CHAPTER-III

Validation of the *ex vivo* observations in guinea pig model
**Background**

In the previous chapter it was observed in A549 cells that CSE-exposure causes downregulation of LAT1 and inactivation of mTOR. This mTOR inactivation was prevented by either LAT1 overexpression or by growing the cells with excess of leucine prior to CSE-exposure. These observations indicated a possible role of leucine in preventing CS-induced emphysema in animal model. The development of emphysema involves different type of cells and thus the observation with A549 cells convey only a part of the total information. Thus, in order to gain an insight into the disease, animal model is absolutely necessary. Guinea pig is a good model for studying cigarette smoke induced lung damage because like human this animal cannot synthesize vitamin C, which plays a major protective role in preventing damage caused by CS-induced oxidative stress (Panda et al., 2000; Banerjee et al., 2008). Cigarette smoke exposure causes destruction of alveoli or air sacs in the lungs, (Banerjee et al, 2007) this destruction of air sacs is a clear indication of emphysematous changes in the lung. Thus, in order to validate the results obtained with A549 cells, the effect of pretreatment and post treatment of guinea pigs with dietary leucine supplement was investigated in CS-exposed guinea pig lungs.
Materials and Methods

Reagents and antibodies

Vitamin C and L-Leucine (99% pure, research grade) was obtained from Sigma, USA. Primary antibodies against mTOR, p-mTOR, 4E-BP1 and p-4EBP1 were from Cell Signaling Technology; p70S6 kinase, p-p70S6 kinase, MAP-LC3, Mad1 and Tubulin were from Santa Cruz; LAT1 was from Gene Tex. Secondary antibody anti-mouse HRP, anti-rabbit HRP and anti-rabbit FITC were from Santa Cruz. Chemiluminescent kit was from Thermo Scientific.

Exposure of guinea pigs to cigarette smoke (CS)

All animal care procedures were as per NIH (National Institute of Health) guidelines and approved by the Institutional Animal Ethics Committee. Two to three month old male guinea pigs (250-350 g) were used for all the experiments. 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 (Panda et al, 2000; Banerjee et al, 2008). Thereafter, 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 (3 cigarette/animal/day, with 2 puffs/cigarette). Control guinea pigs were not exposed to CS.

In a separate experiment, guinea pigs were first exposed to CS for of 14 days as mentioned before. Thereafter the smoke treatment was ceased and animals were fed normal diet along with either vitamin C (1 mg/day) or vitamin C and leucine supplement (48 mg/kg/day) for next 10 days. For each group, a minimum of 3 animals were used. Lung tissue sections were stained with hematoxylin and eosin (H & E) for
histological analysis. Enlargement of air spaces was evaluated by Mean Linear Intercept Measurement (Dunnill, 1962).

Immunohistochemistry

For immunohistochemistry lung tissue from differentially treated guinea pigs was fixed in formaldehyde. Fixed tissues were paraffin-embedded, and serially sectioned at 5 µm and stained with hematoxylin and eosin (H&E). For identification of specific antigens these sections were deparaffinized with xylene and rehydrated with a series of ethanol-water mixture with decreasing concentration of ethanol (90%, 80%, 70%, 60%, 50% ethanol).

Sections were permeabilised by treating with 10mM Na-citrate buffer, pH-6.0 at 95°C for 10 minutes followed by 0.1% Triton X-100 in the same buffer at room temperature. The permeabilised sections were blocked with 5% Bovine serum for 1 hour. The sections were then incubated overnight at 4°C with MAP LC3β primary antibody (Santa Cruz). Sections were then incubated with FITC conjugated secondary antibody at room temperature for 1 h, washed and stained with DAPI. Fluorescent signals were viewed under fluorescence microscope (Olympus IX71, Tokyo, Japan).

In situ ROS measurement in lung sections

The in situ ROS level was measured by oxidation of DCFDA to highly fluorescent DCF. Lungs obtained from the differentially treated CS-exposed animals were kept frozen and cryo sections were made. Thereafter these sections were incubated with DCFDA for 20 mins at 37°C, washed with PBS and viewed under fluorescent microscope (Olympus IX71, Tokyo, Japan).
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TUNEL assay

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 prior to labeling sections were deparaffinized with xylene washed and permeabilised. Such treated sections were washed with PBS and DNA fragmentation was detected by labeling with fluorescein-dUTP using terminal deoxynucleotidyl transferase. Slides were stained with DAPI for detecting the nuclei. The slides were viewed under fluorescence microscope (Olympus IX71, Tokyo, Japan) at excitation wavelength of fluorescein and DAPI. Digital images were captured with cool CCD camera.

Western blot analysis

Tissue extract were prepared from lung samples (100mg) in 900ul of lysis buffer containing 25mM Tris (pH-7.4), 1mM DTT, 0.01% Tween-20, 20% Glycerol, 100uM PMSF, 4mM NaVO4. The homogenate was centrifuged at 13,000 rpm at 4°C for 15 mins and the supernatant was collected for western blot analysis. Followed by protein estimation, equal amout of proteins were resolved by SDS-PAGE and electro-transferred to a PVDF membrane. After blocking with 5% non-fat milk (Hi-Media), membranes were probed with specific primary antibodies at 4°C overnight. Thereafter, the blots were washed, incubated with HRP-conjugated secondary antibody for 1 hr and finally treated with chemi-luminescence reagent and developed by X-Ray film exposure.
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Statistical analyses

Data are represented as means ± SD. For each experiment a minimum of 3 animals were used per group. Statistical significance between the groups was evaluated by One Way Analysis of Variance (ANOVA). P value of <0.05 was considered significant.
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Results

Effect of dietary supplement leucine of CS-induced lung damage

To verify the role of excess dietary leucine in animals exposed to CS guinea pigs were administered with leucine supplements (48 mg/kg body weight/day) for 7 days, prior to exposure to CS. Thereafter, guinea pigs were exposed to cigarette smoke (3 cigarette/animal/days with 2 puffs/cigarette) for duration of 14 days, and finally the animals were sacrificed and the lung tissue sections were examined by staining with H&E. During the course of CS exposure all the animals were fed with normal diet, along with supplementation of L-Leucine in one set of experimental animals. All the animals were given an oral dose vitamin C (1 mg/day) to prevent of scurvy. Consistent with the previous results, it was observed that while control animals that did not receive leucine supplements showed extensive tissue damage following CS exposure, the extent of such damage was greatly reduced in leucine-supplemented animals (Fig. 3.1).

It was observed in A549 cells that leucine and LAT1 mediated cell death prevention in CSE-treated cells were mediated through mTOR signaling pathway. Therefore it was further investigated whether the reduced lung tissue damage in leucine supplemented animals was a result of mTOR activation or not. Consistent with the results obtained with yeast and A549 cells, western blot analysis of lung tissue homogenates showed that CS-exposure resulted in marked reduction in the levels of phospho-mTOR, phospho-S6 kinase and phospho-4E-BP1 whereas, this reduction was far less in leucine-supplemented animals (Fig. 3.2). The level of phospho-mTOR was also confirmed by immunohistochemistry in the lung sections (Fig. 3.3)
Figure 3.1. Leucine supplement in diet prevents CS-induced tissue damage in guinea pig lung. A. Leucine-supplemented diet prevents lung tissue damage by CS. Guinea pigs were administered leucine-supplemented diet and exposed to CS for 14 days as indicated. Formaldehyde fixed lung sections were stained with H&E and viewed under microscope. B. Alveolar size determination. Alveolar size was assessed by alveolar length determined by mean linear intercepts (8 fields, n = 3 animals in each group). Data are represented as mean ± SD. Mean value with different letters indicate significant difference among different treatments. P<0.05 was considered significant. Scale bars: 300 µm.

Figure 3.2 Leucine counters mTOR inactivation in the lung of CS-exposed guinea pig. Tissue homogenates were prepared from lungs obtained from differentially treated guinea pigs as indicated. Homogenates were analyzed by western blotting for different proteins as mentioned in the figure.
Figure 3.3. Immunohistological analysis of mTOR and p-mTOR in guineapig lung sections. Lung sections from differentially treated guinea were stained with mTOR and p-mTOR primary antibody and FITC-tagged secondary antibody.

Effect of dietary leucine supplement on CS-induced ROS in guinea pig lung

It was observed in A549 cells that leucine pre treatment was not being able to reduce the cellular ROS level generated by cigarette smoke, therefore this observation was further investigated in animal model. In order to detect the ROS level in animal lungs, the animals after subjecting to the treatment as mentioned above were sacrificed and the lungs after cryo sectioning stained with DCFDA. In congruent with the result in A549 cells it was observed that leucine failed to decrease ROS levels in in vivo studies. No significant reduction in ROS level was observed in DCFDA stained lung sections of animal’s pre treated with excess leucine prior to CS-exposure compared to the control animals treated with only CS. (Fig 3.4).
Figure 3.4. Leucine fails to prevent CS-induced elevation of intracellular ROS in guinea pig lung. Guinea pigs were exposed to CS in the presence or absence of excess leucine in diet. Cryo sections of lung tissue from the animals were incubated with DCFH-DA and fluorescence was viewed under microscope.

Effect of dietary leucine supplement on pre-existing emphysematous lung damage

It has been observed in the previous experiment that leucine pretreatment can prevent CS induced tissue damage and thus far no therapeutic agent has been reported to reverse pre-existing emphysematous condition of lung. Therefore the effect of leucine supplement on pre-existing emphysema was investigated.

To test this hypothesis, guinea pigs were exposed to CS for fourteen days and thereafter treated with leucine for ten days during which time animals were not exposed to CS. Control animals were either left untreated or treated with vitamin C, an antioxidant known to prevent CS-induced emphysema (Banerjee et al., 2008; Panda et al., 2000).

Thereafter, lung sections were examined. It was observed that lung sections of the leucine-treated animals displayed considerably less alveolar damage compared to the control animals. (Fig.3.5A). However, as expected, no significant reversal of lung tissue damage was observed in vitamin C treated animals (Fig 3.5A, Panel 4 and Fig 3.5B). Thus these results indicated that leucine was able to partially reverse even
existing emphysematous condition of lung and this reversal may involve mTOR activity as significantly increased S6 and 4E-BP1 phosphorylation was observed in lung extracts obtained from leucine treated guinea pig compared to untreated (Fig. 3.5 C). To gain further confidence ethidium bromide staining was performed for examining cellular death in lung sections obtained from the differentially treated animals. In addition to tunnel assay to examine the level of apoptosis and immunostaining for autophagic marker LC3-II was also performed. Result showed considerable reduction in cellular death in lung sections of leucine treated animals compared to control animals (Fig 3.6).
Figure 3.5. 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 with vitamin C (5 mg/day) for 10 days before sacrifice. 5. Animals exposed to CS for 14 days and then fed with leucine for 10 days before sacrifice. Formaldehyde fixed lung sections from these animals were stained with H&E and viewed under microscope. A minimum of three animals were used for each experiment. Scale bars: 300 µm. B. Alveolar size determination. Alveolar sizes of panel 1-5 were assessed by mean linear intercepts (8 fields). Data are represented as mean ± SD. Mean value with same letter represents no significant difference whereas different letters indicate significant difference among different treatments. P<0.05 was considered significant. C. Leucine treatment induces p70S6 kinase and 4EBP1 phosphorylation in the lung of CS-exposed guinea pig. Tissue homogenates were prepared from lungs obtained from differentially treated guinea pigs and was analyzed by western blotting. The blots shown are representative of six experiments done independently from the lung tissue homogenate of three animals in each group.
Figure 3.6. Dietary leucine supplement confers resistance to CS-mediated cellular death in guinea pig lung. Guinea pigs were treated as follows: 1. Control animals not exposed to CS. 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. A minimum of three animals were used for each group. Formaldehyde fixed lung sections from these animals were stained with ethidium bromide to examine cellular death (A). Sections were also subjected to TUNEL assay for apoptosis and immuno stained with anti-LC3 antibody for autophagy (B).
**Discussion**

It has been observed that treating A549 cells with excess leucine or overexpression of LAT1 prevents CSE-induced cell death. This cell survival was related with the mTOR signaling. CSE treatment caused mTOR inactivation which was regained with excess leucine and LAT1 overexpression. Validation of this data in the guinea pig model showed that indeed cigarette smoke exposure causes a decrease in expression of LAT1 which was associated with mTOR inactivation and leucine supplementation can help to prevent CS-induced lung damage. Moreover the study with the animal model came up with the interesting observation that leucine treatment in animals after exposure to CS can revert the lung tissue damage upto some extent. Hence, 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 (Banerjee et al, 2008; Banerjee et al, 2007). 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 of 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 has already been used as a therapeutic agent for skeletal muscle atrophy, Diamond-Blackfan anemia and obesity (Nicastro et al., 2011; Payne et al., 2012; Layman et al., 2006). Thus the current study documents a new mechanism by which CS smoke 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.