ELSEVIER

European Journal of Pharmacology 431 (2001) 151–161

ejp

www.elsevier.com/locate/ejphar

Glycyrrhizin and some analogues induce growth of primary cultured adult rat hepatocytes via epidermal growth factor receptors

Mitsutoshi Kimura [a], Hideo Inoue [b], Kazuhiro Hirabayashi [b], Hideshi Natsume [a], Masahiko Ogihara [a,*]

[a] *Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Josai University, 1-1, Keyakidai, Sakado City, Saitama 350-0295, Japan*

[b] *Research Laboratory, Minophagen Pharmaceutical Co. Ltd., 2-2-3, Komotsubara, Zama, Kanagawa 228-0002, Japan*

Received 3 May 2001; received in revised form 20 September 2001; accepted 25 September 2001

Abstract

We investigated the effects of glycyrrhizin (GL-1) and some analogues on DNA synthesis and proliferation in serum-free primary cultures of adult rat hepatocytes. The hepatocytes underwent DNA synthesis and proliferation in response to GL-1 and some analogues. The effects of these agents occurred in a time- and dose-dependent manner. The proliferative potency as judged by half-maximal effective concentrations was in the following order: 18-β-*H*-glycyrrhetinic acid (GL-3; 4.5×10^{-9} M) < 18-β-*H*-glycyrrhizin (GL-1; 4.4×10^{-8} M) < 18-α-*H*-glycyrrhetinic acid (GL-6; 6.0×10^{-8} M). The analogue 18-α-*H*-glycyrrhetinic acid 3-*O*-β-D-monoglucuronide (GL-5; 1.0×10^{-7} M) weakly stimulated hepatocyte DNA synthesis and proliferation, whereas 18-α-*H*-glycyrrhizin (GL-4) and 18-β-*H*-glycyrrhetinic acid 3-*O*-β-D-monoglucuronide (GL-2) did not. The growth-promoting effects of GL-1, GL-3 and GL-6 were significantly inhibited at higher initial plating densities (7.0×10^4 and 10×10^4 cells/cm^2). A monoclonal antibody against epidermal growth factor (EGF) receptor (1–100 ng/ml), but not that against EGF (1–100 ng/ml), dose-dependently inhibited glycyrrhizin- and analogue-induced hepatocyte DNA synthesis and proliferation. Specific inhibitors of growth-related signal transducers, such as genistein, PD98059 (2′-amino-3′-methoxyflavone) and rapamycin, completely blocked glycyrrhizin- and analogue-induced hepatocyte DNA synthesis and proliferation. Treatment of hepatocytes with GL-1, GL-3 and GL-6 rapidly stimulated tyrosine phosphorylation of the EGF receptor and p42 MAP kinase, which were inhibited by genistein and PD98059, respectively. These results suggest that glycyrrhizin and some analogues are primary hepatocyte mitogens that bind to EGF receptors and subsequently stimulate the receptor tyrosine kinase/mitogen-activated protein kinase pathway to induce hepatocyte DNA synthesis and proliferation.

Keywords: DNA synthesis; Proliferation; Glycyrrhizin; Primary culture; Hepatocyte; Adult, rat

1. Introduction

We have previously demonstrated that epidermal growth factor (EGF) (Kimura and Ogihara, 1997a), insulin (Kimura and Ogihara, 1997b), hepatocyte growth factor (HGF) (Kimura and Ogihara, 1997c) and transforming growth factor-α (TGF-α) (Kimura and Ogihara, 1999) stimulate DNA synthesis and proliferation in serum-free primary cultures of quiescent adult rat hepatocytes. Moreover, the effects of these growth factors are modulated differently by the initial plating densities and comitogens (e.g., catecholamines). In these studies, we demonstrated that the serum-free primary cultured hepatocyte system is an useful in vitro model with which to assess hepatocyte growth and to investigate the mechanism of action induced by primary mitogens and/or comitogens. The basic machinery for the regulation of hepatocyte proliferation is being elucidated (Michalopoulos, 1994; Diehl and Rai, 1996; Michalopoulos and DeFrances, 1997). However, the relative importance of each factor and interactions between them remain poorly understood.

Glycyrrhizin is a triterpene glycoside extracted from licorice root (*Glycyrrhiza glabra*). It consists of an 18-β-*H*-oleanane-type structure that combines with two sugar molecules (glucuronic acids) at the C-3 position (Fig. 1). Glycyrrhizin and some analogues have various pharmacological effects such as anti-inflammatory, antitumorigenic and antihepatotoxic activities (Finney and Somers, 1958;

* Corresponding author. Tel./fax: +81-492-71-7052.
E-mail address: ogiharam@josai.ac.jp (M. Ogihara).

0014-2999/01/$ - see front matter
PII: S0014-2999(01)01424-8

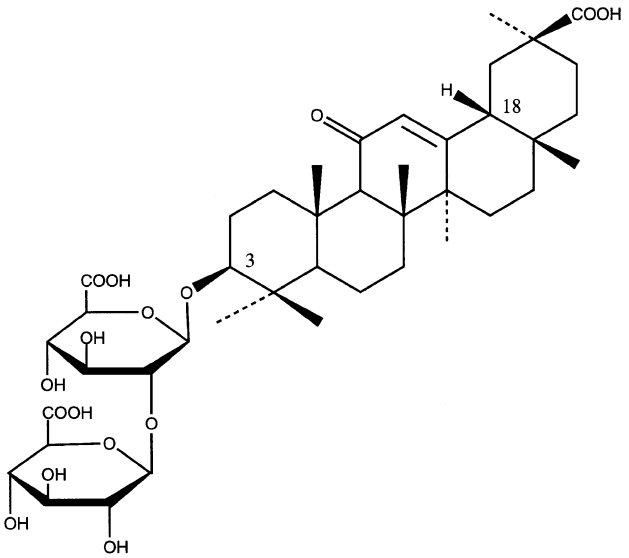

Fig. 1. Chemical structure of glycyrrhizin (GL-1).

Capasso et al., 1983; Nishino et al., 1986; Shibata et al., 1987; Monder et al., 1989; Nose et al., 1994; Inoue et al., 1996). The study of the structure–activity relationships among glycyrrhizin and some analogues will allow the exploitation of more valuable therapeutic agents. For instance, glycyrrhetinic acid (the aglycone of glycyrrhizin) analogues enhance the therapeutic effects and suppress adverse actions (pseudo-aldosteronism) of the parent compound, glycyrrhizin (Kumagai et al., 1987; Shibata et al., 1987).

In Japan, Stronger Neo-Minophagen C (SNMC), the active ingredient of which is glycyrrhizin, has been used intravenously to treat chronic hepatitis for over 20 years. The intravenous administration of SNMC decreases elevated serum alanine aminotransferase levels in patients with chronic viral hepatitis C (Arase et al., 1997; van Rossum et al., 1999), improves liver function with occasional complete recovery from hepatitis and reduces the progression of liver disease to hepatocellular carcinoma (Arase et al., 1997). The effect and safety of SNMC in patients with chronic hepatitis C have recently been evaluated in Europe (van Rossum et al., 1999).

The hepatoprotective effects evoked by glycyrrhizin are thought to be related mainly to its anti-inflammatory action. On the other hand, few studies have focused on the effects of glycyrrhizin and analogues on hepatocyte growth (DNA synthesis and proliferation). Examination of the effects of these agents on hepatocyte growth response is of particular importance, because they may improve liver failure by stimulating liver regeneration. Furthermore, if this is so, the mechanism of action by which glycyrrhizin and analogues improve hepatitis, should be elucidated to develop more effective therapeutic agents. The present study investigates whether or not glycyrrhizin and its analogues stimulate the growth of quiescent adult rat hepatocytes using a serum-free primary culture system.

The results demonstrated that glycyrrhizin and some analogues induce DNA synthesis and proliferation in primary cultures of adult rat hepatocytes in a time- and dose-dependent manner. Like EGF, the growth-promoting effects of glycyrrhizin and the active analogues are cell density-dependent and potentiated by cAMP-elevating agents such as glucagon, forskolin and 8-bromo-cAMP. Furthermore, glycyrrhizin and its active analogues can function as EGF receptor agonists, since hepatocyte DNA synthesis and proliferation were blocked by a monoclonal antibody against the EGF receptor. In addition, we directly demonstrate that treatment of hepatocytes with GL-1, GL-3 and GL-6 rapidly stimulated tyrosine phosphorylation of the EGF receptor (Tyr 1068) and p42 mitogen-activated protein (MAP) kinase, which were inhibited by genistein and PD98059, respectively. These results suggest that glycyrrhizin and some analogues are primary hepatocyte mitogens that bind to EGF receptors and subsequently stimulate the receptor tyrosine kinase/MAP kinase pathway to induce hepatocyte DNA synthesis and proliferation. To our knowledge, this is a novel mechanism of action of glycyrrhizin and its active analogues on the growth of primary cultured hepatocytes.

2. Materials and methods

2.1. Animals

Male Wistar rats (weight 200–220 g) obtained from Saitama Experimental Animal (Saitama, Japan) were allowed to adapt to a humidity- and temperature-controlled room for at least 3 days before experimentation. They were fed a standard diet and given tap water ad libitum. The rats were handled in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Hepatocyte isolation and culture

The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (45 mg/kg). Hepatocytes were isolated from normal livers by two-step in situ collagenase perfusion to facilitate disaggregation of the adult rat liver as described (Seglen, 1975). In brief, hepatocytes were washed three times by slow centrifugation ($50 \times g$, 1 min) of the cell suspension to remove cell debris, damaged and nonparenchymal cells. Viability as tested by Trypan blue exclusion was over 97%. Unless otherwise indicated, isolated hepatocytes were plated onto collagen-coated plastic culture dishes (Sumitomo Bakelite, Tokyo, Japan) at a density of 3.3×10^4 cells/cm^2 (3.1×10^5 cells/35-mm dish) and allowed to attach for 3 h on collagen-coated dishes in Williams' medium E containing 5% newborn calf

serum, 0.1 nM dexamethasone, 100 U/ml penicillin, 100 μg /ml streptomycin and 0.10 μg/ml aprotinin in 5% CO_2 in air at 37 °C. The medium was changed and the cells were cultured in serum- and dexamethasone-free Williams' medium E supplemented with glycyrrhizin and analogues. When appropriate, the following agents were added: glycyrrhizin and analogues with or without EGF, cAMP-elevating agents, inhibitors of signal transducing elements (e.g., genistein, wortmannin, PD98059 and rapamycin) and monoclonal antibodies against EGF or EGF receptors.

2.3. Measurement of DNA synthesis

Hepatocyte DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into acid-precipitable materials (Morley and Kingdon, 1972). Briefly, after an initial attachment period of 3 h, the hepatocytes were washed twice with serum-free Williams' medium E and cultured in medium containing glycyrrhizin and analogues for an additional 4 or 21 h. The cells were pulsed at 0, 1, 2 or 19 h post-glycyrrhizin (or analogue) stimulation for 2 h with [^{3}H]thymidine (1.0 μCi/well) followed by 10% trichloroacetic acid precipitation, as described. [^{3}H]Thymidine incorporation into DNA was measured in a liquid scintillation counter and normalized for cellular protein. Aphidicolin (10 μg/ml) was added to some wells to establish the level of nonreplicative DNA synthesis. Hepatocyte protein content was measured by a modified Lowry procedure with bovine serum albumin as a standard (Lee and Paxman, 1972). Data are expressed as dpm/h/mg cellular protein.

2.4. Counting nuclei

The number of nuclei were counted utilizing a slightly modified version of the procedure described by Nakamura et al. (1983). Briefly, primary cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4), then exposed to 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 (pH 7.4) and the nuclei were counted in a hemocytometer. This procedure was performed because the hepatocytes firmly attached to the collagen-coated plastic culture dishes and were not sufficiently dispersed by 0.02% EDTA–0.05% trypsin.

2.5. Immunoblot analysis

2.5.1. Measurement of EGF receptor tyrosine kinase activity

Tyrosine phosphorylation of the EGF receptor was identified by Western blotting analysis using anti-phosphotyrosine antibody. The phospho-EGF receptor (Tyr 1068) antibody detects EGF receptor only when tyrosine 1068 in the carboxyl-terminal region is phosphorylated. This antibody does not cross-react with other tyrosine-phosphorylated proteins (Rojas et al., 1996).

In brief, hepatocytes were cultured at the density of 3.3×10^4 cells/cm^2 in serum-containing Williams' medium E as described above. After changing to serum-free Williams' medium E, hepatocytes were stimulated with GLs for 3 min at 37 °C. After stimulation, the cells were lysed with lysis buffer [20 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride]. Cell lysates were centrifuged at $3000 \times g$, for 10 min at 4 °C in a microcentrifuge. The supernatant (30 μg protein/lane) was resolved by SDS/6% polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane at 4 °C. The filters were blocked with blocking buffer [5% BSA in 20 mM Tris-buffered saline (pH 7.4), 0.1% Tween-20] for 3 h at 25 °C. Tyrosine phosphorylation of the EGF receptor (Tyr 1068) was detected by immunoblotting using a 1:1000 dilution of rabbit polyclonal phosphotyrosine-specific antibody at 4 °C for 15 h. Blots were developed by enhanced chemiluminescence following incubation with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG monoclonal antibody (1:3000 dilution) for 1 h at 25 °C and exposure to X-ray film according to the manufacturer's instruction. The intensity of the band corresponding to the phosphorylated EGF receptor was quantified by densitometric analysis using the NIH Image program version 1.61 for Macintosh. The data were calculated in arbitrary units and expressed as means ± S.E.M.

2.5.2. Measurement of MAP kinase activity

Phosphorylated MAP kinase isoform, p42 MAP kinase (ERK-2) and p44 MAP kinase (ERK-1), were identified by Western blotting analysis using anti-phospho MAP kinase antibody. In brief, cultured hepatocytes were washed with ice-cold phosphate-buffered saline (pH 7.4) and then 0.2 ml of lysis buffer (10 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) was added, then the hepatocytes were harvested. After centrifugation at $16{,}000 \times g$ for 30 min at 4 °C, the cell lysates were denatured in boiling water for 5 min. Samples of the supernatant (20 μg of protein/lane) were subjected to SDS–PAGE using a 10% polyacrylamide resolving gel by the method of Laemmli (1970). After electrophoresis, proteins were transferred to PVDF membranes at 4 °C.

For the detection of phosphorylated p42 MAP kinase and p44 MAP kinases, the sheets were immersed in blocking buffer containing 5% skim milk, 20 mM Tris-buffered

saline (pH 7.4), 0.1% Tween-20 for 3 h at 25 °C. Phosphorylation of p42 or p44 MAP kinase was detected by protein immunoblotting using a 1:1000 dilution of rabbit polyclonal dual phospho-specific antibodies. Blots were developed by the enhanced chemiluminescence following incubation with HRP-conjugated secondary anti-rabbit IgG monoclonal antibody (1:3000 dilution) for 1 h at 25 °C according to the manufacturer's instructions. The intensity of the band corresponding to phosphorylated MAP kinases was quantified by densitometric analysis as described above. The data were calculated in arbitrary units and expressed as mean ± S.E.M.

2.6. Materials

Glycyrrhizin and the following analogues were prepared by Dr. H. Matsumoto at Research Laboratory of Minophagen Pharmaceutical (Kanagawa, Japan): 18-β-*H*-glycyrrhizin (GL-1, ammonium salt), 18-β-*H*-glycyrrhetinic acid 3-*O*-β-D-monoglucuronide (GL-2, sodium salt), 18-β-*H*-glycyrrhetinic acid (GL-3, sodium salt), 18-α-*H*-glycyrrhizin (GL-4, ammonium salt), 18-α-*H*-glycyrrhetinic acid 3-*O*-β-D-monoglucuronide (GL-5, potassium salt) and 18-α-*H*-glycyrrhetinic acid (GL-6, potassium salt). The following reagents were obtained from Sigma (St. Louis, MO, USA): dexamethasone, aprotinin, aphidicolin, glucagon, forskolin, 8-bromo-cAMP, 2,4-dideoxyadenosine (DDA), genistein and wortmannin. PD98059 (2′-amino-3′-methoxyflavone) was obtained from Calbiochem-Behring (La Jolla, CA, USA). H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) was obtained from Biomol Research Laboratories, (Plymouth Meeting, PA, USA). Rapamycin was obtained from Research Biochemicals (Natick, MA, USA). Monoclonal antibodies against EGF and EGF receptors were from Biomol Research Laboratories. Phospho-EGF receptor (Tyr 1068) antibody and phospho-MAP kinase antibody were purchased from New England Biolabs (Beverly, MA, USA). Chemiluminescence kit was purchased from DuPont NEN (Boston, MA, USA). Williams' medium E and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical (Freehold, NJ, USA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was obtained from DuPont-New England Nuclear. All other reagents were of analytical grade. Phospho-EGF receptor (Tyr 1068) antibody and phospho-MAP kinase antibody were purchased from New England Biolabs. Chemiluminescence kit was purchased from DuPont NEN.

2.7. Statistical analysis

Data are expressed as means ± S.E.M. and were analyzed using the unpaired Student's *t*-test. *P* values below 0.05 were regarded as statistically significant.

3. Results

3.1. Time course associated with stimulation of hepatocyte DNA synthesis and proliferation induced by glycyrrhizin and some analogues

We investigated the time course associated with the effects of glycyrrhizin (GL-1) and analogues on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes in the absence of the additional primary growth factors, insulin and EGF. 18-β-*H*-glycyrrhizin (GL-1; 10^{-6} M), 18-β-*H*-glycyrrhetinic acid (GL-3; 10^{-7} M), 18-α-*H*-glycyrrhetinic acid (GL-6; 10^{-6} M) significantly induced DNA synthesis (Fig. 2A). The DNA synthetic activity induced by each agent was during the first 3.0 h, reached a plateau at about 4 h and was sustained for a further 17 h. In contrast, 18-α-*H*-glycyrrhetinic acid 3-*O*-β-D-monoglu-

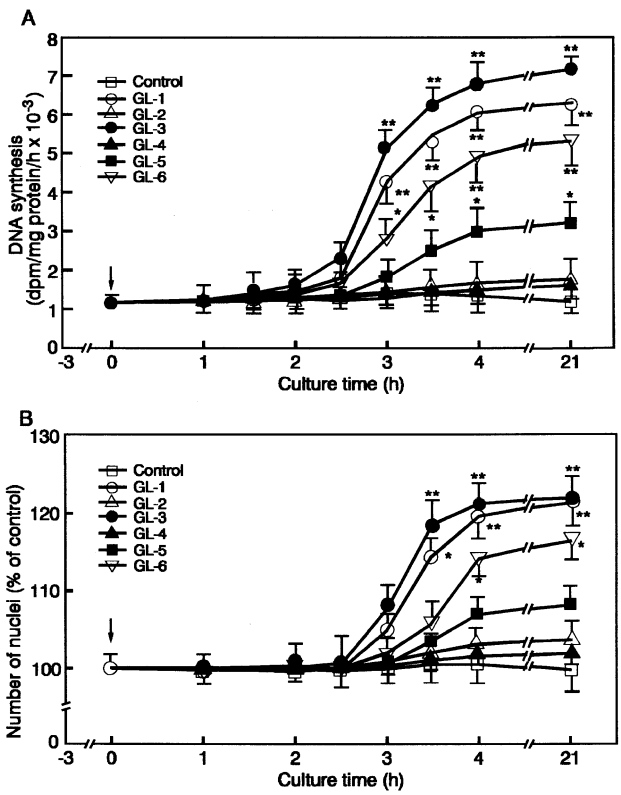

Fig. 2. Time course associated with stimulation of hepatocyte DNA synthesis and proliferation induced by glycyrrhizin and analogues. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured in Williams' medium E supplemented with 5% newborn calf serum and 0.1 nM dexamethasone for 3 h. After attachment for 3 h (zero time), the medium was replaced with serum- and dexamethasone-free Williams' medium E with or without glycyrrhizin (GL-1; 10^{-6} M) and the following analogues (GL-2, 10^{-6} M; GL-3, 10^{-7} M; GL-4, 10^{-6} M; GL-5, 10^{-6} M; GL-6, 10^{-6} M) and cells were cultured for various periods. Hepatocyte DNA synthesis and proliferation were determined as described in Section 2. Data are expressed as means ± S.E.M. of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the respective control (medium alone).

curonide (GL-5; 10^{-6} M) weakly stimulated hepatocyte DNA synthesis, while 18-α-*H*-glycyrrhizin (GL-4; 10^{-6} M) and 18-β-*H*-glycyrrhetinic acid 3-*O*-β-D-mono-glucuronide (GL-2; 10^{-6} M) did not produce any such effects. Hepatocyte proliferation induced by glycyrrhizin and some analogues, as assessed by an increase in the number of nuclei, significantly increased at about 3.5 h (GL-1 and GL-3) and 4.0 h (GL-6) after the addition of each agent, reached a plateau around 4 h and was sustained for an additional 17 h (Fig. 2B). GL-5 weakly stimulated hepatocyte proliferation, whereas GL-2 and GL-4 had no effect.

3.2. Dose-dependent effects of glycyrrhizin and analogues on hepatocyte DNA synthesis and proliferation

We investigated the dose-dependent relationship between glycyrrhizin (GL-1) and analogues GL-2–GL-6 on hepatocyte DNA synthesis and proliferation at 4 h of culture. Fig. 3A shows that GL-1, GL-3 and GL-6 dose dependently stimulated hepatocyte DNA synthesis. The maximal effective concentrations of GL-1, GL-3 and GL-6 were 2.0×10^{-7}, 6.5×10^{-8} and 1.0×10^{-6} M, respectively. The maximal DNA synthetic activities of the cultured hepatocytes induced by GL-1 and GL-3 were 5.5-fold higher than control values; those induced by GL-6 were 4.5-fold higher than controls (Fig. 3A). The estimated half-maximal effective concentrations (ED_{50}) of GL-1, GL-3 and GL-6 required to induce hepatocyte DNA synthesis were 4.4×10^{-8}, 5.2×10^{-9} and 5.5×10^{-8} M, respectively. GL-5 weakly stimulated hepatocyte DNA synthesis with an ED_{50} value of 6.5×10^{-8} M. GL-2 and GL-4 induced no significant DNA synthesis within a concentration range of 10^{-10}–10^{-6} M. Thus, among the compounds tested, GL-3 was the most potent, followed by GL-1, GL-6 and GL-5.

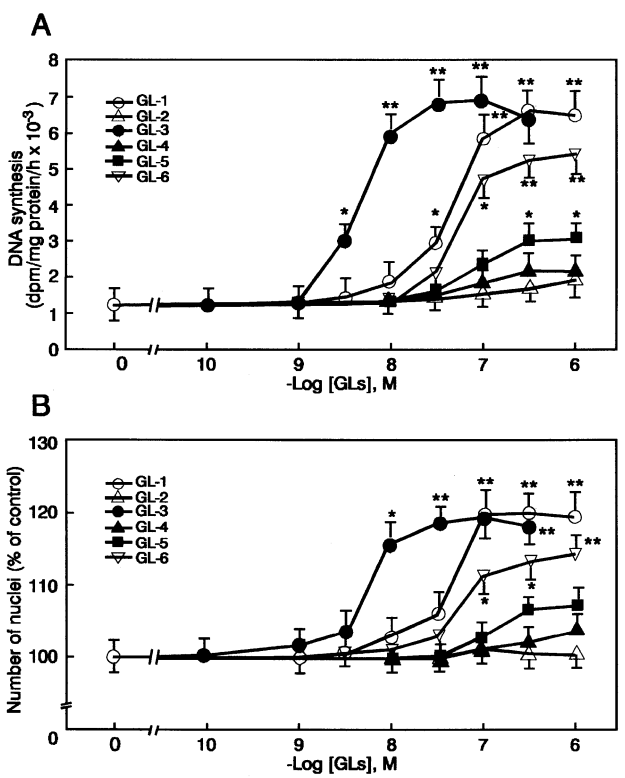

Fig. 3. Dose-dependent effects of glycyrrhizin and analogues on hepatocyte DNA synthesis and proliferation. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured as described in the legend to Fig. 2. After attachment for 3 h (zero time), the medium was replaced with serum- and dexamethasone-free Williams' medium E with or without various concentrations of glycyrrhizin (GL-1) and analogues GL-2–GL-6, then cells were cultured for 4 h. Hepatocyte DNA synthesis and proliferation were determined as described in Section 2. Data are expressed as means ± S.E.M. of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with respective control (medium alone).

Fig. 3B shows a dose-related increase in the number of nuclei (i.e., proliferation) induced by glycyrrhizin (GL-1) and the analogues, GL-2–GL-6. The maximal proliferating activities of the cultured hepatocytes induced by GL-1 and GL-3 were 1.2-fold higher than the control values and those induced by GL-6 were 1.15-fold higher than the control. The maximal effective concentrations of GL-1 and GL-3 and GL-6 were approximately 2.0×10^{-7}, 6.5×10^{-8} and 1.0×10^{-6} M, respectively. The estimated ED_{50} of GL-1, GL-3 and GL-6 required to induce hepatocyte proliferation was 4.4×10^{-8}, 4.5×10^{-9} and 6.0×10^{-8} M, respectively. GL-5 induced weak hepatocyte proliferative activity, while GL-2 and GL-4 did not. Therefore, the relative magnitude of the proliferating effects of each compound largely corresponded to their activities as stimulators of DNA synthesis.

3.3. Influence of cell density on hepatocyte DNA synthesis and proliferation induced by glycyrrhizin and some analogues

We examined whether or not the proliferative effects of glycyrrhizin (GL-1) and of GL-3, GL-5 and GL-6 are affected by initial plating densities. Hepatocyte DNA synthesis and proliferation induced by these agents was measured at 4 h of culture. Fig. 4A shows that hepatocyte DNA synthesis induced by GL-1, GL-3, GL-5 and GL-6 significantly increased with increasing initial plating densities ($1.0–3.3 \times 10^4$ cells/cm^2), reaching a maximal value at a cell density of 3.3×10^4 cells/cm^2. The growth-promoting effects of these agents were inhibited significantly at higher initial plating densities (7×10^4 and 10×10^4 cells/cm^2). In general, the correlation between the ability of these agents to stimulate DNA synthesis and to increase the number of nuclei (proliferation) at various cell densities was close (Fig. 4A and B). The apparent cell density-dependent nature of GL-1 and its analogues at high initial plating densities is similar to that of epidermal growth factor (EGF) (Kimura and Ogihara, 1997a), hepatocyte growth factor (HGF) (Kimura and Ogihara, 1997c) and

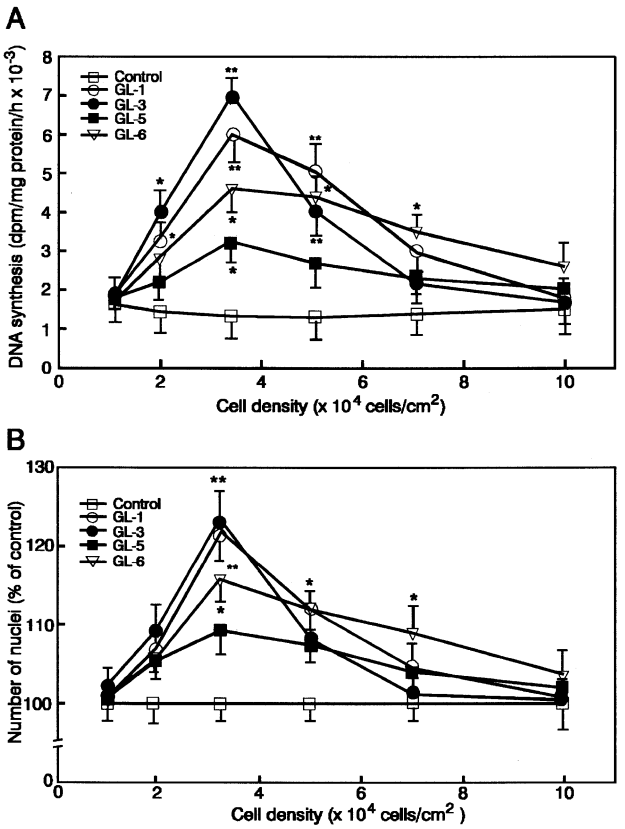

Fig. 4. Influence of cell density on hepatocyte DNA synthesis and proliferation induced by glycyrrhizin and analogues. Hepatocytes at various plating densities (1.0×10^4–1.0×10^5 cells/cm^2) were cultured as described in the legend to Fig. 2. After attachment for 3 h (zero time), the medium was rapidly replaced with serum- and dexamethasone-free Williams' medium E with or without glycyrrhizin (GL-1, 10^{-6} M) and analogues (GL-3, 10^{-7} M; GL-5, 10^{-6} M; GL-6, 10^{-6} M) and cells were cultured for 4 h. Hepatocyte DNA synthesis and proliferation were determined as described in Section 2. Data are expressed as means ± S.E.M. of three experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with respective control (medium alone).

insulin-like growth factor-I (IGF-I) (Kimura and Ogihara, 1998).

3.4. Effects of cAMP-elevating agents, specific inhibitors of adenylate cyclase and protein kinase A on hepatocyte DNA synthesis and proliferation induced by glycyrrhizin and analogues

To characterize the involvement of the adenylate cyclase/protein kinase A system in the signal transduction of the glycyrrhizin (GL-1) and some analogue (GL-3 and GL-6)-induced hepatocyte DNA synthesis and proliferation, we investigated the effects of a direct inhibitor of adenylate cyclase 2,4-dideoxyadenosine (Holgate et al., 1980) and a specific protein kinase A inhibitor H-89 (Zusick et al., 1994) on these responses at 4 h of culture. Fig. 5A and B show that these inhibitors alone did not significantly affect hepatocyte mitogenesis. Incubating hepatocytes with either 10^{-6} M 2,4-dideoxyadenosine or 10^{-7} M H-89 did not significantly inhibit the hepatocyte DNA synthesis and proliferation induced by GL-1, GL-3 and GL-6. Therefore, activation of the adenylate cyclase/cAMP/protein kinase A pathway is not involved per se in the induction of hepatocyte mitogenesis stimulated by GL-1, GL-3 and GL-6.

We investigated the effects of cAMP-elevating agents on GL-1 and derivative-induced hepatocyte DNA synthesis and proliferation. These stimulators of the cAMP pathway (i.e., 10^{-7} M glucagon, 10^{-7} M forskolin and 10^{-7} M 8-bromo-cAMP), which have different mechanisms of action did not significantly induce hepatocyte mitogenesis. However, when combined, hepatocyte DNA synthesis and proliferation induced by GL-1, GL-3 and GL-6 were potentiated by these cAMP-elevating agents. These pharmacological findings indicated that the cAMP modulation of GL-1, GL-3 and GL-6-induced hepatocyte mitogenesis was very similar to that of EGF (Kimura and Ogihara, 1997a).

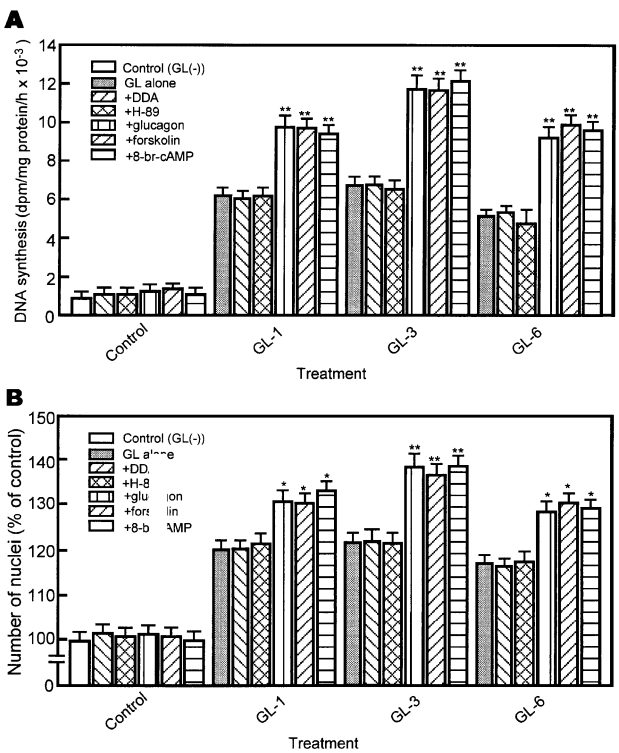

Fig. 5. Effects of cAMP-elevating agents, specific inhibitors of adenylate cyclase and a protein kinase A on hepatocyte DNA synthesis and proliferation induced by glycyrrhizin and analogues. Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2. After attachment for 3 h, the medium was changed and the cells were further cultured for 4 h with glycyrrhizin (GL-1, 10^{-6} M) and analogues (GL-3, 10^{-7} M; GL-6, 10^{-6} M) alone or with glucagon, 10^{-7} M; forskolin, 10^{-7} M; 8-bromo-cAMP, 10^{-7} M; 2, 4-dideoxyadenosine (DDA), 10^{-6} M or H-89, 10^{-7} M. Data are expressed as means ± S.E.M. of three experiments. Values significantly different from respective GL alone are indicated by $^{*}P<0.05$, $^{**}P<0.01$.

3.5. Effects of a monoclonal antibody against EGF receptors on hepatocyte DNA synthesis and proliferation induced by glycyrrhizin and its analogues

Although the pharmacological profiles of GL-1, GL-3 and GL-6 on hepatocyte DNA synthesis and proliferation were very similar to those of EGF, the exact mechanism of how GL-1, GL-3 and GL-6 induces hepatocyte DNA synthesis and proliferation remained to be determined. We postulated that the action of these agents is mediated through binding to hepatocyte EGF receptors, which stimulate DNA synthesis and proliferation. To investigate this notion, we examined the effects of monoclonal antibodies against EGF receptor and EGF on glycyrrhizin and analogue-induced hepatocyte DNA synthesis and proliferation. Fig. 6A and B show that GL-1, GL-3 and GL-6-induced hepatocyte DNA synthesis and proliferation were inhibited dose-dependently by an anti-EGF receptor monoclonal antibody (1–100 ng/ml). On the other hand, neutralizing monoclonal antibodies against EGF (1–100 ng/ml) did not abolish the growth-promoting effects of GL-1, GL-3 and GL-6.

To investigate the specificity of each antibody, we compared MAP kinase responses to GLs or EGF in the absence or presence of the antibodies in primary cultured hepatocytes. The results showed that 10^{-6} M GL-1, 10^{-7} M GL-3 and 10^{-6} M GL-6 significantly stimulated phosphorylation of p42 MAP kinase, but not of p44 MAP kinase, 5 min after the addition of active GLs (Fig. 6C). The effects of active GLs were completely abrogated by the treatment with monoclonal anti-EGF receptor antibody, but not with monoclonal anti-EGF antibody. In contrast, 5-min exposure to 10^{-6} M GL-2 and 10^{-6} M GL-4 did not cause a significant increase in phosphorylation of p42 and p44 MAP kinases (data not shown). As a positive control, EGF (20 ng/ml) also increased the phosphorylation of p42 MAP kinase significantly, but not that of p44 MAP kinase. The effects of EGF on the phosphorylation of p42 MAP kinase were almost completely inhibited by the treatment with anti-EGF receptor antibody as well as anti-EGF antibody (Fig. 6C).

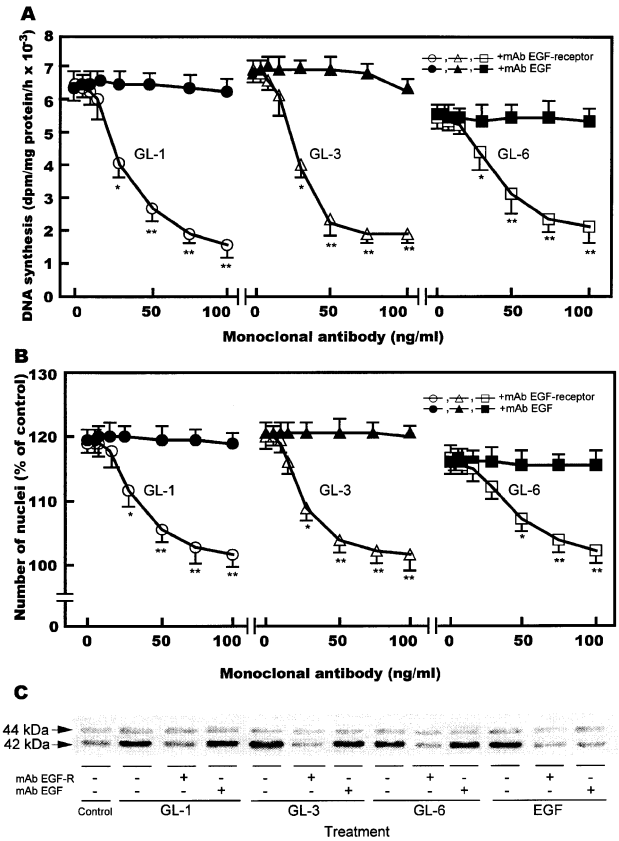

Fig. 6. Effects of monoclonal anti-EGF receptor antibody and of anti-EGF antibody on hepatocyte DNA synthesis, proliferation and MAP kinase activity induced by glycyrrhizin and analogues. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured as described in the legend of Fig. 2. After attachment for 3 h (zero time), the medium was replaced with serum- and dexamethasone-free Williams' medium E with EGF (20 ng/ml), glycyrrhizin (GL-1; 10^{-6} M) and analogues (GL-3; 10^{-7} M, and GL-6; 10^{-6} M) with or without various concentrations of a monoclonal anti-EGF receptor (mAb EGF-R; 1–100 ng/ml) or anti-EGF antibody (mAb EGF; 1–100 ng/ml), and the cells were cultured for 4 h. Hepatocyte DNA synthesis (A) and proliferation (B) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the respective GL alone. (C) Hepatocytes were seeded and cultured as described in the legend of Fig. 2. After changing the medium, the cells were treated with the indicated agonists (10^{-6} M GLs or 20 ng/ml EGF) for 5 min in the absence or presence of the antibodies and lysed. Cell lysates were centrifuged and the supernatant was resolved in SDS/10% PAGE (20 μg/lane). Protein was transferred to a PVDF membrane and immunoblotted with an anti-phospho-MAP kinase antibody and the blot was probed using horseradish peroxidase (HRP)-conjugated secondary antibody as described in Section 2. A typical Western blot image is indicated. Molecular weight markers are indicated on the left.

3.6. Effects of specific inhibitors of growth-related signal transducers on DNA synthesis and proliferation induced by glycyrrhizin and analogues

We investigated whether or not the mitogenic responses of primary cultured hepatocytes to glycyrrhizin (GL-1), GL-3 and GL-6 are mediated by signal transducers, such as receptor tyrosine kinase, phosphatidylinositol 3-kinase, mitogen-activated protein (MAP) kinase kinase and p70 S6K by using their corresponding specific inhibitors, genistein (Akiyama et al., 1987), wortmannin (Baggiolini et al., 1987), PD98059 (Alessi et al., 1995) and rapamycin (Chung et al., 1992; Price et al., 1992). Fig. 7A and B show that the hepatocyte DNA synthesis and proliferation induced by GL-1, GL-3 and GL-6 were almost completely blocked by 5×10^{-6} M genistein, 10^{-6} M PD98059 and 10 ng/ml rapamycin. In contrast, 10^{-7} M wortmannin did not affect significantly the hepatocyte growth response induced by GL-1, GL-3 and GL-6. The specific inhibitors of signal transducers alone did not significantly affect hepatocyte DNA synthesis and proliferation.

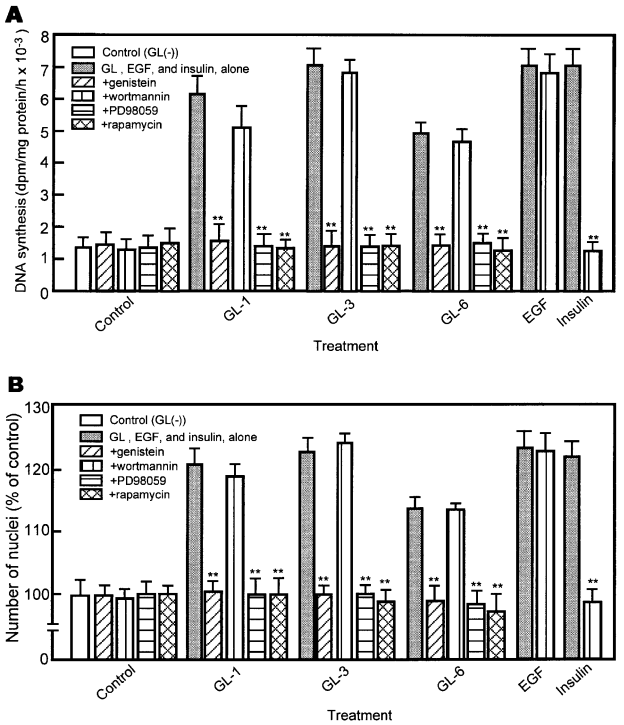

Fig. 7. Effects of specific inhibitors of growth-related signal transducers on DNA synthesis and proliferation induced by glycyrrhizin and analogues. Hepatocytes were plated at a density of 3.3×10 cells/cm^2. After attachment for 3 h, the medium was changed and cells were further cultured for 4 h with glycyrrhizin (GL-1) and analogues (GL-3 and GL-6) alone or with genistein, 5×10^{-6} M; wortmannin, 10^{-7} M; PD98059, 10^{-6} M or rapamycin, 10 ng/ml; EGF (20 ng/ml) alone or EGF (20 ng/ml) with wortmannin (10^{-7} M); insulin (10^{-7} M) alone or insulin (10^{-7} M) with wortmannin (10^{-7} M). Data are expressed as means ± S.E.M. of three experiments. Values significantly different from respective GL alone are indicated by $^{*}P<0.05$, $^{**}P<0.01$.

3.7. Phosphorylation of EGF receptor and p42 MAP kinase by glycyrrhizin and its active analogues

To test directly the notion that GL-1 and some analogues act via the EGF receptor, we compared EGF receptor tyrosine kinase responses to GLs or EGF in primary cultured hepatocytes. Exposure to 10^{-6} M GL-1, 10^{-7} M GL-3 and 10^{-6} M GL-6 significantly increased phosphorylation of the EGF receptor (Tyr 1068) at 1 min (data not shown) and reached a peak value after 3 min. When hepatocytes were stimulated with GL-1 and GL-3 for 3 min, an approximately 2-fold increase in EGF receptor phosphorylation was observed (Fig. 8A). Treatment with 10^{-6} M GL-5 for 3 min gave a smaller increase in EGF receptor tyrosine phosphorylation. The response then declined to the basal level within 10 min (data not shown). In contrast, there was no detectable tyrosine phosphorylation of the EGF receptor (Tyr 1068) after exposure to 10^{-6} M GL-2 and 10^{-6} M GL-4 for 3 min (Fig. 8A). EGF (20 ng/ml) also increased tyrosine phosphorylation of the EGF receptor (Tyr 1068), as expected. Active GL- or EGF-stimulated phosphorylation of EGF receptor was almost completely inhibited by the receptor tyrosine kinase inhibitor, genistein (5×10^{-6} M), a concentration that completely inhibited the GL- or EGF-stimulated hepatocyte mitogenesis (Fig. 7A and B). These findings demonstrate that GL binding to the EGF receptor resulted in EGF

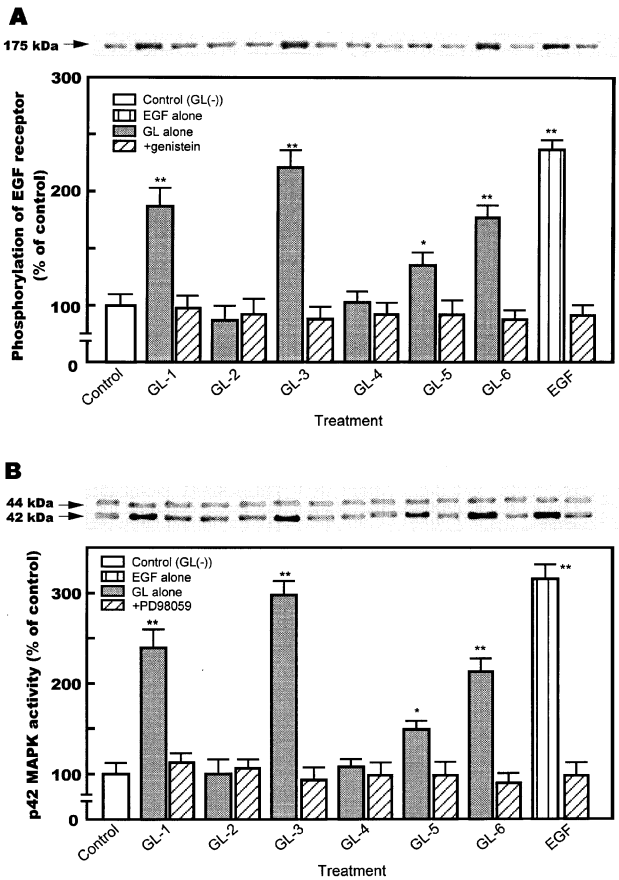

Fig. 8. Phosphorylation of EGF receptor and p42 MAP kinase by GL-1 and its active analogues. (A) Hepatocytes were seeded and cultured as described in the legend of Fig. 2. After changing the medium, the cells were treated with the indicated agonists for 1 or 3 min and lysed. Genistein (5×10^{-6} M) was added 5 min before the medium change and GLs (10^{-6} M) or EGF (20 ng/ml) was added after the medium change for a further 3 min. Cell lysates were centrifuged and the supernatant was resolved in SDS/6% PAGE (30 μg/lane). Protein was transferred to a PVDF membrane and immunoblotted with a phospho-EGF receptor (Tyr 1068) antibody and the blot was probed using HRP-conjugated secondary antibody as described in Section 2. A typical Western blot image is indicated on the top of the figure. A molecular weight marker is indicated on the left. Results are expressed as a percentage of the respective control value (mean + S.E.M. of three experiments). (B) Hepatocytes were seeded and cultured as described in the legend of Fig. 2. After changing the medium, the cells were treated with the indicated agonists for 3 or 5 min and lysed. PD98059 (10^{-6} M) was added 5 min before the medium change and GLs (10^{-6} M) or EGF (20 ng/ml) was added after the medium change for a further 5 min. Phosphorylation of MAP kinases by EGF, GL-1, and its analogues was determined as described in the Section 2. Typical Western blot images are indicated on the top of the figure. Molecular weight markers are indicated on the left of each figure. Results are expressed as a percentage of the respective control value (mean ± S.E.M. of three experiments).

receptor phosphorylation (Tyr 1068) and subsequent stimulation of hepatocyte DNA synthesis and proliferation.

To examine whether GL-1 and some analogues act via MAP kinase molecules, we compared MAP kinase responses to GLs or EGF in primary cultured hepatocytes. The results showed that 10^{-6} M GL-1, 10^{-7} M GL-3 and 10^{-6} M GL-6 significantly stimulated phosphorylation of p42 MAP kinase, but not of p44 MAP kinase 3 min after the addition of active GL (data not shown) and reached a peak value after 5 min. When hepatocytes were stimulated with active GLs for 5 min, 2.5- to 3.0-fold increases in phosphorylation of p42 MAP kinase were observed (Fig. 8B). Treatment with 10^{-6} M GL-5 for 5 min yielded a smaller increase in the phosphorylation of p42 MAP kinase. Next, phosphorylation of p42 MAP kinase declined to the basal value 10 min after the addition of active GL (data not shown). As a positive control, EGF (20 ng/ml) also increased the phosphorylation of p42 MAP kinase significantly, but not that of p44 MAP kinase. In contrast, 5-min exposure to 10^{-6} M GL-2 and 10^{-6} M GL-4, did not cause a significant increase in phosphorylation of p42 and p44 MAP kinase (data not shown). The GL- or EGF-stimulated p42 MAP kinase activation was completely blocked by 5×10^{-6} M PD98059, a concentration that completely inhibited the GL or EGF activation of hepatocyte DNA synthesis and proliferation (Fig. 7A and B).

4. Discussion

Figs. 2 and 3 show that glycyrrhizin (GL-1), the analogues, GL-3 and GL-6 and to a lesser extent GL-5, alone stimulated hepatocyte DNA synthesis and proliferation in the absence of exogenous primary growth factors. We, therefore, investigated structure–activity relationships to exploit more valuable therapeutic agents. We removed glucuronic acid or changed the configuration of the hydrogen atoms at C-18 in the parent compound, GL-1. The study of the structure–activity relationships showed that replacing the β-hydrogen at the 18-position of the 18-β-*H*-oleanane-type structure in glycyrrhizin by α-hydrogen (cf. GL-1 vs. GL-4) negated the ability to cause hepatocyte DNA synthesis and proliferation. Accordingly, it appears that the stereospecificity of hydrogen atom at C-18 position is essential for the potent stimulation of hepatocyte mitogenesis. When one glucuronic acid was removed from a sugar moiety of GL-1 or GL-3 (cf. GL-2 vs. GL-4), the growth-promoting activity of both 18-α-*H*- and 18-β-*H*-type monoglycosides was almost completely lost. Thus, the stereospecificity of the sugar moiety also seemed essential for the potent stimulation of hepatocyte mitogenesis. When two glucuronic acid molecules were removed from a sugar moiety of GL-1 or GL-4 (cf. GL-3 vs. GL-6), analysis of the ED_{50} showed that GL-3 was more potent than GL-6 as a hepatocyte growth stimulator. In addition, the maximal stimulation produced by GL-3 is significantly higher than GL-6. Thus, it appeared that both the stereospecificity of aglycone and the sugar moiety of the parent compound (GL-1) are essential for the potent stimulation of hepatocyte mitogenesis.

Fig. 3A and B show that GL-1, GL-3 and GL-6 induced hepatocyte DNA synthesis and proliferation in a dose-dependent manner at concentration ranges of 10^{-9} M–10^{-6} M. The amounts of these substances bound to the liver have been evaluated in guinea pigs after the i.v. administration of glycyrrhizin (Yamamura et al., 1997). Glycyrrhizin and glycyrrhetinic acid 3-*O*-monoglucuronide were detected at concentrations of 1.3–31.8 μg/g of liver, but glycyrrhetinic acid was undetectable (Sato et al., 1996). Sato et al. showed that the threshold plasma glycyrrhizin concentration for a sufficient therapeutic effect is near 5 μg/ml (i.e., 5×10^{-6} M) in patients with chronic hepatitis and in chronically CCl_4-intoxicated rats. We found that when GL-1, GL-3 and GL-6 reached about 5×10^{-6} M in primary cultures of adult rat hepatocytes, hepatocyte DNA synthesis and proliferation were maximally induced.

The growth-promoting effects of GL-1, GL-3 and GL-6 were cell density-dependent (Fig. 4A and B). The cell density-dependent nature of GL-1 and the active derivatives was similar to that of EGF (Kimura and Ogihara, 1997a). In addition, we showed that cAMP-elevating agents potentiate EGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes (Kimura and Ogihara, 1997a). In the present study, we investigated whether or not agents that affect cellular cAMP levels can modulate the hepatocyte DNA synthesis and proliferation induced by GL-1, GL-3 and GL-6. Fig. 5A and B show that the growth-promoting effects of GL-1, GL-3 and GL-6 were not mediated directly by an increase in either adenylate cyclase activity or protein kinase A activity, since 2,4-dideoxyadenosine (a direct inhibitor of adenylate cyclase) and H-89 (a direct inhibitor of protein kinase A) did not affect the mitogenic effects of glycyrrhizin and their analogues. On the other hand, when combined with GL-1, GL-3 and GL-6, cAMP-elevating agents such as glucagon, forskolin and 8-bromo-cAMP potentiated hepatocyte growth induced by glycyrrhizin and its analogues, although cAMP-elevating agents on their own had no effect on hepatocyte DNA synthesis and proliferation (Fig. 5A and B). These results suggest that stimulators of the cAMP pathway cross-talk with the glycyrrhizin-signaling pathway. Taken together, these pharmacological findings confirmed that the cAMP modulation of GL-1, GL-3 and GL-6-induced hepatocyte mitogenesis was similar to that of EGF (Kimura and Ogihara, 1997a). Therefore, we postulated that the mitogenic action of glycyrrhizin and its analogues is mediated through EGF receptors. This notion was supported by our results indicating that GL-1, GL-3 and GL-6 can function as EGF receptor agonists, as anti-EGF receptor antibody is exerting inhibitory effects (Fig. 6A and B). The EGF receptor mediation of GL-1, GL-3

and GL-6 action and their modulation by cAMP-elevating agents may promote liver regeneration (hepatoprotective action) and may be applied in the treatment of acute or chronic hepatitis in vivo.

The intracellular mediation of cell proliferation occurs mainly through the inositol 1,4,5-trisphosphate/Ca^{2+} (Berridge, 1993) and tyrosine kinase pathways (Ullrich and Schlessinger, 1990; Davis, 1993). We revealed an EGF-signaling system in an earlier study, using pharmacological manipulation of signal transduction to identify pathways that activate EGF-induced hepatocyte DNA synthesis and proliferation. The present study also investigated whether or not the mitogenic responses of cultured hepatocytes to GL-1, GL-3 and GL-6 are mediated by signal transducers, such as receptor tyrosine kinase, phosphatidylinositol 3-kinase, MAP kinase kinase and p70 S6K using corresponding specific inhibitors of the signal transducers. Fig. 7A and B show that genistein, PD98059 and rapamycin inhibited GL-1-, GL-3- and GL-6-induced hepatocyte growth by interfering with corresponding signal transducers that mediate the transduction of the mitogenic signals. These results suggest that tyrosine kinase, MAP kinase kinase and p70 S6K mediate the growth-related signal transduction induced by GL-1, GL-3 and GL-6. In contrast, Fig. 7A and B showed that active GL- or EGF-stimulated hepatocyte DNA synthesis and proliferation are not inhibited by 10^{-7} M wortmannin, a concentration that completely inhibits insulin activation of hepatocyte DNA synthesis and proliferation. Therefore, phosphatidylinositol-3-kinase activity may not be an intermediate in the active GL- or EGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

In many cells, mitogenic signaling stimulated by growth factors such as EGF, requires the intrinsic tyrosine kinase activity of its transmembrane receptors. Several autophosphorylation sites have been identified in the carboxyl-terminal region of the EGF receptor. Phosphotyrosine 1068 of activated EGF receptor (170-kDa protein) is a direct binding site for the Grb2, SH2 domain (Rojas et al., 1996). The signal transduction pathways by which the receptor tyrosine kinase activate extracellular signal-regulated kinase (ERK) subfamily of MAP kinase are well established (Ullrich and Schlessinger, 1990; Davis, 1993).

However, it is unclear whether the intrinsic tyrosine kinase activity of the EGF receptor is directly involved in GL-induced hepatocyte DNA synthesis and proliferation. To evaluate the role of tyrosine kinase activity of the EGF receptor in GL-stimulated hepatocyte mitogenesis, we examined the effects of GLs or EGF on phosphorylation of the carboxyl-terminal region of the EGF receptor (tyrosine 1068). The results in Fig. 8A showed that GL-1, GL-3 and GL-6, but not GL-2 and GL-4, increased tyrosine phosphorylation of the EGF receptor. Three-minute GL treatment was sufficient to trigger tyrosine phosphorylation of the EGF receptor in primary cultured hepatocytes (Fig. 8A). These results demonstrate that activation of the EGF receptor tyrosine kinase by GL-1 and its active analogues was involved in the induction of hepatocyte DNA synthesis and proliferation.

In addition, immunoblotting analysis directly demonstrated that active GLs or EGF stimulated phosphorylation of p42 MAP kinase (Fig. 8B), but not of p44 MAP kinase (data not shown). The GL- or EGF-stimulated p42 MAP kinase activation was completely blocked by 10^{-6} M PD98059, a concentration that completely inhibited the GL or EGF activation of hepatocyte mitogenesis. These results indicate that activation of p42 MAP kinase is involved in the GL or hepatocyte DNA synthesis and proliferation. To study further the role of EGF receptor tyrosine kinase in the GL- or EGF-mediated p42 MAP kinase pathway, we examined the effects of genistein on the p42 MAP kinase activity stimulated by GLs or EGF. Inhibition of EGF receptor tyrosine kinase activity by genistein (5×10^{-6} M) completely abolished the GL- or EGF-induced p42 MAP kinase activation (data not shown). These results support the notion that GL-1 and its active analogues act on EGF receptors with tyrosine kinase activity to proliferate these cells, through an EGF receptor tyrosine kinase-dependent activation of p42 MAP kinase.

In conclusion, the mitogenic effects of glycyrrhizin (GL-1) and the analogues, GL-3 and GL-6 were mediated through activation of EGF receptors. These mitogens subsequently stimulated tyrosine kinase/MAP kinase pathways to produce hepatocyte DNA synthesis and proliferation in vitro. In addition, cAMP-elevating agents (e.g., 8-bromo-cAMP, forskolin and glucagon) potentiated the growth-promoting effects of glycyrrhizin and its active analogues. Since a significant increase in the intracellular cAMP levels is involved in the regenerating rat liver (Tsujii et al., 1993), GL-1, GL-3 and GL-6 could promote liver regeneration in acute or chronic hepatitis, as well as liver cirrhosis by these novel mechanisms of action in vivo independently of their anti-inflammatory effects.

Acknowledgements

We are grateful to Dr. H. Matsumoto at the Research Laboratory of Minophagen Pharmaceutical for providing glycyrrhizin and its analogues.

References

Akiyama, T., Ishida, J., Nakagawa, H., Ogawara, S., Watanabe, N., Itoh, M., Shibuya, M., Fukami, Y., 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J. Biol. Chem. 262, 5592–5595.

Alessi, D., Cuenda, A., Cohen, P., Dudley, D., Staltiel, A., 1995. PD098059 is a specific inhibitor of MAP kinase kinase-1 in vitro and in vivo. J. Biol. Chem. 270, 27489–27494.

Arase, Y., Ikeda, K., Murashima, N., Chayama, K., Tsubota, A., Koida, I., Suzuki, Y., Saitoh, S., Kobayashi, M., Kumada, H., 1997. The long term efficacy of glycyrrhizin in chronic hepatitis C patients. Cancer 79, 1494–1500.

Baggiolini, M., Dewald, B., Schnyder, J., Ruch, W., Cooper, P.H., Payne, T.G., 1987. Inhibition of the phagocytosis-induced respiratory burst by the fungal metabolite wortmannin and some analogues. Exp. Cell Res. 169, 408–418.

Berridge, M.J., 1993. Inositol trisphosphate and calcium signalling. Nature 361, 315–325.

Capasso, F., Mascolo, N., Autore, G., Duraccio, M.R., 1983. Glycyrrhetinic acid, leucocytes and prostaglandins. J. Pharm. Pharmacol. 35, 332–335.

Chung, J., Kuo, C.D., Crabtree, G.R., Blenis, J., 1992. Rapamycin-FKBP specifically blocks growth-dependent activation and signaling by the 70 kD S6 protein kinases. Cell 69, 1227–1236.

Davis, R.J., 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 268, 14553–14556.

Diehl, A.M., Rai, R.M., 1996. Regulation of signal transduction during liver regeneration. FASEB J. 10, 215–227.

Finney, R.S.H., Somers, G.F., 1958. The anti-inflammatory activity of glycyrrhetinic acid and derivatives. J. Pharm. Pharmacol. 10, 613–620.

Holgate, S.T., Lewis, R.A., Austen, K.F., 1980. Role of adenylate cyclase in immunologic release of mediators from rat mast cells: agonist and antagonist effects of purine- and ribose-modified adenosine analogs. Proc. Natl. Acad. Sci. U. S. A. 77, 6800–6804.

Inoue, H., Nagata, N., Shibata, S., Koshihara, Y., 1996. Inhibitory effect of glycyrrhetinic acid derivatives on capsaicin-induced ear edema in mice. Jpn. J. Pharmacol. 71, 281–289.

Kimura, M., Ogihara, M., 1997a. Density-dependent proliferation of adult rat hepatocytes in primary culture induced by epidermal growth factor is potentiated by cAMP-elevating agents. Eur. J. Pharmacol. 324, 267–276.

Kimura, M., Ogihara, M., 1997b. Proliferation of adult rat hepatocytes in primary culture induced by insulin is potentiated by cAMP-elevating agents. Eur. J. Pharmacol. 327, 87–95.

Kimura, M., Ogihara, M., 1997c. Proliferation of adult rat hepatocytes by hepatocyte growth factor is potentiated by both phenylephrine and metaproterenol. J. Pharmacol. Exp. Ther. 282, 1146–1154.

Kimura, M., Ogihara, M., 1998. Effects of insulin-like growth factor I and II on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Eur. J. Pharmacol. 354, 271–281.

Kimura, M., Ogihara, M., 1999. Stimulation by transforming growth factor-α of DNA synthesis and proliferation of adult rat hepatocytes in primary cultures: modulation by α- and β-adrenoceptor agonist. J. Pharmacol. Exp. Ther. 291, 171–180.

Kumagai, A., Hirabayashi, K., Yamamoto, M., Nagata, N., 1987. Chemical modification of glycyrrhetinic acid in relation to the biological activities. Chem. Pharm. Bull. (Tokyo) 35, 1910–1918.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lee, M.B., Paxman, S., 1972. Modification of the Lowry procedure for the analysis of proteolipid protein. Anal. Biochem. 47, 184–192.

Michalopoulos, G.K., 1994. Control mechanisms of liver regeneration. J. Gastroenterol. 29, 23–29.

Michalopoulos, G.K., DeFrances, M.C., 1997. Liver regeneration. Science 276, 60–66.

Monder, C., Stewart, P.M., Lakashmi, V., Valentino, R., Burt, D., Edwards, C.R.W., 1989. Licorice inhibits corticosteroid 11β-dehydrogenase of rat kidney and liver: in vivo and in vitro studies. Endocrinology 125, 1046–1053.

Morley, C.G.D., Kingdon, H.S., 1972. Use of ^{3}H-thymidine for measurement of DNA synthesis in rat liver—a warning. Anal. Biochem. 45, 298–305.

Nakamura, T., Tomita, Y., Ichihara, A., 1983. Density-dependent growth control of adult rat hepatocytes in primary culture. J. Biochem. 94, 1029–1035.

Nishino, H., Yoshida, K., Iwashima, A., Takizawa, H., Konishi, S., Okamoto, H., Okabe, H., Shibata, S., Fujiki, H., Sugimura, T., 1986. Glycyrrhetic acid inhibits tumor-promoting activity of teleocidin and 12-*O*-tetradecanolyphorbol-13-acetate in two stages mouse skin carcinogenesis. Jpn. J. Cancer Res. 77, 33–38.

Nose, M., Ito, M., Kamimura, K., Shimizu, M., Ogihara, Y., 1994. A comparison of the antihepatotoxic activity between glycyrrhizin and glycyrrhetinic acid. Planta Med. 60, 136–139.

Price, D.J., Grove, J.R., Calvo, V., Avruch, J., Bierer, B.E., 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. Science 257, 973–977.

Rojas, M., Yao, S.-Y., Lin, Y.-Z., 1996. Controlling epidermal growth factor (EGF)-stimulated ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor. J. Biol. Chem. 271, 27456–27461.

Sato, H., Goto, W., Yamamura, J., Kurokawa, M., Kageyama, S., Takahara, T., Watanabe, A., Shiraki, K., 1996. Therapeutic basis of glycyrrhizin on chronic hepatitis B. Antiviral Res. 30, 171–177.

Seglen, P.O., 1975. Preparation of isolated liver cells. Methods Cell Biol. 13, 29–83.

Shibata, S., Takahashi, K., Yano, S., Harada, M., Saito, H., Tamura, Y., Kumagai, A., Hirabayashi, K., Yamamoto, M., Nagata, N., 1987. Chemical modification of glycyrrhetinic acid in relation to the biological activities. Chem. Pharm. Bull. (Tokyo) 35, 1910–1918.

Tsujii, H., Okamoto, Y., Kikuchi, E., Matsumoto, M., Nakano, H., 1993. Prostaglandin E and rat liver regeneration. J. Gastroenterol. 105, 495–499.

Ullrich, A., Schlessinger, J., 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61, 203–212.

van Rossum, T.G., Vulto, A.G., Hop, W.C., Brouwer, J.T., Niesters, H.G., Schalm, S.W., 1999. Intravenous glycyrrhizin for the treatment of chronic hepatitis C: a double-blind, randomized, placebo-controlled phase I/II trial. J. Gastroenterol. Hepatol. 14, 1093–1099.

Yamamura, Y., Kotaki, H., Tanaka, N., Akikawa, T., Sawada, Y., Iga, T., 1997. The pharmacokinetics of glycyrrhizin and its restorative effects on hepatic function in patients with chronic hepatitis and in chronically carbon-tetrachloride-intoxicated rats. Biopharm. Drug Dispos. 18, 717–725.

Zusick, M.J., Puzas, J.E., Rosier, R.N., Gunter, K.K., Gunter, T.E., 1994. Cyclic-AMP-dependent protein kinase activity is not required by parathyroid hormone to stimulate phosphoinositide signaling in chondrocytes but is required to transduce the hormone's proliferative effect. Arch. Biochem. Biophys. 315, 352–361.