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Role of inner mitochondrial protein OPA1 in mitochondrial dysfunction by tobacco smoking and in the pathogenesis of COPD

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ABSTRACT

Background: Chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) are linked to several mitochondrial alterations. Cigarette smoke (CS) alters the structure and function of mitochondria. OPA1 is the main inner mitochondrial GTPase responsible for the fusion events. OPA1 undergoes proteolytic cleavage from long to short forms during acute stress and mitophagy. However, the exact role of OPA1 isoforms and related proteins during CS-induced mitophagy and COPD is not clear.

Methods: Lung tissues from non-smokers, smokers, COPD and IPF were used to determine the relative expression of OPA1 and related proteins. Additionally, we used mouse lungs from chronic (6 months) CS exposure to evaluate the status of OPA1. Primary lung fibroblasts from normal and COPD patients and naked mole rat (NMR) lung fibroblasts, human fetal lung fibroblast (HFL1), mouse embryonic fibroblast from wild type (WT), OPA1$^{-/-}$, MFN1 and MFN2$^{-/-}$ were used to determine the effect of CS on OPA1 isoforms. Various mitochondrial fusion promoters/activators (BGP-15, leflunomide, M1) and fission inhibitor (DRP1) were used to determine their effect on OPA1 status and cigarette smoke extract (CSE)-induced lung epithelial (BEAS2B) cell damage, respectively. Seahorse flux analyzer was used to determine the effect of these compounds in BEAS2B cells with and without CSE exposure.

Findings: Short OPA1 isoforms were predominantly detected and significantly increased in COPD subjects. Acute CSE treatment in various cell lines except NMR was found to increase the conversion of long to short OPA1 isoforms. CSE treatment significantly increased mitochondrial stress-related protein SLP2 in all the cells used. OPA1 interacting partners like prohibitins (PHB1 and 2) were also altered depending on the CS exposure. Finally, BGP-15 and leflunomide treatment were able to preserve the long OPA1 isoform in cells treated with CSE.

Interpretation/conclusion: The long OPA1 isoform along with SLP2 and prohibitins play a crucial role in CS-induced lung damage, causing mitophagy/mitochondrial dysfunction in COPD, which may be used as a novel therapeutic target in COPD.

1. Introduction

Mitochondrial dysfunction is the key event which occurs in the pathogenesis of chronic lung diseases like chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) [1,2]. Altered mitochondrial (membrane potential, OXPHOS, proteins) dynamics are often associated with these diseases [3]. Cigarette smoke (CS) is the main causative factor that influences many chronic lung diseases like COPD, fibrosis and associated lung viral and bacterial infections, including but not limited to the recently identified COVID-19 [4–7]. Stress factors like CS and other environmental pollutants are shown to increase the free radicals formation causing oxidative stress, where mitochondria play a very crucial role in regulating superoxide radical production [8]. We and others have shown that CS causes mitochondrial dysfunction as observed by changes in mitochondrial membrane potential, mitochondrial mass, and mitochondrial superoxide production (9–13). Additionally, CS exposure alters the abundance of several crucial mitochondrial proteins, such as PINK1, PARKIN, RHOT1 and DRP1, which are essential for mitochondrial dynamics and quality control mechanisms [9–13]. We have also reported that chronic

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CS exposure causes mitochondrial dysfunction/mitophagy leading to peri-nuclear mitochondrial accumulation in human lung epithelial cells and fibroblasts [11].

In vitro and *in vivo* studies have shown that CS exposure (low vs. high) and duration (acute vs. chronic) are of utmost importance in the mitochondrial damage response [10,14]. Acute CS exposure increases the levels of DRP1 and FIS1 reflecting the increase in mitochondrial fragmentation [14]. Concurrent decrease in outer mitochondrial membrane (OMM) fusion proteins like MFN1 and 2 were also observed [15]. While the chronic low dose CS exposure in BEAS2B cells and primary bronchial epithelial cells (PBECs) from COPD patients show altered mitophagy associated with accumulation of damaged mitochondria and increase in fusion proteins (MFN1, MFN2 and OPA1), which can be seen as fused mitochondria in the PBECs [10]. Coordinated fission and fusion process are required to maintain the normal cellular process. Excessive or deficiency in either of these processes results in altered cellular physiology and mitochondrial dysfunction [16].

Among the mitochondrial fusion proteins, MFN1 and MFN2 are related to the outer mitochondrial membrane (OMM), while OPA1 is a sole inner mitochondrial membrane (IMM) protein identified in coordinating fusion events. OPA1 has several isoforms (8 different splice variants), which are mainly divided into two: long (L-) and short (S-) isoforms. Several reports show differential roles played by long and short isoforms [17]. L-OPA1 is widely assigned to its role in elongation/fusion activity, while shorter isoform plays an important role in mitochondrial energetics and cristae maintenance [18,19]. L-OPA1 is processed to S-OPA1 by two main proteases like OMA1 and YME1L and their activity increases during mitochondrial dysfunction and fragmentation [20]. OPA1 plays an important role in mitochondrial fusion, energetics/OXPHOS, cristae/mt-DNA maintenance, while all these processes are affected during CS exposure. Studies involving the role of various forms of OPA1 and its related signaling proteins like SLP2, prohibitins (PHB1 and 2), OMA1 and YME1L, etc. remain unexplored. Here, we have made an effort to determine the levels of OPA1 isoforms after CSE treatment using different cell types, such as epithelial cells and fibroblasts from human, mouse and naked mole-rat (NMR).

Further, mitochondrial processes and pathways as druggable targets are gaining wide importance due to their involvement in various diseases and disorders ranging from Alzheimer's to chronic infections [21,22]. Several studies were published reporting a wide range of chemicals/drugs, which can be used to target specific mitochondrial processes like fusion (promoters) and fission (inhibitors) in various pre-clinical models of disease. Mdivi1 was most widely studied for its protective action and is a reversible fission (Drp1) inhibitor in various contexts of mitochondrial dysfunction associated diseases where fission plays a crucial role, including CS-associated mitochondrial dysfunctions [11]. Recently, other strategies, apart from fission inhibitors are being developed for various reasons like feasibility and improvement over the existing strategies [23]. Among them, fusion promoters like M1, leflunomide which targets MFN proteins and BGP-15 targeting OPA1 were recently reported [24–26]. Altered mitochondrial dynamics play a profound role in various chronic inflammatory diseases that show time-dependent (acute vs. chronic) responses and may have a significant role in altering the drug therapeutics. For instance, OPA1 and DRP1 were found to be altered in various types of cancers, including but not limited to lung cancers. Cancers with decreased OPA1 levels (with increased fragmentation) were found to be drug-resistant and the ones with decreased DRP1 (with increased fusion/length) were found to be drug-sensitive [27]. Drug therapeutics based on various mitochondrial dynamic phases may potentially alter the treatment outcomes and play a crucial role in the progression of disease. Therefore, it is imperative to determine the relation of these tightly regulated processes in chronic lung disease like COPD, which may improve the current treatment strategies.

It is now well established that CS causes mitochondrial dysfunction, and restoration of the mitochondrial function proved to be beneficial in various models of COPD [11]. However, the underlying molecular mechanisms and the key alterations during the progression of COPD in particular relation to mitophagy remain elusive. Keeping all the above findings in view, we are interested in understanding the importance of various OPA1 isoforms and related proteins following CS exposure (acute vs. chronic) and in chronic lung diseases, such as COPD. We hypothesized that CS exposure influences OPA1 protein conversions depending on the concentration and duration of the CS exposure subsequently leading to mitophagy/mitochondrial dysfunction in lung cells *in vitro and in vivo*. To understand this phenomenon, we used several pharmacological strategies employing the two recently reported activators (OPA1 activator: BGP-15 and MFN2 activator: leflunomide). The results from this work add to the current understanding and support that mitochondrial dysfunctions are characteristics of smoking-associated chronic lung diseases, such as COPD.

2. Materials and methods

2.1. Ethical approval: Institutional biosafety and review board approvals

2.1.1. Ethics statement

The current study was approved for the procurement of the human lung tissues as de-identified tissues by the Materials Transfer Agreement (MTA), and laboratory protocols by the Institutional Biosafety Committee (IBC) of the University of Rochester Medical Center, Rochester, NY, with Project Code: DRAI1 001 Protocol: 004, Date of approval and IBC approval includes: October 2, 2017, November 2, 2017, 9/29/2017, and, July 2, 2018 University agreement signed on the above dates as well. Patients' data or patients are not directly involved in this study as the lung tissues were procured from several agencies (see below). All patients/subjects were of age 21 and above. All methods were carried out in accordance with the Institutional Biosafety Committee guidelines and regulations of the University of Rochester, Rochester, NY.

2.1.2. Chemicals/reagents

Pharmacological compounds used in the current study like BGP-15, leflunomide, M1 promoter/activator were procured from Sigma Aldrich and Mdivi-1 was from Merck Millipore. The stocks for these compounds were made in cell culture grade DMSO and subsequently diluted in the respective media to their final concentration as reported. The used drug doses were based on the earlier reports for their therapeutic activity for their fusion promotion or fission inhibition activity.

2.1.3. Cell culture and treatments

Human immortalized bronchial epithelial cells (BEAS2B) and fibroblasts (HFL1) were maintained in DMEM + F12 containing either 5% or 10% FBS (fetal bovine serum) and 1% antibiotics (penicillin and streptomycin), respectively. Human primary lung cells like NHBE, SAEC, normal and diseased (COPD) fibroblasts were maintained as per the manufacturer's protocol (Lonza). Mouse embryonic fibroblasts (WT, OPA1$^{-/-}$, MFN1, 2 $^{-/-}$) were maintained in DMEM glutamax with 10% FBS and 1% antibiotics. Young naked mole-rat (NMR, ~2 years old) lung fibroblasts from early passages were kindly provided by Dr. Vera Gorbunova lab at the Department of Biology, University of Rochester and were maintained in EMEM (ATCC) culture media containing 15% FBS at 32 °C incubator with 5% CO2 and 3% O2 [28].

Cigarette smoke extract (CSE) was prepared as described previously [12]. CSE treatments were done with no FBS or 1% FBS or 5% FBS in case of NMR lung fibroblasts or as reported earlier in other cases [12]. The duration of CSE exposure varied and is indicated accordingly. Based on our earlier reports and unpublished observations, cells were treated with 1% or 2% CSE for 4 h [14]. Lower doses (0.25 and 0.5%) of CSE when tested for a duration of 4 h had no significant effect on the OPA1 processing dynamics. To determine for the chronic exposure effects on OPA1 signaling, BEAS2B cells were treated with a low dose (0.25% CSE on alternate days) for 15 days, this treatment causes significant

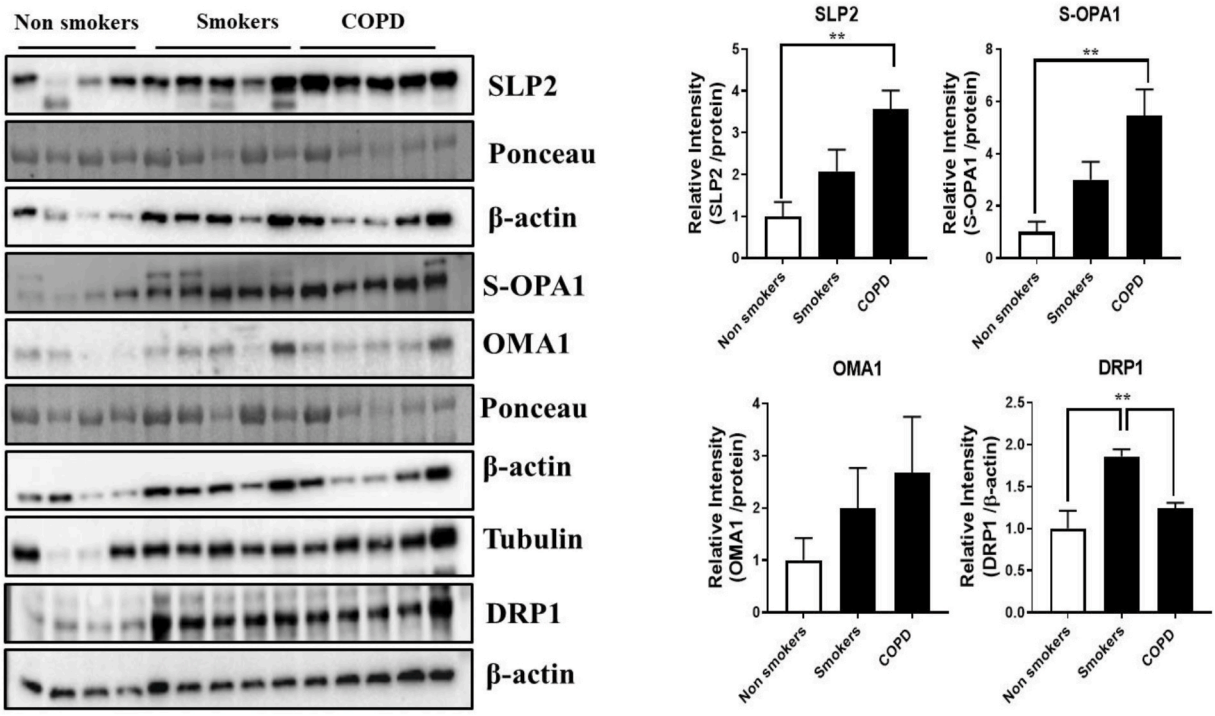

Fig. 1. Altered proteins involved in mitochondrial dynamics among non-smokers, smokers and patients with COPD. Lung homogenates (mostly from the lower peripheral lobes or as supplied) were prepared in the RIPA buffer and probed for various proteins as indicated. Equal amount of proteins were loaded and relative protein expressions were reported with either using β-actin or tubulin or non-specific ponceau band. All the data were represented as mean ± SEM (n = 4 non-smokers, n = 5 smokers and n = 5 COPD). $^{**}P < 0.01$.

mitochondrial alterations as reflected by several changes in mitochondrial protein complexes [13].

2.1.4. Human lung tissue samples

Human lung tissues were procured from the NDRI and the University of Helsinki, as described previously ([29], and were randomly processed (8–12 lung samples per group) for the lung protein extraction in RIPA buffer protease/phosphatase inhibitor cocktail. The details of the subjects and samples were recently reported [29]. The study consisted of four different groups which were classified based on their smoking status/history as non-smoker and smokers or depending on disease condition as either COPD or lung fibrosis. The extracted proteins were denatured in a 4X gel loading buffer containing SDS and β-mercaptoethanol at 95 °C for 5–7 min. These samples were separated on SDS-PAGE and transferred onto nitrocellulose membrane and probed for proteins of interest. The following are the details of the antibodies used in the current study OPA1 (80471S), DRP1 (11925S), PHB1 (2426S), PHB2 (14085S), SLP2 (ab191884), TMPRSS2 (ab92323), Furin (ab183495), β-actin-HRP (ab20272), α-tubulin (ab7291) and GAPDH (ab9484). The membranes were stripped at room temperature and further probed for different proteins/relevant controls.

2.1.5. Animal exposures

The animal (mouse) lung tissue/homogenates used in the current study from various chronic air and CS exposed groups were reported earlier [30]. The tissues were processed and probed for various proteins of interest as indicated above.

2.1.6. Oxygen consumption rate

Oxygen consumption rate (OCR) in BEAS2B cells was measured after different drug/compound treatments as reported earlier [13], using mitostress kit on a Seahorse XFp model flux analyzer. Three wells per group were used along with the parallel controls to compare the effects of the compounds used on CSE-induced OXPHOS related changes.

2.1.7. Statistical analysis

All the values were given as mean ± S.E.M, two-tailed *t*-test was performed to find out the levels of significance when comparing two groups, while one-way ANOVA with Tukey's post hoc analysis was used to compare multiple groups. GraphPad Prism (ver 7 and 8) was used for performing the statistical analysis. $P < 0.05$ is considered as statistically significant.

3. Results

3.1. Increased short OPA1 forms in lungs of smokers and COPD subjects

To determine the clinical relevance of the study, human samples from different diseased cohorts (nonsmokers, smokers and COPD) were used to assay for their OPA1 levels in lung homogenates. As shown in Fig. 1, the OPA1 levels were altered depending on the disease group. Short OPA1 isoforms were significantly increased in lungs of patients with COPD as compared to their respective controls (long OPA1 isoforms were identifiable only in some of the samples used in this study), while the smoking group has a non-significant increase in the S-OPA1 protein levels. This pattern is also true with the SLP-2 protein levels in COPD. A similar kind of observation was seen with the other chronic lung disease; idiopathic pulmonary fibrosis (IPF) samples compared to their controls (Supplementary Figure 1). While the OPA1-related processing proteases like OMA1 and PARL remained unchanged in these samples. We also evaluated the DRP1 levels, which were found to be significantly increased in the smoking groups compared to the rest of the samples/groups.

3.2. Regulation of OPA1 isoforms in chronic CS exposed mouse lungs

We have also determined the expression levels of some of these proteins in the established mouse model of emphysema/COPD caused by chronic exposure to CS. As expected, the short isoforms were

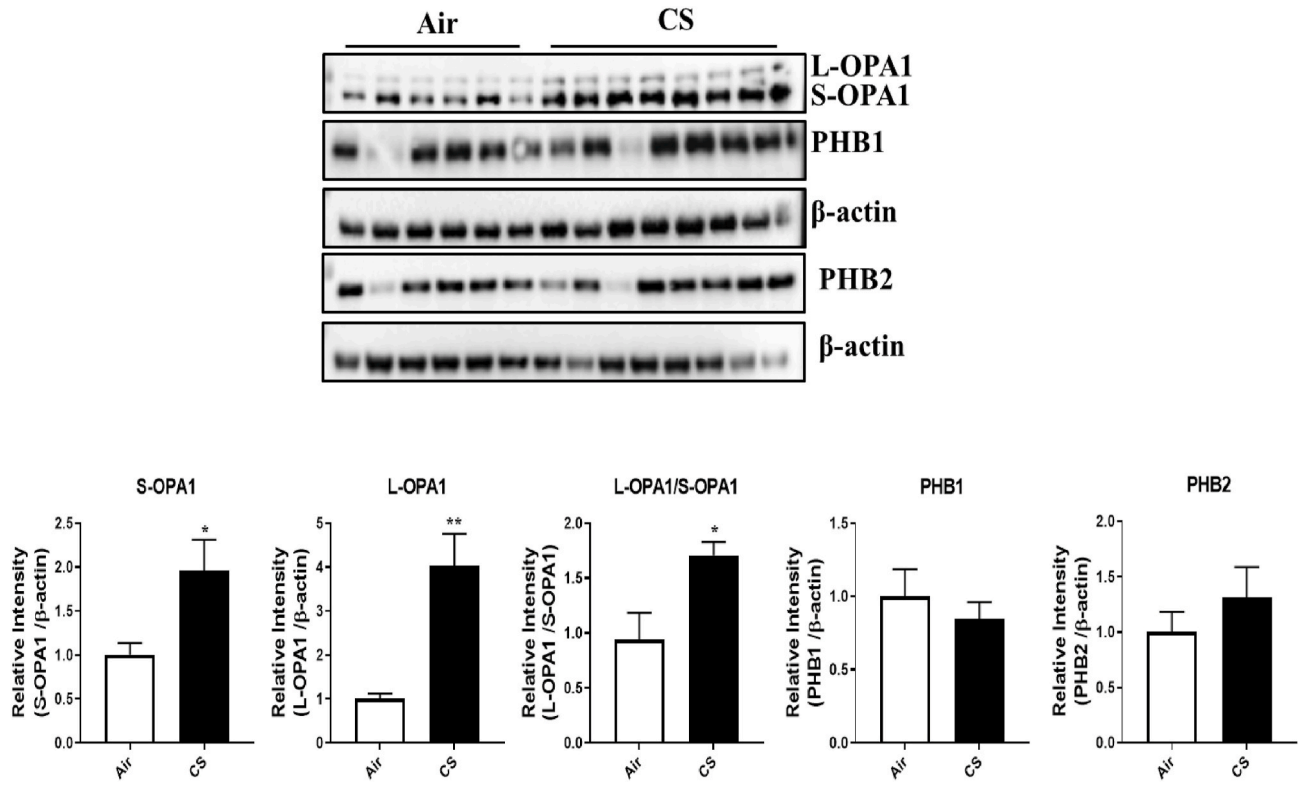

Fig. 2. Altered proteins involved in mitochondrial dynamics following chronic air or CS exposure in mice. Eight-weeks-old mice were exposed to chronic CS for 6 months. Lung homogenates were prepared in RIPA buffer and probed for various proteins as indicated. Relative protein expressions were reported using β-actin as loading control. All the data were represented as mean ± SEM (n = 6 air and n = 8 CS). $^{*}P < 0.05$, $^{**}P < 0.01$.

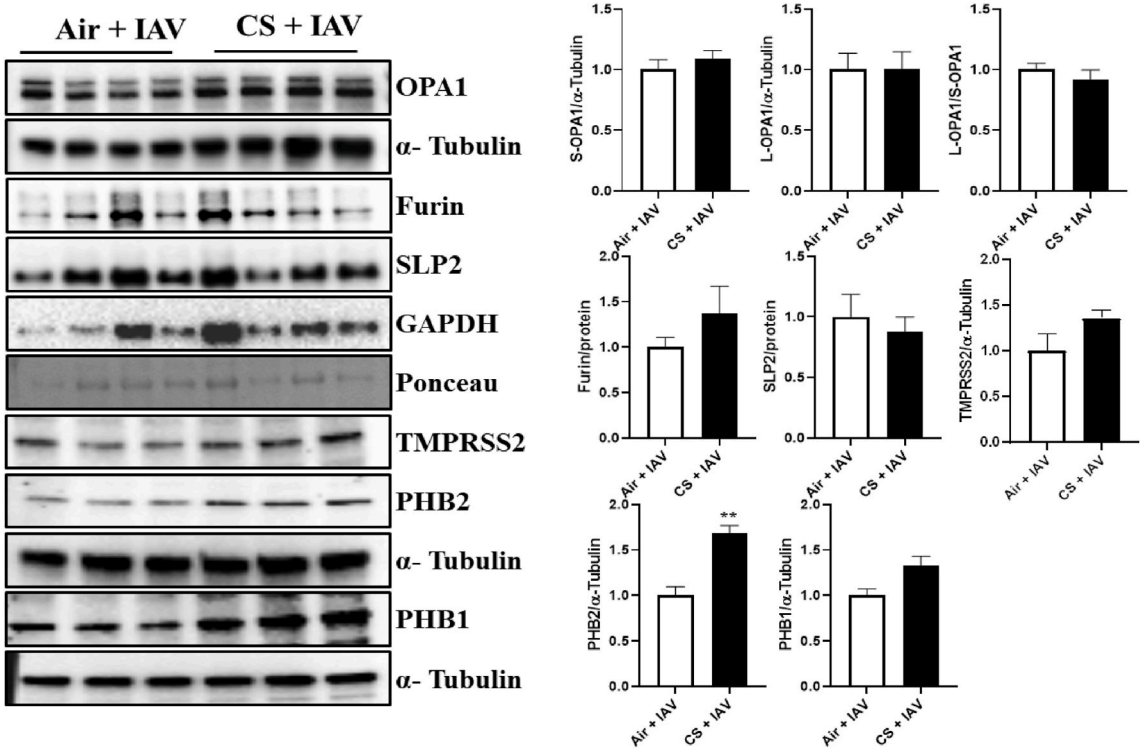

Fig. 3. Altered proteins involved in mitochondrial dynamics following chronic air or CS exposure infected with influenza A virus (IAV) in mice. Eight-weeks-old mice were exposed to CS generated from 3R4F reference cigarettes using Teague TE-10 smoking machine and then inoculated with IAV and lungs were harvested on day 9 post-infection. Lung homogenates were prepared in RIPA buffer and probed for various proteins as indicated. Relative protein expressions were reported using α-tubulin or ponceau as loading control. All the data were represented as mean ± SEM (n = 3–4/group from Air + IAV and CS + IAV). $^{**}P < 0.01$.

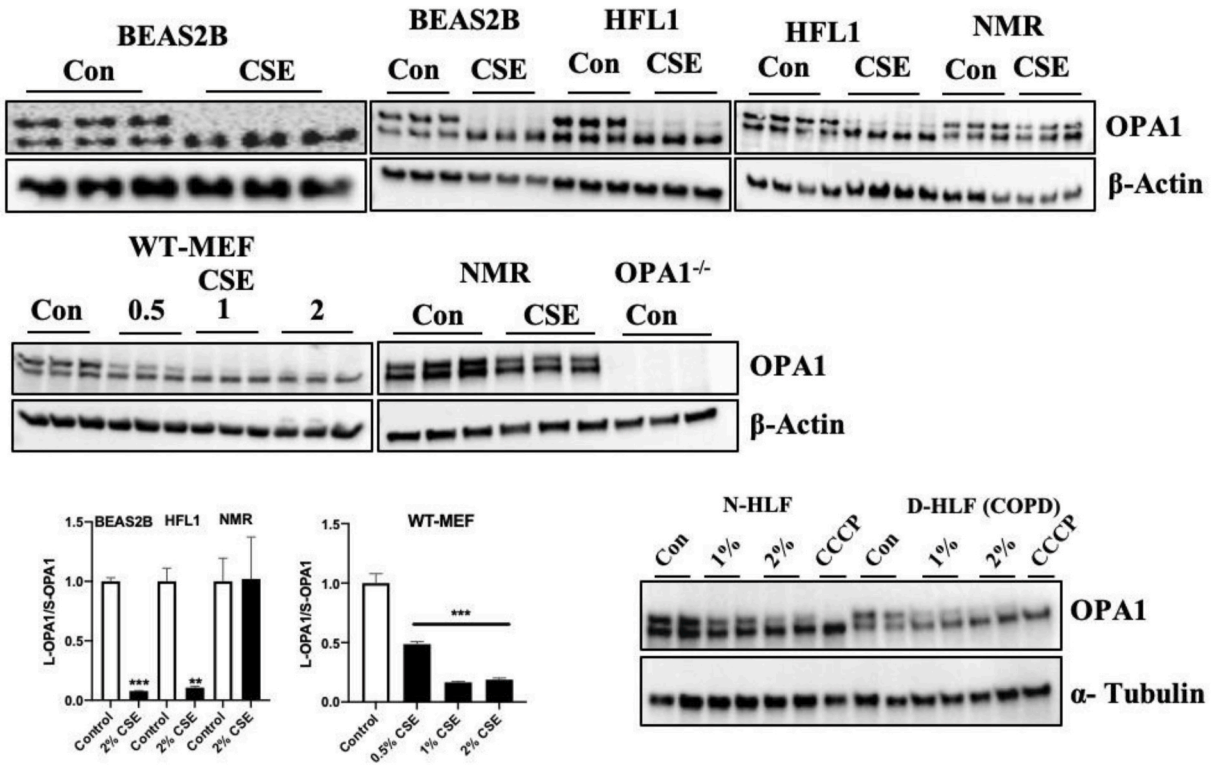

Fig. 4. Effect of cigarette smoke extract (CSE)-induced alterations in protein abundance of mitochondrial dynamics using different cell lines. Cells were exposed to 2% CSE for 4 h (acute) in their respective growth media with or without FBS depending on the cell type and probed for OPA1 expression. Cells include the following immortalized human lung epithelial cells (BEAS2B), human fetal lung fibroblasts (HFL1), naked mole-rat (NMR) lung fibroblasts, normal human lung fibroblasts (N-HLF), diseased/COPD lung fibroblasts (D-COPD), wild type mouse embryonic fibroblasts (WT-MEF), OPA1 KO mouse embryonic fibroblasts (OPA1$^{-/-}$). The blot represents three individual experiments performed in those cell lines, except for the primary human lung fibroblasts (n = 2 samples each/group for N-HFL and D-HFL) were used. $^{**}P < 0.01$ and $^{***}P < 0.001$.

significantly increased in the CS group compared to the air-only group. However, the long isoforms were also increased in these samples, which was reflected in the L/S- OPA1 ratio. While other proteins like PHB1 and PHB2 remain unchanged (Fig. 2).

Increasing interests in the role CS played during viral infections led us to examine the OPA1 expression patterns in our previously established chronic CS exposure and influenza A virus, IAV (CS + IAV)-induced COPD-exacerbation models. In contrast to the air and CS only

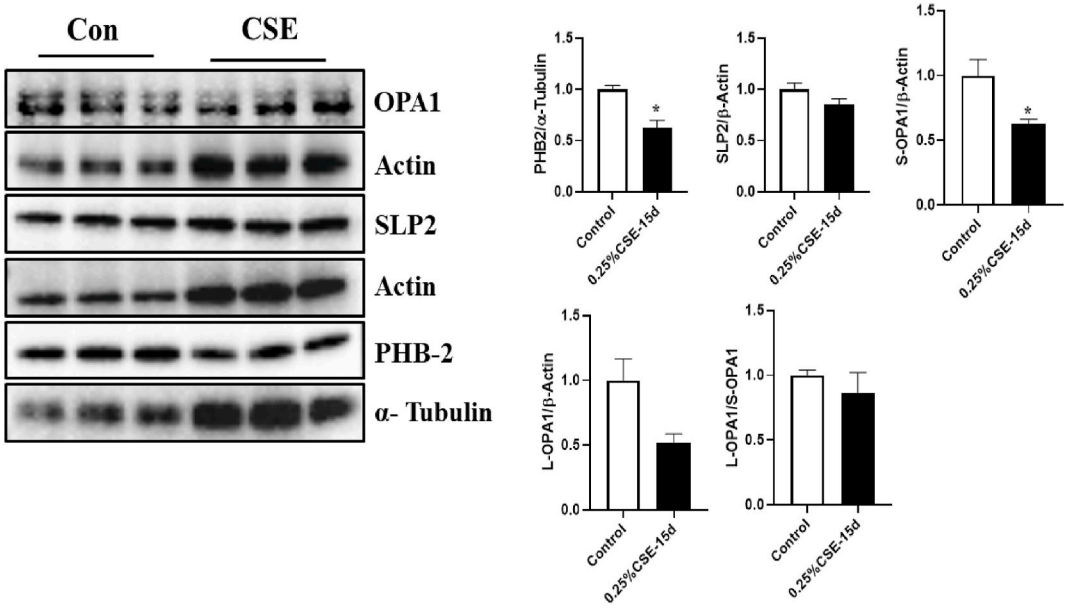

Fig. 5. Effect of chronic cigarette smoke extract (CSE)-induced alterations in protein abundance of mitochondrial dynamics using BEAS2B cells. Human lung epithelial cells (BEAS2B) were exposed to a low dose CSE (0.25%; every alternative days) for 15 days. All the data were represented as mean ± SEM (n = 3/group). $^{*}P < 0.05$.

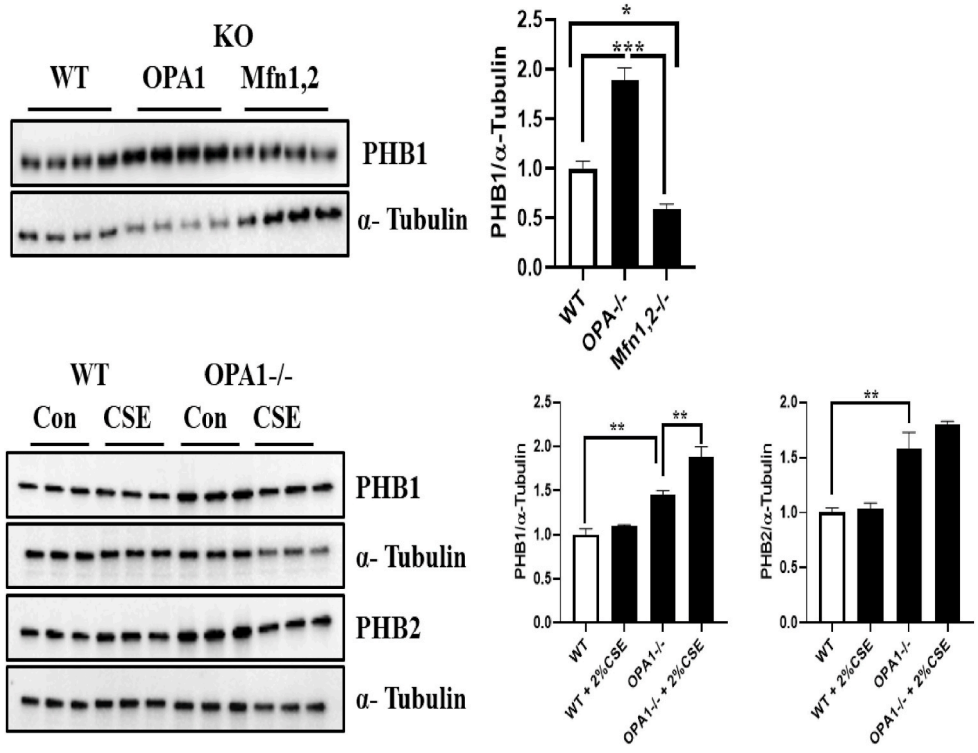

Fig. 6. Effect of acute cigarette smoke extract (CSE)-induced alterations in protein abundance of mitochondrial dynamics using different cells. Human fetal lung fibroblasts (HFL1), wild type mouse embryonic fibroblasts (WT-MEF) and OPA1 knockout mouse embryonic fibroblasts (OPA1$^{-/-}$) were exposed to 2% CSE for 4 h. All the data were normalized using protein and were represented as mean ± SEM (n = 3–4/group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

model, the CS + IAV group does not affect the OPA1 isoforms and L/S ratio, whereas PHB1 ($p = 0.05$) and PHB2 ($p < 0.05$) levels were altered in CS + IAV compared to Air + IAV only group (Fig. 3).

3.3. Effect of CSE on the OPA1 levels in various lung cells

To investigate the mechanism of CSE-induced epithelial cell damage, BEAS2B cells were exposed to different doses of CSE and at varied time periods ranging from 1 to 24 h. There was a dose-dependent decrease in the long isoforms and an increase in the short isoforms of OPA1 protein when BEAS2B cells were exposed to CSE. There was also a significant decrease in the ratio of L/S- isoforms, implying an acute stress response and it may explain the reason for the ATP/energy deficits in the acute exposure to CSE. The conversion of long to short isoforms is also evident in human fetal lung fibroblasts cells like HFL1. Patient-derived fibroblasts (n = 2) from normal vs COPD also showed a similar kind of dose-response in altering the OPA1 isoforms in response to the acute CSE treatment.

In parallel, we have used fibroblasts from different species like mouse embryonic fibroblasts (MEFs) and naked mole-rat (NMR) lung fibroblasts to compare the effect of acute CSE exposure in these cells. Mice were used as an experimental model for the development of CS-induced emphysema/COPD. NMR was reported to have a difference in the mitochondrial OXPHOS proteins [31,32]. To determine whether they produce a similar kind of stress response towards acute CSE exposure, we treated WT-MEFs and NMR lung fibroblasts with CSE. It was interesting to observe that WT-MEFs produced a similar response towards the CSE treatment as seen with HFL1, while NMR lung fibroblasts, maintained the L/S-OPA1 ratio like the unexposed cells as shown in Fig. 4.

While chronic low dose (0.25% CSE for 15 days) exposure in BEAS2B cells decreased the S-OPA1 levels, but there was no significant difference observed in the ratio of the long to short forms. This was further reflected in the decrease of PHB2 levels, with no alterations in the SLP2 levels, which are otherwise increased during the high dose of CSE acute exposure (Fig. 5).

3.4. Effect of CSE on mouse embryonic fibroblasts (MEFs)

To determine the influence of the fusion proteins MFN1 and MFN2 and OPA1 levels on the PHB1 levels, we probed for the protein expressions of OPA1 in WT, OPA1$^{-/-}$ and MFN1 and 2 $^{-/-}$ double knockout MEFs. It was interesting to observe that prohibitin (PHB1) levels were increased in the untreated OPA1$^{-/-}$ cells, compared to MFN1 and MFN2$^{-/-}$ MEFs as shown in Fig. 6. We found that complete removal of inner mitochondrial fusion protein OPA1 resulted in a significant increase in the levels of PHB1 levels, whereas complete removal of MFN1 and MFN2 proteins significantly decreased the PHB1 levels compared to WT-MEF PHB1 expression. OPA1 response remains the same in the acute CSE exposure in WT-MEFs. The levels of prohibitins especially PHB1 were further increased when the OPA1$^{-/-}$ MEFs were exposed to acute CSE (Fig. 6).

3.5. Effect of CSE on NMR lung fibroblasts

Recently, it was reported that the longevity of the naked mole rats is linked to their mitochondrial antioxidant defenses [31]. Growing evidence suggests that celllar senescence is linked to smoking, COPD and fibrosis and influences its progression. Here, for the first time, we used young NMR lung fibroblasts to determine the effects of CSE in this species. It was interesting to note that under the NMR fibroblast cell growth conditions, CSE treatment did not show much significant effect on the OPA1 levels, which was reflected in the maintenance of L-OPA1 and L/S-OPA1 ratios. However, the rest of the outcomes, like DRP1 and SLP2 were increased in a dose-dependent manner as similar to the other cell types used in the study. While significant changes only in PHB1 were observed during CSE treatment as shown in Fig. 7.

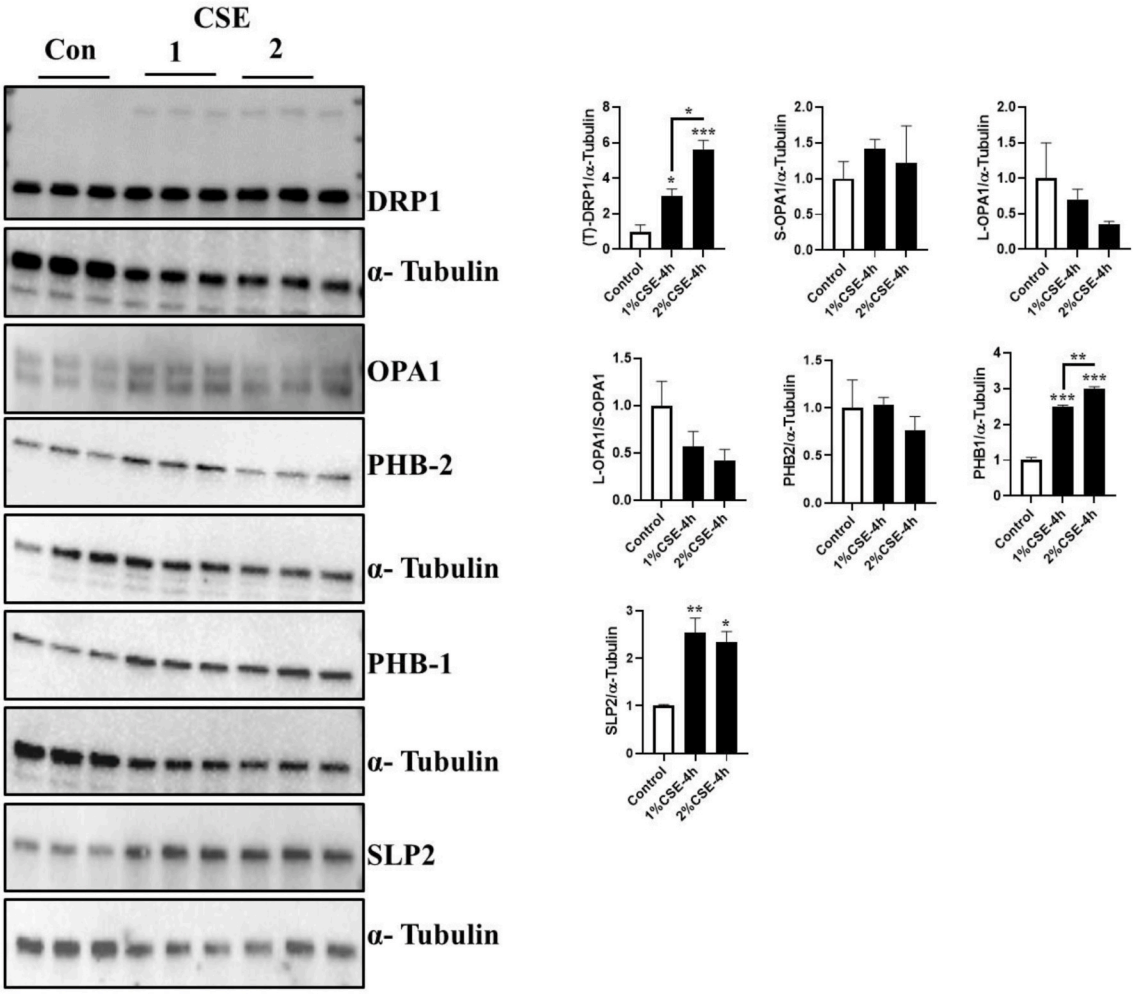

Fig. 7. Effect of acute cigarette smoke extract (CSE)-induced alterations in protein abundance of mitochondrial dynamics using naked mole-rat (NMR) lung fibroblasts. NMR lung fibroblasts were exposed to 1 and 2% CSE for 4 h and the cells were lysed in RIPA buffer and were probed for different proteins as indicated. Low passage numbers (3–9) were used for the experiments. All the data were represented as mean ± SEM (n = 3/group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3.6. Effect of BGP-15 and other fusion promoters on the OPA1 levels during CSE treatment

Next, we used recently reported OPA1 (BGP-15) and MFN2 (leflunomide) activators/promoters in the acute CSE exposure setup. BEAS2B cells were pre-treated with these compounds and subsequently treated with CSE. BGP-15 as reported recently maintained the long OPA1 isoforms. Leflunomide, which was also recently reported to be MFN2/fusion promoter, increased/maintained the long OPA1 isoforms during acute CSE exposure. These compounds also decreased the CSE-induced SLP-2 levels. However, it is interesting to observe that PHB1 and PHB2 levels were differentially altered during treatment using these compounds with or without acute CSE treatment (Fig. 8).

Seahorse XFp was used to analyze the effect of the compounds (BGP-15, Leflunomide, M1 fusion promoter and Mdivi-1) in CSE-induced mitochondrial dysfunction and mitochondrial OXPHOS. BEA2B cells were pre-treated with these compounds for 24 h, after which they were treated with 0.5% CSE for additional 24 h, which is the minimum non-toxic dose at which OXPHOS changes can be seen predominantly in these cells without much change in the OPA1 isoforms. The results indicate that BGP-15 *per se* did not have any effect on the OXPHOS at the tested concentration. Among the four compounds tested, Mdivi-1 showed the enhanced preservation of the OXPHOS compared to the other compounds, signifying the role of DRP1 related mitochondrial dysfunctions as an earlier event during the low dose treatment (Fig. 9).

4. Discussion

Mitochondrial quality control is a key attributing factor for the healthy lifespan of the cells [33]. Mitochondrial dysfunction occurs in the pathogenesis of chronic/age-related diseases and disorders. COPD and pulmonary fibrosis affect millions across the globe and are responsible for several mortalities and morbidities per year [2]. Even though most of the studies have linked mitochondrial dysfunctions as a key contributor to the development and progression of these diseases, yet there is a lack of clear understanding of the mechanism of how these changes take place at a varied length of intervals. Smoking is one of the key causative factors which causes COPD and mitochondrial dysfunction in the lungs [4,11,12]. As smoke contains thousands of highly complex mixture of chemicals it is highly challenging to attribute the key toxic events to a particular chemical/dose apart from oxidative and carbonyl stresses. Nevertheless, CSE exposures are the most common and widely used methods to study the molecular mechanisms involved in COPD-related dysfunctions. In the current study, we used several validated *in vitro* and *in vivo* models to understand the importance of OPA1 related mitochondrial dysfunctions.

The striking difference can be observed in the levels of long and short OPA1 isoforms when the cells are exposed to acute CSE. This is reflected by the processing and changes in the levels of long to short isoforms. Recently, several key studies have attributed varied functional relevance for these isoforms [18–20,27]. Long isoforms are most important in the

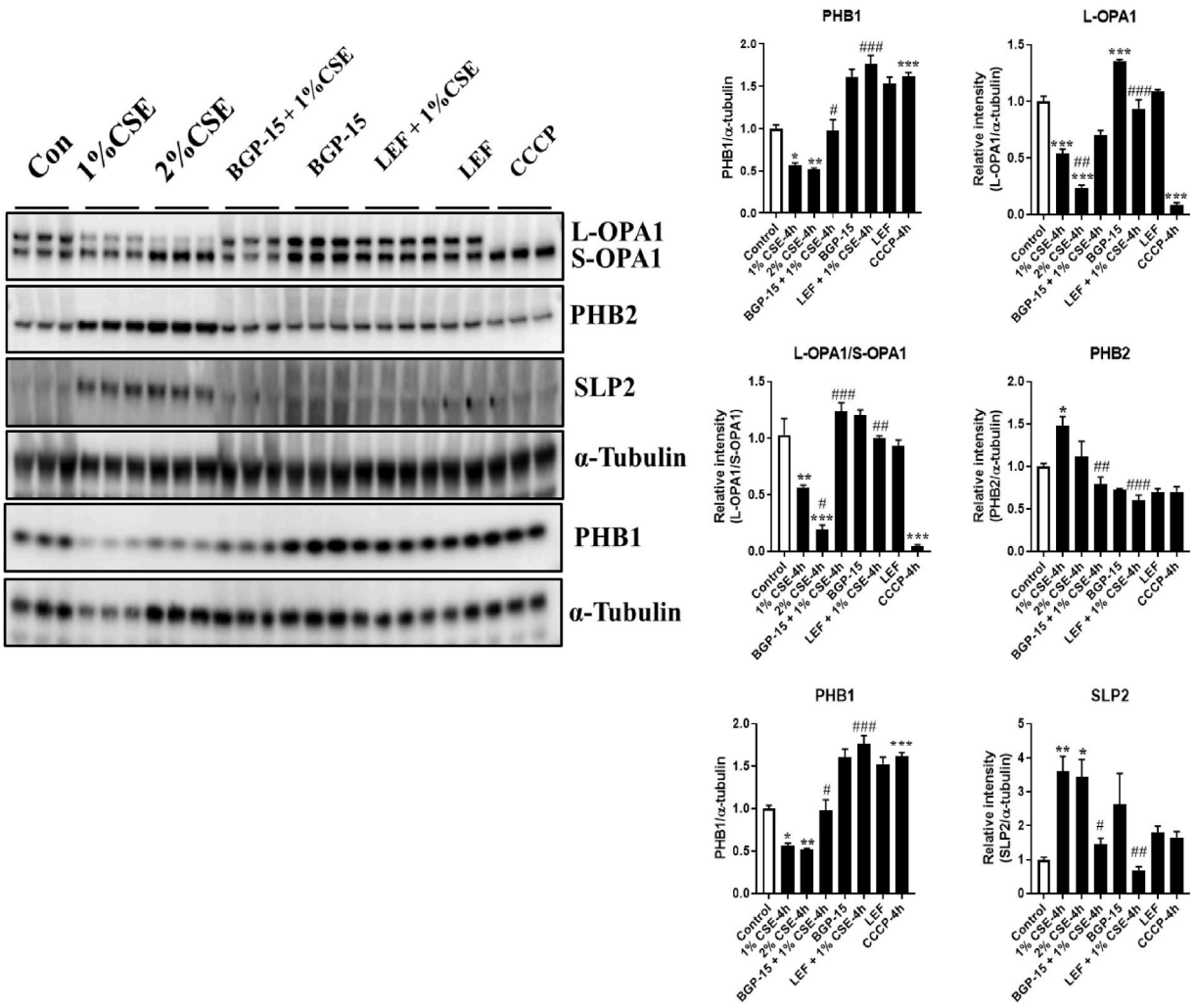

Fig. 8. Effect of various BGP-15 and leflunomide on CSE-induced protein alterations in BEAS2B cells. Immortalized human lung epithelial cells (BEAS2B) were pre-treated with different compounds that promote fusion through OPA1 (BGP-15) and MFN2 (leflunomide) for 24 h prior to 1 and 2% CSE exposure for 4 h. Cells were lysed and probed for different proteins as indicated. All the data were represented as mean ± SEM (n = 3/group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

fusion process, whereas short isoforms play an important role in cristae maintenance and energy homeostasis. However, previous reports claim changes in the OPA1 levels and much is not known about how smoking influences the isoform levels during the progression of COPD. The current study was aimed to understand this gap by using the strength of various acute and chronic *in vitro* and *in vivo* models of CS exposure.

OPA1 related interacting proteins include IMM proteases like OMA1, YME1L1 and PARL, SLP-2 and prohibitins, which are altered during cellular stress and activate many pathways to process and act on OPA1 [20,34–36]. Some of these related proteins were included in this study for their relative expression. SLP-2 is essential during stress-related mitochondrial hyperfusion [37], and is also an oncogenic, anti-apoptotic protein upregulated in cancers [38]. We found a similar and consistent increase of SLP-2 protein not only during acute CSE treatment, but also in the lung homogenates of COPD and IPF when compared to their respective controls. This highlights the importance of OPA1 associated pathways during the acute response and in chronic lung diseases. Moreover, most of these proteins were found to alter the drug treatment regime, especially OPA1, DRP1 and SLP2 were found to increase the resistance to the drugs during anti-cancer drug regime [27,38].

The current study provides an insight into the important directions involved in certain aspects of early stress responses in terms of acute CS exposure and its role in COPD. The study also employs recently reported drug modulators of fusion and fission, which we found to protect/maintain the lung epithelial cells against the CS-induced acute stress responses. BGP-15, a PARP inhibitor was recently reported to have OPA1 binding activity and can help in increasing the OPA1 levels, including the long isoform [26]. Thus, we included BGP-15 as an experimental compound to determine for its effect on OPA1 levels during CSE-induced acute stress conditions. Moreover, BGP-15 also has an additional PARP inhibitory activity, which is usually high in smokers and COPD subjects. BGP-15 was reported to enter phase II clinical trials for indicated use in type 2 diabetes mellitus. It was also proposed to be evaluated in inappropriate sinus tachycardia [39]. Among the other compounds, we used leflunomide, which is used clinically as an immunosuppressant and was recently found to promote MFN2 activity [25]. M1 compound was also reported to have fusion promoter activity in the MEFs [40]. Apart from the compounds showing effects on fusion activity, we also used one of the well-studied and reported (DRP1-induced) fission inhibitors Mdivi-1. Mdivi-1 was found to be superior in inhibiting the OXPHOS changes induced by CSE exposure, compared to other compounds. M1 compound also showed improvement in some of the OXPHOS related parameters. Neither BGP-15 nor leflunomide showed much improvement in any of the OXPHOS parameters measured in BEAS2B treated cells in the current study. This signifies and agrees with the role of fission as an early response during the CS exposure which is in accordance with the previous reports [14]. Nonetheless,

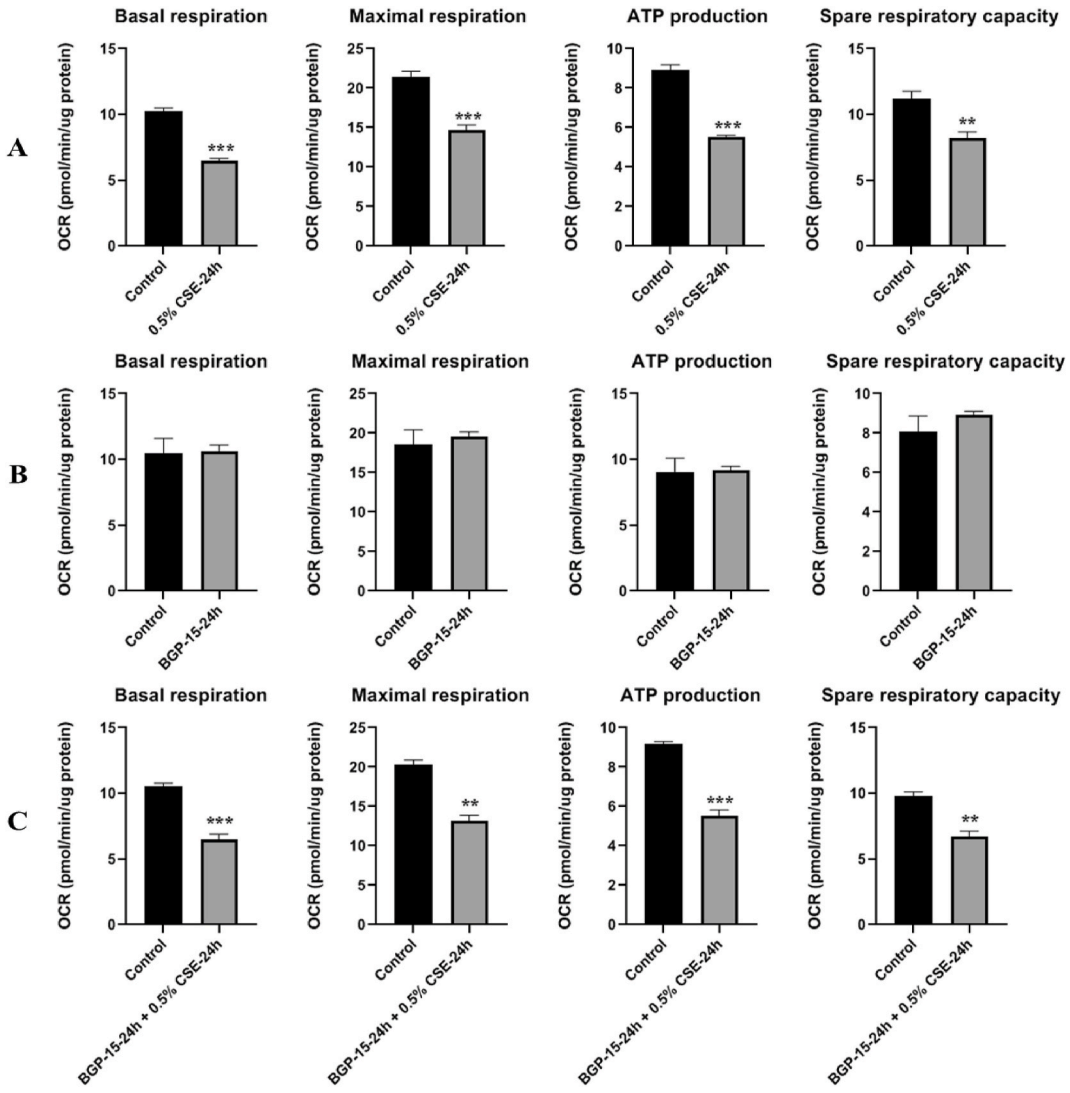

Fig. 9. Effect of various fusion promoters/activators (BGP-15, Leflunomide and M1 compound) and fission Drp1 inhibitor (Mdivi-1) in CSE-induced mitochondrial (OXPHOS) dysfunction using Seahorse XFp system with 3 wells for samples/group, n = 6 wells/group for 0.5% CSE-24h group. Around 20,000 BEAS2B cells/well were plated on a Seahorse flux analyzer plates and pre-treated with the compounds for 24 h prior to CSE exposure for an additional 24 h. The media was changed to the assay medium conditions 1 h prior to the readings. The graphs were represented along with the untreated control for each assay performed on the same day. A-G represents the data derived from the line graphs (a–g). All the data were normalized using protein and were represented as mean ± SEM. =$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

there are reports which show non-toxic stress response due to CSE induces mitochondrial elongation and also discusses the contrast/varied observation based on different cell types used in the study [41]. Apart from lung diseases, the most common inherited and observed optic neuropathies Dominant optic atrophy (DOA) and Leber hereditary optic neuropathy (LHON) are linked to the mutations in the mitochondrial related genes [42]. Among them, the DOA is associated with the mutations in the OPA1 and its isoforms [43]. Recent clinical evidence suggests that cigarette smokers are at relatively high risk for DOA and LHON and the smoke triggers these mitochondrial-associated genetic disorders [44,45]. Recent toxicity report indicates reduced OPA1 oligomerization or increase in OPA1 cleavage, which again signifies the importance of maintaining long isoforms for cellular homeostatis [46]. Furthermore, Lai et al., recently reported that restoring L-OPA1 during acute ischemic stroke improves the conditions by inhibiting neuronal apoptosis [47].

Even though BGP-15 and leflunomide do not affect OXPHOS parameters, they increased and maintained the L-OPA1 protein levels, which are more important in maintaining the length/fusion of the mitochondria rather than bio-energetics maintained by S-OPA1. This again emphasizes the need to determine and understand the role of several important mitochondrial proteins in CS-induced lung diseases. Among the OPA1 related and regulated proteins prohibitin, especially PHB1 was found to be decreased in smokers associated with COPD without any change in the levels of PHB2 [48]. On contrary, it was shown that acute lung injury induced by LPS was found to increase PHB levels [49]. Here, we have found that in acute CSE exposure PHB2 levels were increased, with decreased or no change in PHB1 expression in the cells. This highlights the necessity to further establish the differential role of prohibitins in acute and chronic lung exposure conditions.

NMR was found to have a differential mitochondrial response capability [31,32]. We sought out to determine the effect of CS on the NMR lung fibroblasts, which are widely used in cellular senescence and longevity studies. Since COPD is highly associated with lung cellular senescence, it can reveal a new angle to the current studies with CS. It was interesting to note that OPA1 levels were maintained in NMR lung

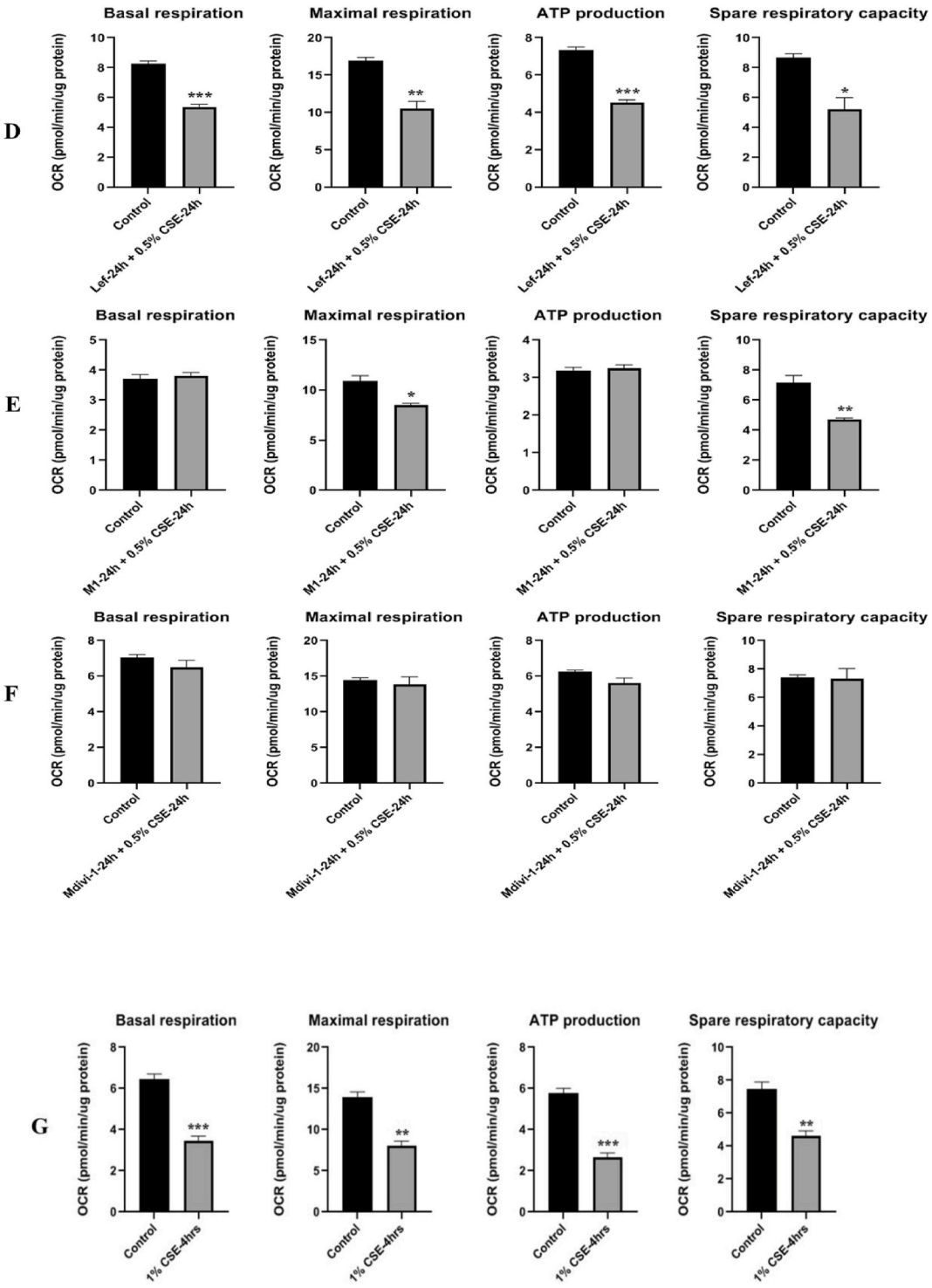

Fig. 9. (*continued*).

fibroblasts, with little processing of L-to S-OPA1 isoforms. While the rest of the proteins has a similar expression pattern compared to human lung and mouse embryonic fibroblasts. Future studies are required which are in progress to further understand the link of OPA1 with cellular senescence as they play a crucial role in mtDNA maintenance and mitochondrial energetics.

Overall, this study establishes the expression patterns of the OPA1 isoforms along with some of its interacting partners, which play a very crucial role in the maintenance of mitochondrial quality control and mitophagy (Fig. 10). Disruptions in any of these forms/interactions eventually lead to mitochondrial dysfunctions, which ultimately affect the overall lung health and its response towards the drugs used. Further studies are ongoing to understand these crucial relationships in COPD and fibrosis models with a major focus on the mitochondrial targets.

Author contributions

KPM and IR designed the experiments. IR sought funding for research. KPM performed, analyzed and interpreted all the experiments. All the animal exposure/experiments were done by IKS. KPM wrote the

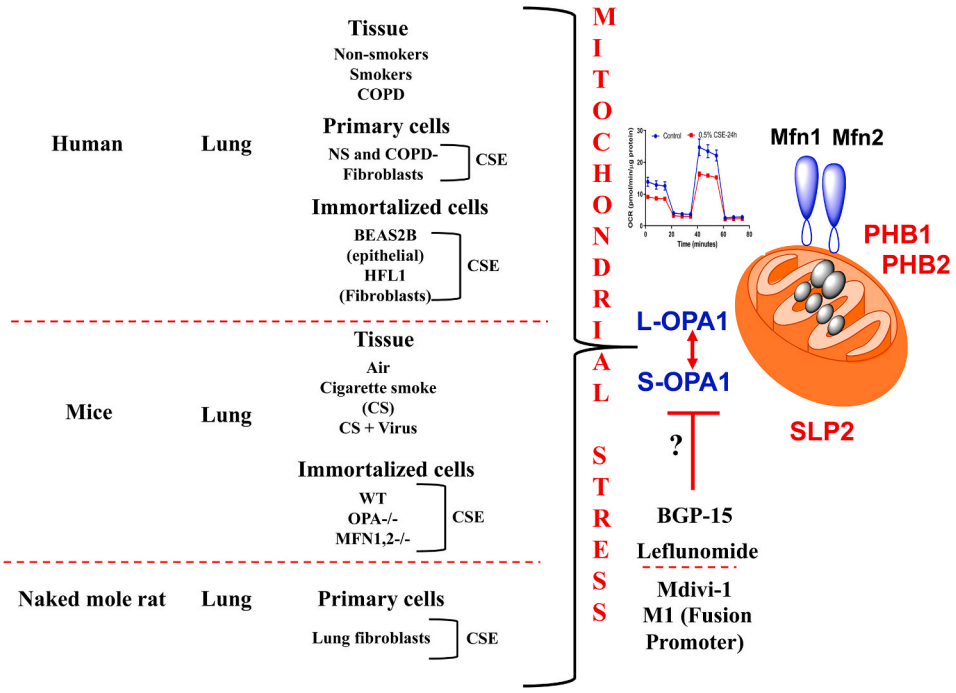

Fig. 10. Overall schematic for the involving of OPA1 in mitochondrial dysfunction by CS and in the pathogenesis of COPD. This schematic depicts the important role of OPA1 (fusion) interacting partners and their possible associations during acute vs. chronic cigarette smoke-induced mitochondrial dysfunction in the lungs. These coordinate events demonstrate the altered mitochondrial quality control mechanism (mitophagy) and mitochondrial dysfunction caused by smoking-induced lung injury *in vitro* and *in vivo*.

manuscript, IKS and IR interpreted, edited and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102055.

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