



Leucine and its transporter provide protection against cigarette smoke-induced cell death: A potential therapy for emphysema



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ABSTRACT

Cigarette smoke (CS) is a major risk factor for emphysematous changes in the lungs and the underlying mechanism involves CS-induced cell death. In the present study we investigated the ability of nutrients to rescue CS-induced cell death. We observed that pre-treatment with excess leucine can partially rescue CS extract-induced cell death in *Saccharomyces cerevisiae* and alveolar epithelial A549 cells. Excess dietary leucine was also effective in alleviating effects of CS in guinea pig lungs. Further investigation to understand the underlying mechanism showed that CS exposure causes downregulation of leucine transporter that results in inactivation of mTOR, which is a positive regulator of protein synthesis and cell proliferation. Notably, leucine supplemented diet ameliorated even existing CS-induced emphysematous changes in guinea pig lung, a condition hitherto thought to be irreversible. Thus the current study documents a new mechanism by which CS affects cellular physiology wherein leucine transporter is a key target.

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Abbreviations: CS, cigarette smoke; CSE, cigarette smoke extract; ROS, reactive oxygen species; mTOR, mammalian target of rapamycin; H & E, hematoxylin and eosin; ChIP, chromatin immune precipitation; Mad1, Max dimerization protein 1; Lat1, L-type amino acid transporter 1; YCM, yeast complete media; Bap2, branched-chain amino acid permease; Tat1, tyrosine and tryptophan amino acid transporter 1; PCR, polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; EtBr, ethidium bromide; V, valine; L, leucine; I, isoleucine; S, serine; K, lysine; Y, tyrosine; W, tryptophan; T, threonine; F, phenylalanine; H, histidine; M, methionine; R, arginine; N, asparagine; E, glutamic acid.

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1. Introduction

Emphysema, a major cause of morbidity and mortality, is a type of inflammatory disease defined by abnormal and permanent enlargement of the airspaces of alveolar cells. A primary risk factor for this disease is cigarette smoking [1,2]. An underlying reason for the harmful nature of cigarette smoke (CS) is that it is a very rich source of oxidants. It contains more than 10^{15} free radicals and ~4700 reactive chemical compounds [3]. In addition, CS induces the generation of reactive oxygen species (ROS) within cells, which cause oxidative stress [4]. Oxidative stress in turn leads to inflammation and the resulting apoptosis contributes to alveolar epithelial cell loss leading to emphysema [4,5]. Consistently, studies have documented the presence of apoptotic cells in the lungs of emphysema

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patients [5,6]. In addition to apoptosis, a number of studies also document autophagic cell death in the lungs of both emphysema patients and mouse exposed to CS [7,8]. The process of autophagy is highly regulated and nutritional deprivation is one of the major signals for its induction [9,10]. Thus CS-induced cell death is a complex process that involves multiple pathways and the underlying regulatory mechanisms are yet to be fully understood.

There is a relationship between stress response and nutritional status as better nutrient availability has been shown to combat the effects of stress [11]. Therefore, the current study investigated whether nutrient availability modulates cellular response to CS. Using both *in vitro* and *in vivo* approaches, we present evidence that increased availability of leucine, an essential amino acid, provides protection against CS-induced cell death. We have also shown that CS exposure causes downregulation of LAT1, a ubiquitously expressed gene encoding a high affinity permease for branched chain amino acids like leucine [12]. Additionally, ectopic expression of the Lat1 protein could partially prevent the CS-induced cell death. This protective function of leucine and Lat1 is mediated by mammalian target of rapamycin (mTOR), which is a Ser/Thr kinase that plays a crucial role in cell growth and proliferation by globally regulating transcription and translation [13]. Interestingly we also observed that dietary leucine supplement reverses pre-existing emphysematous changes of guinea pig lung.

2. Materials and methods

2.1. Preparation of cigarette smoke extracts (CSE)

CSE was prepared using a commercially available filter-tipped cigarette with a tar content of 15 mg and nicotine content of 1 mg manufactured by the Indian Tobacco Company Limited (ITC Ltd.) as described previously [14,15]. The smoke from a cigarette was bubbled through 1 ml of 50 mM PBS, pH 7.4, till the cigarette was consumed completely. The pH of this aqueous CSE was adjusted to 7.4 using NaOH and was filter sterilized by passing the extract through 0.2 µm syringe filter. Different batches of CSE preparations were normalized by measuring their absorbance at 270 nm. The OD₂₇₀ of 0.6 was considered as 100% CSE. This 100% stock solution was diluted to 3% or 20% as per the requirement of the experiment.

2.2. Yeast strain, media, CSE treatment

Saccharomyces cerevisiae strain 301-2B (*MATα Δleu2-3,112 ura3-52 his4Δ34 trp*) [16] was used and unless mentioned, yeast cells were grown in standard synthetic complete medium (YCM) that contains 2% glucose and 0.1% amino acid mixture (containing arginine – 0.9 g, methionine – 0.9 g, tyrosine – 1.35 g, isoleucine – 1.35 g, phenylalanine – 1.35 g, aspartic acid – 4.5 g, glutamic acid – 4.5 g, valine – 4.5 g, threonine – 4.5 g and serine – 4.5 g) along with 0.01% leucine and 0.005% lysine.

Exponentially growing *S. cerevisiae* cells were treated with different doses of CSE for different time periods. Cells were grown in YCM supplemented with either excess of

glucose or excess amino acids followed by CSE treatment and spotted on YCM plates.

Gene knockout yeast strains were made using kanamycin cassette as described previously [17]. Oligonucleotides used to generate the deletion cassettes are listed in Supplementary Table 1.

2.3. Cell culture, CSE treatment and transfection

Alveolar epithelial A549 cells were used for *in vitro* experiments wherein Ham's F12-nutrient mixture was used for culturing the cells. Cells were treated with different concentrations of CSE for different time periods as required. For experiments requiring excess leucine, cells were treated with different concentrations of leucine for 16 h prior to CSE treatment. Transient transfections were performed using PolyFect reagent (Qiagen). To investigate the effect of rapamycin (Sigma Aldrich), cells were treated with 100 nM rapamycin 2 h prior to CSE treatment and then MTT assay was performed after 6 h of CSE treatment.

2.4. siRNA construct

siRNA sequences targeted against LAT1 and MAD1 were designed using siDESIGN software (Thermo Scientific) and cloned into pSuper Retro Puro vector (Oligo-Engine). The primers used are listed in Supplementary Table 1

2.5. MTT assay

Cellular viability was determined by MTT-assay as described previously [18]. Briefly A549 cells were transfected with LAT1 expression construct (phLAT1) or siLAT1. Twenty four hour post-transfection, transfectants were either treated or left untreated with CSE for different periods as indicated in the figure and then MTT assay was performed. Similarly, in order to study the effect of excess leucine on CSE-induced cell death, A549 cells were grown for 16 h in presence of 0.8 mM leucine and then treated with CSE for different time periods. After CSE treatment MTT assay was performed.

In another experiment MTT assay was performed to investigate the effect of mTOR inhibitor rapamycin. A549 cells were either transfected with LAT1 construct or with empty vector (pcDNA3.1). After 24 h of transfection, cells were treated with 100 nM rapamycin for 2 h prior to 3% CSE treatment. After the CSE treatment for 6 h MTT assay was performed.

2.6. Real time PCR

Total RNA from differentially treated yeast and A549 cells was extracted using TRIzol[®] reagent (Invitrogen). First strand cDNA was synthesized using 3 µg of RNA from each sample and MMLV reverse transcriptase (Fermentas). The synthesized cDNA was used for subsequent qPCR analysis. The sequences of primers used were listed in Supplementary Table 1. GAPDH was used as control for LAT1 and gene for actin was used as control for yeast genes *TAT1*, *BAP2* and *TAT2*.

2.7. ChIP assay

A549 cells were treated with 3% CSE for 4 h and thereafter ChIP was performed [19]. The DNA-protein complex was immunoprecipitated using anti-Mad1 antibody (Santa Cruz sc:222). Nonspecific anti-rabbit IgG was used as control. The DNA obtained was analyzed by PCR using specific primers (Supplementary Table 1) designed to target the E-box at LAT1 promoter as indicated in the figure.

2.8. Exposure of guinea pigs to CS

Two to three month old male guinea pigs (250–350 g) were used for all the experiments. Animal care procedures were as per NIH (National Institutes of Health) guidelines and were approved by the Institutional Animal Ethics Committee. Guinea pigs were fed a vitamin C-free diet for 7 days to minimize the vitamin C level in the plasma and tissues as vitamin C is a potential inhibitor of CS-induced oxidative stress [20,21]. Each animal was exposed to 3 cigarettes per day; 2 puffs per cigarette with 1 min rest in smoke-free atmosphere between each puff to allow the animals to breathe air. The duration of exposure was 1 min for each puff. In general each puff produces 2.3 mg suspended particle. During the course of experiment, the guinea pigs were fed with either 1 mg vitamin C/day or 1 mg vitamin C/day and a leucine supplement (48 mg/kg/day) for 7 days. This diet plan was continued for the next 14 days along with CS exposure (three cigarettes/animal/day with two puffs/cigarette). Control guinea pigs were not exposed to CS.

In a separate experiment, guinea pigs were first exposed to CS for 14 days as mentioned above. Thereafter the smoke treatment was ceased and animals were fed normal diet along with either vitamin C (1 mg/day) or leucine supplement (48 mg/kg/day) or both leucine and vitamin C for next 10 days. For experiments involving rapamycin treatment, animals were fed with rapamycin (0.8 mg/kg/day) along with leucine (48 mg/kg/day) after an exposure to CS for 14 days.

Six animals were used in each group for all the experiments. At the end of each experiment animals were sacrificed and lungs were fixed by inflation with 10% buffered formalin. Lung tissue sections were stained with hematoxylin and eosin (H & E) for histological analysis. Morphometric analysis of the lung tissue sections was expressed by mean linear intercept (Lm) [22].

2.9. Histology and TUNEL assay

For histology, fixed tissues were paraffin-embedded and serially sectioned at 5 μ m. Sections were stained with H & E or ethidium bromide (EtBr) (4 μ g/ml) as per requirement and viewed under microscope using 10 \times objective (Olympus IX71).

The TUNEL assay was carried out on formaldehyde fixed lung sections by fluorescein-dUTP labeling using *In Situ* Cell Death Detection Kit (Roche) according to manufacturer's protocol. In brief, sections were deparaffinized with xylene and incubated with the labeling reagent for 30 min at room temperature. After the incubation, sections were washed

with PBS and viewed under fluorescence microscope using 10 \times objective. The percentages of TUNEL and EtBr positive cells were determined using Image-Pro 6.3 software (Media Cybernetics).

2.10. Western blotting

Western blotting was performed according to standard procedure with loading of 30 μ g protein for each sample. Primary antibodies were against mTOR (Cell Signaling Technology 2983S), p-mTOR (Cell Signaling Technology 5536S), p70 S6 kinase (Santa Cruz sc-230), p-p70 S6 kinase (Santa Cruz sc-7984-R), Lat1 (Gene Tex GTX62591), Mad1 (Santa Cruz sc:222) and tubulin (Santa Cruz sc-23948). Densitometric quantification of the western blots was done by ImageLab software (Bio-Rad).

2.11. Statistical analyses

Data are represented as mean \pm SD. For each experiment six animals were used per group. Statistical significance between the groups was evaluated by One Way Analysis of Variance (ANOVA) and Tukey's posthoc test.

3. Results

3.1. Excess leucine in the medium protects yeast cells against CSE-induced cell death

To investigate the connection between nutrient availability and CSE-induced stress response, we initially used *S. cerevisiae* as a model system. We chose this system for the following reasons: (i) cellular response pathways to many stress factors are known to be conserved between yeast and mammalian cells; (ii) yeast cells grow faster and thus use of this system expedites the screening process; and (iii) while culture of mammalian cells needs serum, a complex media component, yeast cells can be grown in synthetic media where the precise composition allows manipulation of media components. The last factor is especially important in the context of the present study, which is dependent upon modulation of nutrient composition. The fact that yeast cells are known to respond to CSE [23] and are more amenable to genetic analyses compared to cultured mammalian cells provides additional justification of using this system. We have standardized the dose and duration of CSE treatment sufficient to cause lethality of yeast cells. The minimum concentration of CSE that could completely inhibit cell growth was 20% and required an exposure time of 4 h (Supplementary Fig. 1). Hence all subsequent experiments with yeast were performed using this condition. To determine if nutrient availability could rescue the growth defect caused by CSE treatment, cells were grown in medium containing two-fold excess of either glucose or amino acids. While both nutrients protected cells against CSE-induced death, cells grown in excess amino acids were better protected compared to those grown in excess glucose (Fig. 1A). Next we examined the effect of individual amino acids and observed that only two branched chain amino acids, leucine and isoleucine, were able to protect cells from CSE-induced death with leucine being more

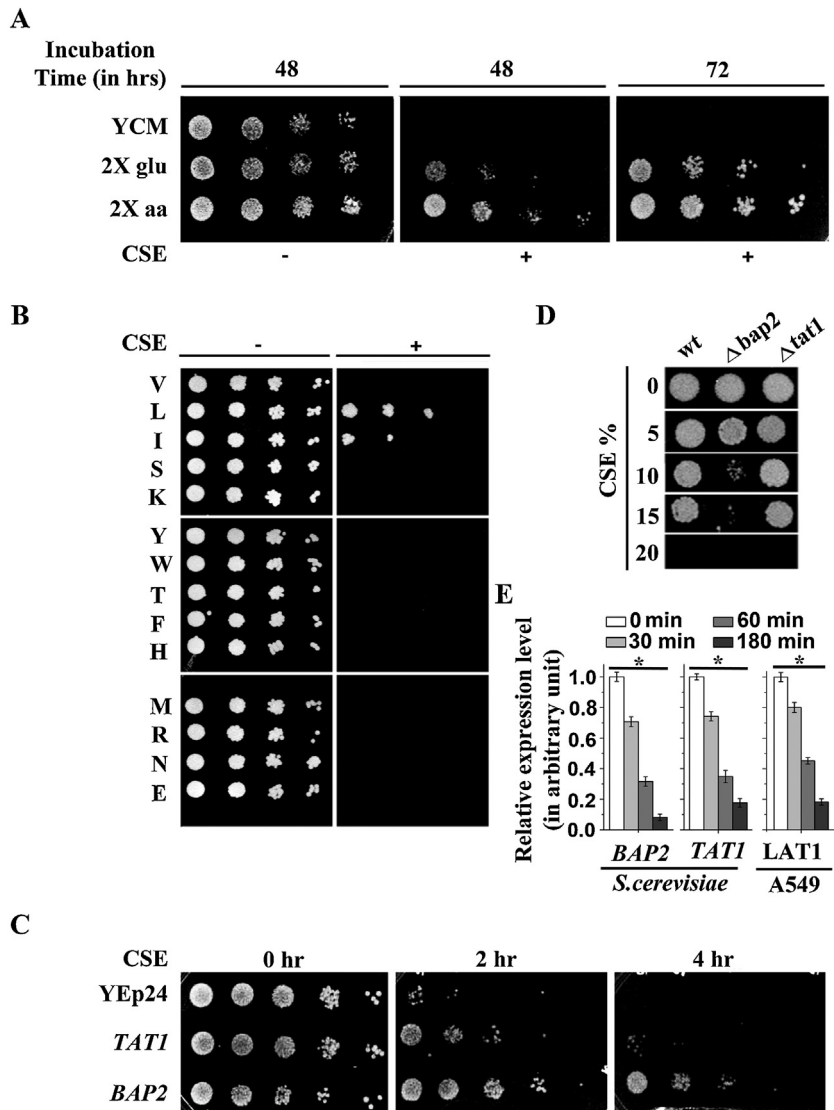


Fig. 1. Excess nutrients prevent CS-induced cell death in *S. cerevisiae*. (A) Effects of excess glucose and amino acid mixture. Exponentially growing cells in YCM or YCM supplemented with either 2× glucose or 2× amino acids, were treated or left untreated with 20% (V/V) CSE for 4 h. Serial dilutions were spotted on YCM plates, following which the plates were incubated at 30 °C for the indicated time periods (48 or 72 h) before scoring for growth. (B) Effects of individual amino acids. Exponentially growing cells in YCM supplemented with 2× concentration of each of the indicated amino acids, were treated as in (a) above. The YCM plates were incubated at 30 °C for 72 h. V: valine; L: leucine; I: isoleucine; S: serine; K: lysine; Y: tyrosine; W: tryptophan; T: threonine; F: phenylalanine; H: histidine; M: methionine; R: arginine; N: asparagine; E: glutamic acid. (C) Effects of overexpressing *TAT1* and *BAP2*. *S. cerevisiae* transformants overexpressing either *TAT1* or *BAP2* from YEp24 vector were treated with 20% CSE as indicated, serially diluted and spotted on YCM plates lacking uracil. Plates were incubated at 30 °C for 72 h. Empty vector (YEp24) transformants were used as control in the experiment. (D) *bap2Δ* is more sensitive to CSE-treatment. Wild-type, *tat1Δ* and *bap2Δ* cells were treated with indicated concentrations of CSE for 4 h, spotted on YCM plates and incubated at 30 °C for 72 h. (E) CSE-treatment downregulates *BAP2* and *TAT1* in yeast cells, and *LAT1* in A549 cells. *S. cerevisiae* and A549 cells were either treated or left untreated with CSE for different time periods (as indicated in the figure) with 20% CSE for yeast and 3% CSE for A549. Expression of *BAP2* and *TAT1* in yeast, and *LAT1* in A549 cells, were determined by qPCR. Results represent mean ± SD of data from three independent experiments; **P* < 0.05.

effective (Fig. 1B). Another branched chain amino acid, valine, failed to rescue growth of CSE-treated cells under our assay conditions.

As excess leucine and isoleucine protected cells from CSE-induced death, it is possible that over-expression of the transporters of these amino acids will have a similar effect. To test this, *BAP2* and *TAT1*, genes encoding two branched chain amino acid transporters [24,25] were expressed from a multicopy vector (YEp24). While

overexpression of both transporters conferred resistance to 20% CSE exposure for 2 h, only *BAP2* overexpressing cells were able to grow following CSE treatment for 4 h (Fig. 1C). Correspondingly, *bap2Δ* cells displayed increased sensitivity to CSE compared to wild-type cells, whereas sensitivity of *tat1Δ* cells was comparable to that of wild-type (Fig. 1D).

Since both excess leucine in the medium and overexpression of *BAP2* rescued CSE-induced growth inhibition of yeast cells, it is possible that CSE exposure results in

downregulation of the leucine transporters. Therefore, we examined the level of *BAP2* and *TAT1* expression in CSE-treated yeast cells and observed a significant reduction in expression of both genes relative to that of untreated cells (Fig. 1E). Since CSE exposure results in death of alveolar epithelial A549 cells [26,27], we examined the expression of LAT1 that encodes a transporter for neutral branched chain amino acids like leucine and isoleucine [12,28] in A549 cells following CSE treatment. Consistent with the results of the studies in yeast, we observed a significant reduction in LAT1 expression level compared to control cells (Fig. 1E). Thus CSE exposure causes downregulation of amino acid transporter(s) of leucine and isoleucine in both mammalian and yeast cells.

3.2. LAT1 overexpression and excess leucine make A549 cells less sensitive to CSE

Since CSE exposure downregulates LAT1 in A549 cells, we examined if the overexpression of LAT1 prevents CSE-induced cell death. We transfected A549 cells with either a construct that constitutively expresses LAT1 or the empty vector (pcDNA3.1). The transfectants were treated with CSE and cell viability was determined by MTT assay. It was observed that cells transfected with the LAT1 construct displayed significantly enhanced viability compared to control transfectants (Fig. 2A). This result indicates that overexpression of LAT1 in A549 cells protected the cells from CSE-induced death. Correspondingly, we verified the effect of extra leucine in the media. Cells were grown in presence of extra leucine ranging from 0.4 mM to 1.6 mM and treated with 3% CSE for 6 h. Consistent with our expectation, addition of extra leucine to the media resulted in better survival of A549 cells following treatment with CSE wherein 0.8 mM leucine exhibited best result (Supplementary Fig. 2). Time kinetics of cell survival with 0.8 mM leucine was performed. The result again demonstrated a protective role of leucine against CSE-induced death (Fig. 2B). However, leucine was found to be less effective than LAT1 (compare Fig. 2A and B). Cell viability was also confirmed by trypan blue dye exclusion assay, which yielded results consistent with those for the MTT assays (Supplementary Fig. 3A and B). In agreement with the results obtained in yeast, excess methionine in the medium failed to provide such protection (Supplementary Fig. 4). To further confirm the role of LAT1, we investigated if downregulation of LAT1 made cells more sensitive to CSE. A549 cells were transfected with a construct expressing a siRNA targeted to LAT1. Consistent with our hypothesis, both MTT and trypan blue assay showed that si-LAT1 transfectants exhibited reduced cell viability compared to control cells (Fig. 2C, Supplementary Fig. 3C).

3.3. LAT1 and leucine-mediated protection against CSE involves mTOR activation

Both excess leucine and overexpression of LAT1 are known to cause activation of mTOR [29–32]. Thus, leucine or Lat1-mediated cellular protection against CSE may be exerted through mTOR activation. Upon activation, mTOR becomes phosphorylated and in turn phosphorylates its

effector p70S6 kinase [13]. Thus, to assess the status of mTOR activation in CSE-treated LAT1 transfectants, we monitored the phosphorylation status of mTOR and p70S6 kinase by western blotting. While mTOR and p70S6 kinase levels remained constant, a significant reduction was observed in their phosphorylated forms after CSE treatment (Fig. 3A). However, over expression of LAT1 prevented this reduction (Fig. 3A). Taken together these results suggest that mTOR is deactivated upon CSE treatment and elevated levels of Lat1 prevents this deactivation. Prevention of mTOR deactivation was also observed in cells treated with excess leucine (Supplementary Fig. 5A). To confirm the involvement of mTOR in both Lat1 and leucine mediated prevention of CSE-induced cell death, mTOR inhibitor rapamycin was added to A549 cells 2 h prior to CSE treatment and both cell survival and p70S6 kinase phosphorylation were examined. Cell survival and p70S6 kinase phosphorylation were significantly reduced upon rapamycin treatment in both LAT1 transfectants and cells grown in the presence of excess leucine (Fig. 3B and Supplementary Fig. 5B). Thus LAT1 downregulation is the key cellular event that leads to mTOR deactivation in CSE-treated cells.

To understand the mechanism of CS-induced LAT1 downregulation, we examined the upstream promoter elements of LAT1 and identified an E-box, a known binding site for Mad family of transcription repressors [33]. Therefore, we investigated if Mad1 level is altered upon CSE-treatment. We observed an elevated level of Mad1 in CSE-treated cells compared to control cells (Fig. 3C). Consistent with our hypothesis that Mad1 negatively regulates LAT1, the level of Lat1 was found to be inversely proportional to Mad1 level in these cells (Fig. 3C). To verify Mad1's involvement in CSE-induced LAT1 downregulation, we examined Lat1 level in A549 cells, wherein MAD1 expression was downregulated by si-MAD1 prior to CSE treatment. Western blot analysis showed higher levels of Lat1 in CSE-treated si-MAD1 transfectants compared to CSE-treated control transfectants (Fig. 3C). Results of MTT assay also showed that these si-MAD1 transfectants were more resistant to CSE-induced cell death (Fig. 3D). These results indicate that exposure to CSE results in Mad1-mediated repression of LAT1. To confirm that Mad1 binds to the promoter of LAT1, we performed ChIP analysis using anti-Mad1 antibody. The result showed that Mad1 binds to the LAT1 promoter element when cells were treated with CSE (Fig. 3E).

3.4. Leucine supplement in diet imparts resistance to CS-induced lung tissue damage

Given that either addition of extra leucine to the growth medium, or overexpression of the high-affinity transporter for leucine, provide protection against CSE-induced cell death in both *S. cerevisiae* and A549 cells, we determined if leucine supplement has the ability to impart similar resistance *in vivo*. We conducted our studies in guinea pig model. Guinea pigs were kept on vitamin C-free diet for 7 days and then administered leucine supplement in diet for another 7 days followed by CS exposure for 14 days, during which the excess leucine diet was

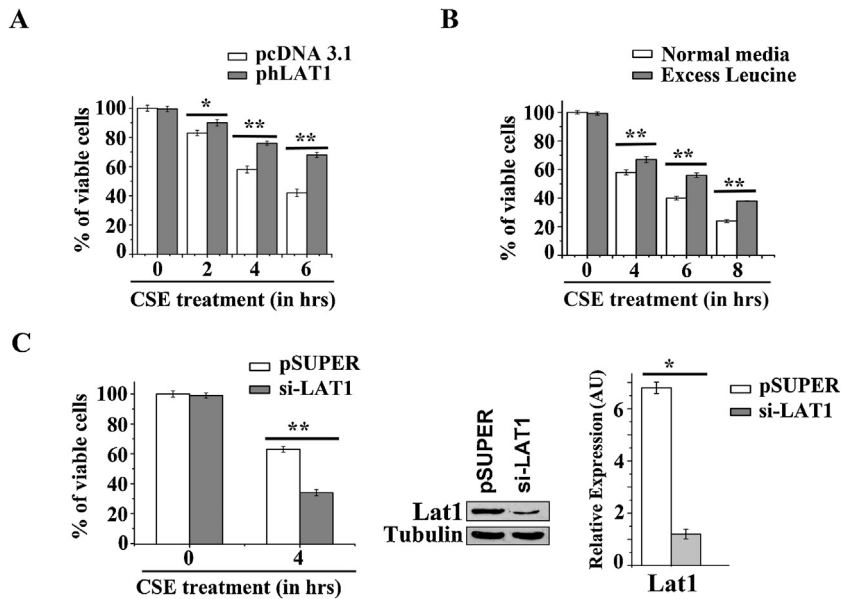


Fig. 2. Ectopic expression of LAT1 and excess leucine protected A549 cells from CSE-induced cell death. (A) A549 cells transfected with LAT1 expression construct exhibit less sensitivity to CSE. A549 cells were transfected either with a construct that expresses LAT1 from viral promoter or with the empty vector (pcDNA3.1). Twenty four hour post-transfection, transfectants were either treated with 3% CSE, or left untreated for different periods as indicated. Cell viability was assessed by MTT assay. (B) Excess leucine in the growth medium partially rescues A549 cells from CSE-induced cell death. A549 cells were grown for 16 h in presence or absence of 0.8 mM leucine. Cells were then treated with 3% CSE for different time periods as indicated and cell viability was assayed by MTT assay. (C) Downregulation of LAT1 makes A549 cells more sensitive to CSE. A549 cells were transfected either with si-LAT1 or pSUPER (empty vector). After 24 h of transfection, transfectants were treated with 3% CSE for 4 h and cell viability was assayed by MTT assay. Western blotting, followed by densitometric quantification was performed to monitor the cellular levels of Lat1. Densitometric quantification for Lat1 was normalized against tubulin. Results are represented as mean \pm SD of data obtained from three independent experiments; * $P < 0.05$, ** $P < 0.005$.

continued. During the entire study the animals were maintained on low vitamin C diet. The animals were sacrificed and the lung tissue sections were examined by staining with H & E. We observed that while tissue sections of control animals without leucine supplement showed extensive damage following CS exposure, the extent of such damage was greatly reduced in lung tissue of animals receiving leucine-supplement (Fig. 4A and B). Consistent with the results obtained in A549 cells, western blot analysis of lung tissue homogenates showed that exposure to CS resulted in significant reduction in the levels of phospho-mTOR and phospho-p70S6 kinase (Fig. 4C). In contrast, this reduction was attenuated in animals receiving leucine-supplement (Fig. 4C). Additionally, we also observed significant reduction of Lat1 level in the lung extract of CS-exposed guinea pig compared to lung extract from guinea pig that was not exposed to CS. Similar to A549 cells, this reduction was found to be inversely related with Mad1 expression (Fig. 4C). Collectively, these results from guinea pigs validate all the *in vitro* results obtained from A549 cells.

3.5. Leucine administration ameliorates pre-existing emphysematous changes in lung tissue

There are no reports of any therapeutic agent capable of reversing pre-existing emphysematous condition of lung. Since leucine administration prevents CS-induced lung damage through mTOR activation, we tested if leucine administration is able to reverse pre-existing CS-induced conditions in lung. First guinea pigs were exposed to CS for

14 days and thereafter administered leucine for 10 days during which animals were not exposed to CS. Control animals were either left untreated or treated with vitamin C, which is known to prevent CS-induced emphysema [21]. The lung sections of leucine-treated animals displayed considerably less pulmonary damage compared to animals exposed to CS (Fig. 5A, compare panels 2 and 3 with panel 5; Fig. 5B). As expected, vitamin C was unable to cause any significant reversal of lung tissue damage (Fig. 5A, compare panels 2 and 3 with panel 4; Fig. 5B). Thus these results indicate that leucine is able to reverse even existing emphysematous condition of lung and this reversal may involve mTOR activity as significantly increased p70S6 kinase phosphorylation was observed in lung extracts obtained from leucine treated guinea pig compared to untreated (Fig. 5C).

To confirm the involvement of mTOR in leucine-mediated reversal of CS-induced lung damage, animals were fed with rapamycin along with leucine for 10 days following 14 days of exposure to CS. Administration of rapamycin, along with leucine, resulted in considerable reduction in the reversal of lung tissue damage compared to the tissue damage reversal observed in animals that were fed with leucine alone following exposure to CS (Fig. 6A and B, compare panels 3 and 4). Consistent with the histology pattern, western blot analysis also showed a significant reduction of phospho-p70S6 kinase level in rapamycin and leucine treated lung tissue extract compared to the corresponding leucine treated extract (Fig. 6C, lanes 3 and 4). Taken together these results demonstrated

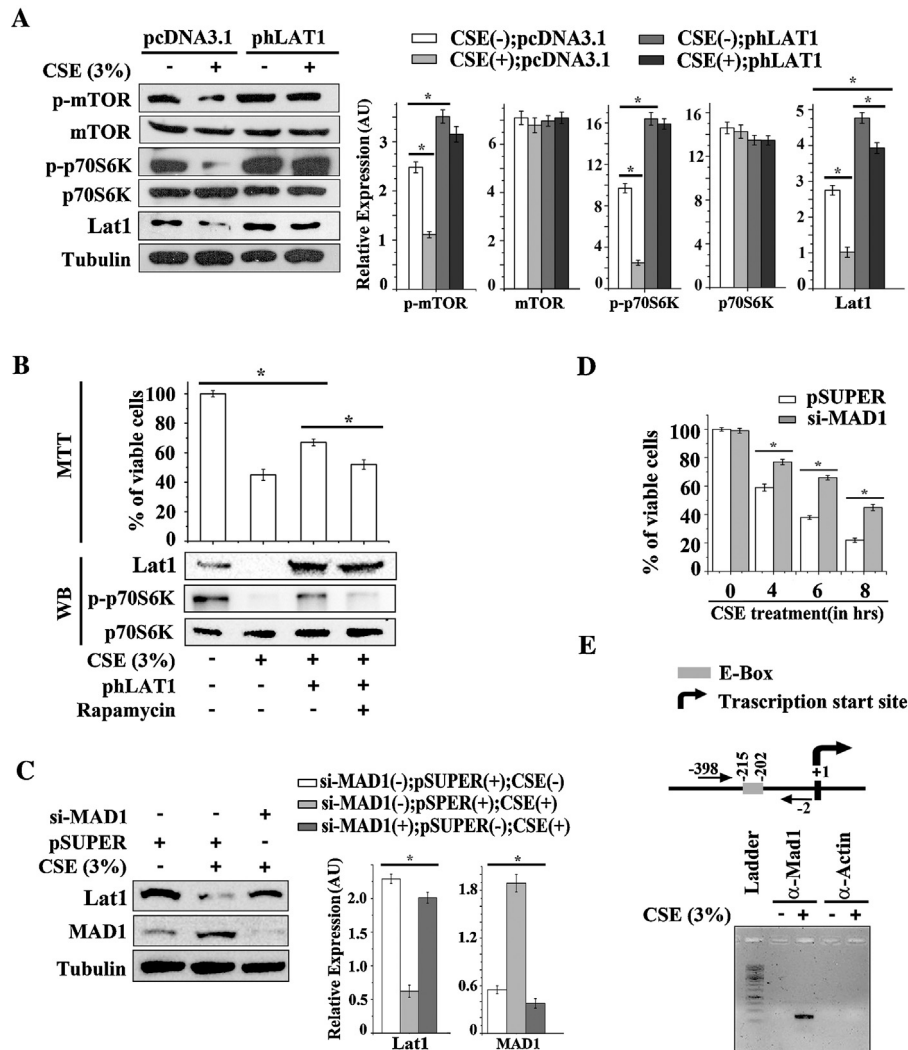


Fig. 3. Downregulation of LAT1 by CSE causes mTOR inactivation and involves Mad1. (A) Ectopic expression of LAT1 in A549 cells prevents CSE-induced mTOR inactivation. A549 cells were transfected with either a construct expressing LAT1 or empty vector (pcDNA3.1). Twenty four hour post-transfection, transfectants were either treated with CSE for 4 h or left untreated. Western blotting and densitometric quantification of different proteins were performed (data normalized against tubulin). Results represent mean \pm SD of data obtained from three independent experiments; * P < 0.05. (B) Rapamycin counters LAT1-mediated cell survival. A549 transfectants were treated with rapamycin for 2 h prior to CSE-treatment for 6 h. Cell viability was examined by MTT assay and phospho-p70S6 kinase level was examined. Data represented as mean \pm SD from three independent experiments; * P < 0.05. (C) Lat1 level is regulated by Mad1. A549 cells were transfected with either si-MAD1 or empty vector. Twenty four hour post-transfection, transfectants were either treated with CSE for 4 h or left untreated. Western blotting and densitometric quantification for Lat1 and Mad1 were performed (data normalized against tubulin). Results represent mean \pm SD of data obtained from three independent experiments; * P < 0.05. (D) MAD1 downregulation provides protection against CSE. A549 cells were transfected with either si-MAD1 or empty vector. Twenty four hour post-transfection, transfectants were either treated with 3% CSE for 8 h or left untreated. Cell viability was assessed by MTT assay. Results represent mean \pm SD of data obtained from three independent experiments; * P < 0.05. (E) ChIP analysis. CSE-treated A549 cells were cross-linked with paraformaldehyde. Chromatin was immunoprecipitated with anti-Mad1 antibody. Immunoprecipitated DNA was amplified with PCR primers corresponding to the E-Box binding site at LAT1 upstream promoter sequence as indicated in the upper panel and analyzed by agarose gel electrophoresis.

that leucine mediated amelioration of pre-existing emphysematous changes in lung tissue was mediated through mTOR activation.

Given that lung tissue damage in CS-exposed animals results from cellular death, we stained lung tissue sections with EtBr to determine if administration of excess leucine in the diet alters the extent of cellular death. Extensive cellular death was observed in lung sections of CS-exposed animals compared to the control animals (Fig. 7A, compare

panels 1 and 2). However, this CS-induced cellular death was reduced in lung sections from animals that were fed with leucine for 10 days after an exposure to CS for 14 days (Fig. 7A, compare panels 2 and 3). Since this CS-induced cellular death is attributed, at least in part, to apoptosis (4), we examined the level of apoptosis in the lung sections by TUNEL assay. Consistent with the results of EtBr staining, we observed considerable reduction of TUNEL-positive apoptotic cells in lung sections of leucine treated

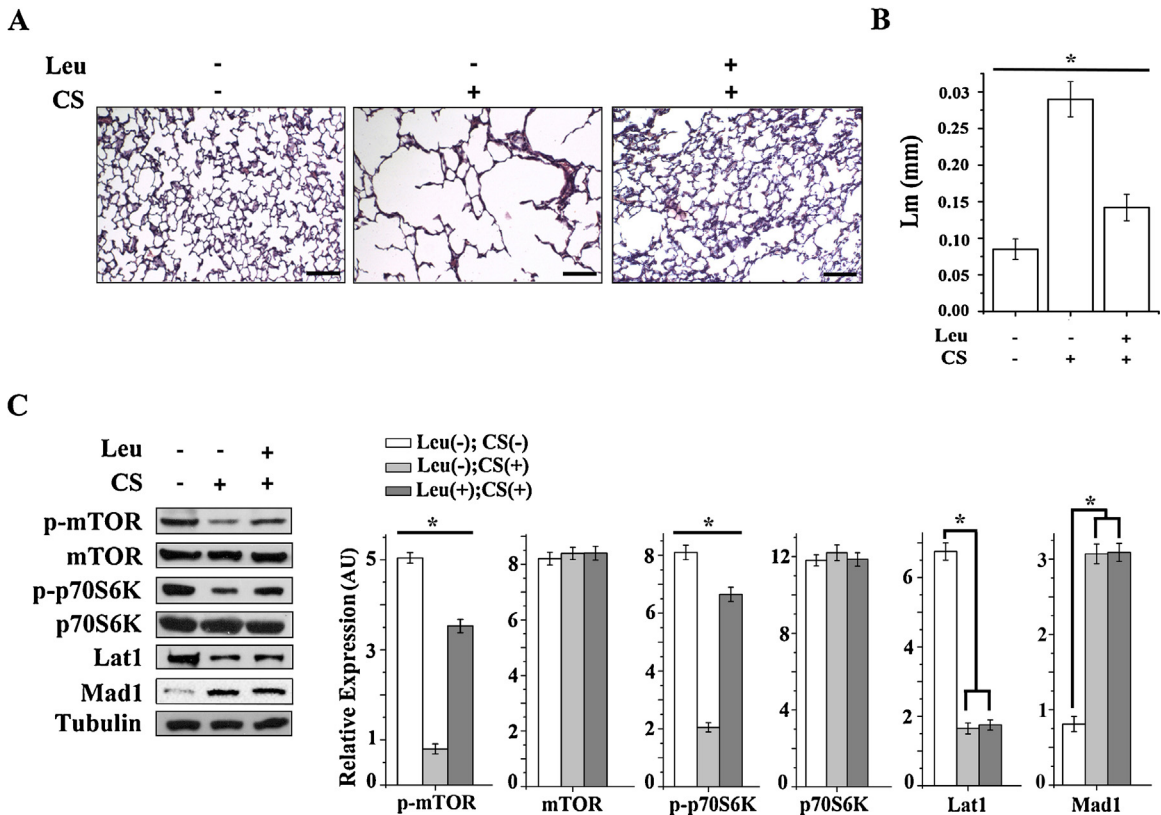


Fig. 4. Leucine supplement in diet prevents CS-induced lung tissue damage in guinea pig by activating mTOR. (A) Leucine-supplemented diet prevents lung tissue damage by CS. Guinea pigs were administered leucine-supplemented diet for 7 days followed by CS exposure for 14 days during which leucine supplement was maintained. Fixed lung sections were stained with H & E and viewed under microscope ($n = 6$ animals in each group). Scale bars: $300 \mu\text{m}$. (B) Morphometric analysis. Morphometric analysis was performed and expressed by mean linear intercept (Lm) of lung tissue sections obtained from animals in panel A (18 fields for each group). Data are represented as mean \pm SD; $*P < 0.05$. (C) Leucine counters mTOR inactivation in the lung of CS-exposed guinea pig. Lung tissue homogenates from animals of panel A were analyzed by western blotting for different proteins. The blots shown are representative of six independent experiments from the lung tissue homogenate of three animals in each group. Densitometric quantification was performed and the data shown was normalized against tubulin. Data are represented as mean \pm SD; $*P < 0.05$.

animals compared to animals that were exposed to CS but not treated with leucine (Fig. 7B). Taken together the above results indicate that leucine can reverse the pulmonary damage caused by exposure to CS through the activation of mTOR pathway.

4. Discussion

The current study demonstrates that leucine supplement can attenuate cellular damage caused by CS. The study was initiated in the genetically facile yeast model wherein nutritional supplement, particularly excess leucine in growth media, made cells resistant to CSE-induced cell death. Similar resistance was also conferred upon overexpression of the branched chain amino acid transporter Bap2 (Fig. 1C). Cells overexpressing Tat1, another branched chain amino acid transporter, were less resistant compared to those overexpressing Bap2. This difference is likely due to the higher affinity of Bap2 for leucine compared to Tat1 [34]. Consistently while *bap2* Δ cells were more sensitive to CSE-induced growth inhibition compared to wild-type, *tat1* Δ cells exhibited sensitivity similar to

wild-type levels. This is most likely because *tat1* Δ cells contain functional Bap2.

Overexpression of the mammalian transporter for leucine, LAT1, as well as excess leucine in the growth medium, protected A549 lung epithelial cells against CSE-induced cell death. This protection stems from the ability of leucine to activate mTOR [29]. There is a positive correlation between mTOR activation and mitochondrial function as increased mitochondrial activity leads to the downregulation of AMPK, a negative regulator of mTOR signaling complex [35]. Leucine can enhance mitochondrial functions in two ways: (i) by serving as a mitochondrial fuel upon undergoing oxidative decarboxylation and (ii) by acting as an allosteric activator of glutamate dehydrogenase that produces α ketoglutarate, a TCA cycle intermediate [30].

Although leucine pre-treatment provides protection against CS-induced cell death, this protection is only partial. This is likely because CS has pleiotropic effects on multiple cellular pathways. For example, CS exposure has been shown to cause protein degradation, inhibit protein synthesis, and induce both DNA damage and oxidative stress [20,36]. Elevated leucine level most likely

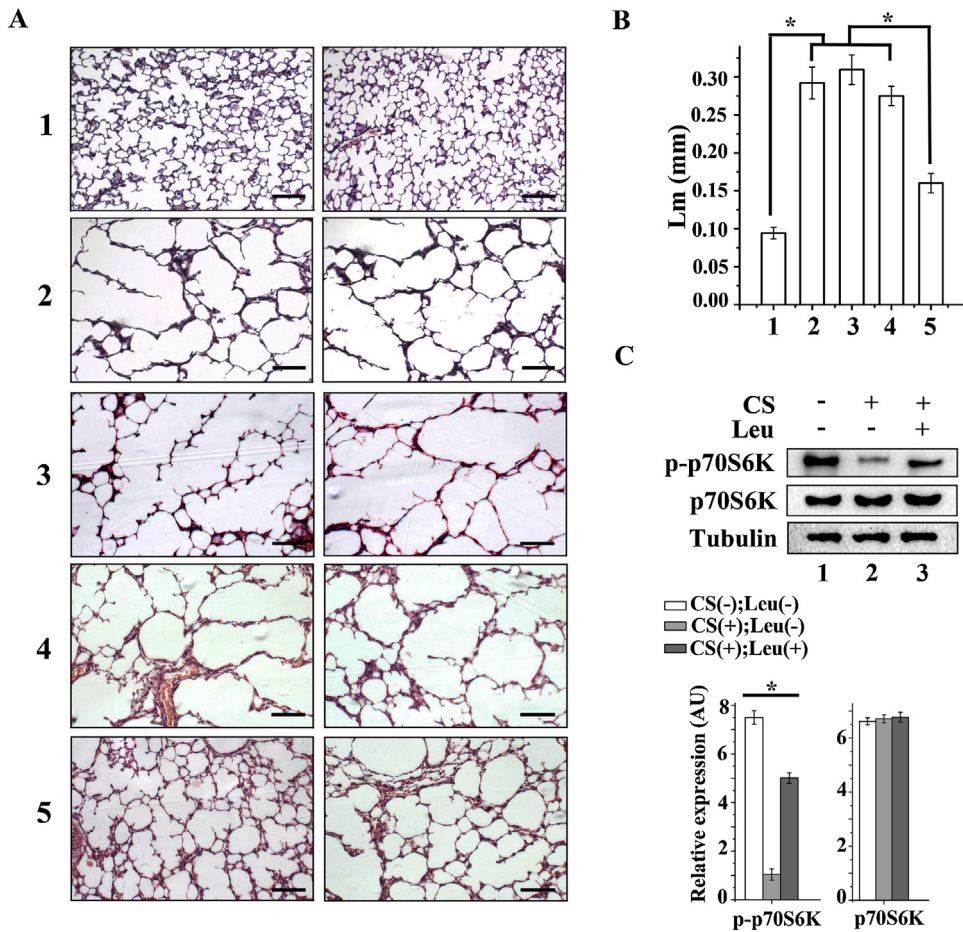


Fig. 5. Dietary leucine supplement reverses pre-existing emphysematous changes in lung tissue. (A) Histology of differentially treated animals. Guinea pigs were differentially treated as follow: (1) Control animals not exposed to CS. (2) Animals exposed to CS for 14 days and sacrificed. (3) Animals exposed to CS for 14 days and then left untreated for another 10 days before sacrifice. (4) Animals exposed to CS for 14 days and then fed vitamin C supplemented diet for 10 days before sacrifice. (5) Animals exposed to CS for 14 days and then fed leucine supplemented diet for 10 days before sacrifice. Fixed lung sections from these animals were stained with H & E and viewed under microscope. Scale bars: 300 μ m. ($n = 6$ animals in each group). (B) Morphometric analysis. Morphometric analysis of lung tissue sections obtained from differentially treated animals (18 fields for each group) was performed and expressed by mean linear intercept (Lm). Data are represented as mean \pm SD; * $P < 0.05$. (C) Leucine treatment induces p70S6 kinase phosphorylation in the lung of CS-exposed guinea pig. Lung tissue homogenates from differentially treated guinea pigs were analyzed by western blotting. Lane1: Control; Lane2: CS treatment (14 days) only; Lane3: CS treatment (14 days) followed by leucine treatment (10 days). The blots shown are representative of six independent experiments ($n = 3$ animals in each group). Densitometric quantification was performed and the data shown was normalized against tubulin. Data are represented as mean \pm SD; * $P < 0.05$.

counteracts only a subset of these pathophysiological effects related to protein synthesis. Consistently, we observed that although leucine could reverse the ROS-mediated downregulation of mTOR, it could not reduce ROS generation (Supplementary Fig. 6). Another reason why leucine can only partially rescue the effects of CS is because CS causes downregulation of the amino acid transporter Lat1, the primary mode of entry of leucine into the cell. Besides leucine, Lat1 also transports other neutral essential amino acids such as isoleucine, valine and aromatic amino acids like phenylalanine, tryptophan and tyrosine [12]. Thus downregulation of LAT1 leads to a deficiency of multiple amino acids and leucine alone is unlikely to be a remedy for such a deprivation of multiple nutrients. Consistently, we observed that ectopic expression of LAT1 is more efficient in combating CS-induced cell death compared to

administration of excess leucine (Fig. 2A). In addition, our results in *S. cerevisiae* showed that besides *BAP2*, other amino acid permeases like *TAT1* (Fig. 1E) and *TAT2* are also downregulated by CSE (Supplementary Fig. 7) indicating that this xenobiotic mixture is likely to cause downregulation of multiple nutrient transporters. Such a repression of the multiple nutrient transporters may also be taking place in human cells because the E-box element, the binding site of Mad1 repressor, is also present in the promoter elements of several human amino acid transporters.

Previous studies reported a reduction in protein synthesis upon exposure to CS but the underlying mechanism has remained unclear [20,37]. One potential mechanism revealed by Yoshida et al. involved inactivation of mTOR [38]. This inactivation is mediated by a stress-related protein Rtp801 that activates TSC2, a negative regulator of

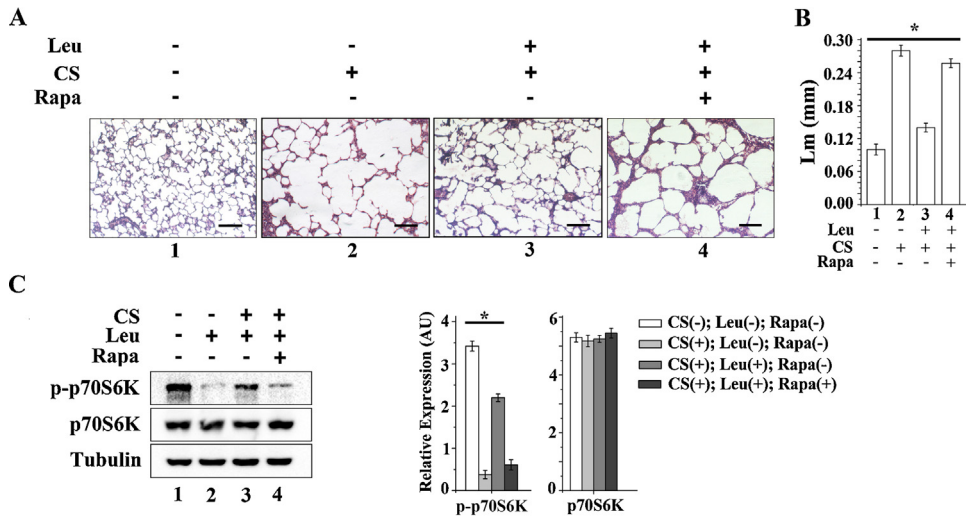


Fig. 6. Rapamycin treatment inhibits the ability of dietary leucine to reverse pre-existing emphysematous changes. (A) Histology of differentially treated animals. Guinea pigs were differentially treated as follows: (1) Control animals. (2) Animals exposed to CS for 14 days and then left untreated for another 10 days before sacrifice. (3) Animals exposed to CS for 14 days and then fed with leucine for 10 days before sacrifice. (4) Animals exposed to CS for 14 days and then fed with leucine and rapamycin for 10 days before sacrifice. Fixed lung sections from these animals were stained with H & E and viewed under microscope. Scale bars: 300 μ m. ($n=6$ animals in each group). (B) Morphometric analysis. Morphometric analysis of lung tissue sections obtained from differentially treated animals (18 fields for each group) was performed and expressed by mean linear intercept (Lm). Data are represented as mean \pm SD; $*P<0.05$. (C) Rapamycin inhibits leucine-induced phosphorylation of p70S6 kinase in CS-exposed guinea pig lung. Tissue homogenates, prepared from lungs of animals in panel A, were analyzed by western blotting. The blots shown are representative of six independent experiments ($n=6$ animals per group). Densitometric quantification was performed and the data shown was normalized against tubulin. Data are represented as mean \pm SD; $*P<0.05$.

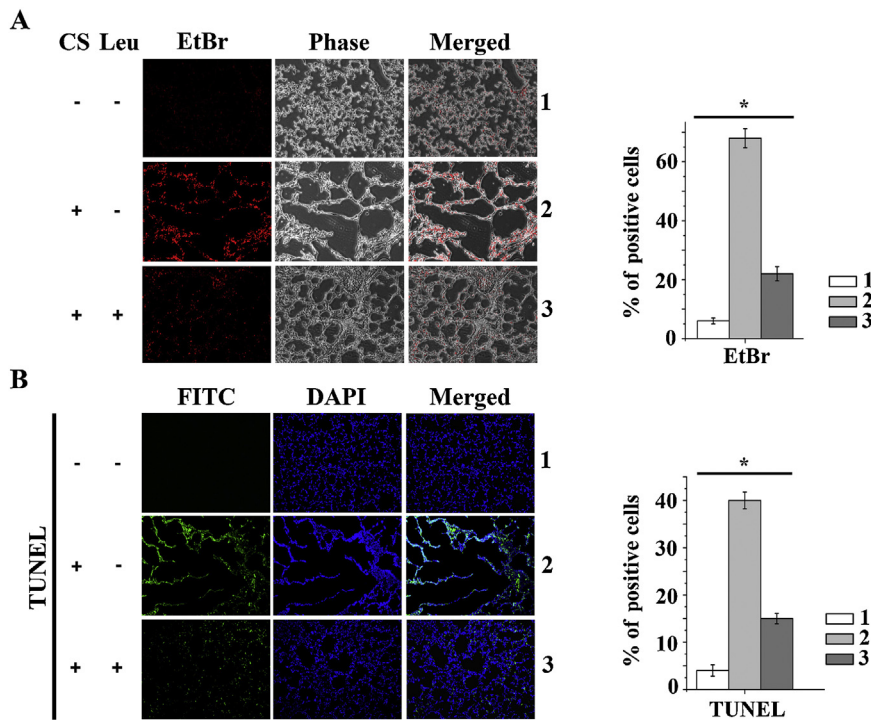


Fig. 7. Effect of leucine supplemented diet on cellular death profile of animals with emphysema. Guinea pigs were treated as follows: (1) Control animals. (2) Animals exposed to CS for 14 days and then left untreated for another 10 days before sacrifice. (3) Animals exposed to CS for 14 days and then fed leucine supplemented diet for 10 days before sacrifice. Six animals were used for each group. Fixed lung sections from these animals were stained with EtBr to examine cellular death. (A) Sections were also subjected to TUNEL assay for detection of apoptosis (B) The percentage of EtBr and TUNEL positive cells were determined (18 fields for each group). Data are represented as mean \pm SD; $*P<0.05$.

mTOR. The current study uncovers an additional mechanism of mTOR inactivation by CS wherein CS causes downregulation of amino acid transporter Lat1.

Previous reports have documented the induction of autophagy following exposure to CS [7,8]. However, the underlying mechanism by which CS induces autophagy is not well understood. Our results showing that exposure to CS results in downregulation of amino acid transporter(s) provides a possible link between CS exposure and autophagy wherein reduced nutrient availability may trigger a starvation-induced autophagic response. Future investigations are necessary to confirm this hypothesis.

The effect of leucine on CS-induced cell death was also validated in the guinea pig. Guinea pig is a good animal model system to study CS-induced damage as, like humans, this animal is unable to synthesize vitamin C, a known antioxidant. Thus dietary supplementation is the only source of this important vitamin. Since CS-induced oxidative stress is crucial in developing emphysema, previous studies have shown that restricting the availability of vitamin C in the diet results in accelerated emphysematous changes [15,21]. Using this same strategy of vitamin C deprivation, we have been able to establish accelerated development of CS-induced atherosclerosis in guinea pig aorta [39].

Using this model, the current study identifies a likely treatment option for emphysema, a disease hitherto thought to be irreversible. Previous reports document that the onset of emphysema may be prevented with antioxidants such as black tea and vitamin C [15,21]. However, until now, no cure has been reported. Lung transplant is the only option, but this is hardly a cure because of organ donor scarcity, the prohibitive cost of the procedure and the poor health of most emphysema patients. The current study is the first to document a non-surgical therapeutic option for this condition as leucine is able to reverse even pre-existing emphysematous changes in CS-exposed guinea pig lung. Leucine therapy is likely to have minimal side effects as it has already been used as a therapeutic agent for skeletal muscle atrophy, Diamond-Blackfan anemia and obesity [40–42].

5. Conclusion

The current study documents a new mechanism by which CS affects cellular physiology wherein amino acid transporters are a key target and that leucine is likely to have significant therapeutic potential for treating emphysema resulting from CS exposure.

Conflict of interest

The authors declare no competing interest.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.toxrep.2014.09.011](https://doi.org/10.1016/j.toxrep.2014.09.011).

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