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# 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 $6.46\times10^{-8}$ M and $3.34\times10^{-8}$ 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 IFG-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 (Fausto, 2000; Michalopoulos and DeFrances, 1997). 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)-α all stimulate DNA synthesis in hepatocytes *in vivo* and *in vitro* (Borowiak et al., 2004; Fausto 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 $B_6$ and $B_{12}$. 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 (Li and Schellhorn, 2007; Tajima and Pinnell, 1982). 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 Ferdinabdy, 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 Buron, 1994; Belin et al., 2009; Koh et al., 1998; 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.

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## 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 $3.3\times10^4$ cells/cm$^2$ in minimum essential medium containing 5% bovine calf serum and $10^{-10}$ M dexamethasone for 3 h in 5% $CO_2$ 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 [$^3$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 [$^3$H]-thymidine (1.0 μCi/well) at 2 h and 19 h following the addition of L-ascorbic acid or its analogues. Incorporation of [$^3$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 $3.3\times10^4$ cells/cm$^2$ 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 β-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 °C) to remove cellular debris, then denatured in boiling water for 5 min. For immunoblotting analysis, samples of the supernatant (30 μg/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 (Okamoto et al., 2009; Towbin et al., 1979). Phosphorylated MAP kinase activity was normalized to the total MAP kinase activity. The data were calculated in arbitrary units and are expressed as means ± 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)-α 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-α in the presence of L-ascorbic acid or L-ascorbic acid 2-glucoside.

### 2.8. Assay of [$^{125}$I]-IGF-I binding

Hepatocytes were isolated and cultured as described in Section 2.2. After 3 h of culture, hepatocytes were washed 3 times with Hanks-10 mM Hepes buffer (pH 7.4) supplemented with 8 mM glucose and 10 mg/ml bovine serum albumin. [$^{125}$I]-IGF-I binding to primary cultures of hepatocytes was measured according to the method as described elsewhere (Caro et al., 1988). Briefly, the