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N-(methylamino)isobutyric acid inhibits proliferation of CFSC-2C hepatic stellate cells

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Abstract

Activation of hepatic stellate cells (HSCs) involves the induction of ECM protein synthesis and rapid cell proliferation. Thus, agents that interfere with either process could potentially mitigate the development of liver disease by reducing the synthesis of proteins associated with fibrosis or by reducing the number of activated HSC. Previously, we described that the non-metabolizable amino acid analog *N*-(methylamino)isobutyric acid (MeAIB) reduced hepatic collagen content of rats in a model of CCl₄-induced liver injury, and in vitro studies using CFSC-2G cells indicated that MeAIB directly reduced collagen synthesis. However, the MeAIB-mediated reduction of hepatic collagen, in vivo, following liver injury was associated with a decrease in hepatic α -smooth muscle actin (α -SMA) which suggested that MeAIB also inhibited the activation of HSCs. Because HSC activation is inseparable from proliferation, the purpose of this study was to examine the effect of MeAIB treatment on the proliferation of HSCs in an in vitro model utilizing CFSC-2G cell cultures. In these studies, MeAIB effectively inhibited the proliferation of CFSC-2G cells by interfering with the progression of the cells through the G₁-phase of the cell cycle which delayed entry into S-phase. MeAIB prevented the phosphorylation of p70S6 kinase (p70S6K) at Thr389 and reduced the phosphorylation at Thr421/Ser424. Because p70S6K is required for G₁-cell cycle progression and is known to be regulated by nutrient availability, this correlates well with MeAIB interfering with the proliferation of CFSC-2G HSCs. In addition, the rate of protein synthesis was reduced by MeAIB treatment following mitogenic stimulation, which agrees with a p70S6K-mediated reduction in translation. These data are consistent with MeAIB inhibiting the proliferation of CFSC-2G cells by altering the mitogen activated pathway(s) leading to phosphorylation of p70S6K by a yet to be described mechanism.

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1. Introduction

Liver fibrosis is characterized by the abnormal production of extracellular matrix (ECM) components in response to a hepatic insult, such that excess quantities of collagens I, III, and IV are deposited in dense bands that distort the normal liver architecture. The preponderance of evidence indicates that hepatic stellate cells (HSC, lipocytes, fat-

storing or Ito cells) play a central role in this process and are a significant source of fibrillar and non-fibrillar matrix proteins. Before liver injury, HSCs are quiescent and produce very low levels of matrix proteins, but following a hepatic insult HSC proliferate rapidly while increasing the expression of α -smooth muscle actin (α -SMA) and procollagen I in a process referred to as HSC activation. In addition, HSC are known to be major sources of collagens and other matrix proteins that are deposited during the development of fibrosis. Therefore, the aberrant accumulation of matrix proteins occurs as a result of an increase in the number of HSC in addition to an increase in the biosynthesis and secretion of matrix proteins [1–4]. As a product of our investigations into the role of amino acid uptake and accumulation on physiological processes of the

Abbreviations: ECM, extracellular matrix; MeAIB, *N*-(methylamino)isobutyric acid; α -SMA, α -smooth muscle actin; p70S6K, p70S6 kinase; ALLN, *N*-acetyl-leu-leu-norleu-al; PSI, *N*-boc-ile-glu-(*O*-*t*-butyl)-ala-leucinal; MAPK, mitogen activated protein kinase

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cell, we have observed that inhibition of proline uptake by the non-metabolizable analog, *N*-(methylamino)isobutyric acid (MeAIB), has a profound inhibitory effect on the production of collagen in a rat model of liver injury [5]. In addition to reducing the hepatic collagen content, a decrease in the expression of α -SMA in liver 1 week following CCl_4 -induced liver injury indicated that MeAIB potentially interfered with the activation of HSC. Furthermore, previous studies by our laboratory indicate that MeAIB can inhibit the rapid proliferation of hepatic cells following partial hepatectomy [6]. Because HSC activation is closely coupled to HSC proliferation [1], MeAIB treatment could have the additional property of interfering with HSC activation, provided the anti-proliferative properties of MeAIB extend to HSC.

We investigated the influence of MeAIB on the proliferation of the CFSC-2G HSC line to determine if MeAIB treatment potentially inhibits the activation process in a well established in vitro HSC model [7,8]. We found that MeAIB inhibits movement of CFSC-2G cells into the S-phase of the cell cycle and that interference with G_1 -cell cycle progression is where MeAIB exerts an anti-proliferative effect. Despite no change in the phosphorylation of mitogen activated protein kinase (MAPK, ERK1/2), down regulation in the phosphorylation of p70S6 kinase (p70S6K) was observed with MeAIB treatment which correlates with the inhibition of G_1 -cell cycle progression. Additionally, the functional implication of hypo-phosphorylation of p70S6K was evaluated by measuring protein synthesis rate following mitogenic stimulation of CFSC-2G cells. The protein synthesis rate was reduced in a dose dependent manner by MeAIB while protein degradation was not affected. These data have led us to conclude that MeAIB inhibits the proliferation of CFSC-2G cells which suggests that MeAIB may inhibit the activation of HSC in vivo following hepatic damage or insult.

2. Experimental procedures

2.1. Cell culture

The cell line designated CFSC-2G [7,8] was derived from rat liver, has a phenotype similar to that of early passage primary HSC and was kindly provided by Dr. M. Rojkind (Albert Einstein College of Medicine, NY). CFSC-2G cells were routinely cultured in standard MEM formulated by combining MEM (Sigma, St. Louis, MO, Cat No. M0268) with $1 \times$ non-essential amino acids (Sigma, Cat No. M-7145), 10 mM HEPES (pH 7.4), 10,000 units penicillin with 10 mg streptomycin per liter and 10% FBS (Gibco-BRL, Grand Island, NY). Incubation conditions were at 37 °C with 5% CO_2 and 95% air. Cells were passaged weekly by dissociation in Ca^{2+} -free Hank's salt solution containing 0.25% trypsin and 0.5 mM EDTA

followed by plating a 1:4 dilution of cells onto plastic in standard MEM containing 10% FBS. The media were changed biweekly.

2.2. Proliferation assay

The entry of serum-starved CFSC-2G cells into the S-phase of the cell cycle was monitored by incorporation of ^3H -thymidine into DNA following mitogenic stimulation with 10% FBS. Cells were dissociated with 0.1% trypsin/1 mM EDTA in Hank's and were plated at 5000 cells per well in 24 well tissue culture dishes in MEM containing 10% FBS. After 24 h of attachment and recovery, the medium was aspirated and replaced with serum-free MEM. Serum-free conditions were maintained for 72 h with the medium changed once after 48 h which resulted in a sub-confluent cell density of 40–50%. Mitogenic stimulation of serum-starved 2G cells was initiated by replacement of serum-free media with MEM containing 10% FBS. DNA synthesis was measured by aspiration of the medium and replacement with 0.25 ml of KRB containing 1 mM MgSO_4 , 0.01 mM CaCl_2 and 10 μCi ^3H -thymidine/ml for 60 min. Incorporation of label into DNA was measured by aspiration of the supernatant, solubilization of the cells in 0.5 ml of 0.1N NaOH/0.1% SDS and precipitation of the macromolecules with 5% TCA with the inclusion of 100 μg herring DNA as a carrier. ^3H -thymidine incorporation into TCA insoluble material was quantified by scintillation spectrophotometry and DPM normalized to protein content.

2.3. Cell cycle analysis by flow cytometry

The movement of cells through the cell cycle was measured by quantifying the nucleic acid content by flow cytometry [9]. Approximately 25,000 CFSC-2G cells were plated onto 60 mm plastic dishes in MEM containing 10% FBS and incubated for 24 h at 37 °C. The medium was aspirated and replaced with serum-free MEM. Serum-free conditions were maintained for 72 h with the media changed once after 48 h which resulted in a sub-confluent cell density of 40–50%. Mitogenic stimulation of serum-starved 2G cells was initiated by replacement of serum-free media with MEM containing 10% FBS. Cells were removed from the plates with Ca^{2+} -free Hank's basal salt solution containing 0.25% trypsin and 0.5 mM EDTA and collected by centrifugation at $600 \times g$ for 15 min. Cells were fixed at 4 °C in 0.1% freshly made paraformaldehyde in PBS, pH 7.4, for 30 min. Cells were then washed twice in PBS before addition of propidium iodide (PI) reagent (100 mM NaCl, 10 mM Tris, 10 mg/l RNAse A, 1 ml/l Nonidet P-40 and 75 mM propidium iodide, pH 8). Cells were incubated at room temperature for 2 h in PI reagent before analysis by flow cytometry using a Beckman FACS Vantage at the University of Nebraska Flow Cytometry Core Facility.

2.4. Measurement of protein synthesis rate

CFSC-2G cells were cultured in 24 well plates and serum-starved as described in the proliferation assays above. The rate at which proteins were synthesized was measured by pulse label experiments following stimulation with MEM containing 10% FBS and 0 or 5 mM MeAIB. At the times indicated in the experiments, the media were aspirated and replaced with identical media containing 20 $\mu\text{Ci/ml}$ ^3H -leucine for 60 min at 37 °C. The media were aspirated and the cells solubilized in 1 ml of 0.1% SDS containing 0.1 M NaOH. Half of the cell lysate was combined with 100 μg carrier protein (BSA) and precipitated with 10% TCA while the protein content of the remainder was determined by the method of Lowery. Precipitated protein was collected by filtration under vacuum over glass fiber disks followed by two washes of ice-cold 5% TCA and two washes of acetone. Radioactivity in macromolecules was measured by scintillation counting and normalized to protein content.

2.5. Determination of protein degradation rate

Protein degradation was measured by the efflux of radioactivity into the medium from cells pre-labeled with ^3H -leucine. CFSC-2G cells were plated at 5000 cells per well in 24 well tissue culture dishes in MEM containing 10% FBS. After 24 h of attachment and recovery, the medium was aspirated and replaced with serum-free MEM. After 48 h, the medium was replaced with 1 ml of identical serum-free medium containing 10 μCi ^3H -leucine, and the cells incubated an additional 24 h to provide for the incorporation of label into proteins. Unincorporated ^3H -leucine was removed from the cells by washing with three changes of serum-free MEM for 20 min. The cells were then stimulated to proliferate by changing the medium to MEM containing 10% FBS with 0 or 5 mM MeAIB. At the times indicated in the individual experiments, the media were aspirated and the macromolecules precipitated by the addition of TCA to 10%. The precipitated material was removed by centrifugation, 10,000 $\times g$ for 10 min, and the radioactivity in the TCA soluble fraction determined by scintillation counting. When indicated, inhibitors were added 2 h before the stimulation of serum-starved cells with medium containing 10% FBS.

2.6. Western blotting

Phospho-specific antibodies for p70S6 kinase, Ser 389 or Thr 421/Ser 424, were used to evaluate the phosphorylation of p70S6 kinase by standard Western blotting (New England BioLabs, Beverly, MA). CFSC-2G cells were cultured exactly as described for the proliferation assay above. After stimulation with 10% FBS, the cells were harvested at defined times by aspirating the medium

and solubilizing in a buffer containing 1% SDS, 0.1 mM EDTA and 10 mM Tris (pH 8) followed by incubation at 80 °C for 20 min. Insoluble material was removed by centrifugation (10 min at 10,000 $\times g$) and protein content determined by Lowry assay. Proteins (100 μg) were separated by denaturing SDS-PAGE and transferred to nitrocellulose by electro-blotting. Nitrocellulose was blocked with 2% BSA in TBST (150 mM NaCl, 10 mM Tris, 0.02% Tween 20, pH 7.5) for 2 h followed by incubation with the indicated antibody at 4 °C for 16–18 h. Visualization of primary antibody was with HRP-conjugated anti-rabbit IgG and autoradiography (ECL, Amersham, England).

2.7. Statistical analysis

Each data point represents the mean \pm standard deviation of a minimum of three determinations or the number of replicates (n -value) indicated in the individual experiments. Significance was evaluated utilizing a Student's t -test with $P < 0.01$ set as statistically significant.

3. Results

3.1. Stellate cell proliferation is inhibited by MeAIB

The proliferation of CFSC-2G cells was evaluated by pulse labeling DNA with ^3H -thymidine. As illustrated in Fig. 1, the stimulation of serum-starved CFSC-2G cells with 10% FBS resulted in the proliferation of the cells with DNA synthesis detectable after 10 h and with maximal synthesis of DNA occurring at 20 h post stimulation. The

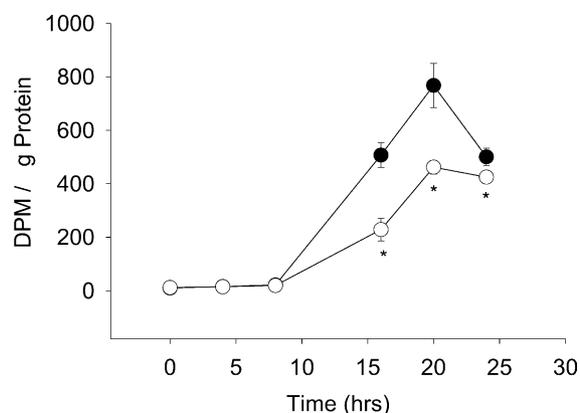


Fig. 1. Inhibition of CFSC-2G proliferation by MeAIB treatment. CFSC-2G cells were plated and serum-starved as described in Section 2. The cells were subjected to mitogenic stimulation by the replacement of serum-free medium with identical medium containing 10% FBS at time 0. MeAIB was included in the medium at 0 (●) or 5 mM (○). The DNA synthesis rate was measured at 0, 4, 8, 16, 20, and 24 h by pulse labeling with ^3H -thymidine as described in Section 2. Data represent the mean \pm S.D. of three measurements ($n = 3$) and are representative of three independent experiments. Asterisks indicate a significant difference at the $P < 0.01$ level.

rate of synthesis was reduced at 24 h. These data are consistent with a synchronous movement of cells from a quiescence state, presumably in the G_{0/1}-phase of the cell cycle, into the S-phase of the cell cycle and finally out of S-phase. The addition of 5 mM MeAIB to the medium significantly reduced the incorporation of thymidine into DNA by 55 ± 9%, 39 ± 11%, and 15 ± 6% at 16, 20, and 24 h following stimulation, respectively. These observations indicate that MeAIB inhibits the proliferation of CFSC-2G cells and suggests that the entry of the cells into the S-phase of the cell cycle is inhibited.

3.2. Cell proliferation is blocked in the G_{0/1}-phase

Flow cytometry of cellular DNA content was utilized for the evaluation of the effect of MeAIB on the entry of CFSC-2G cells into the cell cycle. Following serum starvation, the preponderance of CFSC-2G cells (87.6%) contained a 2N amount of DNA indicating that the cells were effectively arrested in the G_{0/1}-phase of the cell cycle as seen in Fig. 2A. Stimulation of the cells with 10% FBS resulted in the progression of cells through the G_{0/1}-phase, into the S-phase of the cell cycle (Fig. 2B) and eventually to the G₂-phase (Fig. 2D). MeAIB reduced the number of cells entering the S-phase of the cell cycle by almost 40% as seen in Fig. 2C, which reduced the number of cells reaching the G₂-phase by 58% as seen in Fig. 2E. However, MeAIB treatment did not completely block the entry of CFSC-2G cell into S-phase (Fig. 2E). These data confirm the findings obtained by ³H-thymidine incorporation and verify that MeAIB interferes with the entry of CFSC-2G cells into the S-phase of the cell cycle. These data are consistent with MeAIB-mediated interference with G₁-cell cycle progression which delays the entry of CFSC-2G cells into S-phase and subsequently reduces the number of cells reaching the G₂-phase.

3.3. Phosphorylation of p70S6K is inhibited by MeAIB

Because p70S6K is known to be regulated by amino acids and is required for G₁-progression, the phosphorylation of key regulatory residues of p70S6K was investigated by using phospho-specific antibodies. MeAIB treatment completely abrogated the phosphorylation of p70S6K at Thr389, while the timing of p70S6K phosphorylation at Thr421/Ser424 was altered as seen in Fig. 3. p70S6K demonstrated detectable phosphorylation of Thr389 following stimulation with FBS at 2, 4, and 8 h, while the inclusion of 5 mM MeAIB resulted in no detectable phospho-Thr389. By comparison, phospho-Thr421/Ser424 was detected at 2, 4, 8, and 24 h following stimulation, but inclusion of 5 mM MeAIB resulted in detection of phospho-Thr421/Ser424 at 2 and 4 h only. Because the phosphorylation of MAPK (ERK1/2) was not affected by treatment with MeAIB (Fig. 3), the inhibition was reasonably specific for p70S6K and shows that MeAIB treatment

does not produce a general down regulation of all mitogen activated protein kinase activities.

3.4. MeAIB inhibits protein synthesis but not catabolism

Activated p70S6K results in the phosphorylation of the ribosomal S6 subunit which is thought to up regulate the translation of a family of mRNAs containing a 5' poly-pyrimidine tract and is required for G₁-cell cycle progression [10–12]. Since MeAIB inhibits the phosphorylation of p70S6K following stimulation of CFSC-2G cells, the functional implication of reduced activation was evaluated by measuring protein synthesis rates. The incorporation of ³H-leucine into macromolecules was used as a measure of protein synthesis rates which were increased from baseline when CFSC-2G cells were exposed to medium containing 10% FBS. The addition of 0.5 to 5 mM MeAIB reduced the incorporation of ³H-leucine into proteins in a concentration dependent manner as illustrated in Fig. 4 which increased with time after mitogenic stimulation. Addition of MeAIB before the addition of FBS did not change the baseline (time 0) protein synthesis rates indicating that only proliferating cells were affected by MeAIB treatment. MeAIB-mediated inhibition of increased protein synthesis following mitogenic stimulation correlates well with the observed decrease in p70S6K phosphorylation following MeAIB treatment.

As a control, the rate of protein degradation by CFSC-2G cells was also evaluated by measuring the efflux of radioactivity from cellular proteins previously labeled with ³H-leucine. Addition of 5 mM MeAIB to the media during the mitogenic stimulation of serum-starved CFSC-2G cells did not alter the efflux of radioactivity into the medium as seen in Fig. 5. Increasing the leucine concentration in the medium to 2 mM produced representative results (data not shown). Some experiments included 10 mM NH₄Cl, 10 μM *N*-acetyl-leu-leu-norleu-al (ALLN, Sigma, St. Louis, MO) or *N*-boc-ile-glu-(*O*-*t*-butyl)-ala-leucinal (PSI, Peptides International, Louisville, KY) to inhibit protein degradation by lysosome, Ca²⁺-activated proteasome and proteasome activities, respectively. As illustrated in Fig. 5, the relative strength of the inhibitors at reducing the release of radioactivity from proteins was PSI >> ALLN > NH₄Cl. This suggests that the proteasome plays a larger role in protein catabolism relative to the lysosome in CFSC-2G HSCs, and that the protein synthesis rate measurements were not confounded by alterations in the rate of protein catabolism.

4. Discussion

MeAIB inhibits the uptake of amino acids that gain entry into the cell via the "System A" amino acid transporter by a competitive mechanism [13]; therefore, the effect of

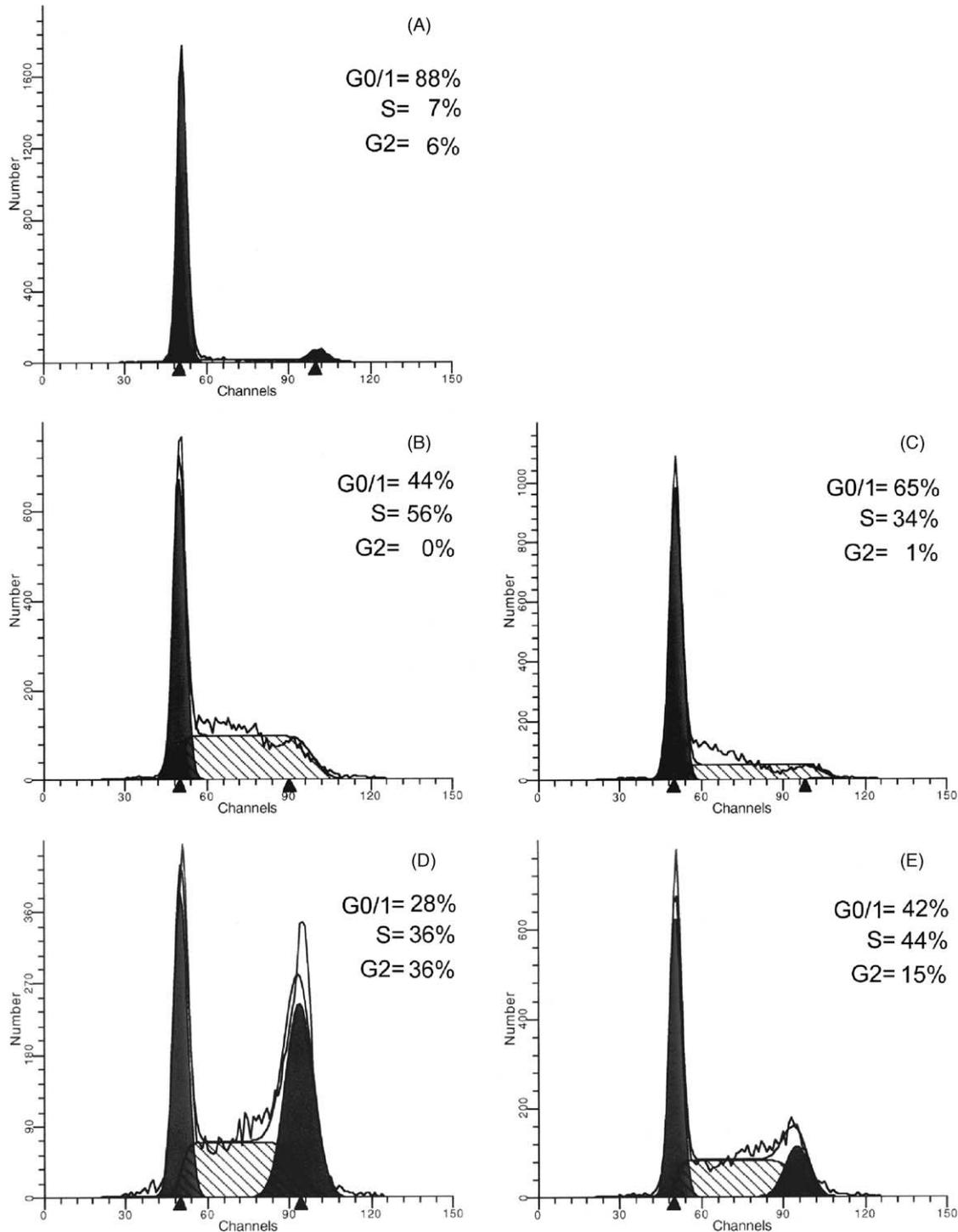


Fig. 2. Cell-cycle analysis of CFSC-2G cells. CFSC-2G cells were plated in pairs and serum-starved as described in Section 2. Cells were stimulated to proliferate by the supplementation of the media with FBS to 10% at time 0. Cells were harvested and analyzed by FACS at 0 (panel A), 16 (panels B and C) or 24 h (panels D and E) as described in Section 2. MeAIB was included at 0 mM (panels A, B, and D) or 5 mM (panels C and E) at the time of mitogenic stimulation. Data are composed of approximately 2×10^4 measurements and are representative of three independent experiments.

MeAIB treatment is a reduction in the uptake and intracellular concentration of System A substrate amino acids such as alanine, glycine and proline. Because the high affinity, active transport of proline in most cell types is largely due to the System A transporter activity, the effect of MeAIB treatment on proline uptake and subsequent

intracellular concentration can be quite significant. For example, MeAIB can essentially eliminate the active concentration of proline in CFSC-2G cells without effecting the intercellular concentration of leucine [5]. While the effect of MeAIB on the cellular concentration of other substrate amino acids has not been evaluated in CFSC-2G

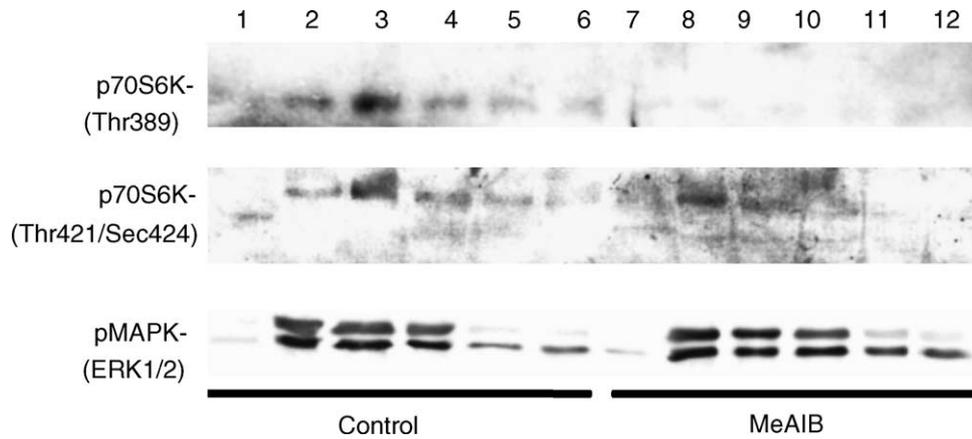


Fig. 3. Phosphorylation of p70S6K and MAPK by CFSC-2G HSCs. The phosphorylation of p70S6K (Thr389 or Thr421/424) and MAPK (ERK1/2) was evaluated by Western blot utilizing phospho-specific antibodies as described in Section 2. Serum-starved cells were stimulated with medium containing 10% FBS and 0 mM (lanes 1–6) or 5 mM MeAIB (lanes 7–12). Cells were harvested 0 (lanes 1 and 7), 2 (lanes 2 and 8), 4 (lanes 3 and 9), 8 (lanes 4 and 10), 24 (lanes 5 and 11) and 36 h (lanes 6 and 12) following mitogenic stimulation.

cells, we hypothesize that a MeAIB-mediated reduction in the intercellular concentration of specific amino acids would trigger the cell's regulatory machinery that is responsible for evaluating adequate nutritional status prior to cell proliferation.

The MeAIB-mediated down regulation of p70S6K phosphorylation state, following mitogenic stimulation of CFSC-2G cells, supports the idea that nutritional parameters can regulate cell proliferation. The p70S6K is a well known regulatory enzyme that is critical for cell cycle progression through G_1 [14] and is known to be modulated by amino acid availability [15–17]. While leucine has been reported as the most effective modulator of p70S6K phosphorylation [16–18], our data indicate the non-metabolizable amino acid analog, MeAIB, is an efficient modulator of p70S6K as well. Because the phosphorylation of p70S6K at Thr389 is critical for kinase function and most

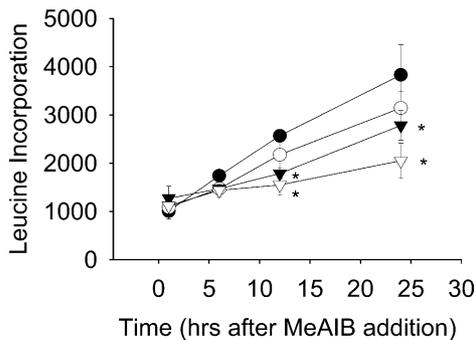


Fig. 4. Effect of MeAIB on incorporation of leucine into proteins of proliferating cells. CFSC-2G cells were plated and serum-starved as described in Section 2. Cells were stimulated to proliferate by the supplementation of the media with FBS to 10% at time 0. MeAIB was included in the medium at 0 (●), 0.5 (○), 2.5 (▼) or 5 mM (▽) and protein synthesis rate determined by pulse labeling with ^3H -leucine. Data are the mean \pm S.D. of four measurements ($n = 4$) and are representative of three independent experiments. Asterisks indicate a significant difference at the $P < 0.01$ level.

closely correlates with p70S6K kinase activity in vivo [11,12], MeAIB-mediated inhibition of this site provides a foundation on which future studies can be based. For example, the phosphorylation of Thr229 by phosphoinositide-dependent protein kinase (PDK1) is required prior to phosphorylation of the Ser/Thr-Pro sites in the autoinhibitory domain and Thr389 in the linker domain [19] which leads us to a testable hypothesis that MeAIB may act by

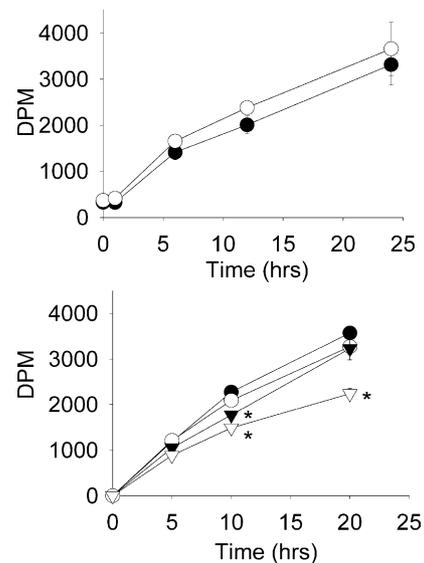


Fig. 5. Effect MeAIB on protein catabolism. CFSC-2G cells were plated, pre-labeled with ^3H -leucine, serum-starved and washed as described in Section 2. Cells were stimulated to proliferate by the supplementation of the media with FBS to 10% at time 0. In the upper panel, MeAIB was included in the medium at 0 (●) or 5 mM (○) and in the lower panel the treatments were control (●), 10 mM NH_4Cl (○), 10 μM *N*-acetyl-leu-norleu-al (▼) or 10 μM *N*-boc-ile-glu-(*O*-*t*-butyl)-ala-leucinal (▽). The release of acid soluble radioactivity was determined as described in Section 2. Data are the mean \pm S.D. of four measurements ($n = 4$) and are representative of three independent experiments. Asterisks indicate a significant difference at the $P < 0.01$ level.

interfering with the mitogen activated pathways responsible for the activation of PDK1, such as reduced PI3 kinase activity. Alternatively, MeAIB could inhibit the phosphorylation of Thr389 by interfering with the activation of a yet to be described kinase responsible for the direct phosphorylation of this site. Unfortunately, the reduced phosphorylation at Thr421/Ser424 following MeAIB treatment is complicated due to the influence of previously phosphorylated residues modifying subsequent phosphorylation of other sites, but the growth factor activation of MAPK can be eliminated because MeAIB did not alter the phosphorylation of ERK1/2. These studies provide a framework for the logical determination of the mechanism by which MeAIB exerts an anti-proliferative effect on CFSC-2G HSCs, and the G₁ blockade of cell cycle progression by MeAIB is consistent with down regulation of p70S6K phosphorylation.

Interestingly, rapamycin, an inhibitor of p70S6K phosphorylation, has been reported to inhibit stellate cell proliferation and limits fibrogenesis in a rat model of liver fibrosis (Zhu et al. 1999) which suggests that reduced activation of p70S6K is a mechanism rapamycin shares with MeAIB. Rapamycin inhibits phosphorylation of p70S6K by interaction with the target of rapamycin (mTOR, FRAP). mTOR is a member of the ATM-related kinase family and is known to be activated by the insulin receptor signaling cascade, PI3 kinase and protein kinase B (PKB, Akt) [20–22]. Also, mTOR activation is distinct from the Ras/MAP kinase cascade components [11], and mTOR can be activated independently of insulin by amino acids through a nutrient signaling pathway [23,24]. Given the similarity between the effect of rapamycin and MeAIB on the deposition of collagen following experimental liver injury and the shared inhibition of p70S6K, the role of p70S6K activation in abnormal HSC function is emphasized.

In conclusion, we report that MeAIB inhibits the proliferation of CFSC-2G cells by retarding the movement of the cells into the S-phase of the cell cycle. The MeAIB-mediated interference in proliferation was accompanied by an alteration in P70S6K phosphorylation and by a reduction in the rate of protein synthesis following mitogenic stimulation. Protein catabolism and the phosphorylation of MAPK were not effected by MeAIB treatment. These observations are consistent with MeAIB treatment producing an anti-proliferative response sometime in the G₀/G₁- to S-phase transition of the cell cycle.

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