

Signal transduction pathway for L-ascorbic acid- and L-ascorbic acid  
2-glucoside-induced DNA synthesis and cell proliferation in primary  
cultures of adult rat hepatocytes

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**Abstract:** We examined the effects of L-ascorbic acid and its analogues on DNA synthesis and cell proliferation. We also investigated the signal transduction pathways involved in the induction of mitogenesis by L-ascorbic acid and its analogues using primary cultures of adult rat hepatocytes. Following a 4-h serum-free cultivation, both L-ascorbic acid and its stable analogue, L-ascorbic acid 2-glucoside, time- and dose-dependently stimulated hepatocyte DNA synthesis and cell proliferation, with  $EC_{50}$  values of  $6.46 \times 10^{-8}$  M and  $3.34 \times 10^{-8}$  M, respectively. Dehydroascorbic acid ( $10^{-6}$  M -  $10^{-5}$  M) weakly stimulated hepatocyte mitogenesis, whereas isoascorbic acid ( $10^{-9}$  M -  $10^{-5}$  M) had no effect. Hepatocyte mitogenesis induced by L-ascorbic acid or L-ascorbic acid 2-glucoside was dose-dependently abolished by treatment with monoclonal antibodies against insulin-like growth factor (IGF)-I receptor, but not by treatment with monoclonal antibodies against insulin receptor or IGF-II receptor. Western blot analysis showed that both L-ascorbic acid and L-ascorbic acid 2-glucoside significantly stimulated IGF-I receptor tyrosine kinase activity within 3 min, and mitogen-activated protein (MAP) kinase activity within 5 min. These results demonstrate that both L-ascorbic acid and L-ascorbic acid 2-glucoside induce DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes by interacting with the IGF-I receptor site and by activating the receptor tyrosine kinase/MAP kinase pathway.

## 1. Introduction

Mature rat liver in its normal state is quiescent. However, after extensive hepatic resection, the remaining hepatocytes proliferate to restore the original mass within 2 weeks (Michalopoulos and DeFrances, 1997; Fasto, 2000). This regenerative process is regulated by multiple factors such as peptide growth factors, cytokines, and intermediary metabolites. For example, epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and transforming growth factor (TGF)- $\alpha$  all stimulate DNA synthesis in hepatocytes *in vivo* and *in vitro* (Borowiak et al., 2004; Fasto et al., 2006). In the past decade, the cellular and molecular mechanisms of action of these multiple factors have been investigated *in vitro* using primary culture systems (Kimura and Ogihara, 1997a,b, 1998, 2005; Kimura et al., 2009). In contrast, the role of vitamins in regulating hepatocyte mitogenesis remains to be elucidated.

L-Ascorbic acid, also known as vitamin C, is a nutritional supplement essential for preventing scurvy. Human and non-human primates cannot synthesize L-ascorbic acid, and therefore, it must be provided exogenously and transported intracellularly, a process that is mediated by transporters located at the cell membrane (Vera et al., 1994; Savini et al., 2008).

L-Ascorbic acid is classified as a water-soluble vitamin, as are vitamins B<sub>6</sub> and B<sub>12</sub>. L-Ascorbic acid is reversibly oxidized in the body to dehydroascorbic acid, which retains full vitamin C activity. Vitamin C is an

essential nutrient for the biosynthesis of collagen and L-carnitine, and for the conversion of dopamine to norepinephrine (Tajima and Pinnel, 1982; Li and Schellhorn, 2007). The liver is an important target for the antioxidant effects of vitamin C, and plays a role in body vitamin homeostasis. Several reviews have summarized our current understanding of the physiology and pharmacology of vitamin C (Arrigoni and Tullio, 2002; Konya and Ferdinandy, 2006; Mandl et al., 2009).

L-ascorbic acid and its derivatives can inhibit or stimulate the growth of normal and tumor cells, depending on the cell type (Alcain and Burton, 1994; Belin et al., 2009; Koh et al., 1998; Pelin et al., 2009; Yang et al., 2006; Shibayama et al., 2008). However, the cellular mechanisms of this inhibition or stimulation are poorly understood. Using primary cultures of adult rat hepatocytes, our aim was to test whether or not L-ascorbic acid and its analogues can stimulate hepatocyte DNA synthesis and cell proliferation, and if so, to analyze the signal transduction pathways involved.

## **2. Materials and Methods**

### **2.1. Animals**

Male Wistar rats (weight 200 – 220 g) were obtained from Saitama Experimental Animal Co. (Saitama, Japan). They were maintained in an alternating 12-h light/dark cycle, with food and water available *ad libitum*. The experimental protocol and handling of the animals during experiments were approved by the Experimental Animal Research Committee at the Josai University of Pharmaceutical Science, Japan.

### **2.2. Hepatocyte isolation and culture**

The methods of hepatocyte isolation and culture have been described elsewhere (Nakamura et al., 1983). In brief, the rats were anesthetized with an 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 (Seglen, 1975). The viability of hepatocytes consistently exceeded 96%, as determined by the trypan blue exclusion assay. Unless otherwise indicated, isolated hepatocytes were plated onto 6-well collagen-coated plastic culture dishes (35 mm diameter; Iwaki Glass Co., Tokyo, Japan) at a density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> in minimum essential medium containing 5% bovine calf serum and  $10^{-10}$  M dexamethasone for 3 h in 5% CO<sub>2</sub> in air (Kimura et al., 2011). The medium was then changed, and the cells were cultured in serum-free

minimum essential medium containing various concentrations of L-ascorbic acid or its analogues with or without specific inhibitors of signal transducers. In some experiments, the hepatocytes were cultured in serum-free minimum essential medium containing various concentrations of L-ascorbic acid or its analogues with or without monoclonal antibodies against several growth factor receptors and growth factors. In this study, minimum essential medium was used in place of Williams' medium E because it does not contain L-ascorbic acid, vitamin E, or vitamin K. L-ascorbic acid, dehydroascorbic acid, isoascorbic acid and L-ascorbic acid 2-glucoside were buffered at pH 7.0 with sodium hydroxide and prepared fresh before each experiment.

### **2.3. Measurement of DNA synthesis**

Hepatocyte DNA synthesis was assessed by measuring [<sup>3</sup>H]-thymidine incorporation into acid-precipitable materials (Morley and Kingdon, 1972). After an initial attachment period of 3 h, the hepatocytes were washed twice with serum-free minimum essential medium and cultured in medium containing L-ascorbic acid or its analogues for a further 4 h or 21 h. The cells were pulse-stimulated for 2 h with [<sup>3</sup>H]-thymidine (1.0 µCi/well) at 2 h and 19 h following the addition of L-ascorbic acid or its analogues. Incorporation of [<sup>3</sup>H]-thymidine into DNA was determined as described previously (Kimura and Ogihara, 1997a). The hepatocyte protein content

was determined using a modified Lowry procedure (Lee and Paxman, 1972) using bovine serum albumin as the standard. Data are expressed as dpm/h/mg cellular protein.

#### **2.4. Counting the number of nuclei**

The number of nuclei rather than the number of cells was counted, as previously described but with minor modifications (Nakamura et al., 1983). Briefly, the primary cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4). Then, the cells were lysed by incubation in 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 nucleus 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.

#### **2.5. Determination of IGF-I receptor tyrosine kinase activity**

Tyrosine phosphorylation of the IGF-I receptor was identified by Western blotting using anti-phosphotyrosine antibody (Li et al., 1994). The phospho-IGF-I receptor antibody detects IGF-I receptor only when tyrosine 1161 in the carboxyl-terminal region is phosphorylated. This antibody does not cross-react with other tyrosine phosphorylated proteins. In brief, hepatocytes were freshly isolated and seeded at a density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> and cultured in minimal essential medium containing 5%

newborn bovine serum. The medium was then aspirated and replaced, and the cells were further cultured in serum-free minimal essential medium with or without L-ascorbic acid or L-ascorbic acid 2-glucoside for various lengths of time. Cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4) and then 0.2 ml of lysis buffer (20 mM Tris buffer, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM  $\beta$ -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride) was added. Cell lysates were obtained by scraping the cells in lysis buffer, followed by sonication for 3 min. Cell lysates were centrifuged ( $3000 \times g$  for 3 min at  $4^{\circ}\text{C}$ ) to remove cellular debris, then denatured in boiling water for 5 min. For immunoblotting analysis, samples of the supernatant ( $30 \mu\text{g}/\text{lane}$ ) were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a 7.5% polyacrylamide resolving gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and immunoblotted with anti-phosphotyrosine antibody (Li et al., 1994). Blots were developed by enhanced chemiluminescence following incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. The tyrosine kinase activity (autophosphorylation) of the phosphorylated p95-kDa protein (P-p95-kDa) was normalized to that of the total p95-kDa protein.



## **2.6. Determination of MAP kinase activity**

Phosphorylated MAP kinase isoforms (P-p42 and P-p44 MAPK) were 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 immunoglobulin G (IgG) as a secondary antibody, as previously described (Towbin et al., 1979; Okamoto et al., 2009).

Phosphorylated MAP kinase activity was normalized to the total MAP kinase activity. The data were calculated in arbitrary units and are expressed as means  $\pm$  standard error of the means (S.E.M.). Statistical significance was set at  $*P < 0.05$  compared with medium alone. The autodiagram is a representation of three experiments using different cell preparations.

## **2.7. Neutralization of growth factor receptors and growth factors**

In experiments employing neutralizing antibodies, serum-free primary cultured hepatocytes were treated with maximum concentrations of monoclonal antibodies against HGF receptor, EGF receptor, insulin receptor, IGF-I receptor, IGF-II receptor, and tumor necrosis factor (TNF)- $\alpha$  receptor-1 in the presence of L-ascorbic acid or L-ascorbic acid 2-glucoside.

In some experiments, serum-free primary cultured hepatocytes were treated with various concentrations of monoclonal antibodies against IGF-I receptors, IGF-I, and TGF- $\alpha$  in the presence of L-ascorbic acid or L-ascorbic

acid 2-glucoside.

### **2.8. Assay of [<sup>125</sup>I]-IGF-I binding**

Hepatocytes were isolated and cultured as described in Section 2.2. After 3h of culture, hepatocytes were washed 3 times with Hanks-10mM Hepes buffer (pH 7.4) supplemented with 8 mM glucose and 10 mg/mL bovine serum albumin. [<sup>125</sup>I]-IGF-I binding to primary cultures of hepatocytes was measured according to the method as described elsewhere (Caro et al., 1988). Briefly, the hepatocytes were incubated at 20°C for 2h with increasing concentrations of [<sup>125</sup>I]-IGF-I (1~150 pM) in the presence or absence of unlabeled IGF-I. Free [<sup>125</sup>I]-IGF-I was separated from bound by washing twice with fresh incubation buffer at 4°C. The cells were solubilized in 0.5 mL of 0.2 M NaOH and radioactivity was counted by a gamma counter (Aloka 7001, Japan). Nonspecific binding was determined by incubation in the presence of 100 nM unlabeled IGF-I. Specific binding was calculated by subtracting nonspecific binding from total binding. Under the conditions employed, specific binding of [<sup>125</sup>I]-IGF-I to primary cultured hepatocytes was 80% of the total radioactivity bound.

### **2.9. Competitive [<sup>125</sup>I]-IGF-I binding assay**

The binding affinity of L-ascorbic acid and its analogues to IGF-I receptors was determined by competitive [<sup>125</sup>I]-IGF-I binding

studies. Briefly, the hepatocytes were incubated at 20°C for 2h with [<sup>125</sup>I]-IGF-I (50 pM) in the presence or absence of increasing concentrations of unlabeled IGF-I or L-ascorbic acid and its analogues. Other experimental conditions were the same as described in Section 2.8.

## 2.10. Materials

The following reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA): AG1478 (*N*-[3-chlorophenyl]-6,7-dimethoxy-4-quinazolinamine), AG538 ( $\alpha$ -cyano-(3-methoxy-4-hydroxy-5-iodocinnamoyl)-(3', 4'-dihydroxyphenyl) ketone), D-(-)-isoascorbic acid, and dexamethasone. Rapamycin and PD98059 were obtained from R & D Systems (Minneapolis, MN, USA). Minimum essential medium and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical Co., (Freehold, NJ, USA). L-ascorbic acid, L-ascorbic acid 2-glucoside, and dehydroascorbic acid were obtained from Wako Pure Chemicals Co. (Osaka, Japan). Monoclonal antibodies against IGF-I and IGF-I receptor (Cat.No.GR11) were obtained from Oncogene Research Products (Cambridge, MA, USA). Polyclonal anti-IGF-I receptor (phospho-Tyr1161) antibody was obtained from Applied Biochemical Materials Inc. (Richmond, BC, Canada). Monoclonal antibodies against TNF receptor-1 and TGF- $\alpha$  were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Other antibodies were

obtained as follows: monoclonal antibody against HGF receptor (c-Met) (Cell Signaling Technology, Beverly, MA, USA), monoclonal antibody against EGF receptor (New England Biolabs, Beverly, MA, USA), monoclonal antibody against insulin receptor ( $\alpha$ -subunit) (Novus Biologicals, LLC, Littleton, CO, USA), and monoclonal antibody against IGF-II receptor (Epitomics, Inc., Burlingame, CA, USA). [Methyl  $^3\text{H}$ ]-thymidine (20 Ci/mmol) and [ $^{125}\text{I}$ ]-IGF-I (2705 Ci/mmol) were purchased from PerkinElmer life sciences (Boston, MA, USA). All other reagents were of analytical grade.

## **2.9. Statistical analysis**

Data are expressed as mean  $\pm$  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. P values less than 0.05 were regarded as statistically significant.

### 3. Results

#### *3.1. Time course of induced stimulation of hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid and its analogues*

We examined the effects of L-ascorbic acid (vitamin C) and its analogues on DNA synthesis and nuclei number (cell proliferation) in primary cultures of adult rat hepatocytes. Cells were cultured in medium, with or without L-ascorbic acid or its analogues, for 4 h and 21 h. As shown in Figure 1, exogenous L-ascorbic acid ( $3 \times 10^{-6}$  M) or L-ascorbic acid 2-glucoside ( $10^{-6}$  M) induced hepatocyte DNA synthesis and cell proliferation. A significant increase in hepatocyte DNA synthesis occurred after 2.5 h of culture with L-ascorbic acid or L-ascorbic acid 2-glucoside. A significant increase in the number of nuclei (cell proliferation) was observed after 3.0 h of culture with L-ascorbic acid 2-glucoside, and after 3.5 h of culture with L-ascorbic acid; in both cases, proliferation peaked at 4 h and was sustained for a further 17 h. Dehydroascorbic acid ( $3 \times 10^{-6}$  M) only weakly stimulated hepatocyte DNA synthesis and cell proliferation, and isoascorbic acid ( $3 \times 10^{-6}$  M), a stereoisomer of L-ascorbic acid, had no effect.

#### *3.2. Dose-dependent effects of L-ascorbic acid and its analogues on hepatocyte DNA synthesis and cell proliferation*

We next examined the dose-dependent effects of L-ascorbic acid and its

analogues on hepatocyte DNA synthesis and cell proliferation. Hepatocytes were treated with various concentrations of L-ascorbic acid or its analogues for 4 h in serum-free culture, then DNA synthesis and cell proliferation were measured. As shown in Figure 2, both L-ascorbic acid ( $10^{-9}$  –  $10^{-5}$  M) and its stable analogue, L-ascorbic acid 2-glucoside ( $10^{-9}$  –  $10^{-5}$  M), dose-dependently stimulated hepatocyte DNA synthesis and cell proliferation within 4 h of culture in serum-free medium. A maximum increase in DNA synthesis was observed with  $3 \times 10^{-6}$  M L-ascorbic acid and with  $10^{-6}$  M L-ascorbic acid 2-glucoside. The 50% effective concentration ( $EC_{50}$ ) occurred at  $6.46 \times 10^{-8}$  M L-ascorbic acid and  $3.34 \times 10^{-8}$  M L-ascorbic acid 2-glucoside, indicating that L-ascorbic acid 2-glucoside is more potent than L-ascorbic acid in stimulating hepatocyte DNA synthesis and cell proliferation. Dehydroascorbic acid ( $10^{-6}$  -  $10^{-5}$  M) only weakly stimulated hepatocyte DNA synthesis and cell proliferation, and isoascorbic acid ( $10^{-9}$  –  $10^{-5}$  M), a stereoisomer of L-ascorbic acid, had no effect. Other lipid-soluble vitamins such as (+)- $\alpha$ -tocopherol (vitamin E;  $10^{-9}$  –  $10^{-5}$  M) and phylloquinone (vitamin K<sub>1</sub>;  $10^{-9}$  –  $10^{-5}$  M) also had no significant effect (data not shown).

*3.3. Effects of specific inhibitors of growth-related signal transducers on hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid and L-ascorbic acid 2-glucoside*

We investigated whether or not the mitogenic responses of hepatocytes to L-ascorbic acid and L-ascorbic acid 2-glucoside were mediated by signal transducers, such as receptor tyrosine kinase, phosphatidylinositol 3-kinase (PI(3)K), mitogen-activated protein (MAP) kinase, and ribosomal protein p70 S6 kinase, by using the corresponding specific inhibitors of the signal transducers. The inhibitors used were AG1478 ( $3 \times 10^{-6}$  M), a specific inhibitor of receptor tyrosine kinase (Levizki and Gazit, 1995), LY294002 ( $10^{-7}$  M), a specific inhibitor of PI(3)K (Vlahos et al., 1994), PD98059 ( $10^{-6}$  M), a specific inhibitor of MAP kinase kinase (Alessei et al., 1995), and rapamycin (10 ng/ml), a specific inhibitor of mammalian target of rapamycin (m-TOR; Chung et al., 1992; Price et al., 1992). These inhibitors alone had no significant effect on hepatocyte DNA synthesis and cell proliferation. However, when combined, AG1478 ( $3 \times 10^{-6}$  M) and LY294002 ( $10^{-7}$  M) essentially blocked L-ascorbic acid ( $3 \times 10^{-6}$  M)-induced or L-ascorbic acid 2-glucoside ( $10^{-6}$  M)-induced hepatocyte DNA synthesis and cell proliferation during 4 h of culture. PD98059 ( $10^{-6}$  M) attenuated L-ascorbic acid- or L-ascorbic acid 2-glucoside-induced hepatocyte DNA synthesis and cell proliferation, whereas, rapamycin (10 ng/ml) blocked the effects induced by L-ascorbic acid ( $3 \times 10^{-6}$  M) or L-ascorbic acid 2-glucoside ( $10^{-6}$  M).

*3.4. Effect of anti-growth factor receptor monoclonal antibodies on hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid and L-ascorbic acid 2-glucoside*

Several intracellular signal-transducing elements are involved in L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced hepatocyte DNA synthesis and cell proliferation, as shown in Figure 3. However, the receptors or binding sites that functionally interact with L-ascorbic acid and L-ascorbic acid 2-glucoside are unknown. To investigate which receptors may interact with the L-ascorbic acid signal transduction pathways, we tested monoclonal antibodies against known growth factor receptors and cytokine receptors to see whether or not they affect L-ascorbic acid- or L-ascorbic acid 2-glucoside-induced hepatocyte DNA synthesis and cell proliferation after 4 h of culture. As shown in Figure 4, hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid ( $3 \times 10^{-6}$  M) or L-ascorbic acid 2-glucoside ( $10^{-6}$  M) were radically inhibited by monoclonal anti-IGF-I receptor antibody (100 ng/ml). In contrast, L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced hepatocyte DNA synthesis and cell proliferation were not significantly inhibited by monoclonal antibodies against IGF-II receptor (100 ng/ml), insulin receptor (100 ng/ml), EGF receptor (100 ng/ml), HGF receptor (100 ng/ml), or TNF- $\alpha$  receptor-1 (100 ng/ml). These results indicate that anti-IGF-I receptor antibody specifically inhibits the effects of L-ascorbic acid ( $3 \times 10^{-6}$  M) and



L-ascorbic acid 2-glucoside ( $10^{-6}$  M) on hepatocyte mitogenesis.

*3.5. Dose-dependent effects of monoclonal antibodies against IGF-I receptor, TGF- $\alpha$ , and IGF-I on hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid and L-ascorbic acid 2-glucoside*

We next examined the dose-response relationship between monoclonal antibody against IGF-I receptor and hepatocyte mitogenesis. As shown in Figure 5, L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced hepatocyte DNA synthesis and cell proliferation were dose-dependently inhibited by monoclonal antibody against IGF-I receptor antibody. The  $IC_{50}$  values for these synthesis and proliferation effects after 4 h of culture were 25 ng/ml and 35 ng/ml, respectively. In addition, to exclude the possibility that the autocrine factors, TGF- $\alpha$  and IGF-I, mediate L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced hepatocyte DNA synthesis and cell proliferation in primary cultures, we examined the effects of neutralizing monoclonal antibodies against TGF- $\alpha$  and IGF-I. Figures 5A and 5B show that the addition of a neutralizing monoclonal antibody against IGF-I (1 – 100 ng/ml) and TGF- $\alpha$  (1 – 100 ng/ml) to cultures did not affect the growth-promoting effects of L-ascorbic acid and L-ascorbic acid 2-glucoside on hepatocyte DNA synthesis and cell proliferation. These monoclonal antibodies by themselves had no significant effect during 4 h of culture (data not shown).

*3.6. Effects of L-ascorbic acid and L-ascorbic acid 2-glucoside on receptor tyrosine kinase and mitogen-activated protein (MAP) kinase activity in primary cultured hepatocytes*

To confirm that L-ascorbic acid and L-ascorbic acid 2-glucoside induce hepatocyte DNA synthesis and cell proliferation through the IGF-I receptor tyrosine kinase/MAP kinase signaling pathway, we investigated whether or not they stimulate receptor tyrosine kinase and MAP kinase activity.

Figure 6A shows that both L-ascorbic acid and L-ascorbic acid 2-glucoside ( $10^{-6}$  M) stimulation increases the phosphorylation of IGF-I receptor tyrosine kinase, with phosphorylation peaking at about 3-fold (compared with the control) 3 min after the addition. The addition of AG1478 ( $3 \times 10^{-6}$  M) significantly inhibited the L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced increase in IGF-I receptor tyrosine kinase activity, whereas the addition of the more specific inhibitor, AG538 ( $10^{-7}$  M), completely abolished the increase. Figure 6B shows that L-ascorbic acid ( $3 \times 10^{-6}$  M) and L-ascorbic acid 2-glucoside ( $10^{-6}$  M) stimulation caused an increase in the phosphorylation of p42 MAP kinase, peaking at about 3-fold (compared with the control) 5 min after the addition. The addition of PD98059 ( $10^{-6}$  M) completely abolished the L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced increase in p42 MAP kinase activity. AG1478, AG538, and LY294002 inhibited the L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced increase in the phosphorylation of p42 MAP kinase

(Fig. 6B), whereas rapamycin had no effect (Fig. 6B).

*3.7. Effects of L-ascorbic acid and its analogues on [<sup>125</sup>I]-IGF-I binding to IGF-I receptors of primary cultured hepatocytes.*

We first characterized [<sup>125</sup>I]-IGF-I binding to IGF-I receptors of primary cultured hepatocytes according to the method described elsewhere (Caro et al., 1988). Specific binding of [<sup>125</sup>I]-IGF-I to IGF-I receptors was almost saturated at a concentration of 75 pM (Fig. 7A) and Scatchard analysis of its curve showed that the K<sub>d</sub> value was 0.35 nM and the receptor density (B<sub>max</sub>) was 52.4 fmol/3.3x10<sup>5</sup> cells.

Fig. 7B shows the competitive binding studies of [<sup>125</sup>I]-IGF-I in primary cultured hepatocytes. This figure demonstrates the specificity of L-ascorbic acid and L-ascorbic acid 2-glucoside to IGF-I receptors, as increasing concentrations of L-ascorbic acid and L-ascorbic acid 2-glucoside compete in a dose-dependent manner with [<sup>125</sup>I]-IGF-I from the binding sites. The IC<sub>50</sub> values for L-ascorbic acid and L-ascorbic acid 2-glucoside were 1.8x10<sup>-7</sup> M and 5.4x10<sup>-8</sup> M, respectively. Isoascorbic acid and dehydroascorbic acid have very low replacement activity.

#### 4. Discussion

As shown in Figures 1 and 2, both L-ascorbic acid and its stable analogue, L-ascorbic acid 2-glucoside, time- and dose-dependently stimulate DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes. Isoascorbic acid, which has antioxidant activity but not the biological activity of vitamin C, did not affect hepatocyte mitogenesis, nor did other lipid-soluble vitamins such as vitamin E ((+)  $\alpha$ -tocopherol;  $10^{-8}$  –  $10^{-5}$  M), which has antioxidant properties, or vitamin K<sub>1</sub> (phylloquinone;  $10^{-8}$  –  $10^{-5}$  M) (data not shown). These results suggest that the stimulatory effects of L-ascorbic acid and L-ascorbic acid 2-glucoside on hepatocyte DNA synthesis and cell proliferation are unrelated to their antioxidant or hydrophilic properties.

In order to clarify how L-ascorbic acid and L-ascorbic acid 2-glucoside induce hepatocyte DNA synthesis and cell proliferation, we investigated the effects of specific inhibitors of signal transducers on these responses in primary cultures of adult rat hepatocytes. Specific inhibitors of the intracellular signaling pathway are useful probes with which to characterize target proteins involved in the activation of hepatocyte DNA synthesis and cell proliferation induced by growth factors or cytokines in cultured adult rat hepatocytes (Kimura and Ogihara, 1997a,b; Okamoto et al., 2009). In the present study, hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid and L-ascorbic acid 2-glucoside

were effectively blocked by AG1478, LY294002, PD98059, and rapamycin, suggesting that hepatocyte mitogenesis induced by L-ascorbic acid and L-ascorbic acid 2-glucoside is mediated, at least partially, through receptor tyrosine kinase, PI(3)K, MAP kinase kinase, and m-TOR (p70 S6K) (Figs. 3 and 4). Although L-ascorbic acid and L-ascorbic acid 2-glucoside are believed to stimulate hepatocyte DNA synthesis and cell proliferation through these signal-transducing elements, their target sites remain unknown.

We hypothesized that L-ascorbic acid and L-ascorbic acid 2-glucoside stimulate hepatocyte DNA synthesis and cell proliferation through known receptors that have functionally active binding sites for these two compounds. To investigate which receptors functionally produce L-ascorbic acid signaling pathways, we used specific monoclonal antibodies against growth-promoting receptors and investigated their ability to inhibit L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced hepatocyte DNA synthesis and cell proliferation following 4 h of culture. As shown in Figures 4 and 5, L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced hepatocyte mitogenesis was completely inhibited by monoclonal antibodies against IGF-I receptor, but not by antibodies against IGF-II receptor. In addition, monoclonal antibodies against insulin, EGF, HGF, and TNF-1 receptor were also ineffective.

To exclude the possibility that L-ascorbic acid and L-ascorbic acid

2-glucoside selectively stimulate the secretion of a primary mitogen in an autocrine manner, thus inducing hepatocyte mitogenesis, we examined the effects of neutralizing antibodies against autocrine factors. Mitogens that could fulfill this requirement are TGF- $\alpha$  and IGF-I, since hepatocytes express mRNA for TGF- $\alpha$  and IGF-I, and cells can synthesize and store these primary mitogens (Michalopoulos and DeFrances, 1997). Both TGF- $\alpha$  and IGF-I are highly active growth factors for stimulating hepatocyte DNA synthesis and cell proliferation (Andus et al., 1991; Diehl and Rai, 1996; Fausto, 2000; Kimura and Ogihara, 1998; Kimura and Ogihara, 1999). As shown in Figures 5A and 5B, the addition of neutralizing monoclonal antibodies against IGF-I and TGF- $\alpha$  to the culture medium did not affect the growth-promoting effect of L-ascorbic acid or L-ascorbic acid 2-glucoside on primary cultured hepatocytes, thereby suggesting that the autocrine hypothesis is not valid. Taken together, these results indicate that L-ascorbic acid and L-ascorbic acid 2-glucoside induce DNA synthesis and cell proliferation in adult rat hepatocytes by interacting with the IGF-I receptor. In agreement with these results (Figs. 3 and 5), we previously demonstrated that IGF-I can induce hepatocyte DNA synthesis and cell proliferation in primary cultures, mediated by signal transducers such as receptor tyrosine kinase, PI(3)K, MAP kinase, and p70 S6 kinase (Kimura and Ogihara, 1998). In addition, when combined with IGF-I treatment (10 nM), L-ascorbic acid ( $10^{-7}$  M) and L-ascorbic acid 2-glucoside ( $10^{-7}$  M)

stimulated hepatocyte DNA synthesis and proliferation in an additive manner (data not shown).

The IGFs are a family of polypeptide hormones that have close structural and functional homologies with insulin (Ullrich et al., 1986). IGF peptides act as autocrine growth factors and are implicated in the regulation of fetal growth and development. The effects of IGF are triggered when these ligands bind to their specific IGF receptor. IGF receptors are members of the tyrosine kinase receptor family (e.g., EGF, HGF, and platelet-derived growth factor receptors). IGF and insulin receptors are highly homologous, heterotetrameric molecules composed of two extracellular  $\alpha$ -subunits (which contain the binding site) and two transmembrane  $\beta$ -subunits (which have tyrosine kinase activity, activated in response to ligand binding). Thus, binding of an IGF to its receptor triggers a cascade of events that includes receptor autophosphorylation, phosphorylation of intracellular substrates, and activation of signaling pathways involved in growth regulation and metabolic processes (Czech, 1989; Davis, 1993; Humbel, 1990; Lund et al., 1986; Myers et al., 1993; Parrizas et al., 1997). IGF-I receptor-mediated signal transduction pathways in normal and cancer cells have been studied in detail (Blume-Jensen and Hunter, 2001; Kurmasheva and Houghton, 2006; Melmed et al., 1996; Zha et al., 2010).

To further confirm that L-ascorbic acid and L-ascorbic acid 2-glucoside induce hepatocyte DNA synthesis and cell proliferation through the IGF-I

receptor-mediated signal transduction pathway, we investigated whether or not these agents actually stimulate IGF-I receptor tyrosine kinase and MAP kinase activities. Western blot analysis showed that L-ascorbic acid and L-ascorbic acid 2-glucoside can stimulate both hepatic IGF-I receptor tyrosine kinase and MAP kinase activities (Fig. 6). These effects were blocked by specific inhibitors of receptor tyrosine kinase, which suggests that both L-ascorbic acid and L-ascorbic acid 2-glucoside act as IGF-I receptor agonists to induce hepatocyte mitogenesis (Fig. 6A). In addition, AG1478, AG538, and LY294002 inhibited L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced increases in the phosphorylation of p42 MAP kinase (Fig. 6B), whereas rapamycin had no effect (Fig. 6B).

The primary event in the action of IGF-I on cultured hepatocytes is the binding to specific receptors on the cell surface. To confirm involvement of IGF-I receptors in the induction of hepatocyte DNA synthesis and proliferation by L-ascorbic acid and L-ascorbic acid 2-glucoside, we performed [<sup>125</sup>I]-IGF-I binding test. As shown in Fig. 7A, we demonstrated the specific bindings of [<sup>125</sup>I]-IGF-I to IGF-I receptor on primary cultured hepatocytes. Then, specificity of L-ascorbic acid and its analogues on the IGF-I receptors was demonstrated by competitive [<sup>125</sup>I]-IGF-I binding study. Fig.7B showed that the IC<sub>50</sub> value of L-ascorbic acid 2-glucoside was about 5-fold potent than L-ascorbic acid in displacing [<sup>125</sup>I]-IGF-I. Therefore, there is a close relation between specific binding of L-ascorbic acid and L-ascorbic



acid 2-glucoside to IGF-I receptor (Fig. 7A, B) and L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes (Fig. 2A, B) Taken together, a novel signal transduction pathway can rationally account for the hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid and L-ascorbic acid 2-glucoside (Fig. 8).

In conclusion, the present results demonstrate for the first time that L-ascorbic acid and its stable analogue, L-ascorbic acid 2-glucoside, can induce hepatocyte DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes. The effects of L-ascorbic acid and L-ascorbic acid 2-glucoside are apparently mediated by their interaction with IGF-I receptor and subsequent activation of IGF-I receptor tyrosine kinase. Other possible interactions involve downstream PI(3)K, MAP kinase, and p70 S6K to induce hepatocyte mitogenesis. Although the physiological significance of L-ascorbic acid cannot be properly gauged from this *in vitro* study alone, the novel signaling mechanisms induced by L-ascorbic acid and L-ascorbic acid 2-glucoside may provide strategies for hepatocyte proliferation therapy during liver regeneration *in vivo*. In addition, L-ascorbic acid and L-ascorbic acid 2-glucoside may be used as safe agonists to cure diseases such as growth failure, muscular dystrophy, and type II diabetes.

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## 6. Legends

Fig. 1 Time course for the stimulation of hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid and its analogues. Hepatocytes at a cell density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> were plated and cultured in minimum essential medium containing 5% newborn calf serum and 0.1 nM dexamethasone for 3 h. After the 3-h attachment period (time zero), the medium was rapidly replaced with serum-free minimum essential medium and the hepatocytes were cultured with L-ascorbic acid ( $3 \times 10^{-6}$  M), L-ascorbic acid 2-glucoside ( $10^{-6}$  M), dehydroascorbic acid ( $3 \times 10^{-6}$  M), or isoascorbic acid ( $3 \times 10^{-6}$  M) for an additional period of time. Hepatocyte DNA synthesis (A) and cell proliferation (B) were determined as described in Section 2. Data are expressed as mean  $\pm$  S.E.M. of three separate experiments. \*P < 0.05, \*\*P < 0.01 compared with the respective control.

Fig. 2 Dose-dependent effects of L-ascorbic acid and its analogues on hepatocyte DNA synthesis and cell proliferation. Hepatocytes at a cell density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> were plated and cultured for 3 h as described in the legend to Fig. 1. After the 3-h attachment period (time zero), the medium was rapidly replaced with serum-free minimum essential medium and the hepatocytes were cultured with various concentrations of L-ascorbic acid or its analogues for an additional 4 h. Hepatocyte DNA

synthesis (A) and cell proliferation (B) were determined as described in Section 2. Data are expressed as mean  $\pm$  S.E.M. of three separate experiments. \*P < 0.05, \*\*P < 0.01 compared with the respective control.

Fig. 3 Effects of specific inhibitors of growth-related signal transducers on hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid or L-ascorbic acid 2-glucoside. Hepatocytes at a cell density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> were plated and cultured for 3 h as described in the legend to Fig. 1. After the 3-h attachment period (time zero), the medium was rapidly replaced with serum-free minimum essential medium and the hepatocytes were cultured with L-ascorbic acid or L-ascorbic acid 2-glucoside in the presence of specific inhibitors of growth-related signal transducers for an additional 4 h. Concentrations were as follows: AG1478 ( $3 \times 10^{-6}$  M), LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M), and rapamycin (10 ng/ml). Hepatocyte DNA synthesis (A) and cell proliferation (B) were determined as described in Section 2. Data are expressed as mean  $\pm$  S.E.M. of three separate experiments. \*P < 0.05, \*\*P < 0.01 compared with the respective control.

Fig. 4 Effect of anti-growth factor receptor monoclonal antibodies on L-ascorbic acid- and L-ascorbic acid 2-glucoside-induced hepatocyte DNA synthesis and cell proliferation. Hepatocytes at a cell density of  $3.3 \times 10^4$

cells/cm<sup>2</sup> were plated and cultured for 3 h as described in the legend to Fig. 1. After the 3-h attachment period (time zero), the medium was rapidly replaced with serum-free minimum essential medium and the hepatocytes were cultured with submaximal concentrations of monoclonal antibodies against hepatocyte growth factor (HGF) receptor (100 ng/ml), epidermal growth factor (EGF) receptor (100 ng/ml), insulin receptor (100 ng/ml), insulin-like growth factor (IGF)-I receptor (100 ng/ml), IGF-II receptor (100 ng/ml) or tumor necrosis factor (TNF)- $\alpha$  receptor-1(100 ng/ml) in the presence of L-ascorbic acid ( $3 \times 10^{-6}$  M) or L-ascorbic acid 2-glucoside ( $10^{-6}$  M) for an additional 4 h. Hepatocyte DNA synthesis (A) and cell proliferation (B) were determined as described in Section 2. Data are expressed as mean  $\pm$  S.E.M. of three separate experiments. \*\*P < 0.01 compared with the respective control.

Fig. 5 Dose-dependent effects of monoclonal antibodies against IGF-I receptor, transforming growth factor (TGF)- $\alpha$ , and IGF-I on hepatocyte DNA synthesis and cell proliferation induced by L-ascorbic acid or L-ascorbic acid 2-glucoside. Hepatocytes at a cell density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> were plated and cultured for 3 h as described in the legend to Fig. 1. After the 3-h attachment period (time zero), the medium was rapidly replaced with serum-free minimum essential medium and the hepatocytes were cultured with various concentrations of monoclonal antibodies against

IGF-I receptor, TGF- $\alpha$ , or IGF-I in the presence of L-ascorbic acid ( $3 \times 10^{-6}$  M) or L-ascorbic acid 2-glucoside ( $10^{-6}$  M) for an additional 4 h. Hepatocyte DNA synthesis (A) and cell proliferation (B) were determined as described in Section 2. Data are expressed as mean  $\pm$  S.E.M. of three separate experiments. \*P < 0.05, \*\*P < 0.01 compared with the respective control.

Fig. 6 Effects of L-ascorbic acid- and L-ascorbic acid 2-glucoside on receptor tyrosine kinase and mitogen-activated protein (MAP) kinase activity in primary cultured hepatocytes. Hepatocytes at a cell density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> were plated and cultured for 3 h as described in the legend to Fig. 1. The phosphorylation of IGF-I receptor and p42 MAP kinase by L-ascorbic acid and L-ascorbic acid 2-glucoside was described in the Materials and Methods section. In brief, hepatocytes were seeded and cultured as described in the legend to Fig. 1. After changing the medium, the cells were treated with L-ascorbic acid or L-ascorbic acid 2-glucoside in the presence or absence of AG1478 ( $3 \times 10^{-6}$  M), LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M), rapamycin (10 ng/ml) or AG538 ( $10^{-7}$  M) for 3 and 5 min, then lysed. Cell lysates were centrifuged and the supernatant proteins were resolved using sodium dodecyl sulfate (SDS)-PAGE (30  $\mu$ g/lane). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and immunoblotted with a phospho-IGF-I receptor or antibody, or with phospho-MAP kinase antibody, and the blots was probed using horseradish peroxidase

(HRP)-conjugated secondary antibody. A typical Western blot image is shown at the top of the figure. Results are expressed as a percentage of the respective control value (mean  $\pm$  S.E.M. of three experiments).

Fig. 7 Specific binding of [ $^{125}$ I]-IGF-I to IGF-I receptor on primary cultures of hepatocytes as a function of [ $^{125}$ I]-IGF-I concentration (A). Specific binding was determined as described under Materials and Methods section. Values are means for two experiments. Inset; Scatchard plot of [ $^{125}$ I]-IGF-I binding to IGF-I receptor on the primary cultured hepatocytes.

Displacement of [ $^{125}$ I]-IGF-I on IGF-I receptors of primary cultured hepatocytes by L-ascorbic acid and its analogues (B). The primary cultures of hepatocytes were incubated for 2h at 20°C with 50 pM [ $^{125}$ I]-IGF-I and various concentrations of L-ascorbic acid and its analogues as described under Materials and Methods section. Values are means for two experiments. The IC<sub>50</sub> is defined as the concentration of competing ligand which inhibits 50% of the specific binding of [ $^{125}$ I]-IGF-I. The IC<sub>50</sub> values for IGF-I, L-ascorbic acid, and L-ascorbic acid 2-glucoside were 4.3x10<sup>-10</sup> M, 1.8x10<sup>-7</sup> M, and 5.4x10<sup>-8</sup> M, respectively.

Fig. 8 A strategy to prove L-ascorbic acid is an agonist of IGF-I receptor in primary cultures of adult rat hepatocytes.

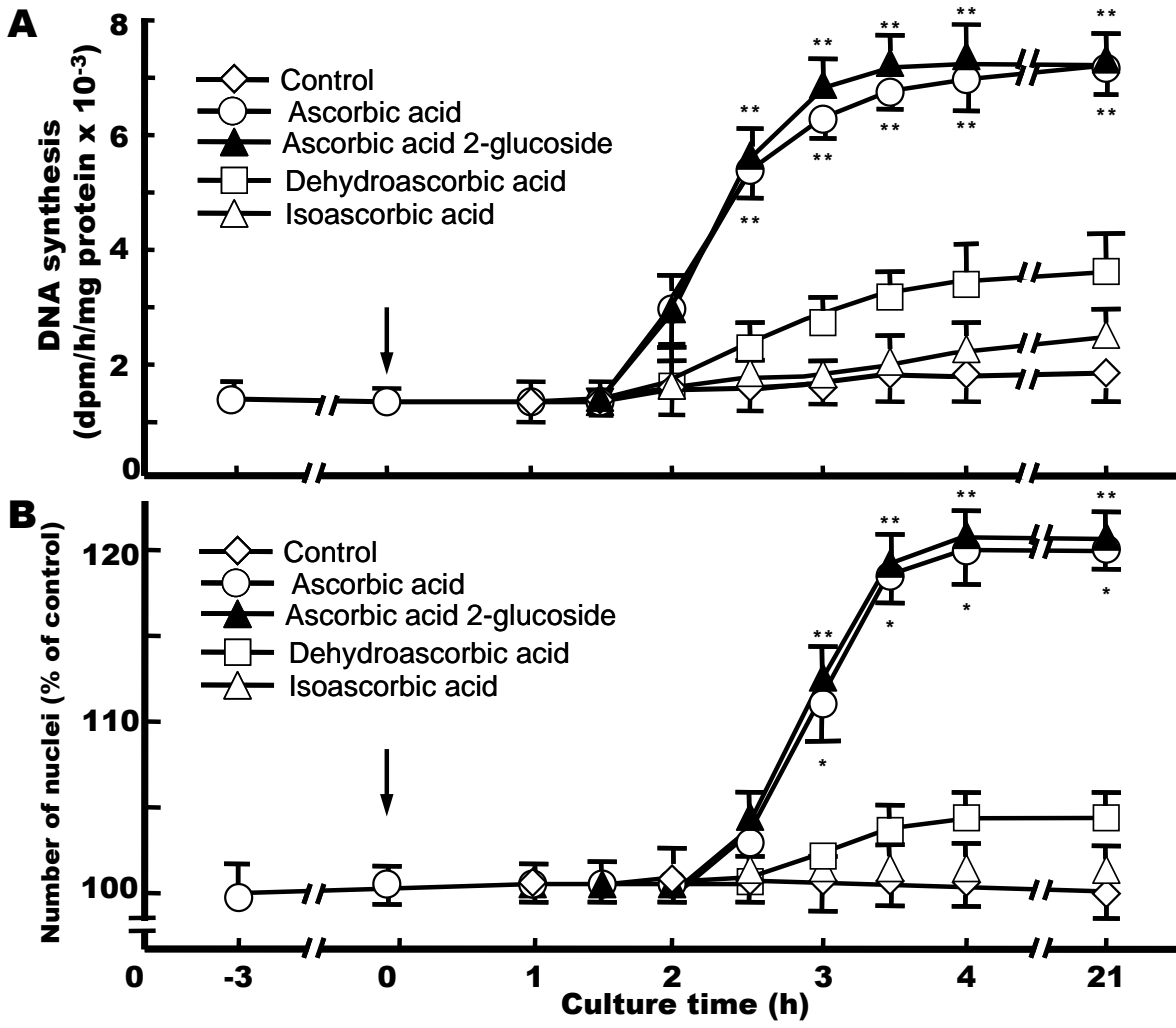


Fig.1



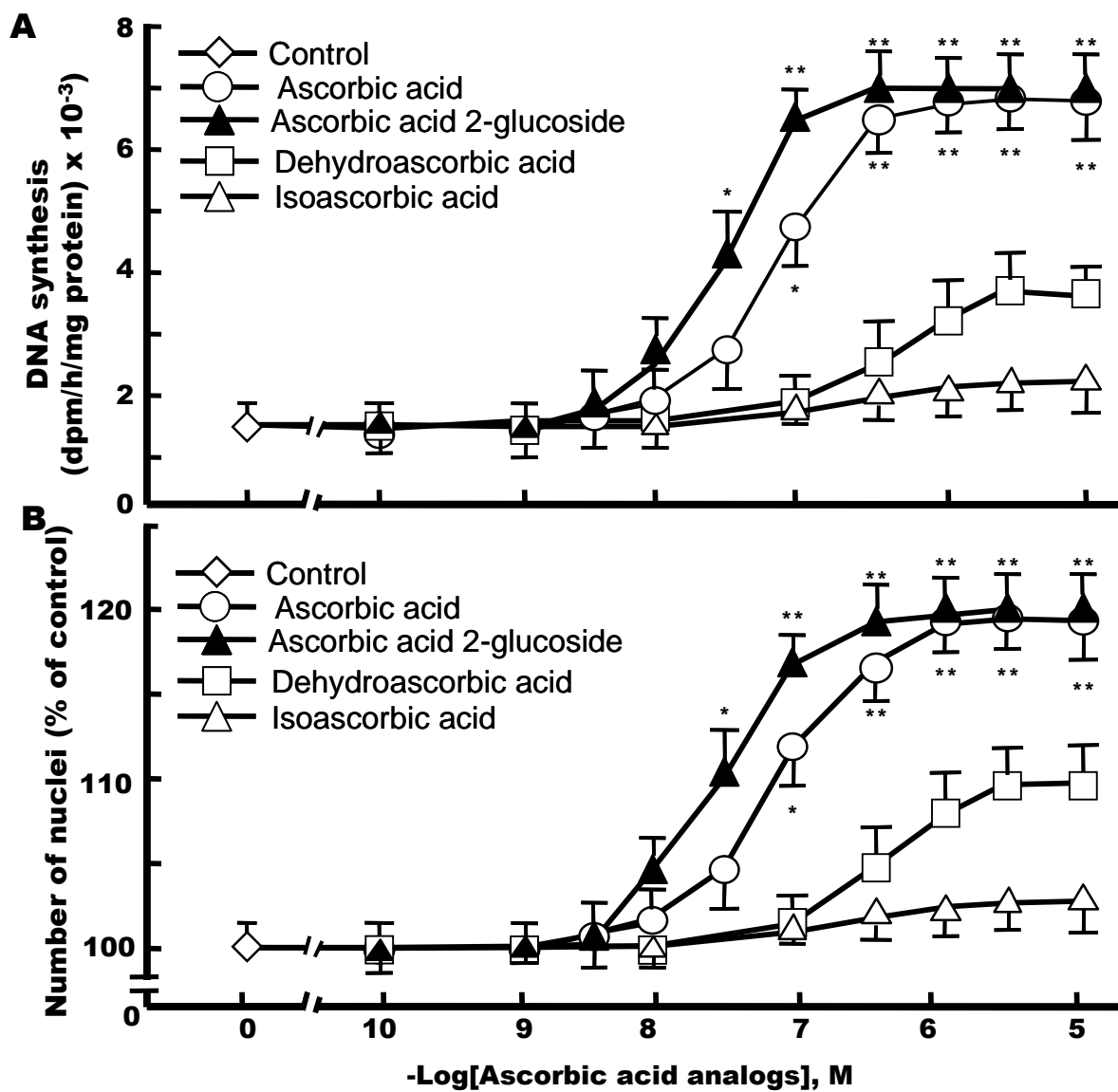


Fig.2

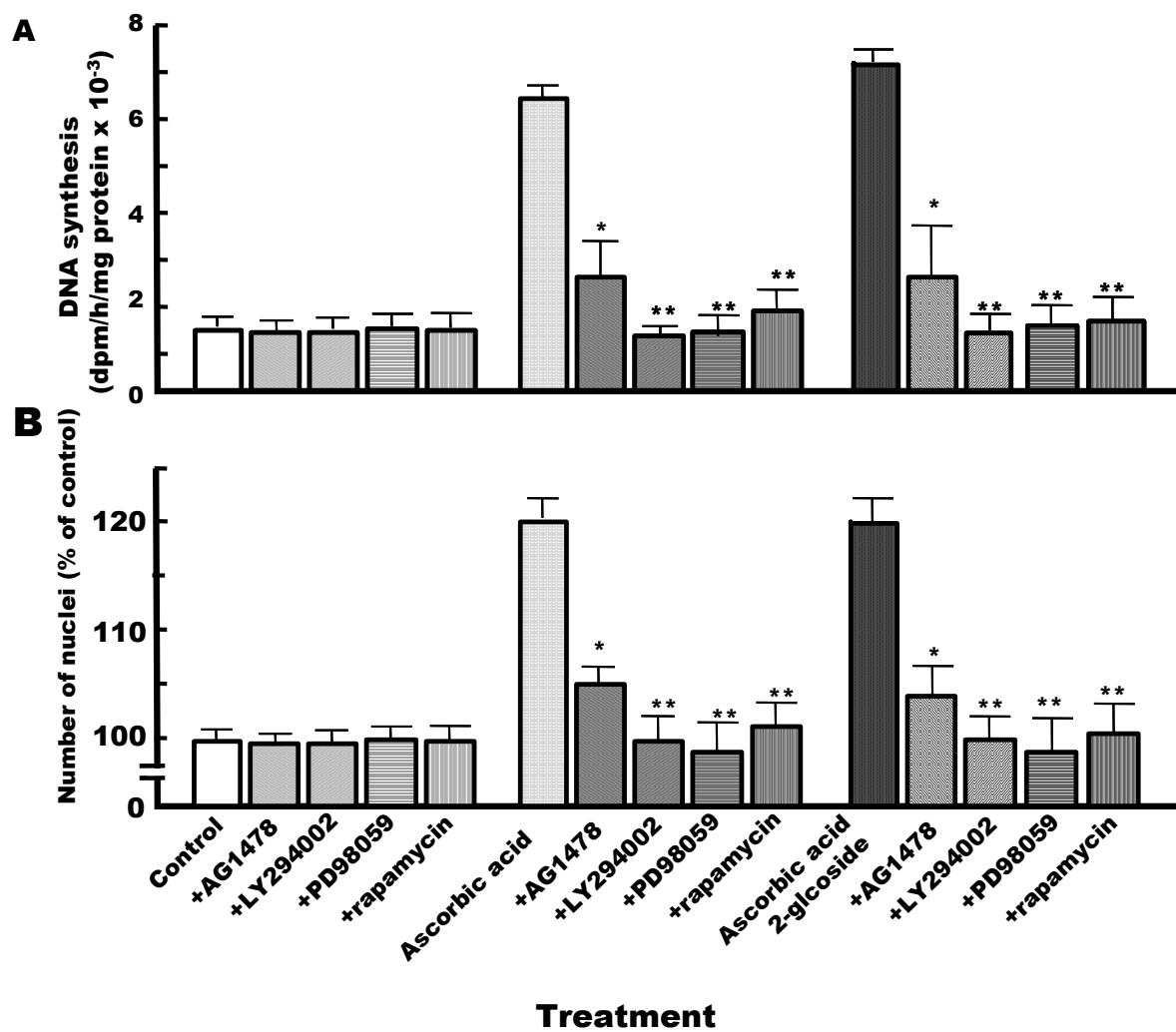


Fig.3

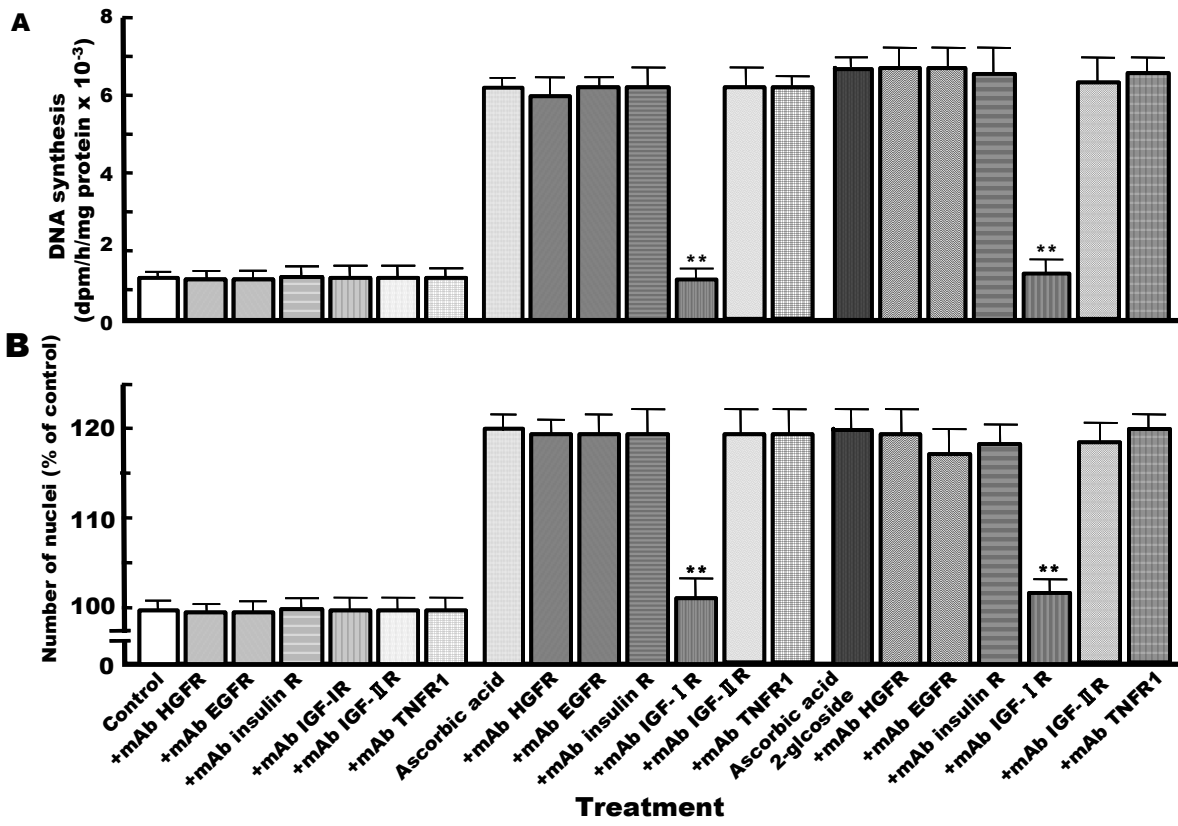


Fig.4

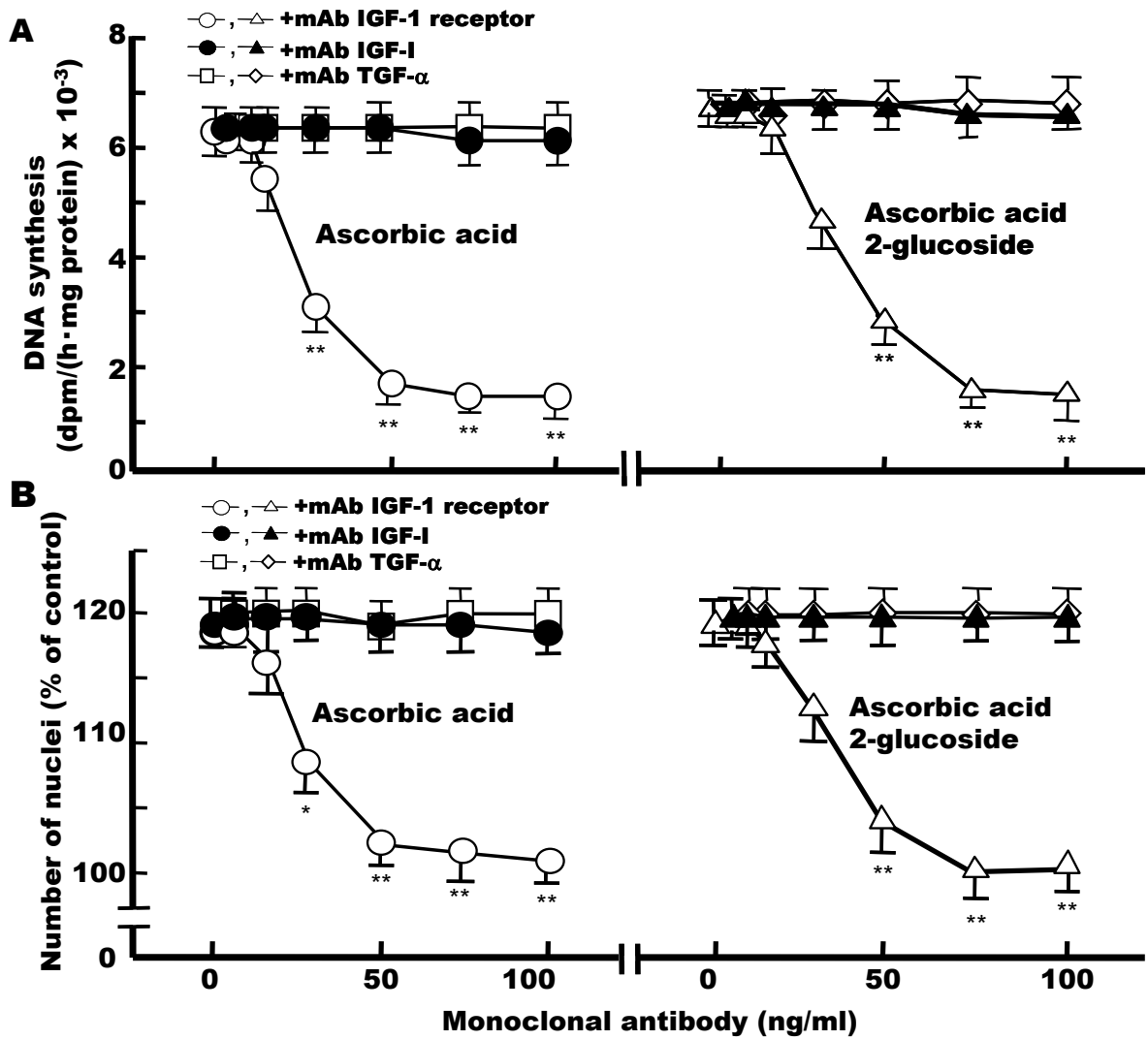


Fig.5

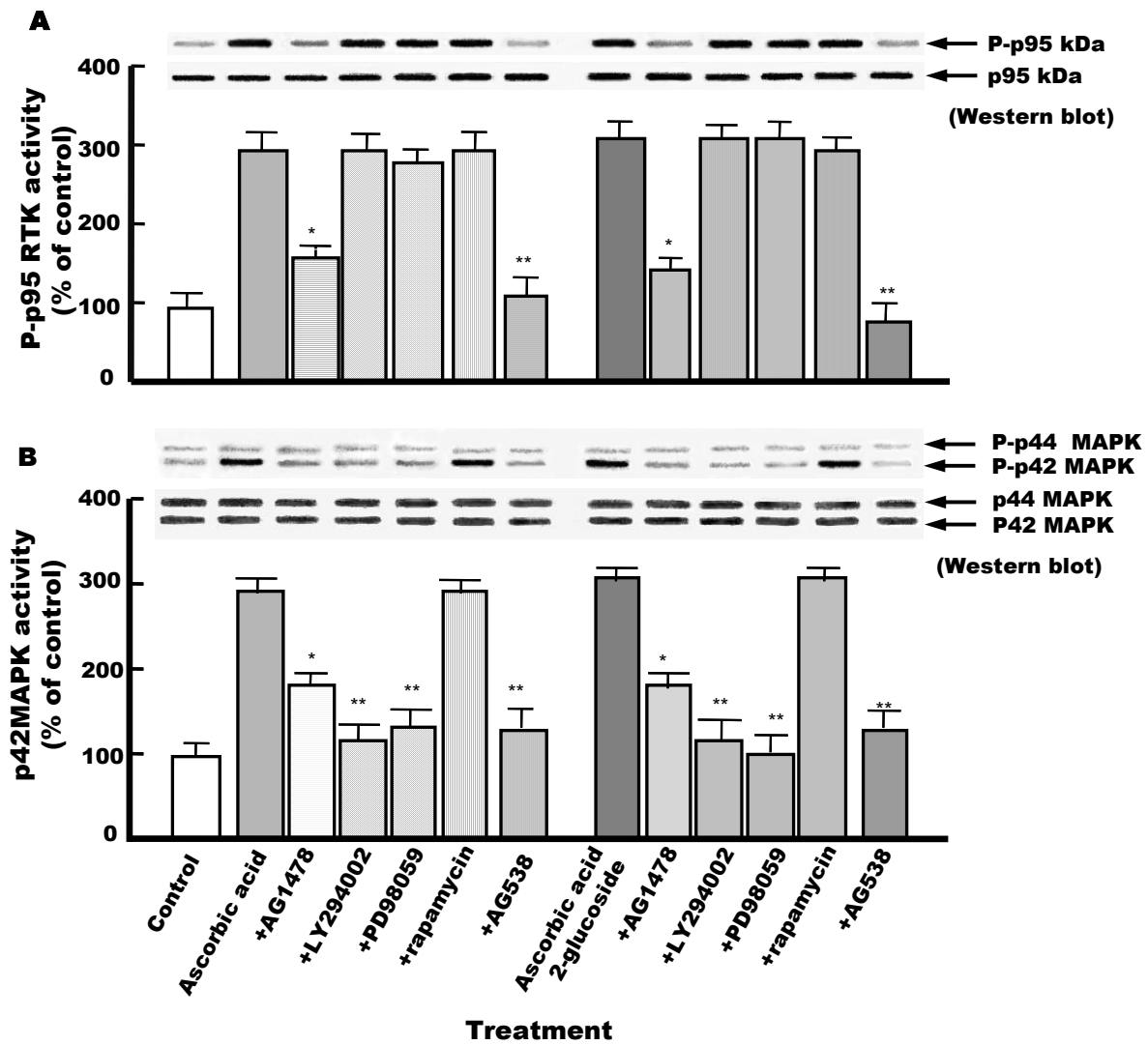


Fig.6

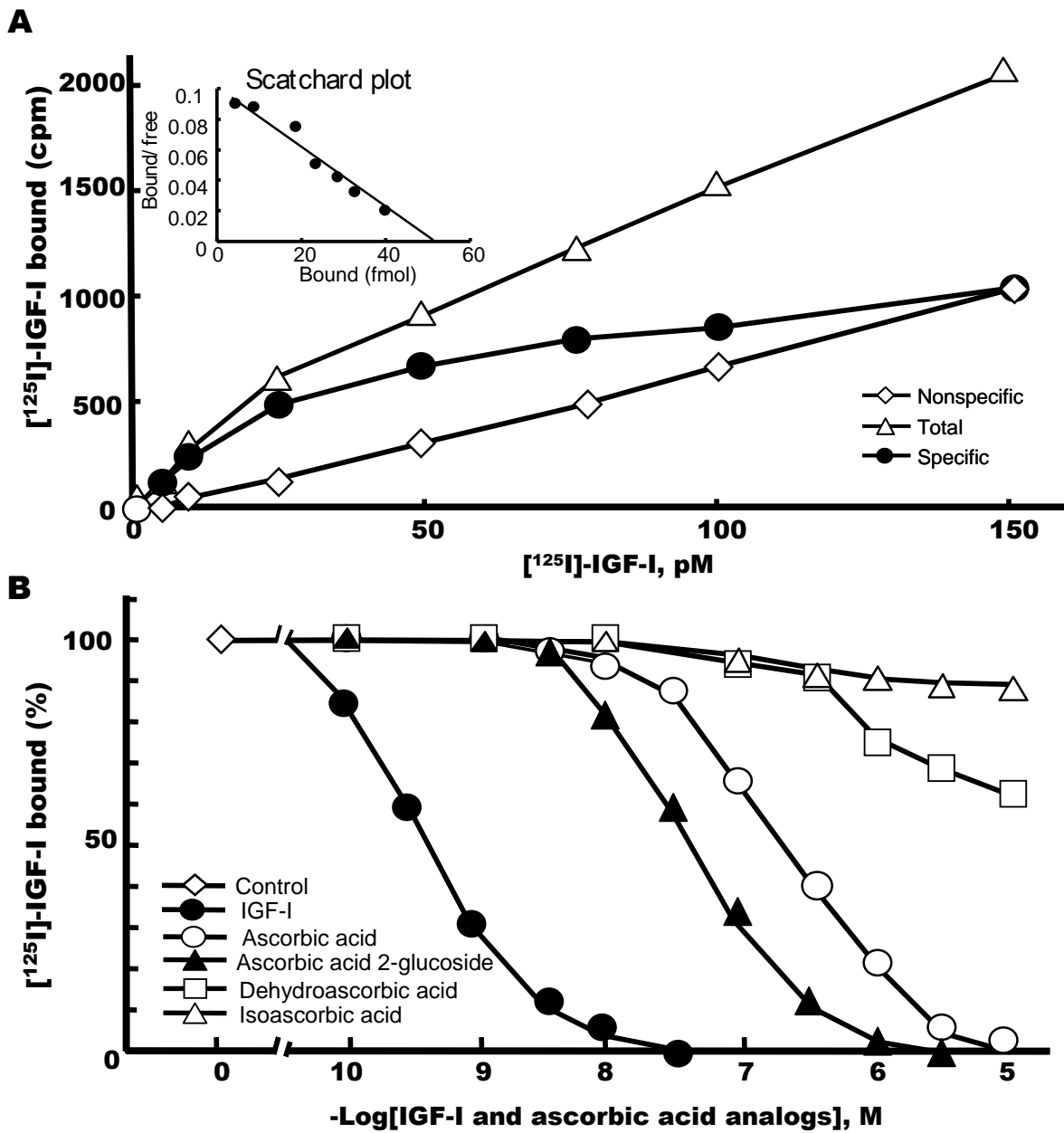


Fig.7

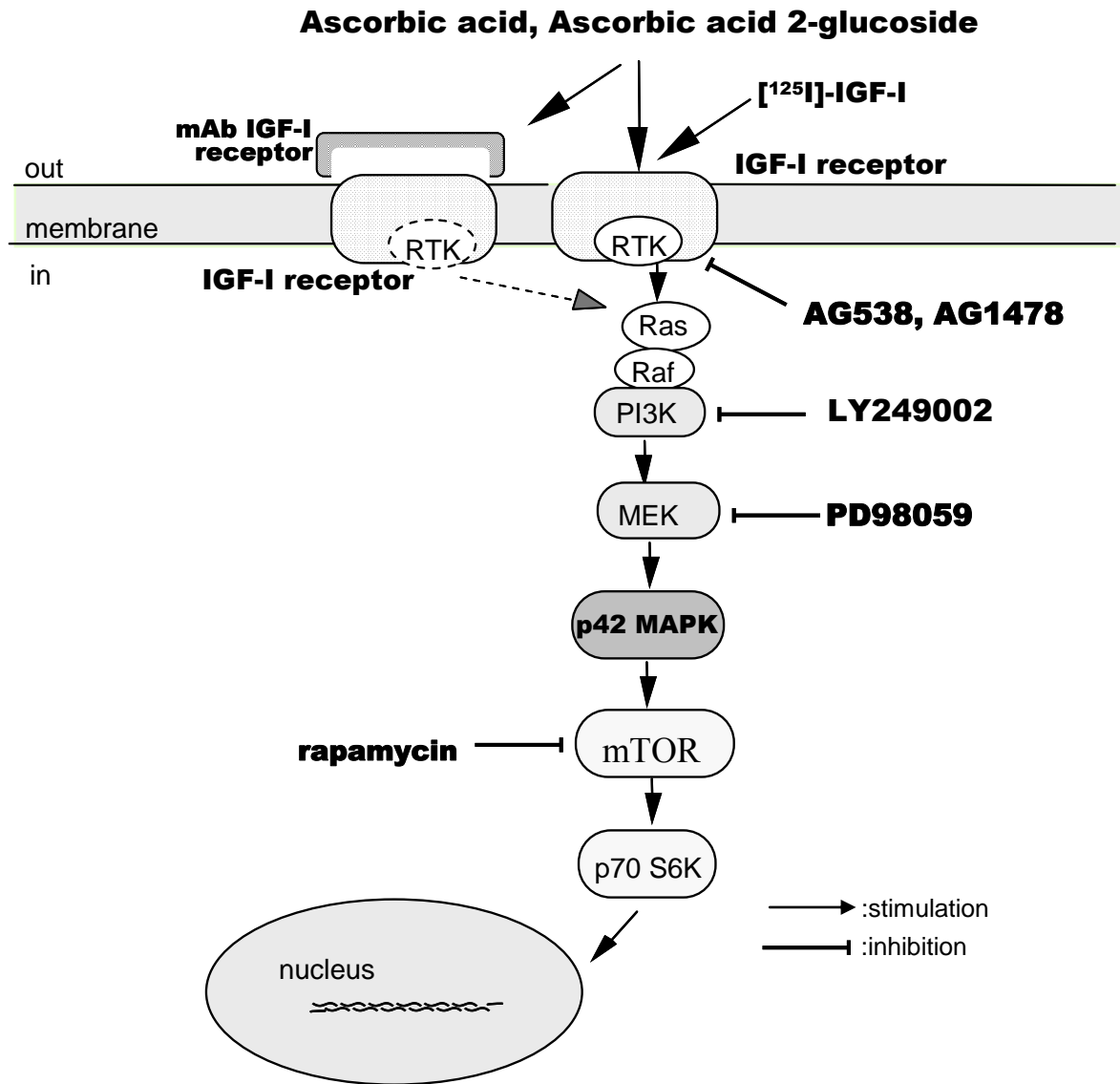


Fig.8