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Reduced DMPC and PMPC in lung surfactant promote SARS-CoV-2 infection in obesity

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ABSTRACT

Objective: Obesity is an established risk factor for higher SARS-CoV-2 viral loads, severe COVID-19 pneumonia requiring hospitalization, and worse outcomes. However, the underlying mechanisms for the increased risk are not well understood. SARS-CoV-2 is a respiratory virus with the primary route of entry through the lungs, where the Spike protein of SARS-CoV-2 binds to the ACE2 receptor on pneumocytes. Lung surfactant produced by type II pneumocytes plays a major role in respiratory defense against infections. Surfactant predominantly contains lipids, especially phosphatidylcholines (PC), and obesity is characterized by aberrant lipid metabolism. We hypothesized that altered lipid composition in lung surfactant in obesity may promote SARS-CoV-2 infection, leading to severe COVID-19 disease.

Methods: Lipidomic analysis of lung tissue and bronchoalveolar lavage fluid (BALF) was performed using LC-MS/MS. The effects of PCs on SARS-CoV-2 pseudovirus infection were studied in HEK293T cells with ACE2 overexpression and in Vero-E6 cells with endogenous ACE2 expression. For the cell-cell fusion assay, HEK293T-ACE2 and HEK293T expressing SARS-CoV-2 Spike/eGFP were used as the target and effector cells, respectively.

Results: Lipidomic analysis revealed that myristic acid-containing dimyristoyl-PC (DMPC) and palmitoylmyristoyl-PC (PMPC) were reduced in lung tissue and BALF from high fat diet-induced obese mice. DMPC and PMPC markedly inhibited wild type and D614G mutant SARS-CoV-2 infection in HEK293T-ACE2 and Vero-E6 cells. Feeding obese mice with trimyristin, the triglycerides of myristic acid, increased DMPC and PMPC levels in lung surfactant. Lipid extract from BALF of trimyristin-treated obese mice mitigated the elevated wild type and D614G mutant SARS-CoV-2 infection. The inhibitory effects of DMPC and PMPC on SARS-CoV-2 infection were reversed by cholesterol.

Conclusions: The reduced DMPC and PMPC in lung surfactant may promote SARS-CoV-2 infection. Increasing DMPC and PMPC in lung surfactant could be an innovative strategy for preventing and treating severe COVID-19 disease in obesity.

1. Introduction

The ongoing COVID-19 pandemic caused by SARS-CoV-2 infection has led to more than 6 million deaths worldwide [1,2]. Although it is well documented that obesity is a major risk factor for higher SARS-CoV-2 viral load, severe COVID-19 disease, and worse outcomes [3–5], the underlying molecular mechanisms are not well understood. The proinflammatory status, impaired immune response, thrombogenic responses to pathogens, and dysregulation of the renin-angiotensin system may be involved [3–5], but experimentally validated evidence for these mechanisms is limited.

COVID-19 is primarily a respiratory disease, and the initial viral

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entry is through the respiratory tract. The lungs are constantly exposed to microbes from aspiration. During evolution, the lung has developed dedicated defense systems to prevent infections. Surfactant produced by type II pneumocytes is a frontline of the lung host defense system [6]. Lung surfactant is a complex mixture of lipids and proteins, with approximately 90% lipids, predominantly phosphatidylcholines (PCs), and 10% proteins by mass [7,8]. The main function of surfactant is to reduce surface tension at the air-liquid interface to prevent alveolar collapse. Surfactant also plays a key role in host defense against infection and inflammation. The lipophilic lung surfactant fraction has antimicrobial and anti-inflammatory properties when applied intratracheally to the lung as well as topically onto the skin [9,10].

Since obesity is characterized by aberrant lipid metabolism, we reasoned that altered lipid composition in lung surfactant may weaken its defense against SARS-CoV-2 infection. Our lipidomic analysis of lung tissue and surfactant revealed that dimyristoyl-phosphatidylcholine (DMPC) and palmitoylmyristoyl-phosphatidylcholine (PMPC) were reduced in obese mice. Importantly, DMPC and PMPC inhibited wild type and D614G mutant SARS-CoV-2 infection. Thus, increasing DMPC and PMPC in lung surfactant may reduce SARS-CoV-2 infection and prevent severe disease of COVID-19.

2. Materials and methods

2.1. Mouse studies

The obese mouse model was generated by feeding C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME, USA) with a high-fat diet (HFD) for 12 weeks starting from 8 weeks of age [11,12]. For the trimyristin treatment, obese mice were fed the HFD containing 5%(wt/wt) trimyristin (J62819, Alfa Aesar, Tewksbury, MA, USA) for two weeks. Glucose and insulin tolerance tests were performed as described previously [11,12]. All mouse studies were conducted in accordance with federal guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.2. Lipidomic analysis

The nontargeted lipidomic analysis of lung tissue and BALF was performed as described, and the data were processed using MS-DIAL (v. 2.78) software program [13]. The targeted lipid measurements used DMPC, PMPC and DPPC from Avanti as standards.

2.3. Generation of SARS-CoV-2 pseudovirus

Two types of SARS-CoV-2 pseudovirus particles were generated for studying Spike-mediated viral infection. The first type was based on the single-cycle lentiviral system using plasmids obtained from BEI Resources repository [14]. The second type of pseudotyped SARS-CoV-2 used the single-cycle vesicular stomatitis viruses (scVSVs) system [14,15]. The mutant D614G SARS-CoV-2 Spike gene was obtained from Addgene (158075).

2.4. Measurements of SARS-CoV-2 pseudovirus infection

HEK293T cells with stable overexpression of ACE2 (HEK293T-ACE2) were kindly provided by Dr. Jesse D. Bloom [14]. Vero E6 cells were obtained from ATCC (CRL-1586). SARS-CoV-2 pseudoviruses with different concentrations of DMPC, PMPC and DPPC dissolved in ethanol were inoculated to HEK293-ACE2 or Vero E6 cells. After 24 h of incubation, SARS-CoV-2 viral infection rates were detected by GFP positive cells or measured by luciferase activity (E1501, Promega, Madison, WI, USA). In the experiments using cholesterol, cells were pre-incubated with cholesterol for 2 h before SARS-CoV-2 pseudovirus and lipids were added.

2.5. Cell–cell fusion assay

Cell–cell fusion assays were performed as described previously [16,17]. Briefly, the target HEK293T cells were transfected with 2019-nCov_pcDNA3.1(+)-P2A-eGFP plasmid (from Haisheng Yu and obtained from Genscript (Piscataway, NJ, USA)), resulting in simultaneous expression of spike protein and eGFP in the same cell. HEK293T-ACE2 cells were used as target cells. The same amount of target and effector cells (1×10^4) were mixed and co-cultured in DMEM with or without lipids for 48 h in the 96-well plate. The cell-cell fusion rate was counted from five randomly selected fields under the fluorescence microscope.

2.6. Statistical analyses

GraphPad Prism software (La Jolla, CA, USA) was used to analyze statistical differences. The two-tailed student's *t*-test was used to compare two groups, and the analysis of variance (ANOVA) test was used to compare three or more groups. Data were displayed as the mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Lipidomic analysis of lung tissue and BALF in obesity

HFD feeding markedly increased body weight, glucose, cholesterol and triglyceride levels, and impaired glucose and insulin tolerance (Supplementary Fig. 1A–F). BALF lipids from obese mice enhanced SARS-CoV-2 infection compared with lipids from lean control mice (Fig. 1A) in both HEK293T with ACE2 overexpression and Vero E6 cells with endogenous ACE2 expression. To identify potential lipids that promote SARS-COV-2 infection in obesity and diabetes, we performed nontargeted lipidomic analysis of lung tissue and BALF from obese and control lean mice [18]. The Principal Component Analysis showed that clustering of lipid profiles from obese and lean mice was readily separated under either the positive or negative ion mode (Supplementary Fig. 2A–D). HFD feeding significantly altered 95 lipids in the lung and 37 lipids in BALF compared with controls (Supplementary Fig. 2E–F). Among the altered lipids, 8 were commonly reduced in lung and BALF, while none was commonly increased (Fig. 1B). The eight reduced lipids included two PCs (14:0–14:0; 14:0/16:0), four PGs (16:0–22:6; 16:0–16:1; 14:0–16:0; 16:0–20:5), one PI (16:0–22:6) and one PE (16:0–16:1) (Fig. 1B). Since PMPC (14:0/16:0) is relatively abundant in BALF [19,20] and another myristic acid containing PC (DMPC, 14:0–14:0) was one of the most reduced lipids in obese BALF, we performed targeted lipidomic studies using the standard compounds. We confirmed that DMPC and PMPC, but not the most abundant DPPC, were reduced in BALF of diet-induced obese mice (Fig. 1C–E). Lysophosphatidylcholine acyltransferases (LPCAT1–4) were not altered in the lung of diet-induced obese mice (Supplementary Fig. 3), suggesting the Land cycle is not involved in regulating DMPC and PMPC levels [21].

We then tested the effects of DMPC and PMPC on SARS-CoV-2 infection using the pseudotyped virus system. Both DMPC and PMPC inhibited SARS-CoV-2 pseudovirus infection in HEK293T-ACE2 cells in a dose-dependent fashion. DMPC appeared to be more potent than PMPC (Fig. 1F–G), while DPPC had no effects on SARS-CoV-2 infection (Fig. 1H). The pseudotyped SARS-CoV-2 also expressed GFP as a marker for virus infection. DMPC and PMPC, but not DPPC clearly reduced positive GFP cells (Fig. 1I). Neither DMPC nor PMPC affected pseudotyped vesicular stomatitis virus (VSV) infection (Fig. 1J), suggesting DMPC and PMPC specifically inhibited SARS-CoV-2 infection.

Similarly, DMPC and PMPC, but not DPPC inhibited SARS-CoV-2 infection in Vero E6 cells with endogenous ACE2 expression (Fig. 1K–M). D614G, the common mutation of the SARS-CoV-2 Spike protein in alpha, beta, gamma, and delta SARS-CoV-2 variants, has been reported to enhance virus replication and transmission [22,23]. DMPC

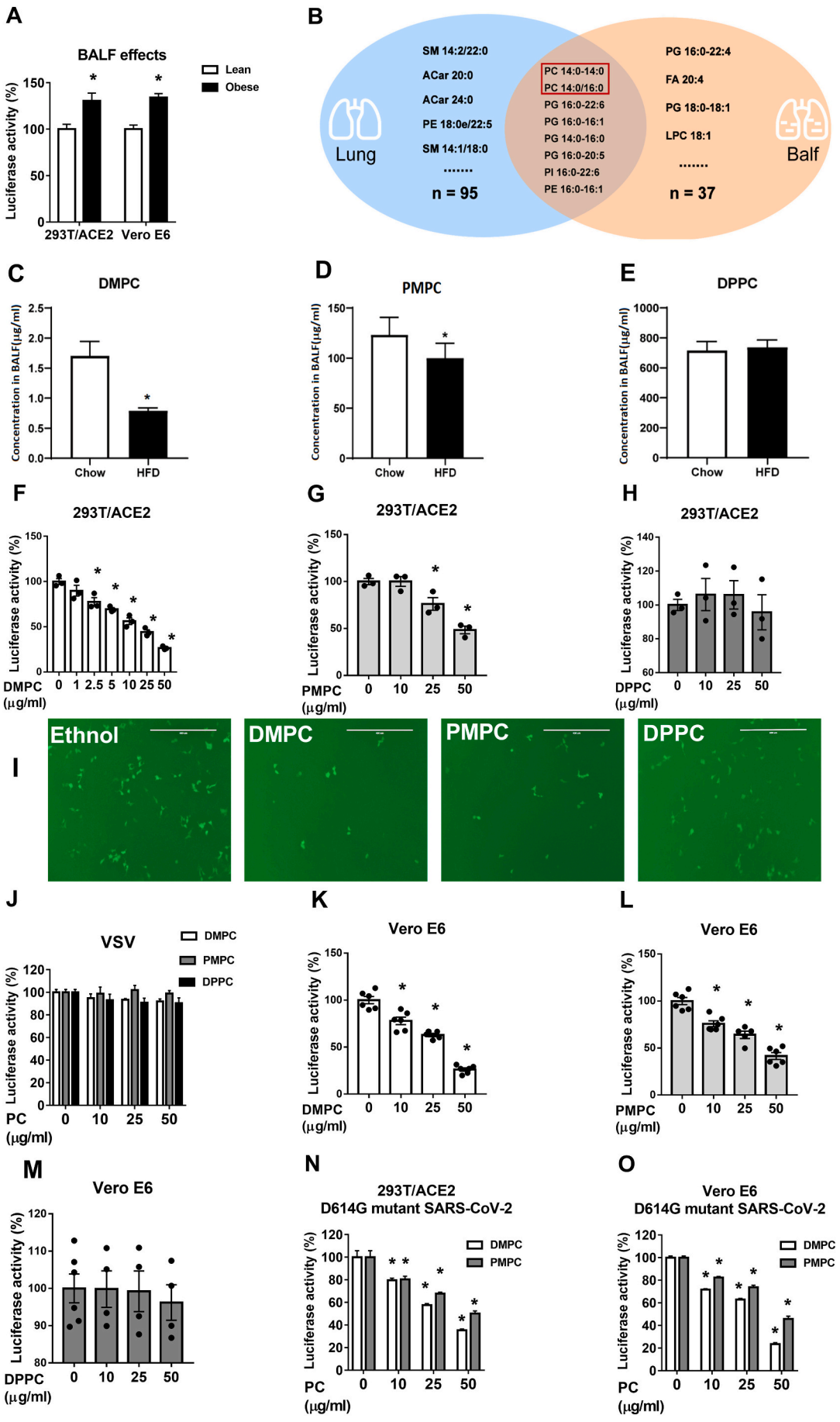

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Fig. 1. A. BALF lipids (25 μg/ml) from diet-induced obese mice enhance pseudotyped SARS-CoV-2 infection compared with lipids from lean control mice in both 293T/ACE2 and Vero E6 cells (n = 6). The pseudotyped SARS-CoV-2 virus particles are generated by transfecting Lenti-X™ 293T cells with plasmids obtained from BEI Resources repository: SARS-CoV-2 Spike (NR-52514), HDM-Hgpm2 (NR-52517), pRC-CMV-Rev1b (NR-52519), HDM-tat1b (NR-52518), and Luciferase-IRES-ZsGreen (NR-52516). The Spike protein on the pseudotype virus serves as a ligand for viral internalization into HEK293T and Vero E6 with ACE2 expression. The luciferase activity is measured as a surrogate for the viral infection rates. B. Commonly altered lipids in lung and BALF of diet-induced obese and control lean mice. C–E. DMPC, PMPC, and DPPC levels measured by targeted lipidomics in BALF from chow or HFD-fed mice (n = 6). F–H. DMPC (F) and PMPC (G), but not DPPC (H), inhibit SARS-CoV-2 pseudovirus infection in 293T/ACE2 cells. The viral infection rate is measured by luciferase activity (n = 3). I. Representative images for the effects of DMPC, PMPC, and DPPC on the infection of SARS-CoV-2 pseudovirus harboring GFP in 293T/ACE2 cells (scale bars, 400 μm). The pseudotyped SARS-CoV-2 viral particles express ZsGreen reporter, allowing the visualization of GFP fluorescence as an indicator for the viral infection. DMPC, PMPC, or DPPC lipids are dissolved in ethanol, and the same volume of ethanol was used as a control. J. Effects of DMPC, PMPC, and DPPC on pseudotyped vesicular stomatitis virus (VSV) infection in 293T/ACE2 cells. K–M. DMPC (K) and PMPC (L), but not DPPC (M), inhibit SARS-CoV-2 infection in Vero E6 cells (n = 4–6). N–O. DMPC and PMPC inhibit the infection of SARS-CoV-2 pseudovirus harboring Spike D614G mutation in 293T/ACE2 (N) and Vero E6 (O) cells (n = 6). *p < 0.05.

and PMPC also potently inhibited infection of SARS-CoV-2 pseudovirus harboring Spike D614G mutation in HEK293T-ACE2 and Vero E6 cells (Fig. 1N–O). Thus, DMPC and PMPC inhibit SARS-CoV-2 infection, which provides initial evidence that reduced DMPC and PMPC in BALF may contribute to the increased SARS-CoV-2 infection in obesity and diabetes.

3.2. Increasing DMPC and PMPC in BALF inhibits SARS-CoV-2 infection

We designed two experiments to investigate whether increasing DMPC and PMPC in BALF may inhibit SARS-CoV-2 infection. First, since DMPC and PMPC were reduced in BALF of obese mice, we added DMPC and PMPC to BALF from obese mice and studied the effects on SARS-CoV-2 infection. In both HEK293T-ACE2 and Vero E6 cells, the addition of DMPC and PMPC to BALF from obese mice reversed the increased SARS-CoV-2 infection (Fig. 2A–B). In the second approach, we treated the HFD-fed obese mice with trimyristin, triglycerides of C14:0 myristic acid, and a precursor for BALF DMPC and PMPC [20]. Two weeks of trimyristin treatment did not alter body weight or glucose levels (Fig. 2C–D). BALF DMPC and PMPC were increased by 8.04 and 1.87 folds, respectively, while DPPC was reduced in the trimyristin-treated obese mice (Fig. 2E–G). BALF from HFD-fed obese mice treated with trimyristin reversed the increased SARS-CoV-2 infection in HEK293T-ACE2 and Vero E6 cells (Fig. 2H–I). Importantly, trimyristin treatment also inhibited D614G mutant SARS-CoV-2 infection (Fig. 2J). These results suggest that increasing BALF DMPC and PMPC may be a promising therapeutic strategy for preventing and treating SARS-CoV-2 infection.

The SARS-CoV-2 virus uses its Spike protein to bind ACE2 receptor on the cell surface to enter target cells. However, DMPC and PMPC did not affect Spike-ACE2 interaction (Supplementary Fig. 4). Cholesterol comprises 30 mol% of lipids in the cell membrane and plays an essential role in mediating SARS-CoV-2 entry to target cells [24–26]. Phosphatidylcholines are also the abundant phospholipids in the mammalian cell membrane [24,25]. We reasoned that DMPC and PMPC may alter cell membrane cholesterol abundance to inhibit SARS-CoV-2 infection. Indeed, cholesterol dose-dependently reversed the inhibitory effects of DMPC and PMPC on SARS-CoV-2 infection in both HEK293T-ACE2 and Vero E6 cells (Fig. 2K–L). The role of cholesterol in regulating the inhibitory effects of DMPC and PMPC on SARS-CoV-2 infection is further studied in the standard cell-cell fusion assay, in which HEK293T cells expressing Spike-eGFP fusion protein serve as target cells, and HEK293T-ACE2 cells work as effector cells [16]. The interaction between Spike and ACE2 fuses the target and effector cells, resulting in syncytia formation [17,25]. DMPC and PMPC disrupted the cell fusion between Spike and ACE expressing HEK293T. The effects were partially reversed by the addition of cholesterol (Fig. 2M–N). These results suggest that DMPC and PMPC may inhibit SARS-CoV-2 infection by replacing cell membrane cholesterol.

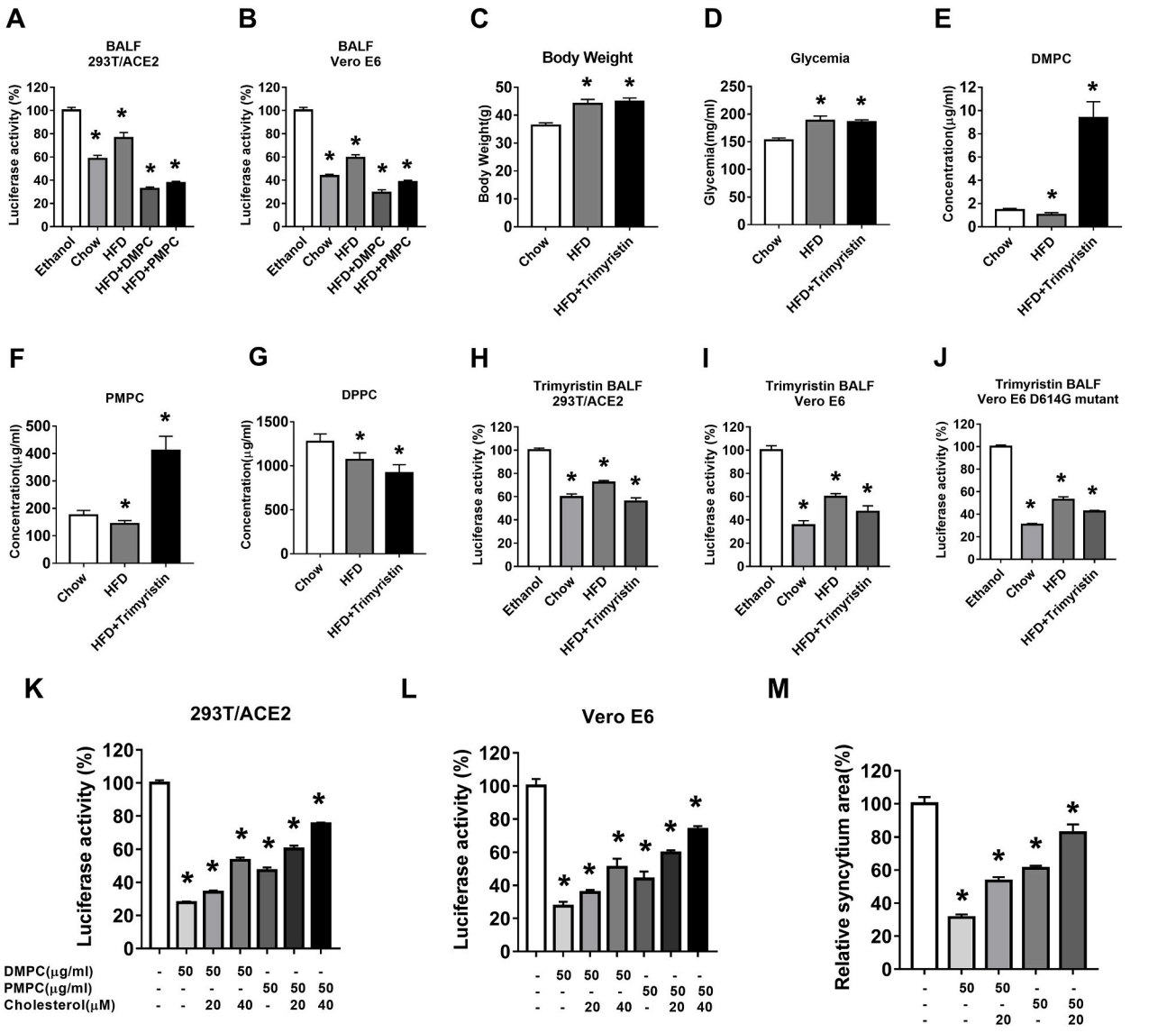
4. Discussion

Since the outbreak of COVID-19 pandemic, epidemiological studies have provided convincing evidence that obesity is associated with higher SARS-CoV-2 viral loads, severe COVID-19 disease, and worse outcomes [3–5]. However, the underlying molecular mechanisms are not well understood. Lung surfactant is a frontliner of host defense against infections. Since obesity is characterized by aberrant lipid metabolism and lung surfactant contains 90% lipids, we hypothesized that altered lipid composition in lung surfactant may impair its protective effects on SARS-CoV-2 infection. Indeed, our lipidomic analysis shows that lung and surfactant DMPC and PMPC are reduced in obesity, which may contribute to the increased SARS-CoV-2 infection and severe COVID-19 disease.

SARS-CoV-2 viruses infect target cells primarily through the interaction between viral surface spike protein and target cell membrane ACE2 [27,28]. The interaction triggers the membrane fusion between virus and target cells, leading to virus internalization. Our data show that DMPC and PMPC do not directly interfere with Spike-ACE2 interaction. Cell membrane cholesterol contents are necessary for membrane fusion and SARS-CoV-2 viral entry. Depleting cellular cholesterol blocks SARS-CoV-2 infection while adding cholesterol enhances SARS-CoV-2 entry to cultured cells [24,29,30]. Multiple clinical studies show that statin use is associated with a reduced risk of developing severe COVID-19 [31,32]. Our data show that cholesterol reversed the inhibitory effects of DMPC and PMPC on membrane fusion, suggesting DMPC and PMPC may deplete or displace membrane cholesterol to inhibit SARS-CoV-2 infection. It is worth noting that cholesterol levels are often increased in obesity. The combination of cholesterol elevation and surfactant DMPC/PMPC reduction in obesity could synergistically enhance SARS-CoV-2 viral infection.

The inhibitory effects of DMPC and PMPC on SARS-CoV-2 infection offer several potential therapeutic options for preventing or treating COVID-19 in obesity. We tested two approaches to increase DMPC and PMPC in the lung surfactant. In the first approach, adding DMPC or PMPC to BALF lipids extracted from obese mice reversed the increased SARS-CoV-2 infection. Natural surfactants from bovine or porcine lungs are FDA-approved drugs for treating pediatric and neonatal acute respiratory distress syndrome (ARDS) [33]. Although natural surfactants are not effective in treating ARDS in adults, DMPC and/or PMPC could be added to the natural surfactants with aerosolized/nebulized surfactant preparations for treating COVID-19, especially at the early phase, to prevent viral entry to cells. In the second approach, treating obese mice with trimyristin increased BALF DMPC and PMPC and inhibited SARS-CoV-2 infection. Potentially, trimyristin could be used as a therapeutic agent to prevent or treat SARS-CoV-2 infection in obese patients. An alternative method is to treat bovine or porcine to increase DMPC and PMPC in the natural surfactants, which can be used for preventing SARS-CoV-2 infection.

The current study has several limitations. Although lipidomic analyses of pulmonary surfactant in humans with asthma, cystic fibrosis and ARDS are reported [34,35], the phospholipid profiles in human obesity have not been studied likely because it is too invasive to obtain lung surfactant for lipidomic analysis from obese patients. Future studies using obese primates will be informative. Trimyristin treatment of obese mice increases DMPC and PMPC in BALF, but we cannot exclude a possibility that other lipids may be involved in inhibiting SARS-CoV-2



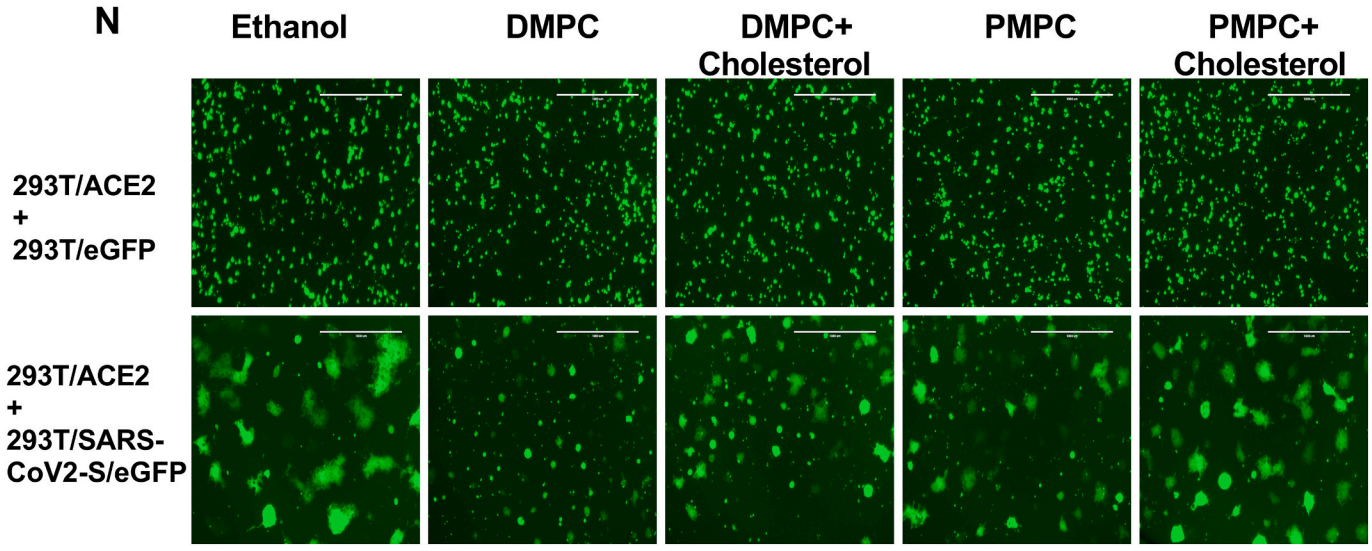


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Fig. 2. A–B. Adding DMPC or PMPC to BALF lipid extraction from obese mice reverses the elevated SARS-CoV-2 infection in 293T/ACE2 (A) and Vero E6 cells (B) (n = 4). C–D. Bodyweight (C) and glucose levels (D) in mice fed with chow, HFD, or HFD with trimyristin for two weeks (n = 5–10). E–G. Trimyristin treatment increases DMPC (E) and PMPC (F) but reduces DPPC (G) in BALF of HFD-fed obese mice (n = 5–8). H–I. BALF lipids from trimyristin-treated obese mice reverse the elevated SARS-CoV-2 infection in 293T/ACE2 (H) and Vero E6 cells (I) (n = 5). J. BALF lipids from trimyristin-treated obese mice reverse the elevated D614G mutant SARS-CoV-2 infection in Vero E6 cells (n = 5). K–L. Cholesterol dose-dependently reverses the inhibitory effects of DMPC and PMPC on SARS-CoV-2 infection in 293T/ACE2 (K) and Vero E6 cells (L) (n = 4). HEK293T-ACE2 or Vero E6 cells are pre-incubated with cholesterol for 2 h. Culture media containing cholesterol are removed, and cells are washed with PBS before SARS-CoV-2 pseudovirus and lipids are added. After 24 h of incubation, SARS-CoV-2 viral infection rates are measured by luciferase activity. M. DMPC and PMPC disrupt cell fusion between Spike and ACE2-expressing HEK293T, and the effects are reversed by the addition of cholesterol (n = 5). *p < 0.05. N. Representative cell fusion assay showing cholesterol reverses the DMPC and PMPC-inhibited cell fusion between Spike-eGFP and ACE2-expressing HEK293T cells (scale bars, 1000 μm). Upper panels: HEK293T cells expressing eGFP are co-cultured with HEK293T-ACE2 cells with or without indicated lipids for 48 h. No cell fusion occurs because Spike protein is not expressed. Lower panels: HEK293T cells expressing Spike-eGFP fusion protein (SARS-CoV2-S/eGFP) are co-cultured with HEK293T-ACE2 cells with or without indicated lipids. The Spike-ACE2 interaction leads to cell membrane fusion and significant syncytia formation in the ethanol-treated group. DMPC and PMPC inhibit syncytia formation, and cholesterol partially reverses the inhibitory effects.

infection. Finally, data interpretation should be limited to males because only male mice are used in this study.

In summary, our lipidomic analysis reveals that lung and surfactant DMPC and PMPC are reduced in obesity. Importantly, DMPC and PMPC inhibit both wild-type and D614G mutant pseudotyped SARS-CoV-2 infection, suggesting the reduced surfactant DMPC and PMPC may contribute to a higher viral load and potentially severe COVID-19 in obesity. Increasing DMPC and PMPC in lung surfactant may be an attractive and affordable strategy to prevent the development of severe COVID-19, especially in obese patients.

CRediT authorship contribution statement

J.S. and Q.Y. conceived and designed the project. K.D. and L.S. performed the majority of the experiments. Z.L., Y.C., Q.S., K.Z., and A. F. helped with some specific experiments. D.P. and X.L. advised the project. K.D. and Q.Y. wrote the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2022.155181.

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