, n=3).

Influence of cell density on PDGF-stimulated hepatocyte DNA synthesis and proliferation with or without phenylephrine

To determine if the proliferative effect of PDGF is affected by the initial plating density, we investigated the density-dependency of hepatocyte DNA synthesis and proliferation induced by 10 ng/ml PDGF with or without phenylephrine (10^{-6} M). Figure 3 shows that hepatocyte DNA synthesis induced by PDGF (10 ng/ml) was not influenced significantly by the initial plating density. However, phenylephrine-stimulated hepatocyte DNA synthesis in the presence of PDGF was significantly inhibited at a higher cell density. As shown in Fig. 4, the increase in the number of nuclei induced by 10 ng/ml PDGF reached a plateau at a cell density of 3.3 x 10^4 cells/cm^2. This increase occurred during the early phase of culture. Hepatocytes cultured without 10 ng/ml PDGF for 4 hr did not show any

significant effect on hepatocyte DNA synthesis or proliferation at various cell densities (Figs. 3 and 4, respectively).

Dose-dependent effects of phenylephrine on PDGF-stimulated hepatocyte DNA synthesis and proliferation during early phase of primary culture

To test the influence of the α_1-adrenoceptor-mediated mechanism on PDGF activity, we examined the dose-dependent effects of phenylephrine on PDGF-stimulated DNA synthesis and proliferation at a low density during the early phase of culture (Fig. 5). Phenylephrine alone had almost no effect on hepatocyte DNA synthesis and proliferation in the range of 10^{-8} -10^{-5} M (data not shown). In contrast, the ability of PDGF (10 ng/ml) to induce hepatocyte DNA synthesis and proliferation was significantly potentiated by the addition of phenylephrine, with maximal potentiation occurring at a concentration of 10^{-6} M (EC_{50} for DNA synthesis: 0.19 ± 0.02 μM, n=3; EC_{50} for nucleus number: 0.3 ± 0.03 μM, n=3). Phenylephrine potentiation was dose dependent up to approximately 10^{-6} M.

Effects of suramin, specific α_1- and α_2-adrenoceptor antagonists on phenylephrine-stimulated hepatocyte DNA synthesis and proliferation in the presence of PDGF

To confirm α_1-adrenoceptor receptor-mediation of phenylephrine-stimulated hepatocyte DNA synthesis and proliferation in

the presence of 10 ng/ml PDGF, we examined the effects of a specific α_1-adrenoceptor antagonist, prazosin, and a specific α_2- adrenoceptor antagonist, yohimbine, on the potentiation of the PDGF effects induced by phenylephrine. As summarized in Table 1, suramin (10^{-5} M), which is known to inhibit PDGF action at the receptor sites (20), significantly blocked PDGF-stimulated hepatocyte DNA synthesis and proliferation in the absence or presence of phenylephrine. Prazosin (10^{-6} M) completely inhibited the phenylephrine effects, whereas yohimbine (10^{-6} M) did not affect hepatocyte DNA synthesis or proliferation during the early and late phases of primary culture. Suramin, prazosin and yohimbine alone had no direct effects on PDGF-stimulated hepatocyte DNA synthesis and proliferation (data not shown). UK-14304 (10^{-6} M), a specific α_2-adrenoceptor agonist (21), did not inhibit phenylephrine-stimulated hepatocyte DNA synthesis and proliferation in the presence of PDGF (10 ng/ml). A β_2-adrenoceptor agonist, metaproterenol, alone had almost no effect on the PDGF-stimulated hepatocyte DNA synthesis and proliferation in the range of 10^{-7} -10^{-5} M. In addition, forskolin (10^{-6} M) and db-cAMP (10^{-5} M) alone did not significantly affect hepatocyte DNA synthesis and proliferation in the presence of 10 ng/ml PDGF (data not shown).

Effects of U-73122, sphingosine, PMA and ionomycin on phenylephrine- or phorbol ester-stimulated hepatocyte DNA synthesis and proliferation in the presence of PDGF

We investigated the possible pharmacological role of phospholipase C (PLC) and its intracellular second messengers (e.g., 1,2-diacylglycerol (DG) and calcium ion) on PDGF-induced hepatocyte DNA synthesis and proliferation during the early and late phases of culture. As summarized in Table 2, the PLC inhibitor, U-73122 (3×10^{-6} M), attenuated PDGF action on hepatocyte DNA synthesis during the 4 hr of culture, but increased the response during the 21 hr of culture. U-73343 (10^{-5} M), a close structural analog of U-73122, did not affect PDGF-induced hepatocyte DNA synthesis and proliferation during the early and late phases of culture. The specific protein kinase C (PKC) inhibitor, sphingosine (10^{-6} M) also inhibited PDGF-induced hepatocyte DNA synthesis during the early phase of culture, but increased the response during the late phase of culture. To further investigate the possible involvement of DG, the hepatocytes were treated with a synthetic analogue, PMA (10^{-7} M), for 4 hr and 21 hr. Although PMA (10^{-7} M) alone did not significantly affect hepatocyte DNA synthesis and proliferation (data not shown), PMA potentiated PDGF-stimulated hepatocyte DNA synthesis during the early phase of culture, but the PMA effects were reduced during the late phase of culture. The PMA effects were significantly blocked by sphingosine (10^{-6} M), but not by U-73122 (3×10^{-6} M). U-73343 did not affect either PMA-induced hepatocyte DNA synthesis or

proliferation up to 10^{-5} M, as expected. Similarly, to determine the possible involvement of intracellular calcium mobilization in hepatocyte DNA synthesis and proliferation, cells were cultured with 10^{-5} M ionomycin for 4 hr and 21 hr. No changes in the PMA-stimulated hepatocyte DNA synthesis and proliferation were observed with ionomycin. Similarly, other calcium mobilizing agents such as angiotensin II and arginine vasopressin (10^{-8} -10^{-6} M) did not affect hepatocyte DNA synthesis and proliferation induced by 10 ng/ml PDGF (data not shown). The results demonstrate that the phenylephrine effects were largely mimicked by PMA, but not by ionomycin.

Effect of specific inhibitors of signal-transducing enzymes on hepatocyte DNA synthesis and proliferation induced by PDGF with or without phenylephrine

Next, we investigated whether or not the mitogenic responses of hepatocytes to PDGF alone and PDGF with phenylephrine were mediated by signal transducers such as receptor tyrosine kinase, phosphatidylinositol 3-kinase (PI(3)K), and ribosomal protein p70 S6 kinase (p70 S6K). To determine whether or not PDGF-stimulated DNA synthesis and proliferation require receptor tyrosine kinase activity, hepatocytes were treated with PDGF (10 ng/ml) in the presence or absence of a specific tyrosine kinase inhibitor, genistein (22), for 4 hr and 21 hr. As summarized in Table 3, genistein (5 x 10^{-6} M) almost completely blocked PDGF-induced stimulation of hepatocyte DNA synthesis and proliferation. The

co-mitogenic effect of phenylephrine in the presence of PDGF was significantly inhibited by genistein (5 x 10^{-6} M) during the early and late phases of culture. Treatment of hepatocytes with a specific PI(3)K inhibitor, wortmannin (10^{-7} M) (23-25), also completely inhibited PDGF-induced stimulation of hepatocyte DNA synthesis and proliferation. Furthermore, the co-mitogenic effect of phenylephrine in the presence of PDGF was blocked by wortmannin (10^{-7} M). Table 3 also shows that the immunosuppressant rapamycin (10 ng/ml) (26-28) almost completely attenuated both the mitogenic effects of PDGF and co-mitogenic effect of phenylephrine on hepatocyte DNA synthesis and proliferation during the early and late phases of primary culture.

DISCUSSION

As shown in Fig.1, we found for the first time that PDGF alone stimulated hepatocytes DNA synthesis and proliferation after a lag of approximately 3-4 hr in primary culture. Similarly, we found that stimulation of hepatocytes with PDGF (10 ng/ml) markedly increased hepatocyte DNA synthesis and proliferation, particularly when PDGF was added in combination with an α_1-adrenoceptor agonist such as phenylephrine (Fig.1). Since phenylephrine alone did not stimulate hepatocyle DNA synthesis and proliferation, the co-mitogenic effects of phenylephrine are likely to be, at least in part, explained by interaction with PDGF signaling mechanisms. Possible mechanisms for this phenomenon will be discussed later. Figure 1

also showed that based on the similarities with the case of insulin (6), the ability of PDGF to induce hepatocyle DNA synthesis and proliferation does not appear to be strictly dependent on the initial plating density in the absence of phenylephrine (Figs. 3 and 4). The density-independent mechanisms of hepatocyte DNA synthesis and proliferation are probably due to production of stimulatory autocrine factor(s) and/or reduced production of inhibitory autocrine factor(s) by the hepatocytes in primary culture (4). Alternatively, it may be a loss of regulation due to cell-cell contact (29-31). However, further studies are required to confirm this hypothesis.

PDGF reportedly acts through tyrosine kinase receptors that phosphorylate and activate phospholipase C (PLC-γ), which leads to enhanced DG and inositol 1,4,5-trisphosphate (IP_3) production (32). Phenylephrine is also known to exert its action through the stimulation of phospholipase C (PLC-β) via Gq-protein. PLC-β catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and DG. Generation of IP_3 leads to mobilization of calcium ions from intracellular stores, whereas DG activates different isozymes of the PKC family (32). Therefore, using two mechanistically distinct inhibitors of signal transducers, U73122 (an inhibitor of PLC) and sphingosine (an inhibitor of PKC), the mechanisms leading to PDGF stimulation of hepatocyte DNA synthesis and proliferation were investigated (33-35). As summarized in Table 2, U73122 (3×10^{-6} M) attenuated the PDGF effect, but not PMA (i.e., cell-permeable synthetic

analogue of DG) effects on hepatocyle DNA synthesis during the early phase of culture. The results suggest that PKC is a downstream signal transduction element of PLC. Furthermore, both PLC and PKC appeal to play an important role in the PDCF regulation of hepatocyte DNA synthesis and proliferation. A point of convergence of these two signaling pathways may be at the level of PKC.

As shown in Fig .1, DNA synthesis became reduced significantly by 21 hr after the addition of PDGF in the absence or presence of phenylephrine. The mechanism underlying the reduction of hepatocyte DNA synthesis may be due to desensitization (or down-regulation) of PKC activity, since this reduction is significantly reversed by the simultaneous addition of the PKC inhibitor sphingosine (10^{-5} M, Table 2). Such agonist-induced desensitization of PKC during long-term culture has been well-documented (36, 37). The sensitivity of PDGF desensitization compared with that of insulin or EGF response may be an unique regulatory device to reduce PDGF action in the absence or presence of α_1-adrenoceptor agonists.

Considering another pathway, if the phenylephrine effects in the presence of PDGF are mediated through calcium ions, phenylephrine can be replaced by the calcium ionophore, ionomycin (38). However, calcium ions do not appear to be involved in the phenylephrine effects, since no changes in hepatocyle DNA synthesis and proliferation were observed upon the addition of 10^{-5} M ionomycin when the cells were cultured with 10^{-5} M ionomycin for 4 and 21 hr (Table 2). In addition, this notion is supported by

the results showing that other calcium-mobilizing agents such as angiotensin II and arginine vasopressin (10^{-8} - 10^{-6} M) did not affect hepatocyte DNA synthesis and proliferation induced by 10 ng/ml PDGF (data not shown).

Tyrosine kinase receptors, such as EGF, HGF and PDGF, are known to generate IP_3 and DG by interacting directly with PLC-γ to stimulate hepatocyte growth and proliferation (9, 39-42). Therefore, we investigated further the possible signal transduction mechanisms that lead to activation of hepatocyte DNA synthesis and proliferation induced by PDGF in primary cultures of adult rat hepatocytes (Table 3). The addition of 5 x 10^{-6} M genistein (22) to hepatocyte cultures to inhibit tyrosine kinase results in the complete attenuation of PDGF-induced hepatocyte DNA synthesis and proliferation, suggesting that the mitogenic effects of this growth factor are mediated through tyrosine kinase activation. Hepatocyte DNA synthesis and proliferation induced by PDGF was almost completely blocked by specific inhibitors of signal transducers such as the specific PI(3)K inhibitor wortmannin (10^{-7} M) and the p70 S6K inhibitor rapamycin (10 ng/ml). Consistent with these findings, Valius and Kazlauskas observed that the activation of PLC-γ and PI(3)K is sufficient to trigger hepatocyte DNA synthesis and proliferation in the human hepatoma cell line Hep G2 (43). Furthermore, although there are data correlating increased cellular proliferation with the activation of P70S6K (26-28), the precise signal

transduction mechanism to explain all these events is not completely defined. Such investigations are now under way in our laboratory.

In conclusion, our results demonstrate for the first time that PDGF can rapidly induce DNA synthesis and proliferation of adult rat hepatocytes in primary culture. This induction has been found to be independent of the initial plating density. The mitogenic effects of PDGF were markedly potentiated by an α_1-adrenoceptor agonist, phenylephrine, which may be mainly mediated through PKC. The present results also suggest that following stimulation with PDGF, activation of tyrosine kinase, PLC. PI(3)K, PKC and P70 S6K is essential for hepatocyte DNA synthesis and proliferation. Taken together, it can be speculated that α_1-adrenoeeptor-mediated action plays a positive role in the regulation of normal liver growth and liver regeneration induced by PDGF *in vivo*.

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FIGURE LEGENDS

Fig. 1. Time course of associated with stimulation of hepatocyte DNA synthesis and proliferation by PDGF with or without phenylephrine. Hepatocytes (3.3×10^4 cells/cm^2) were cultured with or without phenylephrine (10^{-6} M) in presence of PDGF (10 ng/ml) for various lengths of time. PDGF and/or phenylephrine were added at the time of medium change as indicated by the arrow. Data are expressed as mean ± S.E. of three separate experiments. *P< 0.05, **P< 0.01, compared with the control (values just before PDGF addition).

Fig. 2. Dose-dependent effect of PDGF on hepatocyte DNA synthesis and proliferation. Hepatocytes (3.3×10^4 cells/cm^2) were cultured with various concentrations of PDGF for 4 hr. Data are expressed as mean ± S.E. of three separate experiments.

Fig. 3. Influence of cell density on PDGF-stimulated hepatocyte DNA synthesis with or without phenylephrine. Hepatocytes were cultured with PDGF (10 ng/ml) in the presence or absence of phenylephrine (10^{-6} M) at various plating densities for 4 hr. Data are expressed as mean ± S.E. of three separate experiments. *P< 0.05, **P< 0.01, ***P< 0.001 compared with the respective control.

Fig. 4. Influence of cell density on PDGF-stimulated hepatocyte proliferation with or without phenylephrine. Hepatocytes were cultured with PDGF (10 ng/ml) at various plating densities for 4 hr. Data are expressed as mean ± S.E. of three separate experiments. *P< 0.05, **P< 0.01, compared with respective control.

Fig. 5. Dose-dependent effects of phenylephrine on PDGF-stimulated hepatocyte DNA synthesis and proliferation at early phase of primary culture. Hepatocytes (3.3×10^4 cells/cm^2) were cultured with or without various concentrations of phenylephrine (10^{-6} M) in the presence of PDGF (10 ng/ml) for various times. Data are expressed as mean ± S.E. of three separate experiments. *P< 0.05, compared with the control (values with PDGF addition).

Table 1. Effects of suramin, specific α_1- and α_2-adrenoceptor antagonists on phenylephrine-stimulated hepatocyte DNA synthesis and proliferation in the presence of PDGF

Treatment	DNA synthesis (dpm/mg protein/h x 10^{-3})		Number of nuclei (% of control)	
	Culture time (hr)		Culture time (hr)	
	4	21	4	21
Control	0.435±0.212	0.596±0.448	100.0±2.4	99.9±3.7
PDGF	5.030±0.307[b]	2.951±0.569[a]	119.0±3.2[b]	124.6±3.0[b]
+phenylephrine	10.404±0.921[b, d]	3.429±0.889[a]	143.9±3.4[b, d]	149.9±3.0[b, d]
+suramin	1.267±0.322[d]	2.007±0.633	100.9±2.6[c]	103.3±3.7[c]
+phenylephrine+suramin	1.933±1.006[c]	1.064±0.923	103.1±3.5[c]	105.3±3.2[c]
+prazosin	4.952±0.400[b]	2.807±1.005	121.0±3.0[b]	124.3±3.8[a]
+phenylephrine+prazosin	4.854±1.007[a]	2.918±0.829	123.1±3.0[b]	128.5±5.2[a]
+yohimbine	5.429±1.020[b]	2.993±0.758	120.6±5.1[a]	129.0±4.4[b]
+phenylephrine+yohimbine	10.812±0.682[b, d]	3.884±0.834[a]	145.6±2.8[b, d]	149.8±4.4[b, d]
+UK-14304	4.806±0.663[b]	2.932±0.668[a]	120.4±4.4[a]	124.8±5.2[a]
+phenylephrine+UK-14304	10.317±0.832[b, d]	3.863±0.739[a]	142.6±2.8[b, d]	145.8±3.4[b, d]

Hepatocytes were plated at a density of 3.3 x 10^4 cells/cm^2 and cultured as described in legend for Fig. 1. Specific α_1- and α_2-adrenergic antagonists, phenylephrine and cAMP-elevating agents were added with 10 ng/ml PDGF immediately after medium change and cells were cultured for a further 4 hr and 21 hr. Concentrations were as follows: Phenylephrine, 10^{-6} M; suramin, 10^{-5} M; prazosin, 10^{-6} M; yohimbine, 10^{-6} M; UK-14304, 10^{-6} M. Each value is expressed as mean ± S.E. from 3 independent preparations. Values significantly different from control are indicated by [a] $P < 0.05$, [b] $P < 0.01$. Values significantly different from PDGF alone are indicated by [c] $P < 0.05$, [d] $P < 0.01$.

Table 2. Effects of U-73122, sphingosine, PMA and ionomycin on PDGF-stimulated hepatocyte DNA synthesis and poliferation

Treatment	DNA synthesis (dpm/mg protein/h x 10^{-3})		Number of nuclei (% of control)	
	Culture time (hr)		Culture time (hr)	
	4	21	4	21
Control	0.475±0.211	0.596±0.448	100.0±2.4	99.9±3.7
PDGF	5.364±0.833[b]	2.969±0.599[a]	121.1±3.3[b]	126.6±3.9[b]
+phenylephrine	10.211±0.951[b, c]	3.725±0.835[a]	140.3±4.1[b, c]	141.0±3.2[b, c]
+U-73122	1.463±0.533[c]	5.896±1.004[b]	100.3±3.8[c]	126.7±3.7[b]
+U-73343	5.167±0.634[b]	2.972±1.065	121.9±4.2[a]	127.3±4.2[b]
+sphingosine	1.755±0.560[c]	6.338±0.673[b, c]	105.9±3.4[c]	132.9±4.9[b]
+ionomycin	7.034±0.745[b]	3.021±0.822	123.3±3.5[b]	124.0±3.4[b]
+PMA	12.063±0.934[b, d]	3.281±0.633[a]	145.3±4.2[b, c]	145.7±4.5[b, c]
+PMA+U-73122	12.061±1.003[b, d]	3.833±1.011[a]	144.6±4.0[b, c]	146.0±4.6[b, c]
+PMA+U-73343	12.003±0.905[b, d]	3.685±0.621[a]	145.6±4.7[b, c]	145.8±4.8[b, c]
+PMA+sphingosine	6.273±1.301[a]	4.288±1.102[a]	130.6±2.0[b]	139.0±4.0[b]
+PMA+ionomycin	12.236±1.023[b, d]	3.056±0.879	144.3±4.4[b, c]	144.6±4.0[b, c]

Hepatocytes were plated at a density of 3.3 x 10^4 cells/cm^2 and cultured as described in legend for Fig. 1. U-73122, U-73343, sphingosine, ionomycin, phenylephrine and PMA were added with 10 ng /ml PDGF immediately after medium change and cells were cultured for a further 4 hr and 21 hr. Concentrarions were as follows: Phenylephrine, 10^{-6} M; U-73122, 3 x 10^{-6} M; U-73343, 10^{-5} M; sphingosine, 10^{-5} M; ionomycin, 10^{-5} M; PMA, 10^{-7} M. Each value is expressed as mean ± S.E. from 3 independent preparations. Values significantly different from control are indicated by [a] $P < 0.05$, [b] $P < 0.01$. Values significantly different from PDGF alone are indicated by [c] $P < 0.05$, [d] $P < 0.01$.

Table 3. Effect of specific inhibitors of signal-transducing elements on hepatocyte DNA synthesis and number of nuclei induced by phenylephrine in the presence of PDGF

Treatment	DNA synthesis (dpm/mg protein/h x 10^{-3})		Number of nuclei (% of control)	
	Culture time (hr)		Culture time (hr)	
	4	21	4	21
Control	0.683±0.459	0.629±0.391	100.0±3.0	100.3±4.1
PDGF	5.024±0.780[b,]	2.939±0.690	119.7±3.5[a]	125.2±3.0[b]
+phenylephrine	10.226±1.074[b, c]	3.434±1.056	139.4±3.4[b, c]	140.2±4.2[b, c]
+genistein	0.821±0.431[d]	0.779±0.630	101.3±4.1[c]	103.0±3.6[d]
+phenylephrine+genistein	0.972±0.506[c]	0.877±0.401	102.5±3.6[c]	104.7±4.7[c]
+wortmannin	0.742±0.567[c]	0.693±0.573	102.2±4.5[c]	101.3±5.0[c]
+phenylephrine+wortmannin	0.750±0.538[c]	0.622±0.607	103.2±3.5[c]	102.3±4.0[c]
+rapamycin	0.709±0.394[d]	0.579±0.448[c]	100.3±3.1[c]	99.7±3.7[d]
+phenylephrine +rapamycin	0.732±0.323[d]	0.497±0.324[c]	100.8±3.1[c]	100.2±3.1[d]

Hepatocytes were plated at a density of 3.3 x 10^4 cells/cm^2 and cultured as described in legend for Fig. 1. Specific inhibitors of signal-transducing elements were added with 10 ng/ml PDGF immediately after medium change and cells were cultured for a further 4 hr and 21 hr. Concentrations were as follows: Phenylephrine, 10^{-6} M; genistein, 5 x 10^{-6} M; wortmannin, 10^{-7} M; rapamycin, 10 ng/ml. Each value is expressed as mean ± S.E. from 3 independent preparations. Values significantly different from control are indicated by [a] $P < 0.05$, [b] $P < 0.01$. Values significantly different from PDGF alone are indicated by [c] $P < 0.05$, [d] $P < 0.01$.

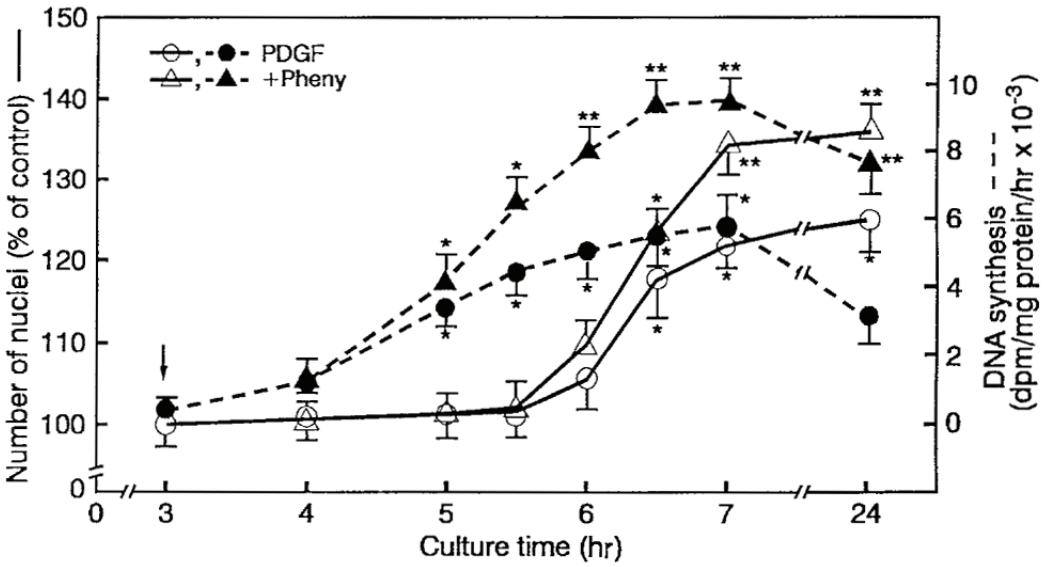


Fig. 1 Kimura M and Ogihara M

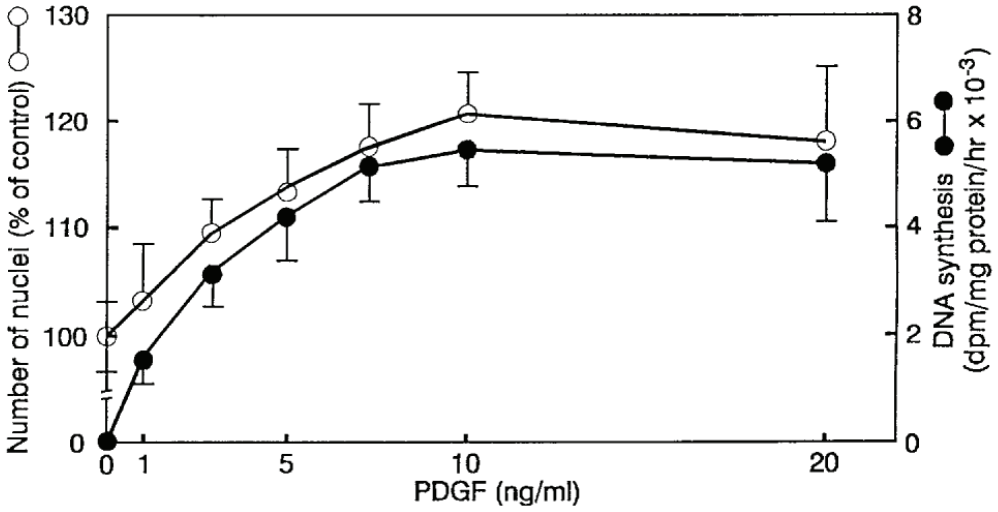


Fig. 2 Kimura M and Ogihara M

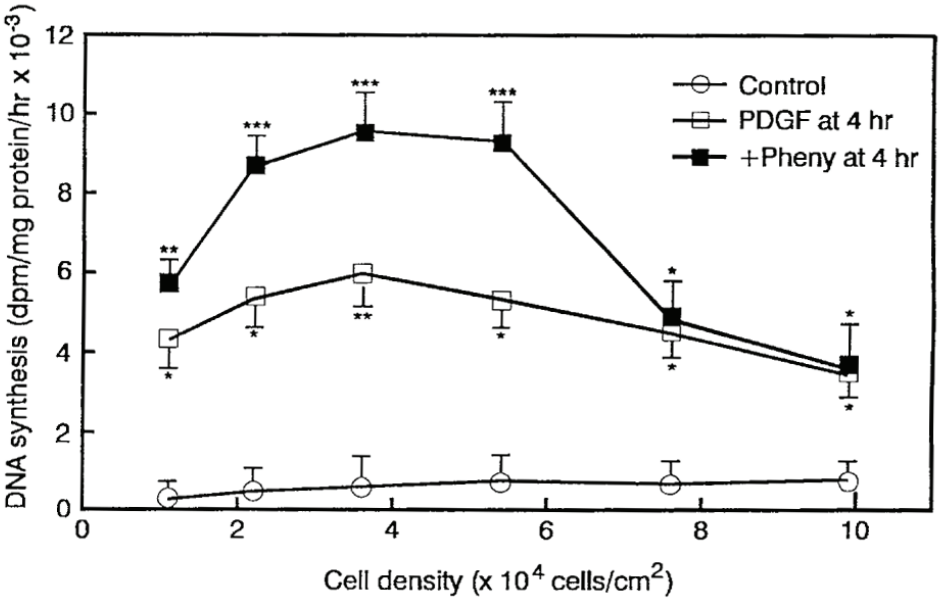
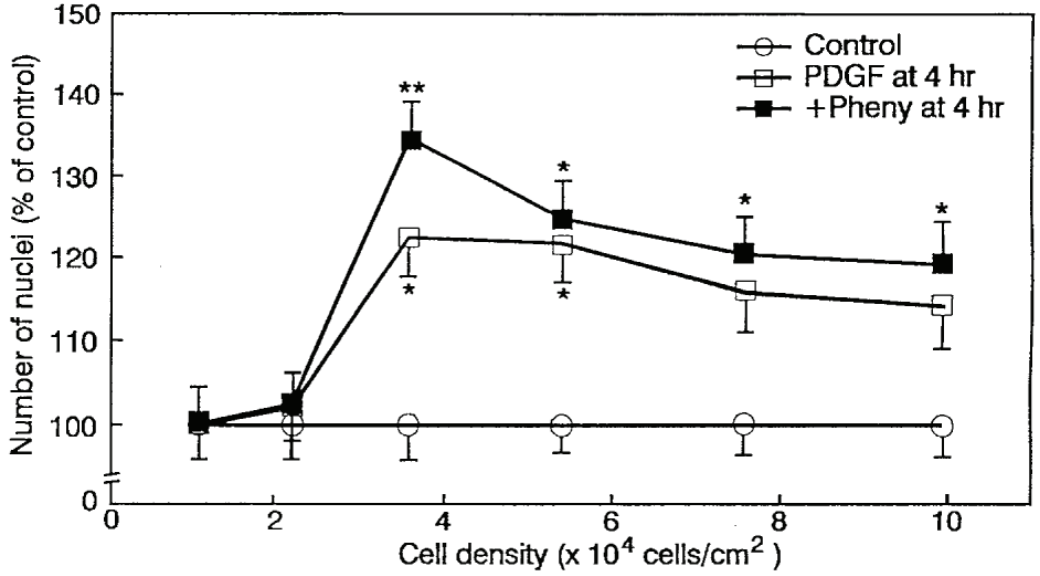


Fig. 3 Kimura M and Ogihara M



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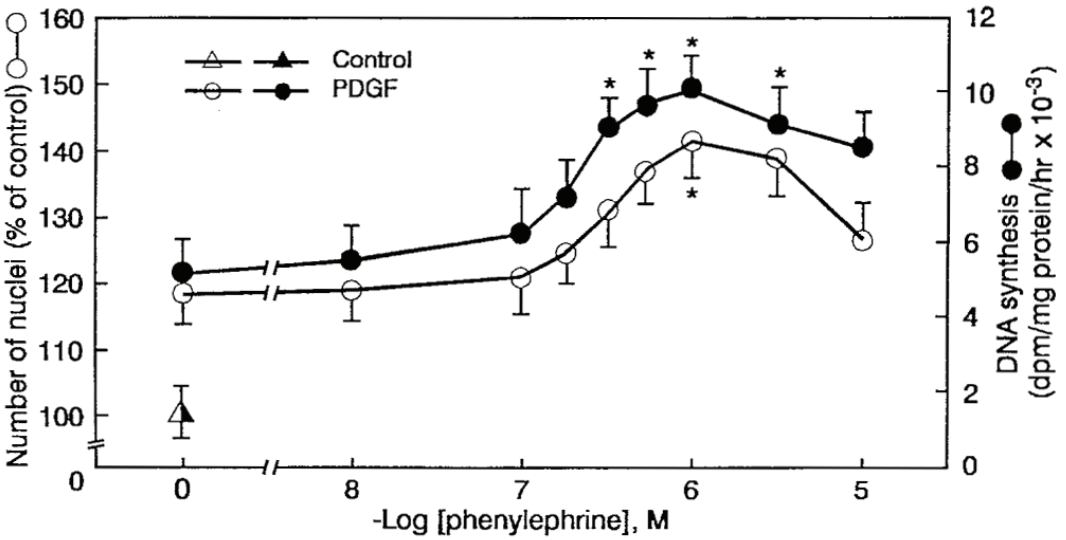

Fig. 5 Kimura M and Ogihara M