Inhibitory Effects of Dexamethasone on Hepatocyte Growth Factor-Induced DNA Synthesis and Proliferation in Primary Cultures of Adult Rat Hepatocytes

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Abstract. We investigated the effects of dexamethasone on hepatocyte growth factor (HGF)-induced DNA synthesis and proliferation in serum-free primary cultures of adult rat hepatocytes. Isolated hepatocytes were cultured at a density of 3.3×10^4 cells/cm^2 in Williams' medium E containing 5% newborn bovine serum and various concentrations of dexamethasone for 1, 2, and 3 h. After a 3-h attachment period, the medium was then changed, and cells were cultured in serum-free, and dexamethasone (10^{-10} M)-contained Williams' medium E with or without glucocorticoid receptor antagonists. After addition of dexamethasone to the culture medium, the growth-stimulating effects of HGF (5 ng/mL) on the primary cultured hepatocytes were time- and dose-dependently inhibited. The mineralcorticoid aldosterone (10^{-7} M) did not produce the same growth-inhibitory effects as dexamethasone (10^{-8} M). The inhibitory effects of dexamethasone were reversed by treatment with the glucocorticoid receptor antagonist mifepristone (RU486, 10^{-6} M) or a monoclonal antibody against glucocorticoid receptor (100 ng/mL). In addition, the growth-inhibitory dose of dexamethasone did not affect HGF-induced receptor tyrosine kinase and extracellular signal-regulated kinase 2 phosphorylation. These results indicate that dexamethasone dose-dependently delays and inhibits HGF-induced DNA synthesis and proliferation through its own intracellular receptor in primary cultures of adult rat hepatocytes.

Keywords: DNA synthesis, proliferation (cultured hepatocyte), hepatocyte growth factor, dexamethasone, signal transduction

Introduction

Much of the research on the proliferation of hepatocytes *in vitro* has been motivated by studies of liver regeneration *in vivo* (1). The factors that initiate and regulate the replication of adult mammalian liver cells following 70% partial hepatectomy have been investigated *in vitro*, and the signal transduction mechanisms of these growth factors have been reviewed (2–4). Maintained in short-term culture, in a defined medium, quiescent hepatocytes undergo one or two rounds of replication in response to mitogens (e.g., epidermal growth factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)- and insulin) (5–11). In addition to the growth factor effects on hepatocyte proliferation, there are various other factors and conditions that modulate the response of hepatocytes to growth stimuli *in vitro* (e.g., co-mitogens, component of basal culture medium, cell density, extracellular matrix materials, and cell-derived autocrine factors) (5,6,12). As *in vitro* systems have been useful for clarifying the mechanism of action of those factors, it is important for investigators to define the conditions under which primary hepatocytes are proliferation-competent *in vitro*.

In routine procedures, hepatocytes are seeded in serum-containing culture medium with a fixed dose of dexamethasone. This is subsequently exchanged for serum-free, dexamethasone-free medium, cells are then

treated with several testing agents, and hepatocyte DNA synthesis and proliferation induced by growth factors such as EGF and HGF are measured in further cultures. With regard to glucocorticoids such as dexamethasone and hydrocortisone within the components added to culture medium, it was observed that they induce cell attachment and viability, but not proliferation (5–7,13,14). However, the concentrations and timing of glucocorticoid addition to cultures varies among investigators (5,15). Thus, optimal culture conditions remain to be identified.

HGF is a potent mitogen first purified from rat platelet and human and rabbit plasma (16). It may act a humoral factor to stimulate liver regeneration in vivo (2–4). The main purpose of the present study was to investigate the effects of glucocorticoid dexamethasone on HGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. In addition, we investigated the glucocorticoid receptor mediation of dexamethasone in relation to the mechanism of intracellular signal transduction during primary culture. Our results show that higher doses of dexamethasone delay and inhibit the HGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes, while lower doses of dexamethasone are optimal for assessing DNA synthesis and proliferation in a defined medium and short-term culture without affecting cell viability and attachment. In addition, glucocorticoid receptor is responsible for the growth-inhibitory actions of dexamethasone in primary

cultured hepatocytes.

Materials and Methods

Animals

Male Wistar rats weighing 200–220 g were obtained from Tokyo Experimental Animal Co. (Tokyo, Japan). Adaptation to a light-, humidity- and temperature-controlled room occurred over a minimum 3-day period prior to the start of experiments. Rats were fed standard diet and given tap water *ad libitum*. The animals used in this study were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals of Josai University.

Hepatocyte Isolation and Culture

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). Hepatocytes were isolated from normal livers by the two-step *in situ* collagenase perfusion technique devised by Seglen in order to facilitate disaggregation of the adult rat liver (17). Briefly, dispersed hepatocytes were washed three times by slow centrifugation (50 × g, 1 min) of the cell suspension to remove cell debris, damaged cells and non-parenchymal cells. Viability, as tested by Trypan blue exclusion, was more than 97%. Unless otherwise indicated, freshly isolated hepatocytes

were plated onto collagen-coated plastic culture dishes (Asahi Techno Glass Co., Tokyo, Japan) at a density of 3.3×10^4 cells/cm^2 (3.0×10^5 cells/35-mm dish), and firstly cultured in Williams' medium E with 5% newborn calf serum and dexamethasone (10^{-10} -10^{-8} M) for 3 h under 5% CO_2 in air at 37 ^oC. Then, the cells were cultured further in serum-free and low concentration of dexamethasone (10^{-10} M)-contained medium with hepatocyte growth factor (HGF, 5 ng/mL). When appropriate, the following agents were added: HGF with or without growth-related signal transducer inhibitors (i.e., PHA665752 (18), PD98059 (19), mifepristone (20)), and a monoclonal antibody against glucocorticoid receptor (21).

Measurement of DNA Synthesis

Hepatocyte DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into acid-precipitable materials. Briefly, after an initial attachment period of 3 h, hepatocytes were washed twice with serum-free, dexamethasone (10^{-10} M)-contained Williams' medium E and cultured in medium containing HGF (5 ng/mL) with or without testing agents for an additional 4 or 21 h. Cells were pulsed at 1, 2, 3, or 19 h after 2-h HGF stimulation with [^{3}H]thymidine (1.0 ∝Ci/well), followed by 10% trichloroacetic acid precipitation, as described previously (22). [^{3}H]Thymidine incorporation into DNA was determined using a scintillation counter in terms of counts per min, and was normalized for cellular protein.

Aphidicolin (10 ∝g/ml) was added to some wells in order to establish the level of non-replicative DNA synthesis. Hepatocyte protein content was measured by a modified Lowry procedure using bovine serum albumin as a standard (23). Data are expressed as dpm/h/mg cellular protein.

Nuclei Counting

The number of nuclei rather than the number of cells was counted using a modified version of the procedure described previously (5,6). Briefly, primary cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (PBS, pH 7.4). Isolated liver cell nuclei were then prepared for quantitation by exposing cultured hepatocytes to 0.25 ml of citric acid (0.1 M) containing Triton X-100 (0.1%) for 30 min at 37 ºC. An equal volume of the nucleus suspension was mixed with Trypan blue (0.3%) in PBS (pH 7.4) and the number of nuclei was counted using a hemocytometer.

Determination of receptor tyrosine kinase activity and extracellular signal-regulated kinase (ERK) Activity

c-Met is a tyrosine kinase receptor for HGF/scatter factor. A 145-kDa protein (HGF receptor subunit) was identified by immunoprecipitation and subsequent immunoblotting with corresponding anti-phospho-receptor tyrosine kinase antibody according to the method described previously (24)

and the manufacturer's instructions. Tyrosine kinase activity (autophosphorylation) of the phosphorylated p145-kDa protein (p-c-Met) was normalized against that of the total p145-kDa protein.

Phosphorylated ERK isoform (ERK1 and ERK2) was identified by Western blot analysis using a 1:1000 dilution of rabbit polyclonal dual phospho-specific antibodies (1 mg/ml) with HRP-conjugated goat anti-rabbit IgG as a secondary antibody, as described previously (25). Briefly, cultured hepatocytes were washed with ice-cold PBS (pH 7.4), and 0.2 ml of lysis buffer (10 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 10 ∝g/ml leupeptin, 10 ∝g/ml aprotinin) was then added and the hepatocytes were harvested. After centrifugation, cell lysates were denatured in boiling water for 5 min. Samples of the supernatant (20 ∝g/lane) were subjected to SDS-PAGE on a 10% polyacrylamide resolving gel using the method of Laemmli (26). After electrophoresis, proteins were transferred to a PVDF membrane at 4 ºC for 10 h. Proteins were quantified by densitometry after the membrane was developed with enhanced chemiluminescence reagent and exposure to Hyperfilm (Kodak, Japan). Densitometric analysis was performed using the NIH image program (ver. 1.6 for Macintosh). Phosphorylated ERK (p-ERK) activity was normalized against total ERK activity. Data were calculated in arbitrary units and are expressed as means ± S.E.M (*P < 0.05 versus medium alone). The

autodiagram is a representation of three experiments using different cell preparations.

Materials

The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA): HGF (human recombinant); mifepristone (RU486); aldosterone; aphidicolin; dexamethasone; hydrocortisone; and aprotinin. PHA665752 was obtained from Calbiochem (Darmstadt, Germany). Monoclonal antibody against glucocorticoid receptor was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal antibody against phospho-c-Met (Tyr1234/1245) was obtained from Cell Signaling Technology (Bevery, MA, USA). Williams' medium E and newborn 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 purchased from DuPont-New England Nuclear (Boston, MA, USA). All other reagents were of analytical grade.

Statistical analyses

Data are expressed as means ± S.E.M. Group comparisons were made by analysis of variance (ANOVA) for unpaired data followed by post-hoc analysis using Dunnett's multiple comparison test. Differences of $P < 0.05$

were considered to be statistically significant.

Results

Time course of inhibitory effects of dexamethasone on HGF-induced hepatocyte DNA synthesis and proliferation

We first examined the effects of glucocorticoid dexamethasone on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes in the presence of hepatocyte growth factor (HGF, 5 ng/mL). Freshly isolated hepatocytes were plated at a low cell density (3.3×10^4 cells/cm^2). Low-dose dexamethasone (10^{-10} M) was added 3 h before HGF addition (time zero), and the change to serum-free, dexamethasone (10^{-10} M or 10^{-8} M)-containing culture medium was made. When cultured for various lengths of time, the hepatic parenchymal cells underwent time-dependent DNA synthesis and proliferation (i.e., an increase in the number of nuclei) in the presence of 5 ng/mL HGF alone. The onset of DNA synthesis was first observed at about 2.0 h after the addition of HGF (Fig. 1A), while mitotic activity in the hepatocytes was first observed at about 3.0 h and peaked at 21 h (Fig. 1B). Maximal stimulation for hepatocyte DNA synthesis and proliferation seen with HGF were approximately 6.0- and 1.3-fold, respectively. DNA synthesis became markedly reduced at 21 h. In contrast, treatment with higher doses of dexamethasone (10^{-8} M) almost completely suppressed the

HGF-induced DNA synthesis and proliferation. Mineral corticoid aldosterone (10^{-7} M) and hydrocortisone (10^{-7} M, a low-efficacy glucocorticoid) did not affect the HGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Cell viability and attachment were not affected by various concentrations of dexamethasone treatment.

Effects of timing of dexamethasone addition on HGF-induced hepatocyte DNA synthesis and proliferation

The effects of timing of dexamethsone addition to the culture on HGF-induced hepatocyte DNA synthesis and proliferation were investigated. High-dose dexamethasone (10^{-8} M) was added at 1, 2, and 3 h before HGF addition, and the change to serum-free, dexamethasone (10^{-10} M)-contained Williams' medium E supplemented with 5 ng/mL HGF was made (time zero). When isolated hepatocytes were treated with low-dose dexamethasone (10^{-10} M, control) for 3 h in serum-containing medium, followed by serum-free, and dexamethasone (10^{-10} M)-contained culture medium, the maximum stimulation of HGF-induced hepatocyte DNA synthesis and proliferation obtained were as shown in Figs. 1A and 1B. When compared with the control, when higher doses of dexamethasone (10^{-8} M) were added at 1 and 2 h before 5 ng/mL HGF addition, the onset of DNA synthesis was significantly delayed depending on the pretreatment time. In

contrast, when dexamethasone (10^{-8} M) was added at 3 h before 5 ng/mL HGF addition, the onset of hepatocyte DNA synthesis was markedly delayed (Fig. 2A). In addition, the hepatic parenchymal cells did not significantly undergo proliferation until 21 h in the presence of 5 ng/mL HGF (Fig. 2B). With high-dose dexamethasone (10^{-8} M) during 24 h culture, the HGF-induced hepatocyte DNA synthesis and proliferation were almost completely suppressed. Cell viability and attachment were not affected by pretreatment with dexamethasone (10^{-8} M).

Dose-dependent inhibitory effects of dexamethasone on HGF-induced Hepatocyte DNA Synthesis and Proliferation: Influence of mifepristone (RU486) and monoclonal antibody against glucocorticoid receptor

The dose-dependent effects of dexamethasone on HGF-induced hepatocyte DNA synthesis and proliferation were investigated after 4 h of culture in the presence of various concentrations of dexamethasone. In addition, to confirm glucocorticoid receptor mediation of the inhibitory effects of dexamethasone, we investigated the effects of a glucocorticoid receptor antagonist mifepristone (RU486) (20), and a monoclonal antibody against glucocorticoid receptor (21), on the HGF-induced hepatocyte DNA synthesis and proliferation. As shown in Figs. 3A and 3B, the DNA synthesis and proliferating effects of 5 ng/mL HGF on primary cultured hepatocytes were significantly inhibited by dexamethasone (3×10^{-9} to 1 ·

10^{-7} M) in a dose-dependent manner (IC_{50} value, 3.0×10^{-9} M). The inhibitory effects of dexamethasone (3×10^{-9} to $1 \cdot 10^{-7}$ M) on the HGF-induced hepatocyte DNA synthesis and proliferation were almost completely reversed by pretreatment with mifepristone (10^{-6} M) or a monoclonal antibody against glucocorticoid receptor (100 ng/mL).

Effects of dexamethasone on HGF-induced receptor tyrosine kinase phosphorylation

In order to confirm the notion that HGF induces hepatocyte DNA synthesis and proliferation through receptor tyrosine kinase activation, we investigated whether 5 ng/mL HGF is able to stimulate receptor tyrosine kinase phosphorylation. The effects of dexamethasone (10^{-8} M) on receptor tyrosine kinase phosphorylation in the presence of 5 ng/mL HGF were also investigated. Figure 4 (Western blot data; upper panel) shows that HGF stimulation caused a significant increase in phosphorylation of c-Met tyrosine kinase (145 kDa) within 3 min. Pretreatment with high-dose dexamethasone (10^{-8} M) did not affect the HGF-induced increase in c-Met phosphorylation. In addition, p145 c-Met phosphorylation induced by 5 ng/mL HGF was abolished by specific c-Met kinase inhibitor PHA665752 (10^{-7} M), but was not affected by aldosterone (10^{-7} M) or PD98059 (10^{-6} M) treatment (lower panel).

Effects of dexamethasone on HGF-induced extracellular signal-regulated kinase phosphorylation

In order to confirm the notion that HGF induces hepatocyte DNA synthesis and proliferation through extracellular signal-regulated kinase (ERK) activation, we investigated whether 5 ng/mL HGF is able to stimulate ERK isoform activity. The effects of dexamethasone (10^{-8} M) on ERK phosphorylation in the presence of 5 ng/mL HGF were also examined. Figure 5 shows that HGF stimulation caused a significant increase in phosphorylation of ERK2, but not ERK1 (Western blot data, upper panel). ERK2 phosphorylation peaked at about 3-fold (as compared with control) at 5 min after addition of 5 ng/mL HGF. Pretreatment with high-dose dexamethasone (10^{-8} M) did not affect HGF-induced increase in ERK2 phosphorylation. In addition, ERK2 phosphorylation induced by 5 ng/mL HGF was abolished by the MAP kinase kinase inhibitor PD98059 (10^{-6} M), but was not affected by aldosterone (10^{-7} M) treatment (lower panel).

Effects of dexamethasone on HGF-induced hepatocyte DNA synthesis and proliferation with or without specific c-Met kinase inhibitor PHA665752 and MAP kinase kinase inhibitor PD98059

In order to confirm the notion that 5 ng/mL HGF induces hepatocyte DNA synthesis and proliferation through receptor tyrosine kinase or ERK activation, we investigated whether or not PHA665752 and PD98059 are

able to inhibit HGF-induced hepatocyte DNA synthesis and proliferation in the presence or absence of dexamethasone or aldosterone. As shown in Fig.6, a specific c-Met inhibitor PHA665752 (10^{-7} M) almost completely blocked HGF-induced stimulation of hepatocyte DNA synthesis and proliferation with or without dexamethasone (10^{-8} M) or aldosterone (10^{-7} M). Treatment of hepatocytes with a specific MAP kinase kinase inhibitor, PD98059 (10^{-6} M), also completely inhibited HGF-induced hepatocyte DNA synthesis and proliferation in the absence or presence of dexamethasone or aldosterone.

Discussion

Primary mitogens (e.g., EGF and HGF) reportedly have growth-stimulating effects on primary cultured hepatocytes through intracellular signal transduction, which in turn brings about changes in the plasma membrane receptor leading to nuclear DNA synthesis and proliferation (2-4). The signal transduction pathway of growth factors has been investigated in vitro. Other substances that modulate the response of hepatocytes to growth stimuli have also been studied. Glucocorticoids such as dexamethasone and hydrocortisone have been reported to affect cell attachment and viability, but not proliferation (5, 15). However, the concentrations (about 10^{-9}-10^{-5} M) and timing of glucocorticoid addition to cultures has varied among investigators (5,6,27,28). Especially, these doses

are higher compared with our experimental condition. Therefore, we investigated the effects of dexamethasone on DNA synthesis and proliferation induced by HGF (5 ng/mL) in primary cultures of adult rat hepatocytes.

As shown in Fig. 1, when lower doses of dexamethasone (10^{-10} M) were added 3 h before 5 ng/mL HGF addition, the hepatic parenchymal cells underwent time-dependent DNA synthesis and proliferation (i.e., an increase in the number of nuclei) in the presence of 5 ng/mL HGF. Onset of DNA synthesis was first observed at about 2.5 h after addition of HGF (Fig. 1A), while mitotic activity of the hepatocytes was first observed at about 3.0 h, and peaked at 4.0 h (Fig. 1B). In contrast, when high-dose dexamethasone (10^{-8} M) was added at 3 h before 5 ng/mL HGF addition, HGF-induced hepatocyte DNA synthesis and proliferation were significantly delayed and inhibited in a time-dependent manner (Figs. 2A and 2B). These results demonstrate that high-dose dexamethasone (10^{-8} M), but not high doses of hydrocortisone (10^{-7} M, a low-efficacy glucocorticoid) or aldosterone (10^{-7} M, mineralcorticoid), significantly suppresses cell cycle progression. In support of this notion, it has been reported that regeneration is crucial for the recovery of hepatic mass following liver regeneration, and glucocorticoids and immunosuppressive agents that are commonly used in transplantation are known to inhibit the expression of specific cytokine and growth factor (e.g., tumor necrosis

factor- and interleukin 6, and HGF). Debonera et al. demonstrated that administration of dexamethasone at the time of transplantation delays expression and nuclear translocation of cyclin D1 and impairs DNA synthesis and mitosis, and inhibits the initiation of the regenerative process *in vivo* (29). Their studies may be particularly relevant to the present results.

However, intracellular signal transduction mechanisms of the growth-inhibitory effects of dexamethasone in the presence of 5 ng/mL HGF remain to be clarified. Specific inhibitors of growth-related signal transducers, mifepristone (10^{-6} M) and a monoclonal antibody against glucocorticoid receptor were found to reverse the inhibitory effects of dexamethasone on HGF-stimulated hepatocyte DNA synthesis and proliferation (Fig. 3A, B, Fig. 6A, B). This suggests that higher doses of dexamethasone (10^{-8} M) inhibit the HGF-induced hepatocyte DNA synthesis and proliferation through binding to its own receptor.

HGF reportedly acts through tyrosine kinase receptors (p145 c-Met) that increase Raf and ERK activity (30, 31). ERKs are serine/threonine kinases that mediate intracellular phosphorylation events triggered by various extracellular stimuli including growth factors, cytokines, and hormones (32, 33). ERKs play a role in numerous intracellular signal transduction pathways (34). Therefore, we examined the growth-inhibitory effects of dexamethasone on HGF-induced c-Met tyrosine kinase and ERK activity.

As shown in Fig. 4A, HGF stimulation caused a rapid increase in the phosphorylation of c-Met tyrosine kinase. In addition, HGF stimulation caused a rapid increase in the phosphorylation of ERK2 (p42 MAP kinase), but not ERK1 (p44 MAP kinase). Pretreatment with high-dose dexamethasone (10^{-8} M) did not affect the HGF-induced increase in c-Met tyrosine kinase and ERK2 phosphorylation. The c-Met tyrosine kinase phosphorylation induced by HGF was abolished by the selective c-Met tyrosine kinase inhibitor PHA665752 (10^{-7} M), but was not affected by aldosterone (10^{-7} M) treatment. In addition, the ERK2 phosphorylation induced by HGF was abolished by the MAP kinase kinase inhibitor PD98059 (10^{-6} M), but was not affected by aldosterone (10^{-7} M) treatment. In addition, the hepatocyte DNA synthesis and proliferation induced by HGF was abolished by PHA665752 (10^{-7} M) or PD98059 (10^{-6} M), but was not affected by aldosterone (10^{-7} M) treatment (Fig. 6). This demonstrates that high-dose dexamethasone (10^{-8} M) inhibits the HGF-induced hepatocyte DNA synthesis and proliferation downstream of ERK2. The present results support the notion that dexamethasone (10^{-8} M) suppresses HGF-induced DNA synthesis and proliferation (i.e., cell cycle progression) at the nuclear level (27). We also found that dexamethasone dose-dependently inhibited EGF- and PDGF-induced hepatocyte DNA synthesis and proliferation (data should be published).

In conclusion, we provide evidence that low-dose dexamethasone (10^{-10} M)

is useful for assessing hepatocyte DNA synthesis and proliferation without affecting cell viability and attachment, thus minimizing the length of time that hepatocyte cultures are maintained. In addition, the glucocorticoid receptor is responsible for the growth-inhibitory effects of dexamethasone on HGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

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Figure Legends

Fig. 1. Time-course of inhibitory effects of dexamethasone on HGF-induced hepatocyte DNA synthesis and proliferation. Freshly isolated hepatocytes were cultured in Williams' medium E containing 5% newborn bovine serum, 10^{-10} M dexamethasone, 0.10 ∝g/ml aprotinin, and antibiotics (100 U/ml penicillin and 100 ∝g/ml streptomycin) at a cell density of 3.3×10^4 cell/cm². After a 3-h attachment period (time zero), the medium was rapidly replaced with serum-free Williams' medium E supplemented with 5 ng/mL HGF without or with dexamethasone (10^{-8} M), hydrocortisone (10^{-7} M), aldosterone (10^{-7} M) and cultured for various lengths of time. Hepatocyte DNA synthesis and proliferation were determined as described in the Materials and Methods section. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei as compared to control culture (B). Results are expressed as means ± S.E.M. of three experiments. *$p < 0.05$, **$p < 0.01$ vs. the respective controls.

Fig. 2. Effects of timing of dexamethasone addition on HGF-induced hepatocyte DNA synthesis and proliferation. Freshly isolated hepatocytes were plated at a density of 3.3×10^4 cells/cm² in Williams' medium E containing 5% newborn bovine serum, 0.10 ∝g/ml aprotinin, and antibiotics.

Dexamethasone (10^{-8} M) was added at 0, 1, and 2 h after the plating, and cells were cultured for 3, 2, and 1 h, respectively. Medium was then replaced by aspiration, and cells were cultured further in serum- and dexamethasone (10^{-10} M) or dexamethasone (10^{-8} M)-containing Williams' medium E supplemented with 5 ng/mL HGF, as described in the legend for Fig. 1. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei as compared to control culture (B). Results are expressed as means ± S.E.M. of three experiments. *$p < 0.05$, **$p < 0.01$ vs. the respective controls (10^{-10} M dexamethasone).

Fig. 3. Dose-dependent inhibitory effect of dexamethasone on HGF-induced hepatocyte DNA synthesis and proliferation: Influence of mifepristone (RU486) and a monoclonal antibody against glucocorticoid receptor. Freshly isolated hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and were cultured as described in the legend for Fig. 1. After the medium change, hepatocytes were cultured with various concentrations of dexamethasone with or without mifepristone (10^{-6} M) or monoclonal antibody against glucocorticoid receptor (100 ng/mL) in the presence of 5 ng/mL HGF for 4 h. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei as compared to control culture (B). Results are expressed

as means ± S.E.M. of three independent experiments. *$p < 0.05$, **$p < 0.01$ vs. HGF-treated controls (HGF alone).

Fig. 4. Effects of dexamethasone on HGF-induced receptor tyrosine kinase phosphorylation. Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and were cultured as described in the legend for Fig. 1. Specific inhibitors were added with 5 ng/mL HGF immediately after the medium change, and hepatocytes were cultured for 3 min. Phosphorylated c-Met protein (HGF receptor subunit; p145 kDa) was identified by immunoprecipitation and subsequent immunoblotting as described in the Materials and Methods section. Concentrations of test agents were as follows: dexamethasone, 10^{-8} M; PHA665752, 10^{-7} M; aldosterone, 10^{-7} M. Results are expressed as means ± S.E.M. of three independent experiments. **$p < 0.01$ vs. the respective HGF-treated controls.

Fig. 5. Effects of dexamethasone on HGF-induced ERK phosphorylation. Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and were cultured as described in the legend for Fig. 1. Specific inhibitors were added with 5 ng/mL HGF immediately after the medium change, and hepatocytes were cultured for 5 min. Hepatocyte ERK 2 (p42 MAP kinase) and ERK 1 (p44 MAP kinase) activity was determined as described in the Materials and Methods section. Concentrations of the test agents were as follows:

dexamethasone, 10^{-8} M; PD98059, 10^{-6} M; aldosterone, 10^{-7} M. Results are expressed as means ± S.E.M. of three independent experiments. **$p < 0.01$ vs. the respective HGF-treated controls.

Fig. 6. Effects of dexamethasone on HGF-induced hepatocyte DNA synthesis and proliferation. Hepatocytes were plated at a cell density of 3.3×10^4 cells/cm^2 and were cultured as described in the legend for Fig. 1. After the medium change, hepatocytes were cultured with dexamethasone (10^{-8} M) with or without PHA665752 (10^{-7} M), PD98059 (10^{-6} M), or aldosterone (10^{-7} M) in the presence of 5 ng/mL HGF for 4 h. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei as compared to control culture (B). Results are expressed as means ± S.E.M. of three independent experiments. *$p < 0.05$, **$p < 0.01$ vs. HGF-treated controls (HGF alone).

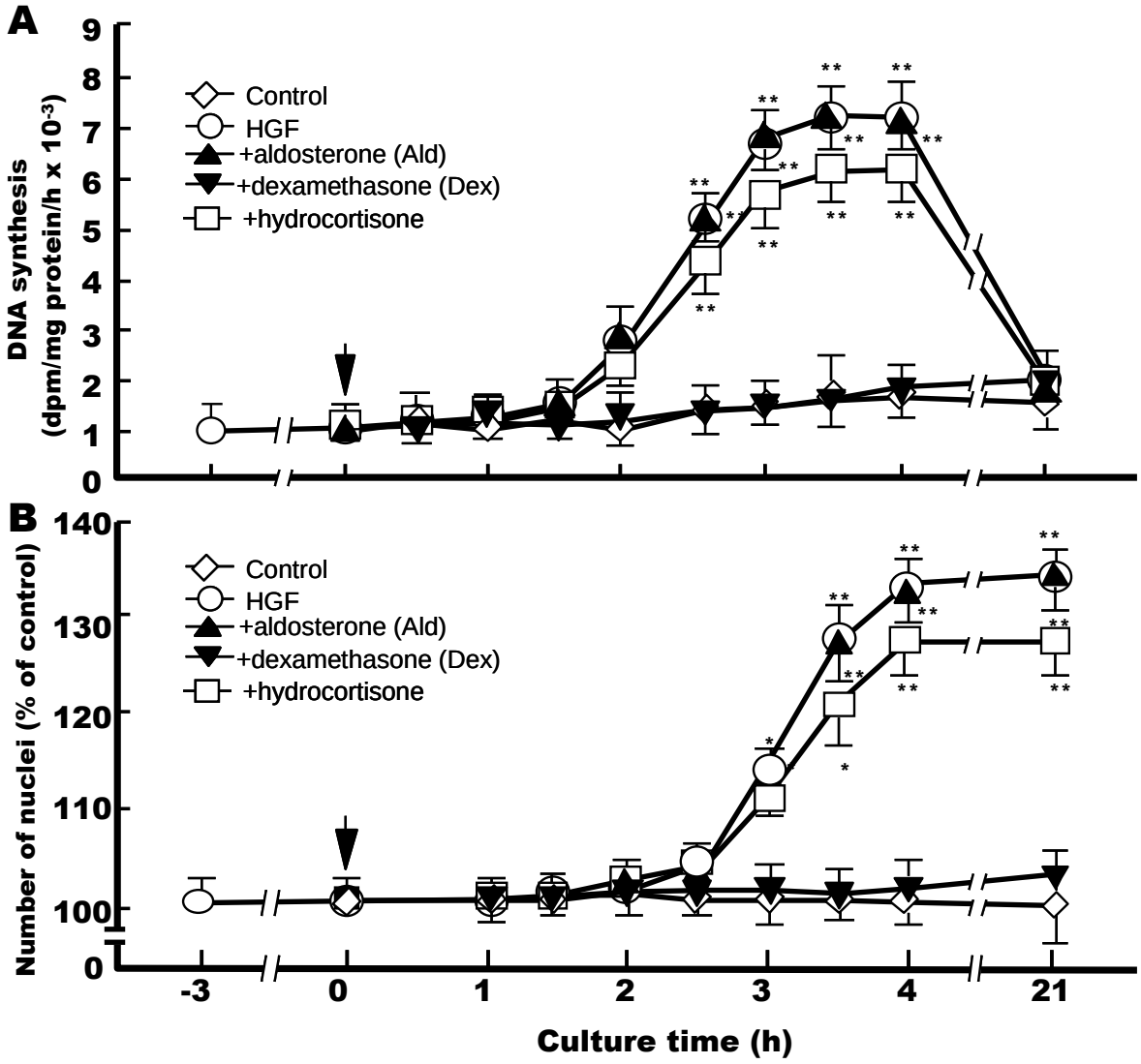

Fig.1

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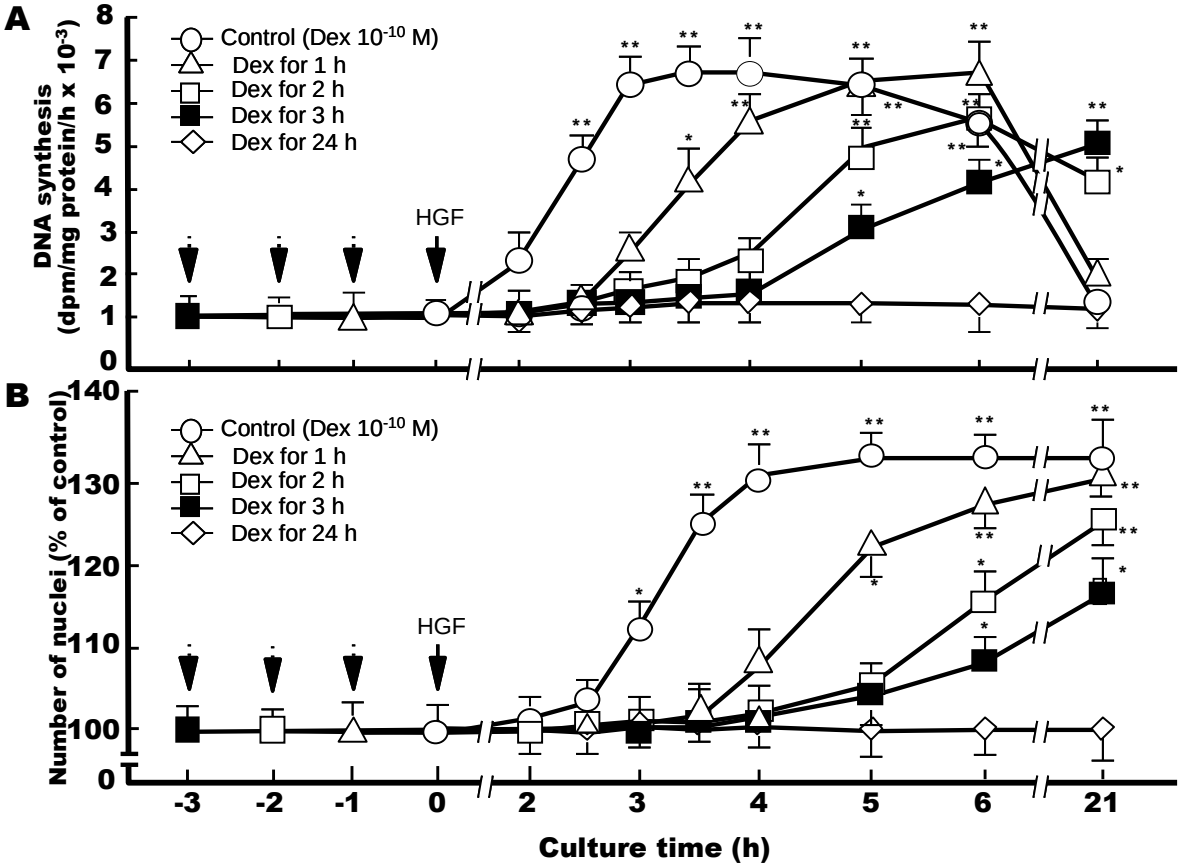

Fig.2

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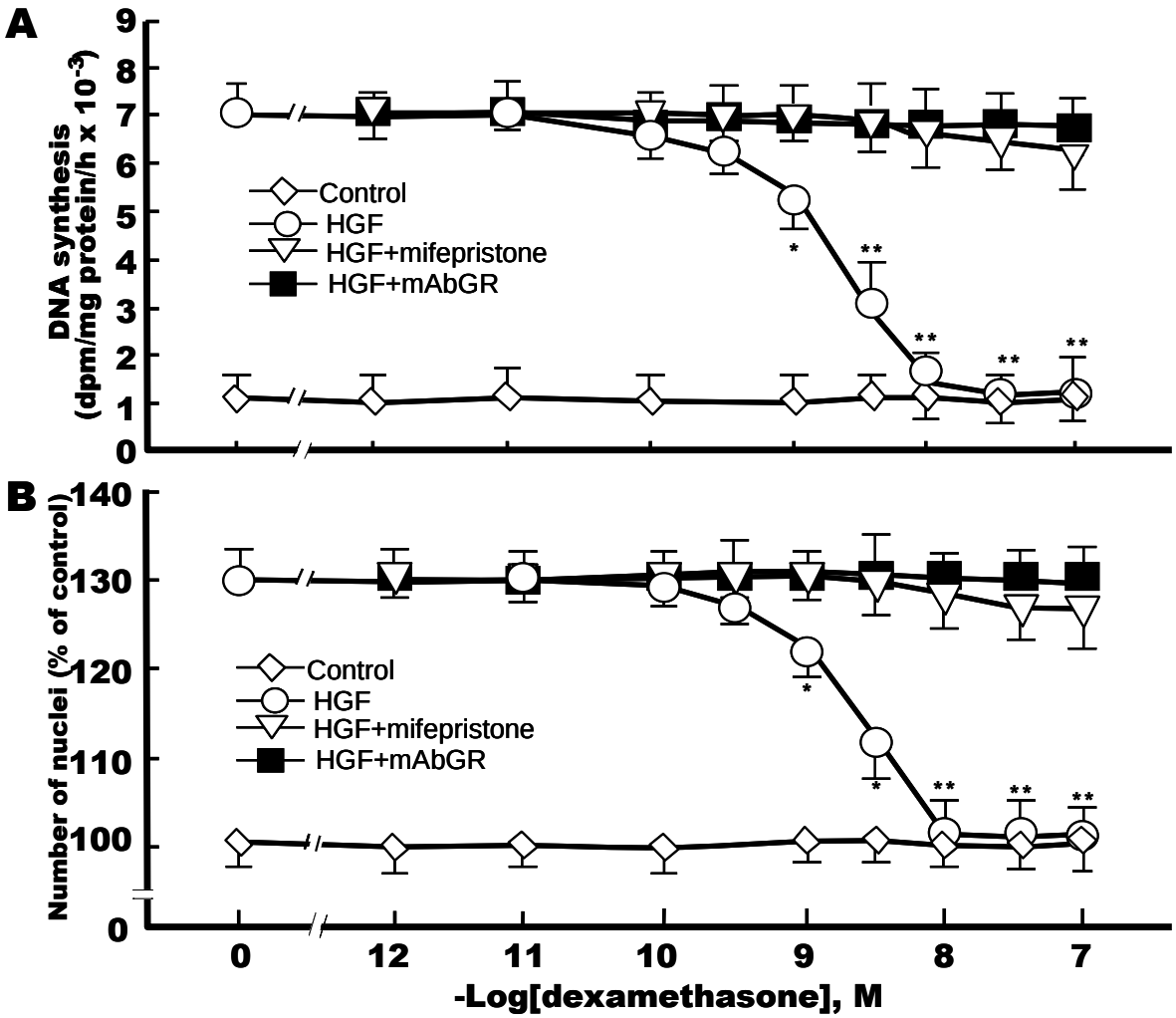

Fig.3

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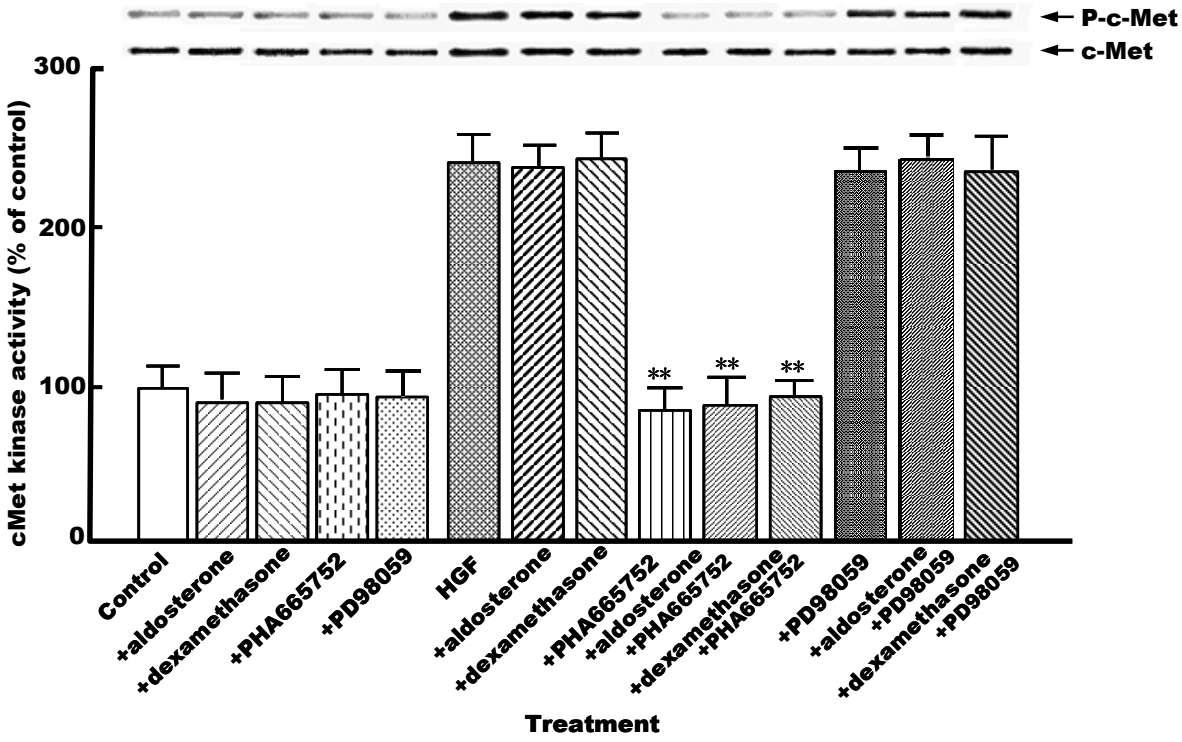

Fig.4

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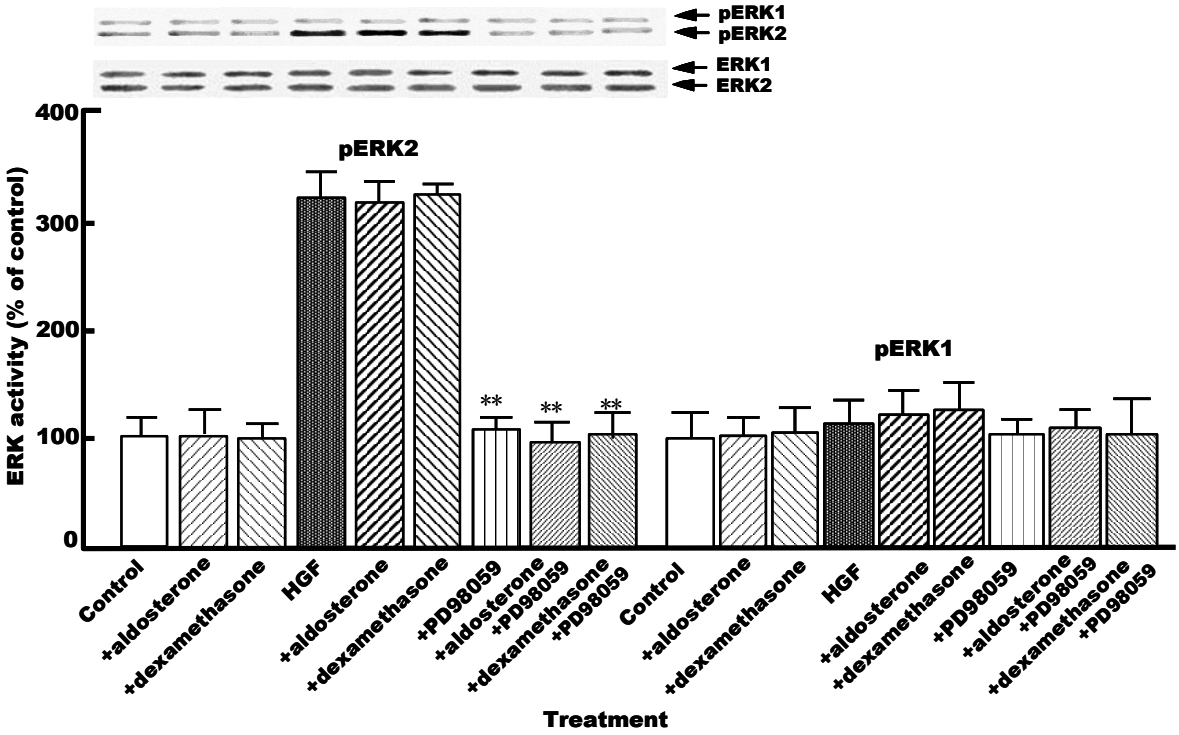

Fig.5

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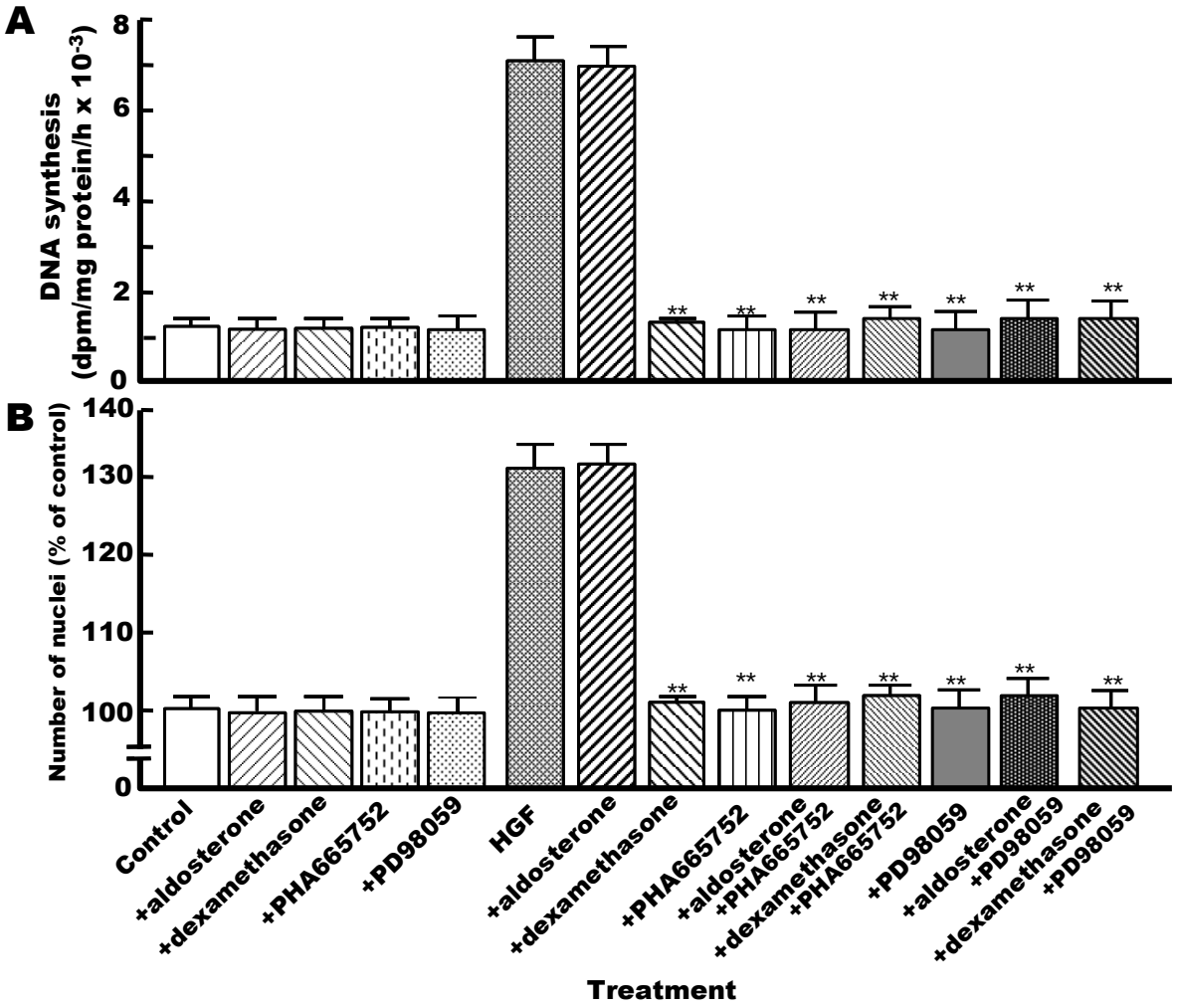

Fig.6

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