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Effects of branched-chain amino acids on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes

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Abstract

We investigated the effects of branched-chain amino acids on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Of the branched-chain amino acids, only leucine (10^{-5}–10^{-3} M) induced hepatocyte DNA synthesis and proliferation in a time- and dose-dependent manner. The addition of valine or isoleucine on its own had no significant effects on the hepatocyte DNA synthesis and proliferation. When combined, isoleucine competitively antagonized leucine-stimulated hepatocyte mitogenesis. U73122 (10^{-6} M), AG1478 (10^{-7} M), wortmannin (10^{-7} M), PD98059 (10^{-6} M) and rapamycin (10 ng/ml) inhibited the ability of leucine to stimulate the hepatocyte DNA synthesis and proliferation, suggesting that phospholipase C, tyrosine kinase, phosphatidylinositol 3-kinase, mitogen-activated protein (MAP) kinase, and p70 S6 kinase are involved in leucine signaling. The mitogenic effects of leucine are completely abolished by the addition of anti-transforming growth factor-α (TGF-α) antibody to the culture medium. Furthermore, leucine stimulated TGF-α secretion into the culture medium and the leucine effect was inhibited by U73122. Isoleucine alone had no significant effect on TGF-α secretion but this agent blocked leucine-induced TGF-α secretion. The results suggest that leucine triggers TGF-α secretion through a putative leucine receptor. The secreted TGF-α then stimulates hepatocyte DNA synthesis and proliferation through activation of TGF-α receptor to induce tyrosine kinase/MAP kinase activity and other downstream growth-related signal transducers.

Keywords: Leucine; DNA synthesis; Proliferation; Hepatocyte

1. Introduction

Branched-chain amino acids (i.e., valine, leucine, and isoleucine) are essential in protein nutrition and are abundant in body proteins. Leucine and isoleucine are known to be good sources of energy, producing 40 mol ATP/mol in specific tissues (Ichihara, 1999). Leucine, however, is poorly metabolized by the liver, since unlike other enzymes branched-chain amino acid transaminase is nearly inactive in the liver. In addition to providing the substrates for protein synthesis or sources of energy, branched-chain amino acids also exert regulatory influences on carbohydrate metabolism, amino acid transport, protein turnover, and gene expression (Harris et al., 2004; Hutson et al., 1987; Ichihara, 2000; Ijichi et al., 2003; Van Sluijters et al., 2000).

Several amino acids, and the branched-chain amino acids in particular, were recently identified as important signaling agents in the initiation and modulation of hepatocyte proliferation. Amino acids exert a mitogenic effect on both adult and fetal hepatocytes, and hepatoma cells when they are placed in combination with insulin, insulin-like growth factor-I (IGF-I), and other growth factors (Dubbelhuis and Meijer, 2002; Krause et al., 2002; Patti et al., 1998). Of the branched-chain amino acids, leucine was reported to activate phosphorylation of two components involved in protein synthesis, ribosomal protein S6 (p70 S6) kinase, a downstream target of rapamycin cell signaling pathway and eukaryotic initiation factor 4E binding protein (4E-BP1), a translational repressor protein (Hara et al., 1998; Wang et al., 1998) even in the absence of additional factors. Other

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investigators have reported that leucine preferentially stimulates the target of rapamycin signaling pathways in adipocytes, a pancreatic cell line, a hepatocyte cell line, and L6 cells (Xu et al., 1998; Ijichi et al., 2003; Peyrollier et al., 2000). Thus, it is clear that amino acid alone and in combination with insulin, IGF-I, and other growth factors exert mitogenic effects (Lynch et al., 2000; Proud and Denton, 1997); however, the cellular mechanism(s) of these effects are not fully defined.

Nevertheless, several attempts have been made to treat patients with liver cirrhosis and hepatic encephalopathy with pure branched-chain amino acids or branched-chain amino acid-enriched formulas (Morgan, 1990; Marchesini et al., 1990; Marchesini et al., 2000; Moriwaki et al., 2004). To gain an understanding of the molecular mechanisms of leucine action, and of the relationship between leucine and the other branched-chain amino acids will be valuable for effective treatment of hepatic injury, such as hepatitis and hepatic cirrhosis (Nishitani et al., 2004). Furthermore, elucidation of the nature of the intracellular signal-transduction pathway should add to our understanding of how branched-chain amino acids modulate or stimulate hepatocyte proliferation.

Previously, we reported that several growth factors and cytokines stimulate DNA synthesis and proliferation in primary cultures of adult rat hepatocytes during early phase of culture (Kimura and Ogihara, 1997, 1998; Kimura et al., 2001a,b). Further, we found that the activities of the growth factors and cytokines are regulated by cell density and modulated differently by α- and β-adrenoceptor agonists. Primary cultures of hepatic parenchymal cells provide a very good model for studying liver functions because the hepatocytes retain most liver functions and these are regulated in vitro by various neurotransmitters, growth factors, hormones, cytokine, and nutrients (Ichihara, 1999).

In the present report, therefore, we examined whether or not branched-chain amino acids and some analogues actually stimulate DNA synthesis and proliferation, and investigate their possible signal transduction pathway in primary cultures of adult rat hepatocytes. The results demonstrate that leucine alone is sufficient to induce autocrine secretion of transforming growth factor-α (TGF-α) by hepatocytes in primary cultures. Secreted TGF-α then stimulates the TGF-α receptor tyrosine kinase/mitogen-activated protein (MAP) kinase pathway to induce hepatocyte DNA synthesis and proliferation. Intriguingly, we also found that isoleucine, but not a valine, antagonized leucine-induced TGF-α secretion and subsequent hepatocyte DNA synthesis and proliferation.

2. Materials and methods

2.1. Animals

Male Wistar rats (weight 200–220 g) obtained from Saitama Experimental Animal Co. (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

Parenchymal hepatocytes were obtained from adult male rats using a two-step collagenase-based perfusion method followed by differential centrifugation as described previously (Seglen, 1975). Most of damaged cells and nonparenchymal cells are removed by a differential centrifugation procedure. Final cell suspension contains practically only parenchymal cells, as detected by microscopic inspection. The cells were then plated on collagen-coated plastic culture dishes at a cell density of 3×10^4 cells/cm^2 in Ham's nutrient mixture F-10 supplemented with 5% newborn bovine serum, 1.0 nM dexamethasone, aprotinin, and antibiotics. In this experiment, we used Ham's nutrient mixture F-10 in place of Williams' medium E (Kimura and Ogihara, 1997) since F-10 contains relatively low concentrations of branched-chain amino acids (L-leucine, 10^{-5} M; L-isoleucine, 2×10^{-5} M; L-valine, 3×10^{-5} M) as compared to Williams' medium E (leucine, 10^{-3} M; isoleucine, 10^{-3} M; valine, 10^{-3} M). After a 3-h attachment period, the culture medium was replaced with serum-free Ham's nutrient medium supplemented with aprotinin and antibiotics. Then 10^{-5}–10^{-3} M valine, leucine, isoleucine, and other test agents were added to the culture medium. The medium was refreshed every 4 h.

2.3. Measurement of hepatocyte DNA synthesis and proliferation

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 Ham's nutrient mixture F-10 and cultured in the Ham's medium containing branched-chain amino acids or other agents for an additional 6 h. The medium was changed to fresh one every 4 h. The cells were pulsed at 0, 1, 2, 3 and 4 h after stimulation by leucine or other agents for 2 h with [^{3}H]thymidine (1.0 mCi/well). These cells were washed twice with Dulbecco's phosphate-buffered saline (PBS, pH 7.4), immersed in 10% trichloroacetic acid (TCA) and solubilized in 1N NaOH, as described (Kimura and Ogihara, 1997). [^{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 non-replicative 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.

The number of nuclei was determined via 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 (PBS), then exposed to 0.25 ml of 0.1 M citric acid containing 0.1% Triton X-100 for 30 min at 37 °C. Hepatocytes firmly attached to the collagen-coated plastic culture dishes and were not sufficiently dispersed by 0.02% EDTA–0.05% trypsin treatment. Thus, an equal volume of the nuclear suspension was mixed with 0.3% Trypan blue in PBS and the nuclei were counted in a hemocytometer in order to obtain an accurate count.

2.4. Neutralization of endogenous growth factors

For experiments with neutralizing antibodies, serum-free primary cultured hepatocytes were treated with varying concentrations of leucine in the presence or absence of monoclonal antibodies against TGF-α and IGF-I (at 5, 10, 20, 30, 50, 70, and 100 ng/ml).

2.5. Measurement of MAP kinase activity

Phosphorylated MAP kinase isoforms (p42 MAP kinase and p44 MAP kinase) were identified by Western blotting analysis using anti-phospho MAP kinase monoclonal antibody as described by Twobin et al., 1979. In brief, after an initial attachment period of 3 h, the hepatocytes were washed twice with serum-free Ham's nutrient mixture F10 and cultured in the serum-free Ham's medium containing branched-chain amino acids or other agents for an additional 20 min. Then hepatocytes were washed once with ice-cold PBS, 0.2 ml of lysis buffer were added, and the hepatocytes were harvested. After centrifugation at 16,300×*g* for 30 min at 4 °C, the cell lysates were denatured in boiling water for 5 min. Samples of the supernatant (50 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide resolving gel by the method of Laemmli (1970). After electrophoresis, proteins were transferred to immobilon-P membranes.

For the detection of phosphorylated extracellular-regulated protein kinase (ERK)1 (p44 kDa) and ERK2 (p42 kDa), membranes were immersed in Tris-buffered saline (pH 7.4) containing 1% bovine serum albumin. The membranes were then incubated with anti-phospho-MAP kinase antibody (1 mg/ml) and washed as described. Antibody binding was visualized by incubation with a horseradish peroxidase conjugated donkey anti-rabbit IgG secondary antibody (1:3000 dilution; Kimura et al., 2001a) followed by enhanced chemiluminescence detection (ECL Kit, Amersham). Densitometric analysis was performed using the NIH Image program version 1.60 for Macintosh. The data were calculated in arbitrary units and expressed as mean±S.E.M.

2.6. ELISA for TGF-α

After an initial attachment period of 3 h, the cultures were washed three times with PBS. The medium was replaced with 1.0 ml PBS containing 1.0 mM $CaCl_2$, and 0.1 μg/ml aprotinin. Hepatocytes were preincubated in the conditioned medium for 5 min in humidified 5% CO_2–95% air at 37 °C and then incubated with branched-chain amino acids and/or test agents for various time periods. Media samples were taken from cultures in six-well plates at 0–0.5 h post-branched-chain amino acid treatment, and the TGF-α concentration was determined by using an enzyme-linked immunosorbent assay (ELISA) kit under manufacture's instruction. Briefly, standard human TGF-α or unknown samples were dispensed into a 96-well microtiter plate coated with a polyclonal antibody against human TGF-α. After incubation for 3 h, it was washed three times with PBS containing 0.05% Tween 20. After the addition of a 0.1 ml aliquot of a polyclonal antibody against human TGF-α, the plate was incubated for 1 h then washed three times. After the addition of 0.2 ml of diluted goat IgG-peroxidase conjugate, the plate was incubated for 0.5 h and then washed. An aliquot (0.1 ml) of 0.25% *o*-phenylenediamine was added and the plate allowed to stand for 45 min. The reaction was stopped by addition of 0.1 ml of 1N sulfuric acid. The absorbance was read at 450 nm by an automatic plate reader with a reference wavelength of 690 nm. The lower detection limit of the TGF-α ELISA was 0.1 ng/ml.

2.7. Materials

The following reagents were obtained from Sigma (St. Louis, MO, USA): aphidicolin, ionomycin, dexamethasone, somatostatin, verapamil hydrochloride, aprotinin, AG1478 (*N*-[3-chlorophenyl]-6, 7-dimethoxy-4-quinazolinamine), wortmannin, rapamycin, and Ham's nutrient mixture F-10. Branched-chain amino acids L-leucine, L-valine, L-isoleucine, and some structural analogues of L-leucine (D-leucine, L-norleucine, and L-leucine amide) were obtained from Wako (Osaka, Japan). U73122 (1-[6-[17β-3-methoxyestra-1, 3, 5(10)-trien-17-yl]-amino] hexyl)-1H-pyrrol-2, 5-dione), U73343 (1-[6-[17β-3-methoxyestra-1, 3, 5(10)-trien-17-yl]-amino] hexyl)-2, 5-pyrrolidine-dione), sphingosine, 2, 4-dideoxyadenosine and H-89 (*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride) were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). PD98059 (2′-amino-3′-methoxyflavone) was obtained from Calbiochem-Behring (La Jolla, CA, USA). Monoclonal antibodies against insulin-like growth factor (IGF-I), and transforming growth factor-α (TGF-α) were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Newborn calf serum and

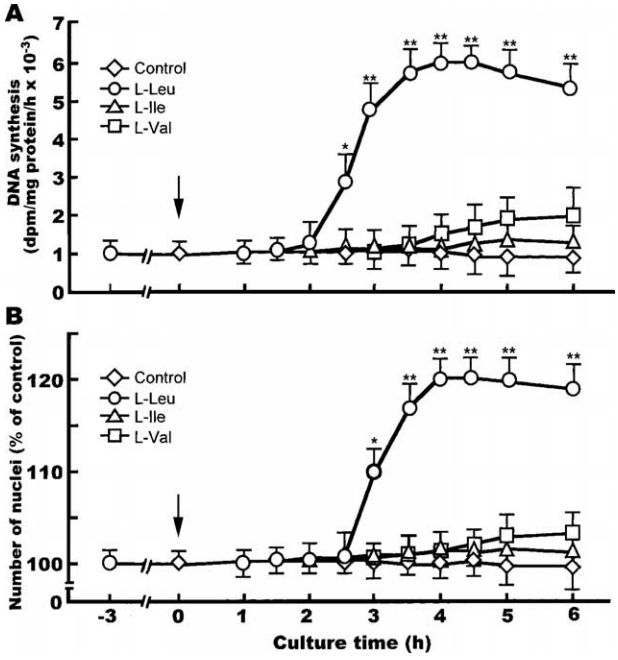

Fig. 1. Time-course associated with effects of branched-chain amino acids on hepatocyte DNA synthesis and proliferation. Hepatocytes were plated in Ham's nutrient mixture F-10 supplemented with 5% newborn calf serum and 0.1 nM dexamethasone at a cell density of 3.3×10^4 cells/cm^2. After an attachment period of 3 h (time zero), the medium was rapidly replaced with serum- and dexamethasone-free Ham's nutrient medium and the cells were cultured with various concentrations of leucine (5×10^{-4} M), isoleucine (10^{-3} M), or valine (10^{-3} M) for an additional 6 h. Hepatocyte DNA synthesis and proliferation were determined, as described in the Materials and methods section. Arrows indicate additions of branched-chain amino acids to the incubation medium. Data are expressed as mean±S.E.M. of the three independent experiments. *P<0.05, **P<0.01 compared with respective control (medium alone).

was purchased from Flow Laboratories (Irvine, Scotland, UK). Collagenase (type II) was obtained from Worthington Biochemical Co. (Freehold, NJ, USA). [Methyl-^{3}H] thymidine (20 Ci/mmol) was purchased from Amersham (Boston, MA, USA). The ELISA kit for TGF-α was obtained from Oncogene Research Products (Boston, MA, USA). All other reagents were of analytical grade.

2.8. Statistics

The differences between two unpaired groups were defined as significant by the Student's t-test when P values were less than 0.05.

3. Results

3.1. Leucine induces hepatocyte DNA synthesis and proliferation

We first examined the effects of branched-chain amino acids (i.e., valine, leucine, and isoleucine) on DNA synthesis and proliferation. The experiments were performed in primary cultures of adult rat hepatocytes in the absence of exogenously added peptide growth factors. Furthermore, freshly isolated hepatocytes were plated at low cell density (3.3×10^4 cells/cm^2) in Ham's nutrient mixture F-10 rather than another medium such as Williams' medium E or Eagle's minimal essential medium. We used F-10 since it contains a relatively low concentration of branched-chain amino acids (see Materials and methods). While they are maintained in a defined medium, hepatic parenchymal cells undergo time-dependent DNA synthesis and proliferation (i.e., an increase in the number of nuclei) in the presence of leucine (5×10^{-4} M). The onset of hepatocyte DNA synthesis and proliferation was observed at about 2.5 h and 3.0 h, respectively, after the addition of leucine (Fig. 1A and B). In leucine-treated cultures, hepatocyte DNA synthesis and proliferation peaked at 3.5 h and 4.0 h, respectively (Fig. 1A and B). Maximal stimulation for hepatocyte DNA synthesis and proliferation was approximately 6.0-fold (DNA synthesis) and 1.2-fold (proliferation). In contrast, the addition of only valine or isoleucine (10^{-4}–10^{-2} M) had no significant effect on hepatocyte DNA synthesis and proliferation.

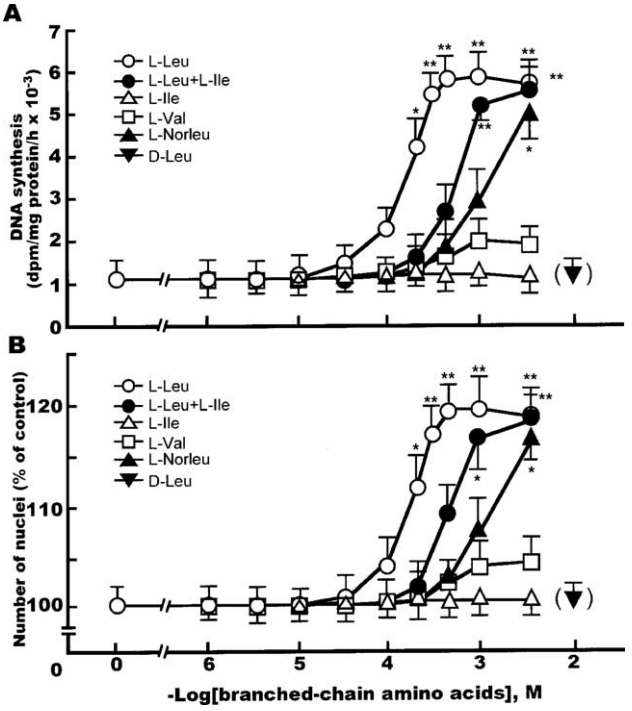

Fig. 2. Dose-dependent effects of leucine and some analogues on hepatocyte DNA synthesis and proliferation. Hepatocytes were cultured in Ham's nutrient mixture at a density of 3.3×10^4 cells/cm^2 as described in the legend of Fig. 1. After an attachment period of 3 h (time zero), the medium was rapidly replaced with serum- and dexamethasone-free Ham's nutrient medium and the cells were cultured with various concentrations of leucine or its analogues for an additional 4 h. DNA synthesis and proliferation were assayed as described in the Materials and methods section. The antagonism of leucine effects by isoleucine (1×10^{-4} M) was also determined. Data are expressed as mean±S.E.M. of the three independent experiments. *P<0.05, **P<0.01 compared with control (medium alone).

3.2. Dose-effects of leucine on hepatocyte DNA synthesis and proliferation

Next we examined the dose-dependent effects of leucine and some of its analogues on hepatocyte DNA synthesis and proliferation. As shown in Fig. 2A and B, L-leucine induced a dose-dependent increase in hepatocyte DNA synthesis and proliferation that is significant at concentrations of 2×10^{-4} M and greater. The maximum increase in DNA synthesis and proliferation was observed with treatment of 1.0 or 2×10^{-3} M leucine for synthesis and proliferation, respectively. The half-maximal effective doses (ED_{50}s) are about 1.58×10^{-4} M and 1.74×10^{-4} M, respectively. Isoleucine and valine had no effect at the concentrations that were tested.

The effects of leucine are stereospecific with an over 100-fold difference in the EC_{50} between values for the L- and D-isomers of leucine and leucine itself. Consistent with this, D-leucine (~10^{-2} M) did not significantly induce hepatocyte DNA synthesis and proliferation at a concentration range of 10^{-6} M–5×10^{-3} M. Next, we tested leucine analogues with side chain modifications. Stimulation in response to norleucine was significantly less potent than that observed with leucine. The reduced agonist activity may be due to the absence of the C-1 carbon. L-leucine amide did not stimulate hepatocyte DNA synthesis and proliferation (data not shown). Intriguingly, when combined with leucine treatment, isoleucine (1×10^{-4} M) but not valine (1×10^{-4} M) inhibited hepatocyte mitogenesis induced by leucine. The agonist dose–response curve shifts to the right in the presence of the fixed concentration of isoleucine, suggesting that isoleucine competitively antagonizes the leucine-stimulated hepatocyte DNA synthesis and proliferation.

3.3. Effects of specific inhibitors of growth-related signal transducer on leucine-induced hepatocyte DNA synthesis and proliferation

To uncover which signaling pathway or pathways are responsible for the leucine effect, we next investigated whether or not the mitogenic response of primary hepatocytes to leucine is mediated by growth-related signal transducers, such as phospholipase C, receptor tyrosine kinase, phosphatidylinositol 3-kinase (PI3K), MAP kinase, and ribosomal protein S6 kinase (p70 S6K). We used four

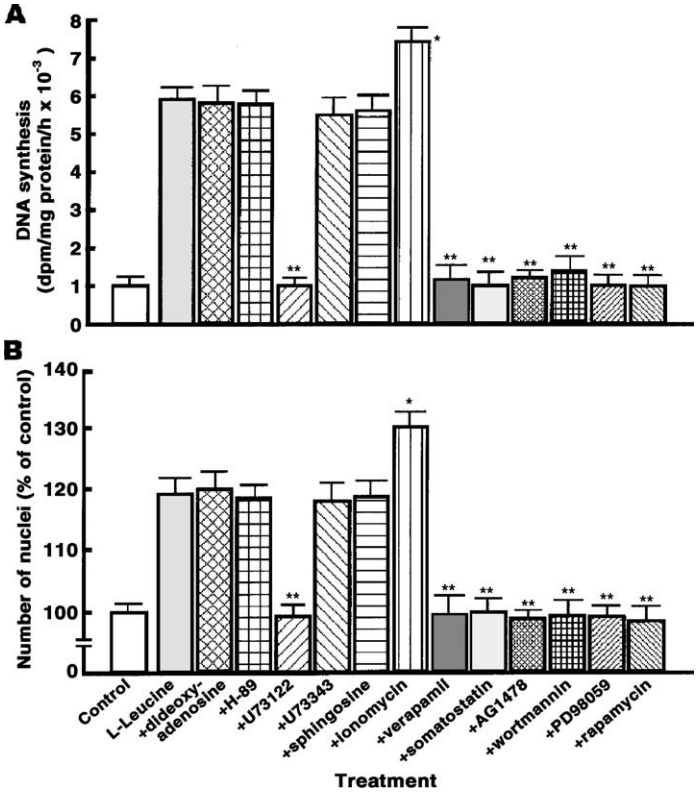

Fig. 3. Effects of specific inhibitors or a stimulator of growth-related signal-transduction on leucine-induced hepatocyte DNA synthesis and proliferation. Hepatocytes were cultured as described in the legend of Fig. 1. After an attachment period of 3 h (time zero), the medium was rapidly replaced with serum- and dexamethasone-free Ham's nutrient medium and the cells were cultured with various concentrations of specific inhibitors with or without leucine for an additional 4 h. Hepatocyte DNA synthesis and proliferation were assayed as described in the Materials and methods section. The inhibitors were as follows: dideoxyadenosine (10^{-6} M), U73122 (10^{-6} M), sphingosine (10^{-6} M), ionomycin (10^{-6} M), verapamil (10^{-6} M), somatostatin (10^{-6} M), U73314 (10^{-6} M), AG1478 (10^{-7} M), wortmannin (10^{-7} M), PD98059 (10^{-6} M), and rapamycin (10 ng/ml). Data are expressed as mean±S.E.M. of the three experiments. *P<0.05, **P<0.01 compared with control (medium alone).

specific inhibitors of these signal transducers: AG1478 (Levitzki and Gazit, 1995), wortmannin (Baggiolini et al., 1987), PD98059 (Alessi et al., 1995), and rapamycin (Chung et al., 1992; Price et al., 1992). AG1478 is an effective tyrosine kinase inhibitor that blocks growth factor-stimulated tyrosine kinase activation. Wortmannin is a specific inhibitor of PI3K. PD98059 is a specific inhibitor of MAP kinase kinase (MEK). Finally, rapamycin is a potent immunosuppressant and antiproliferative agent that mediates its effects through the formation of an active complex with its intracellular receptor, FK506-binding protein.

As shown in Fig. 3A and B, hepatocyte DNA synthesis and proliferation induced by leucine (5×10^{-4} M) was almost completely blocked by U73122 (10^{-6} M), AG1478 (10^{-7} M), wortmannin (10^{-7} M), PD98052 (10^{-6} M), and rapamycin (10 ng/ml). On their own, these inhibitors had no effect on hepatocyte DNA synthesis or proliferation after 4 h of culture. These observations suggest that tyrosine kinase, PI3 kinase, MAP kinase, and target of rapamycin (and other p70S6K downstream targets) are involved in the leucine-stimulated hepatocyte mitogenesis.

To determine the possible involvement of the cAMP/protein kinase A pathway, hepatocytes were treated with an adenylate cyclase inhibitor dideoxyadenosine (Holgate et al., 1980), or a protein kinase A inhibitor H-89 (Zusick et al., 1994) in the absence or presence of leucine. Alone, these inhibitors had no effect on hepatocyte DNA synthesis or proliferation after 4 h of culture. When combined with leucine treatment, neither dideoxyadenosine (10^{-6} M) nor H-89 (10^{-6} M) had an effect on leucine-induced hepatocyte DNA synthesis and proliferation (Fig. 3A and B), suggesting that adenylate cyclase and protein kinase A do not make a significant contribution to leucine-induced hepatocyte mitogenesis.

To characterize the possible involvement of the phospholipase C/Ca^{2+}/protein kinase C pathway in leucine-stimulated hepatocyte DNA synthesis and proliferation, we investigated the effects of the phospholipase C-specific inhibitor, U73122 (Thompson et al., 1991) and a protein kinase C (PKC) inhibitor, sphingosine (Merrill et al., 1989). U73122 (10^{-6} M) markedly inhibited leucine stimulation of hepatocyte DNA synthesis and proliferation during 4 h of culture. In contrast, U73343 (10^{-6} M), a close structural analogue of U73122 that has no such inhibitory action on phospholipase C, does not significantly affect leucine-induced hepatocyte DNA synthesis and proliferation during 4 h of culture; the same was true for sphingosine (10^{-6} M). To ask if Ca^{2+} mobilization is involved in leucine-stimulated hepatocyte DNA synthesis and proliferation, we treated hepatocytes with a Ca^{2+} ionophore ionomycin or a Ca^{2+} channel blocker verapamil together with somatostatin, which decrease cytosolic Ca^{2+} levels. Both verapamil (10^{-6} M) and somatostatin (10^{-6} M) abolished leucine-induced hepatocyte mitogenesis. In contrast, ionomycin (10^{-6} M) significantly potentiated the leucine-induced hepatocyte DNA synthesis and proliferation, even though treatment with ionomycin (10^{-6} M) alone had no effect on hepatocyte DNA synthesis or proliferation. As to the mechanism of intracellular signal transduction pathway, these results suggest that both the putative leucine receptor/G-protein/phospholipase C/Ca^{2+} pathway (possibly acting via Gq) and the tyrosine kinase/MAP kinase signalling pathway are involved in the mitogenic effects of leucine. However, the relationship between these two pathways remains to be elucidated.

3.4. Activation of p42 MAP kinase by leucine and its blockade by PD98059

The MAP kinase pathway is important in hepatocyte proliferation (Alessi et al., 1995; Ullrich and Schlessinger, 1990; Xiaomei et al., 1995). However, the significance of MAP kinase activation for hepatocyte DNA synthesis and proliferation induced by leucine remains to be elucidated. To that end, we investigated whether or not leucine can stimulate MAP kinase activity. As shown in Fig. 4A, leucine (5×10^{-4} M) caused an increase in p42 MAP

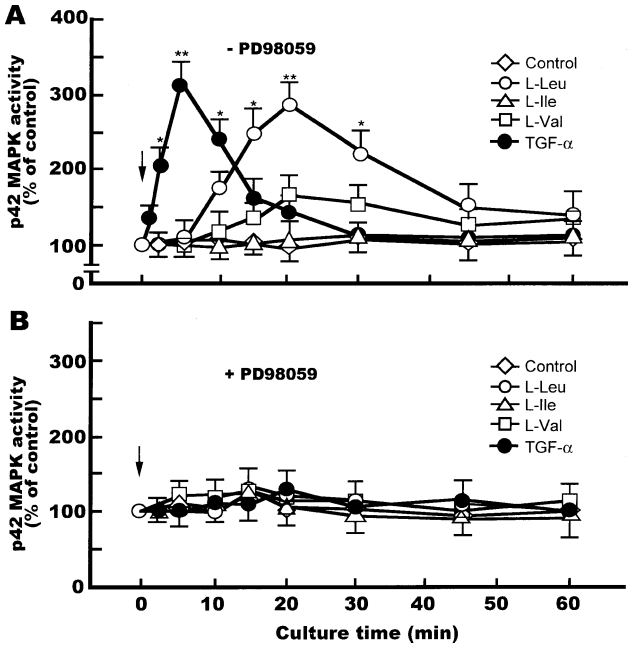

Fig. 4. Activation of p42 MAP kinase by leucine and its blockade by PD98059. Hepatocytes were cultured in Ham's nutrient mixture F-10 supplemented with leucine (5×10^{-4} M) for 0~1 h as described in the legend of Fig. 1. Hepatocyte MAP kinase activity stimulated by leucine or TGF-α was determined at various time points in the absence (A) or presence of PD98059 (10^{-6} M) (B). Phosphorylated MAP kinase isoforms (ERK 1: p44 kDa and ERK 2: p42 kDa) were identified by Western blotting analysis using anti-phospho MAP kinase monoclonal antibody as described in Materials and methods section. TGF-α (5 ng/ml), isoleucine (10^{-3} M), valine (10^{-3} M). Arrows indicate additions to the incubation medium. Data are mean±S.E.M of the three independent experiments. *P<0.05 and **P<0.01 compared with the values without adding branched-chain amino acids.

kinase phosphorylation that reaches a peak at 20 min and gradually declines to the basal level at 60 min after leucine addition. In contrast, leucine (5×10^{-4} M) did not affect p44 MAP kinase (data not shown). Exogenously added TGF-α caused a rapid increase in the phosphorylation of p42 MAP kinase that peaked at 5 min after the addition and was followed by a sharp decrease to the basal level within 30 min (Fig. 4A). Isoleucine and valine on their own did not significantly stimulate p42 MAP kinase activity. Phosphorylation of p42 MAP kinase by either leucine or TGF-α was almost completely blocked by the MEK inhibitor PD98059 (Fig. 4B).

3.5. Effects of monoclonal antibodies against TGF-α or IGF-I receptor on leucine-induced hepatocyte DNA synthesis and proliferation

We hypothesized that leucine selectively stimulates the secretion of primary mitogen(s) in an autocrine manner to induce hepatocyte mitogenesis, since there is little or no evidence that leucine acts as a primary mitogen. The major hepatic autocrine growth factors TGF-α and IGF-I are potential candidates for this primary growth factor, since parenchymal hepatocytes express TGF-α and IGF-I mRNA, and since hepatocytes can synthesize and store the growth factors (Michalopoulos and DeFrances, 1997).

To ask if the growth factors play a role in leucine signaling, we measured the mitogenic function of leucine in the presence of a monoclonal antibody against TGF-α or IGF-I, major hepatic autocrine growth factors. Fig. 5A and B shows that the addition of a neutralizing monoclonal antibody against TGF-α dose-dependently inhibited the growth-promoting effect of leucine (5×10^{-4} M). The IC_{50} values for DNA synthesis and proliferation at 4 h of culture were 26.4 ng/ml and 29.9 ng/ml, respectively. In contrast, the DNA synthetic and proliferative effects of leucine were not significantly affected by treatment with various concentrations of a monoclonal antibody against IGF-I (1–100 ng/ml). Not surprisingly, the anti-IGF-I monoclonal antibodies did not significantly affect hepatocyte DNA synthesis and proliferation in the absence of leucine treatment (data not shown). The results indicate that this elimination of the mitogenic effects of leucine is specific to the antibody against TGF-α. Accordingly, a novel model can be proposed in which the mitogenicity of the leucine is mediated by the autocrine secretion of TGF-α, which then stimulate DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

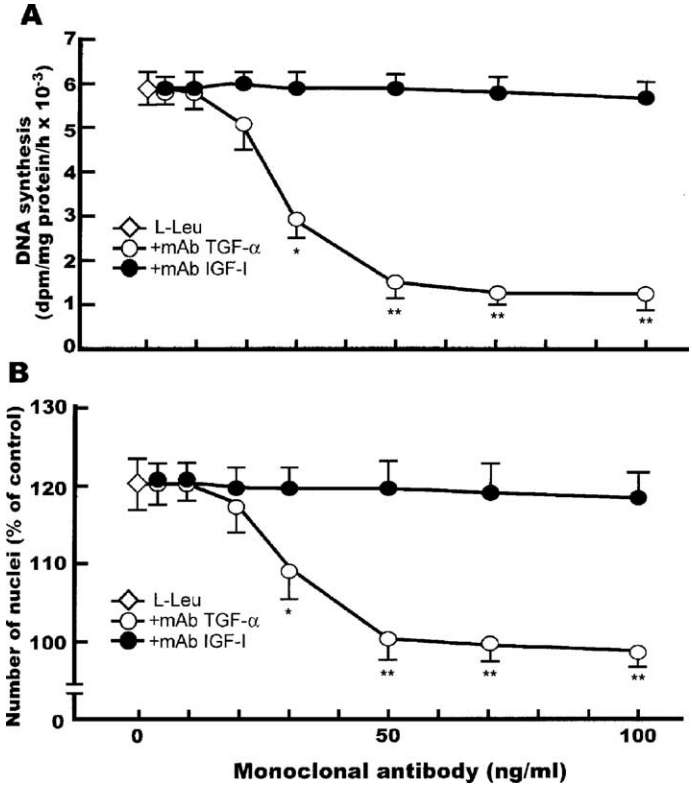

Fig. 5. Effects of monoclonal antibodies against TGF-α or IGF-I on leucine-induced hepatocyte DNA synthesis and proliferation. Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend of Fig. 1. After an attachment period of 3 h, the medium was rapidly replaced with serum- and dexamethasone-free Ham's nutrient medium and the cells were cultured with various concentrations of monoclonal antibodies against transforming growth factor-α (TGF-α) or insulin-like growth factor-I (IGF-I) in the presence of leucine (5×10^{-4} M) for an additional 4 h. DNA synthesis and proliferation were assayed as described in the Materials and methods section. Data are expressed as mean±S.E.M. of the three independent experiments. *P<0.05, **P<0.01 compared with the respective control (leucine alone).

3.6. Leucine-induced secretion of TGF-α by primary cultured hepatocytes: time-course and dose–response relationship

To test the model, it is important to ask if treatment of primary cultured hepatocyte with leucine does in fact lead to increased secretion of TGF-α into cultures. As shown in Fig. 6A and B, when primary hepatocytes were cultured in Ham's nutrient mixture F-10 supplemented with leucine at increasing concentrations, the TGF-α levels in the culture medium increased in a time- and dose-dependent manner. A time-course study showed that a detectable increase began within 2 min of treatment with leucine (5×10^{-4} M). The level of TGF-α in the medium reached a maximum at 10 min, and then remained constant for the duration of the experiment. As shown in Fig. 6B, the ED_{50} value was 8.10×10^{-5} M. The addition of valine (10^{-3} M) or isoleucine (10^{-3} M) had no significant effect on TGF-α levels in the culture medium. These results support the idea that leucine-induced DNA synthesis and proliferation are mediated through the increased release of TGF-α by primary cultures of adult rat hepatocytes.

3.7. Leucine-induced TGF-α secretion and p42 MAP kinase activation by primary cultured hepatocytes: effects of specific inhibitors or stimulator of signal transducers

To confirm links between the leucine-induced TGF-α secretion and hepatocyte mitogenesis, we investigated the regulatory mechanisms associated with the rapid TGF-α secretion induced by leucine. To determine if the phospholipase C/Ca^{2+}/PKC pathway is involved in the leucine-induced TGF-α secretion, we investigated the effects of a phospholipase C inhibitor, U73122 and PKC inhibitor, sphingosine. As shown in Fig. 7A, U73122 markedly attenuated leucine-stimulated TGF-α secretion after 4 h of culture. The U73343, however, did not affect leucine-induced TGF-α secretion. Similarly, PD98059 did not affect

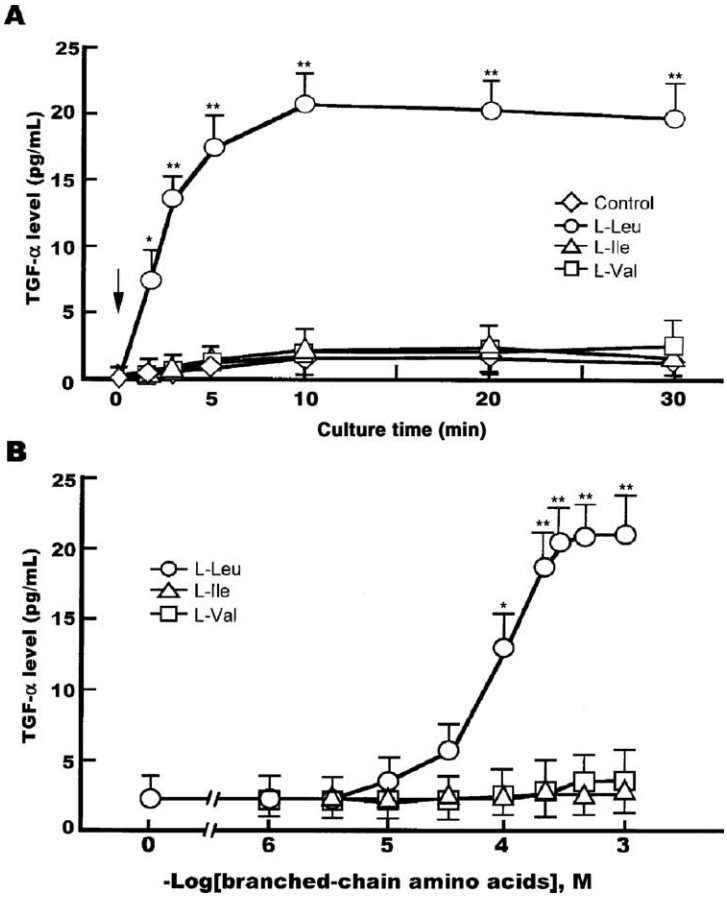

Fig. 6. Time-course of TGF-α levels in the culture medium as stimulated by leucine (A), and dose-dependent effects of leucine on TGF-α levels (B). Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend of Fig. 1. After an initial attachment period of 3 h, the cultures were washed three times with PBS (pH7.4). The medium was replaced with 1.0 ml PBS containing 1.0 mM $CaCl_2$, and 0.10 μg/ml aprotinin. Hepatocytes were preincubated in the conditioned medium for 5 min in humidified 5% CO_2–95% air at 37 °C. Then, leucine (5×10^{-4} M), isoleucine (10^{-3} M), or valine (10^{-3} M) was added to the culture and hepatocytes were further cultured for various time periods. At each time point, medium levels of TGF-α were determined with an ELISA kit for TGF-α (A). Dose-dependent effects of each branched-chain amino acid on the medium levels of TGF-α were determined at 20 min after incubation (B). Data are mean±S.E.M. of the three independent experiments. *P<0.05 and **P<0.01 compared with the values without adding branched-chain amino acids.

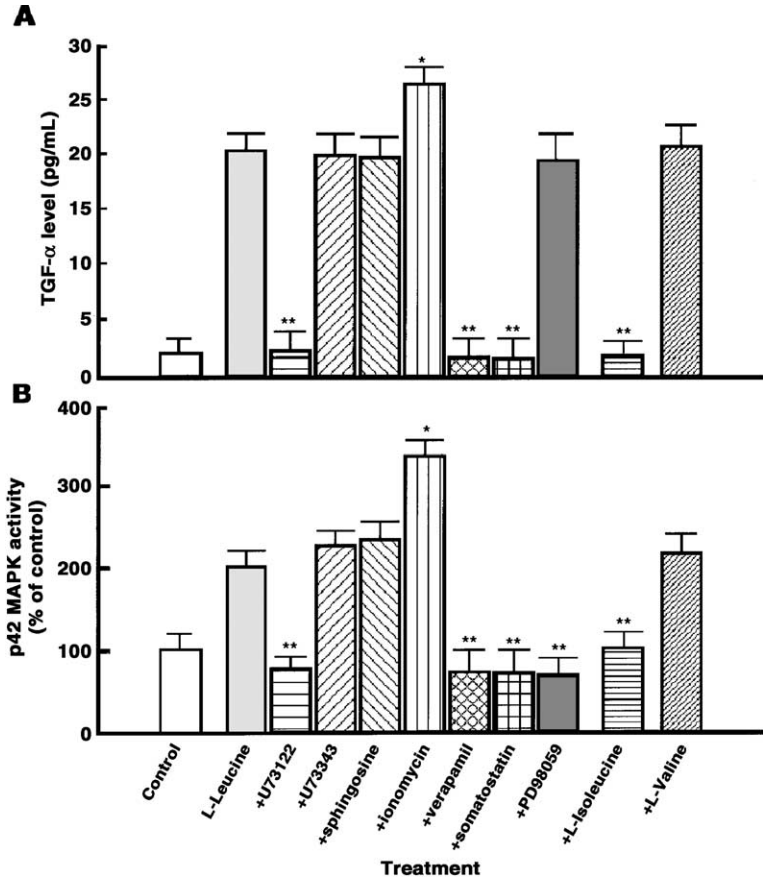

Fig. 7. Effects of specific stimulator or inhibitors of signal transducing elements on leucine-stimulated TGF-α levels and p42 MAP kinase activity. Hepatocytes were cultured at a density of 3.3×10^4 cells/cm^2 as described in the legend of Fig. 1. After an attachment period of 3 h, the medium was rapidly replaced with serum- and dexamethasone-free Ham's nutrient medium and the cells were cultured with specific inhibitors with or without leucine (5×10^{-4} M) for an additional time (10 or 20 min). The inhibitors used are as follows: U73122 (10^{-6} M), U73343 (10^{-6} M), sphingosine (10^{-6} M), ionomycin (10^{-6} M), verapamil (10^{-6} M), somatostatin (10^{-6} M), PD98059 (10^{-6} M), isoleucine (5×10^{-4} M), valine (10^{-3} M), wortmannin (10^{-7} M), and rapamycin (10 ng/ml). For measurement of the level of TGF-α in the culture medium, hepatocytes were cultured in PBS (pH7.4) containing $CaCl_2$ and aprotinin supplemented with leucine for 10 min as described in the legend of Fig. 8 (A). Phosphorylated MAP kinase isoforms (p42 MAP kinase and p44 MAP kinase) were identified by Western blotting analysis using anti-phospho MAP kinase monoclonal antibody as described in Materials and methods section (B). Data are mean±S.E.M of the three independent experiments. *P<0.05 and **P<0.01 compared with the values without adding branched-chain amino acids.

leucine-induced secretion of TGF-α. Leucine-induced TGF-α secretion was unaffected by AG1478 (10^{-7} M), wortmannin (10^{-7} M), rapamycin (10 ng/ml) (data not shown). Leucine-induced TGF-α secretion was blocked by somatostatin and verapamil. As expected, leucine-stimulated TGF-α secretion was significantly potentiated by the activator, ionomycin (10^{-6} M). These results suggest that activation of a putative leucine receptor or recognition sites and subsequent mobilization of extracellular Ca^{2+} led to rapid secretion of TGF-α into culture. Intriguingly, when combined with leucine treatment, isoleucine (5×10^{-4} M), but not valine (5×10^{-4} M), did block leucine-induced TGF-α secretion.

To characterize involvement of the phospholipase C/Ca^{2+}/PKC pathway in induction of p42 MAP kinase by leucine, we investigated the effects of the phospholipase C inhibitor U73122 and a PKC inhibitor sphingosine. Fig. 7B shows that the phosphorylation of p42 MAP kinase induced by leucine was almost completely blocked by U-73122 or PD98059. Treatment of cultured hepatocytes for 20 min with isoleucine (5×10^{-4} M), but not with valine (5×10^{-4} M), caused a marked inhibition of p42 MAP kinase phosphorylation induced by leucine. These results suggest that p42 MAP kinase activity is regulated by putative leucine receptor or recognition sites/Gq/phospholipase C pathway and mobilization of intracellular Ca^{2+}.

3.8. Effects of isoleucine and valine on leucine-induced TGF-α secretion and p42 MAP kinase activity: dose–response relationship

Next we examined the dose-dependent effects of isoleucine and valine on leucine-induced TGF-α secretion and MAP kinase activation in primary cultured hepatocytes. As shown in Fig. 8A, isoleucine, but not valine, dose-dependently inhibited the TGF-α secretion induced by leucine (5.0×10^{-4} M). The effect was significant at concentrations of 3.3×10^{-5} M and greater and maximum

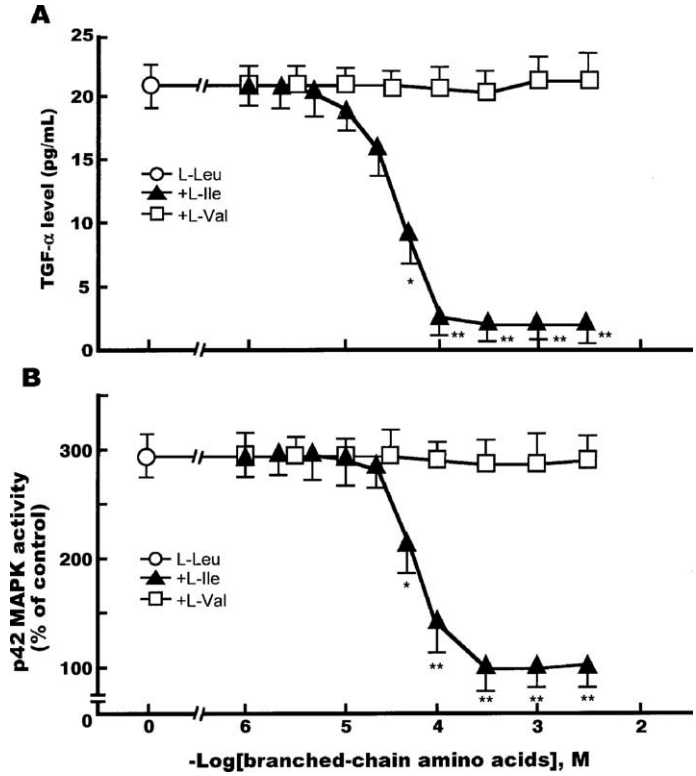

Fig. 8. Dose-dependent inhibition of leucine-stimulated increase in TGF-α levels (A) and p42 MAP kinase activity by isoleucine (B). Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend of Fig. 1. After an attachment period of 3 h, the medium was rapidly replaced with serum- and dexamethasone-free Ham's nutrient medium and the cells were cultured with various concentrations of isoleucine and valine in the presence of leucine (5×10^{-4} M) for an additional 10 or 20 min. TGF-α concentration was determined at various time points by enzyme-linked immumosorbent assay (ELISA) as described in Materials and methods section. Phosphorylated MAP kinase isoforms (ERK 1 and ERK 2) were identified by Western blotting analysis using anti-phospho MAP kinase monoclonal antibody as described in Materials and methods section. Data are expressed as mean±S.E.M. of the three independent experiments. *P<0.05, **P<0.01 compared with control the respective control (leucine alone).

inhibition was observed with 1.0×10^{-4} M isoleucine. The 50% inhibitory concentration value (IC_{50}) of activity is about 3.33×10^{-5} M. Similarly, isoleucine, but not valine, dose-dependently inhibited p42 MAP kinase activation induced by leucine (5.0×10^{-4} M; Fig. 8B). The effect was significant at concentration of 3.0×10^{-4} M and greater and maximum inhibition of p42 MAP kinase activation was observed with 3.0×10^{-4} M isoleucine. The IC_{50} is 6.07×10^{-5} M.

3.9. Effects of isoleucine and valine on leucine-induced hepatocyte DNA synthesis and proliferation: dose–response relationship

To examine the intracellular consequence of the isoleucine block of leucine-induced TGF-α secretion, we looked at the dose-dependent effects of isoleucine and valine on hepatocyte DNA synthesis and proliferation. As shown in Fig. 9A and B, isoleucine, but not valine, dose-dependently inhibited the hepatocyte DNA synthesis and proliferation induced by leucine (5×10^{-4} M). The effect was significant at concentrations of 5.0×10^{-5} M for DNA synthesis and 1.0×10^{-4} M for proliferation. Maximum inhibition was observed with 1.0 and 1.0×10^{-3} M isoleucine, respectively. The IC_{50} values were about 4.50×10^{-5} M and 7.51×10^{-5} M, respectively. Taken together, these results suggest that isoleucine, but not valine, inhibits the autocrine release of TGF-α, presumably by antagonizing effects of leucine at the putative leucine receptor or leucine recognition sites.

4. Discussion

We found that, among branched-chain amino acids, only leucine significantly induces DNA synthesis and proliferation of hepatocytes in a time- and dose-dependent manner (Figs. 1 and 2). The effects are stereospecific, with an over 100-fold difference in the EC_{50} values for the L- and D-isomers of leucine. In addition, the results suggest that isoleucine, a close structural analogues of leucine, competitively antagonizes leucine-stimulated hepatocyte DNA synthesis and proliferation. The ability of isoleucine to inhibit the effects of leucine implies the existence of a leucine receptor or recognition site(s) whose occupation is linked to hepatocyte DNA synthesis and proliferation. Thus, we set out to elucidate some of the structural requirements for agonist activity. Norleucine, which lacks the branched-

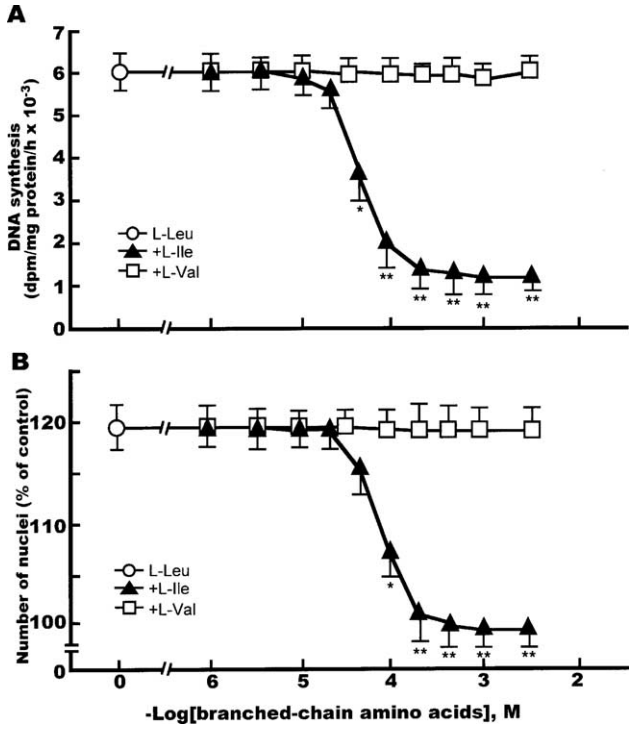

Fig. 9. Dose-dependent inhibition of leucine-induced DNA synthesis and proliferation by isoleucine. Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend of Fig. 1. After an attachment period of 3 h, the medium was rapidly replaced with serum- and dexamethasone-free Ham's nutrient medium and the cells were cultured with various concentrations of isoleucine or valine in the presence of leucine (5×10^{-4} M) for an additional 4 h. Hepatocyte DNA synthesis and proliferation were assayed as described in the Materials and methods section. The cells were pulsed at 2 h post-leucine stimulation for 2 h with [^{3}H]thymidine (1.0 mCi/well). Data are expressed as mean±S.E.M. of the three independent experiments. *P<0.05, **P<0.01 compared with control the respective control (leucine alone).

chain group found in leucine, produces a significant response only at a high concentration (5×10^{-3} M), suggesting that the branched-chain is required for potent agonist activity. It is interesting to note that the existence of a functional leucine receptor has been reported for another cell culture system (Lynch et al., 2000).

A central issue that remains unsolved concerns the mechanism by which leucine stimulates hepatocyte DNA synthesis and proliferation. In exploring the signaling pathway(s) responsible for the leucine effect, we asked if inhibitors of growth-related signal-transducing elements (AG1478, wortmannin, PD98059, rapamycin, and U73122) have an effect on leucine-stimulated hepatocyte DNA synthesis and proliferation. We found that the receptor tyrosine kinase, PI3 kinase, MAP kinase, p70 S6 kinase, and phospholipase C pathways are involved in the leucine action (Fig. 3A and B).

The tyrosine kinase/MAP kinase pathway is known to be an important growth-related signaling pathway for hepatocyte proliferation and can be activated by several different growth factors, including the three most important hepatocyte mitogens, epidermal growth factor, TGF-α (Kimura and Ogihara, 1999) and hepatocyte growth factor (HGF) (Michalopoulos and DeFrances, 1997). In exploring the signaling pathway responsible for these effects, we investigated whether or not leucine directly stimulates MAP kinase activity in primary cultured hepatocytes. The results demonstrate that leucine directly triggers p42 MAP kinase activation, but not p44 MAP kinase activation, which is blocked by the MEK inhibitor PD98049 and by isoleucine (Fig. 4A and B). Leucine has been reported to stimulate target of rapamycin (Ijichi et al., 2003; Van Sluijters et al., 2000). Target of rapamycin appears to be directly responsible for at least some of the phosphorylation on the translational repressor 4E-BP1 in adipocytes (Lynch et al., 2000). In agreement to these reports, leucine induction of hepatocyte DNA synthesis and proliferation is sensitive to rapamycin (Fig. 3).

To determine the possible involvement of Ca^{2+} mobilization in leucine-induced DNA synthesis and proliferation, primary cultured hepatocytes were treated with a Ca^{2+} ionophore ionomycin or a Ca^{2+} channel blocker verapamil, and with somatostatin, which decreases intracellular Ca^{2+} levels. As shown in Fig. 3, verapamil and somatostatin inhibited the leucine-stimulated hepatocyte mitogenesis. In contrast, ionomycin significantly potentiated the leucine-induced hepatocyte DNA synthesis and proliferation, although ionomycin on its own did not have an effect. Thus, it seems possible that the leucine receptor/G-protein (and possibly the Gq-protein/phospholipase C/Ca^{2+} pathway) and the tyrosine kinase/MAP kinase signalling pathway are both involved in intracellular transduction of the signal that mediates the mitogenic effects of leucine. However, there is little evidence as yet that the leucine receptor pathway stimulates the tyrosine kinase/MAP kinase signaling pathway directly to induce hepatocyte mitogenesis. One likely possibility is that the leucine pathway stimulates secretion of a mitogen by primary hepatocytes in an autocrine manner, which, in turn, induces the hepatocyte DNA synthesis and proliferation via the MAP kinase pathway (Fig. 10).

To examine the possibility that TGF-α and/or IGF-I mediates leucine-induced DNA synthesis and proliferation of hepatocytes in primary cultures, we investigated the effects of neutralizing monoclonal antibodies against TGF-α and IGF-I. As shown in Fig. 5A and B, the addition of a neutralizing monoclonal antibody against TGF-α dose-dependently inhibited the growth-promoting effect of leucine (5×10^{-4} M) on DNA synthesis and proliferation. In contrast, a monoclonal antibody against IGF-I had no effect on the hepatocyte DNA synthesis and proliferation triggered by leucine (5×10^{-4} M). These results strongly support the idea that TGF-α mediates the growth-promoting actions of leucine, possibly through autocrine secretion by primary cultured hepatocytes. These data are consistent with the previous finding that leucine serves as a cytokine secretagogue under some conditions (Tomiya et al., 2002) and potentiates glucose-stimulated insulin secretion from pancreatic beta cells (Swenne, 1992; Xu et al., 1998).

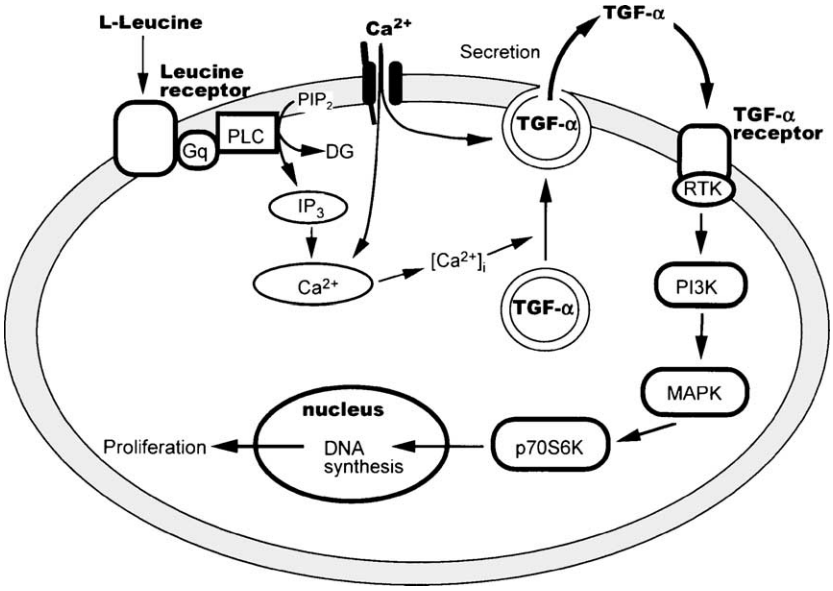

Fig. 10. A proposed mechanism for the leucine-induced hepatocyte DNA synthesis and proliferation. See text for description.

If the model is correct, then treatment of primary cultured hepatocytes with leucine should lead to increased secretion of TGF-α into the culture medium. Indeed, leucine does induce TGF-α in a dose-dependent manner, with an ED_{50} value of 8×10^{-5} M (Fig. 6). The value is about two times more responsive than that for hepatocyte DNA synthesis and proliferation. These findings suggest that leucine binds to a specific receptor on the hepatocyte membrane and triggers the signal transduction pathway leading to autocrine secretion of TGF-α. Consistent with this, previous work from our lab has shown that the growth-promoting actions of prostaglandin E_2 and some analogs on hepatocyte DNA synthesis and proliferation depend on autocrine secretion of TGF-α (Kimura et al., 2001b). Furthermore, Tomiya et al. (2002) demonstrated that, among branched-chain amino acids, only leucine has ability to stimulate the secretion of HGF by hepatic stellate cells.

To confirm links between the leucine-induced hepatocyte mitogenesis and TGF-α secretion, it is important to investigate the regulatory mechanisms associated with rapid secretion of TGF-α. TGF-α secretion ultimately depends on the intracellular concentration of Ca^{2+}, and thus elevation of intracellular free Ca^{2+} concentrations can occur in response to stimulation of phospholipase C by Ca^{2+}-mobilizing agents. In our model, we propose that the leucine receptor is linked to a G-protein (possibly Gq) and stimulates the activity of phospholipase C. Stimulation of phospholipase C triggers breakdown of phosphatidylinositol 4, 5, bisphosphate (PIP_2), resulting in cytoplasmic diacyl glycerol (DG) and phosphatidylinositol 1, 4, 5, trisphosphate (IP_3) (Berridge, 1993). These mediators trigger a cascade of other intracellular events, including activation of PKC and mobilization of Ca^{2+} from intracellular stores. Calcium ionophores mimic the effects of the messenger.

Since leucine-stimulated TGF-α secretion is specifically blocked by isoleucine, TGF-α secretion by hepatocytes may be regulated by the leucine receptor/Gq-protein/phospholipase C/Ca^{2+} pathway. This notion is supported by the facts that the stimulatory effects of leucine were blocked by the phospholipase C inhibitor U73122, a calcium channel blocker, verapamil, and somatostatin, and were potentiated by ionomycin (Fig. 3). Our data suggest that PKC plays only a minor role in leucine signaling, since the PKC inhibitor sphingosine is not effective in the leucine-induced TGF-α secretion and MAP kinase activation. Together, these results suggest that the leucine signal is mediated indirectly through TGF-α and the receptor tyrosine kinase/MAP kinase pathway. Notably, prostaglandin E_2 and other EP_1 receptor agonists have been reported to function via a similar mode of action, TGF-α release and subsequent receptor tyrosine kinase/MAP kinase phosphorylation in primary cultured hepatocytes (Kimura et al., 2001b).

Here, we have demonstrated for the first time that leucine rapidly stimulates TGF-α secretion into the conditioned medium (Fig. 6). We hoped to further determine if isoleucine and/or valine treatment can antagonize leucine-induced TGF-α secretion. Indeed, TGF-α secretion and subsequent hepatocyte DNA synthesis and proliferation are competitively antagonized by isoleucine, but not by valine, suggesting the existence of putative leucine receptor or recognition sites in primary cultured hepatocytes (Figs. 8 and 9). These data are consistent with the previous finding that in adipocytes, leucine primarily mediate phosphorylation of ribosomal protein S6 and the translational repressor, eIF-4E binding protein-1 via a putative L-leucine binding/recognition sites (Lynch et al., 2000).

Previous reports showed that plasma branched-chain amino acid levels are decreased in patients with liver

cirrhosis, owing to an increase in branched-chain amino acid tissue uptake and/or catabolism and a decrease in branched-chain amino acid production from proteins. Several attempts have been made to treat patients who have liver cirrhosis or hepatic encephalopathy with pure branched-chain amino acids (e.g., valine, leucine, and isoleucine) or with branched-chain amino acid-enriched formulas (Marchesini et al., 1990, 2000; Morgan, 1990). Branched-chain amino acids may provide an energy source to prevent endogenous catabolism, which contributes to plasma amino acid imbalance in liver cirrhosis and hepatic encephalopathy. However, the present study shows that different branched-chain amino acids are likely to have different effects. Whereas leucine has agonistic effects on hepatocyte DNA synthesis and proliferation, isoleucine competitively antagonizes those leucine effects. Therefore, branched-chain amino acid-enriched formulas should be carefully reconsidered. Hereafter, leucine might be used to promote regeneration and recovery of damaged hepatocytes, and to reverse acute hepatic failure in vivo.

In summary, this report provides the first biochemical evidence for the mitogenic effects of leucine and the transduction of a leucine-induced signal in primary cultures of adult rat hepatocytes. The results demonstrate that high physiologic concentrations of leucine, acting through autocrine secretion of TGF-α, plays a significant role in the initiation of hepatocyte DNA synthesis and proliferation. In addition, isoleucine, but not valine, is a competitive antagonist for leucine-stimulated hepatocyte mitogenesis. These observations support the notion that, like prostaglandin E_1, leucine is an indirect hepatocyte mitogen.

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