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Letter

Mitochondrial Dysfunction: A Prelude to Neuropathogenesis of SARS-CoV-2

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ABSTRACT: The SARS-CoV-2 virus is notorious for its neuro-invasive capability, causing multiple neurological conditions. The neuropathology of SARS-CoV-2 is increasingly attributed to mitochondrial dysfunction of brain microglia cells. However, the changes in biochemical content of mitochondria that drive the progression of neuro-COVID remain poorly understood. Here we introduce a Raman microspectrometry approach that enables the molecular profiling of single cellular organelles to characterize the mitochondrial molecular makeup in the infected microglia cells. We found that microglia treated with either spike protein or heat-inactivated SARS-CoV-2 trigger a dramatic reduction in mtDNA content and an increase in phospholipid saturation levels. At the same time, no significant changes were detected in Golgi apparatus and in lipid droplets, the organelles that accommodate biogenesis and storage of lipids. We hypothesize that transformations in mitochondria are caused by increased synthesis of reactive oxygen species in these organelles. Our findings call for the development of mitochondria-targeted therapeutic approaches to limit neuropathology associated with SARS-CoV-2.

KEYWORDS: *Microglia, mitochondria, ROS, SARS-CoV-2, neuro-COVID, Raman spectrometry*
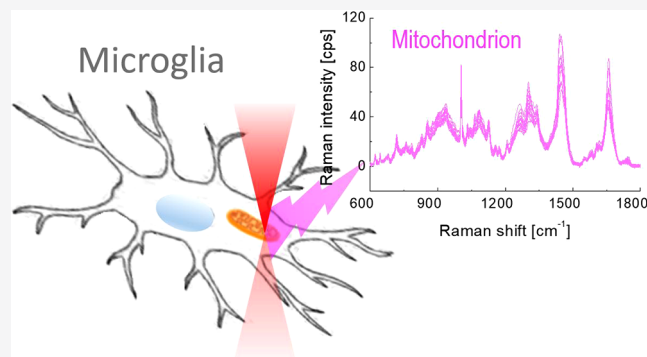
INTRODUCTION

A significant number of COVID-19 patients develop neurological symptoms, attributed to viral encephalitis, resulting in neuroinflammation, neuronal damage, and neurocognitive impairment. The microglia, which are the resident macrophages in the central nervous system, are the major players in the brain's immune response to SARS-CoV-2 infection. Furthermore, it has been shown that functional mitochondria are integral to initiation and maintenance of immune responses by microglia, while neurological damage in COVID patients is attributed to mitochondrial dysfunction. Mitochondria are the primary site of ATP production and also regulate basic metabolic functions and participate in homeostasis, cellular proliferation, and apoptosis as well as in the synthesis of amino acids, lipids, and nucleotides. In microglia these organelles also mediate the antiviral immune response by releasing pro-inflammatory cytokines, which limit viral survival and viral replication and trigger inflammation.[1−4] Strikingly, SARS-CoV-2 can evade the innate immune response of host cells via the modulation of mitochondrial functions. The spike protein of SARS-CoV-2 binds to the angiotensin-converting enzyme-2 (ACE-2) receptor on the human host cell[3] to enter the host, and the transmembrane serine protease 2 (TMPRESS 2) facilitates this attachment by priming the spike protein.[5] Notably, the ACE-2 receptor regulates mitochondrial function.[6] Reduced expression of ACE-2 is correlated with

decreased ATP synthesis and activation of NADPH oxidase 4, which contributes to the production of reactive oxygen species (ROS).[4] Consistent with that, an invasion of SARS-CoV-2 via the ACE-2 receptor compromises mitochondrial regulation. Excessive ROS production exacerbates neuro-inflammation, initiating apoptosis in infected cells, which results in neurocognitive impairments. It is known that SARS-CoV-2 infection results in a massive inflammatory response in the brain by triggering the release of cytokines such as interleukin (IL)-10, TNF-α, and INF-γ, which in turn further increase mitochondrial ROS production through upregulation of mitochondrial genes and modulation of the electron transport chain (ETC).[7] The mitochondrial ROS stimulate additional proinflammatory cytokine production[8] in the face of viral persistence, leading to a "cytokine storm syndrome", which underlies viral encephalopathy.[7] We recently observed an increased oxygen consumption rate (OCR) in microglial cells treated with SARS-CoV-2 spike protein.[9] Our data suggested that SARS-CoV-2 induced a robust inflammatory

response, significantly increasing oxidative stress and OCR, all of which contributed to neuroinflammation and associated neuropathology of an encephalitic coronavirus infection.

In order to evade host cell immunity and facilitate virus replication, SARS-CoV-2 viral open reading frame (ORF) 9b localizes in mitochondria and can directly modulate mitochondrial function, thereby contributing to COVID-19 disease progression.[4]

Thus, we hypothesize that modulating mitochondrial activity may prevent mitochondrial dysfunction following SARS-CoV-2 infection and that mitochondria-targeted pharmacological interventions may enhance an immune response in SARS-CoV-2 associated neuropathogenesis.

Toward verification of this hypothesis, we analyzed the molecular composition in the mitochondria of infected cells. It is worth noting that characterization of the mitochondria metabolic variations by standard biochemical approaches is extremely challenging. Traditional molecular profiling approaches rely on cellular fractioning and extraction of the analyte protein or lipid molecules from the studied organelles, which is a cumbersome procedure that is prone to contamination. Additionally, the molecular extraction approach inherently produces data averaging, thus masking heterogeneity between organelles obtained from different cells.

Remarkably, the capabilities of biochemical analysis have been recently expanded with optical biosensing tools. Raman spectrometry, one of the most valuable biosensing technologies, relies on analysis of molecular vibrational spectra and enables the identification of diverse molecular groups in biological samples. Because of their inherently noninvasive properties and independence from labels, Raman-based techniques have opened new dimensions in systemic studies of cells and tissues.[10,11] The recently developed biomolecular component analysis (BCA) of Raman spectra enables selective detection and concentration measurements of the major categories of biomolecules, including lipids, proteins, nucleic acids, and saccharides,[12,13] in the studied samples. The high three-dimensional resolution available in modern confocal Raman spectrometry setups has been validated for characterization of microscopic subcellular structures, such as single organelles, including the identification of abnormal biomolecular signatures associated with disease progression.[14−20]

In this study, we employed Raman spectrometry together with the BCA algorithm to characterize the changes in the molecular composition of mitochondria in response to treatment with heat-inactivated SARS-CoV-2 or the SARS-CoV-2 spike protein. In addition, we studied key organelles involved in lipid metabolism: Golgi apparatus (GA) and lipid droplets (LD). The roles lipids play in viral infection include viral endocytosis and exocytosis, viral entry into the host cell via membrane fusion, and viral replication, and therefore, we were interested in potential changes of the lipid signatures in these organelles.

Our data indicate that infection with SARS-CoV-2 causes mitochondrial dysfunction in microglia cells, which triggers metabolic alterations that result in a substantial increase in glycolysis.[9] These findings suggest that a metabolic switch to glycolysis compensates for mitochondrial dysfunction and an energy deficit in microglia and that a consequence of this metabolic change is an enhanced inflammatory response that contributes to neuropathology associated with COVID-19. At the same time, the molecular content of GA and LD was not significantly changed, apparently because of the lack of specific

interactions between these organelles and the components of SARS-CoV-2.

Overall, our findings support a view that viral infection of host cells results in higher metabolic alterations to cope with the increased anabolic demand of the cell for viral replication. Furthermore, SARS-CoV-2-induced manipulation of the host-cell metabolic machineries alters transcriptional regulation of key metabolic pathways.

■ METHODS

Cell Culturing and Sample Preparation. Human microglia cells (HMC3) were obtained from ATCC (cat. no. ATCC CRL-3304) and grown in luminescence-free 35 mm glass-bottom dishes (Fisher Scientific Co., Hanover Park, IL). The culture medium used was Eagle's Minimum Essential Medium (EMEM) (cat. no. ATCC 30-2003) supplemented with 5% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin, and the cells were grown to 70% confluence at 37 °C in a humidified atmosphere containing 5% CO_2. The mitochondria and GA were labeled with MitoTracker Green FM and NBD C6 ceramide-BSA (Thermo Fisher Scientific), respectively, as per the manufacturer-provided protocols. After labeling, the cells were thoroughly washed in sterile phosphate-buffered saline (PBS).

The cells were treated with the following viral constructs: 0.5 μg/mL recombinant spike protein from SARS-related Coronavirus 2 Wuhan-Hu-1 (BEI Resources Inc., cat. no. NR-52308, lot no. 70034410) or 5 μL/mL heat-inactivated SARS-Coronavirus 2 (HI-SARS), isolate USA-WA1/2020, (BEI Resources Inc., cat. no. NR-52286, lot no. 70033548, pre-inactivation titer by $TCID_{50}$ assay in Vero E6 Cells = 1.6×10^5 $TCID_{50}$/mL), as specified.

To target acquisition of Raman spectra to specific organelles, the mitochondria, endoplasmic reticulum (ER), and GA were labeled using MitoTracker Green FM, ER-Tracker Green, and NBD C6 ceramide-BSA (ThermoFisher Scientific), respectively, as per the manufacturer-provided protocols. Then the cells were thoroughly washed in sterile PBS, and Raman spectra were acquired in the labeled organelles.

The Raman Microscope. The spectra were measured on a DXR2 Raman microscopy setup (Thermo Fisher Scientific, Madison, WI), equipped with a laser source unit emitting ~60 mW at 633 nm (ROUSB-633-PLR-70-1, Ondax), a 50 μm pinhole to shape the laser beam to a 0.7 μm × 0.7 μm × 1.5 μm full width at half-maximum (fwhm), and a Plan N 100× Olympus objective lens (NA = 1.25). In addition, the Raman microscope was equipped with a fluorescence illumination system (5-UR7005, Olympus), a green fluorescence cube (488/561EX), and a fluorescence lamp (X-Cite 120 PC, Photonic Solutions).

Acquisition of Raman Spectra. Prior to the measurements, live cells were transferred into optically transparent Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) and mounted on the microscope stage. The spectra were acquired from the labeled organelles in live cells as recently described.[15] Fluorescence-labeled organelles were visualized using the 488/561EX fluorescence cube. To generate the spectra, the Raman excitation laser was overlapped with single labeled organelles. To warrant a high-quality signal/noise ratio, the spectra accumulation parameter was set to 6 × 20 s; importantly, no measurable phototoxicity was observed at this irradiation dose. During the experiments, the cells were maintained under physiological conditions at 37 °C. We visually verified the *XYZ* position of the cell before and after each measurement to ensure the spatial precision of Raman spectra acquisition.

Biomolecular Component Analysis of Raman Spectra. The calibration of Raman band intensities on the concentrations of biomolecules in the sample was performed as previously described.[15,21] Quantitative analysis of cellular spectra was performed using BCAbox software (ACIS LLC, Buffalo, NY). The description, interface of the BCAbox software, and schematics for the spectrum processing algorithm are shown in Figures S1 and S2. Representative

examples of raw and preprocessed mitochondria spectra are shown in Figure S3.

RESULTS AND DISCUSSION

In our experiments, we incubated cultured microglia cells with SARS-CoV-2 spike protein or heat-inactivated SARS-CoV-2 to imitate viral neuroinvasion. Untreated microglia were used as an experimental control. Mitochondria and GA were stained with specific fluorescence probes, thus enabling acquisition of Raman spectra in these organelles, while LD were identified by transmitted light imaging.

The obtained Raman spectra were processed with the BCA algorithm to quantify the concentrations of major groups of biomolecules (Figures S6 and S7). The measurements were performed as recently described.[18] It is worth noting that although the Raman spectra were collected within a submicron volume of an excitation laser focused on specific organelles, the adjacent cytoplasm may also overlap with the laser probe and contribute to the spectra. Nevertheless, despite this potential contribution, there were statistically significant differences between the molecular profiles obtained in various organelles, which supports the sensitivity of Raman microspectrometry to the subcellular biochemical environment. The measured values obtained in single mitochondria of control and treated cells are shown in Tables S1−S3.

We found that treatment with SARS-CoV-2 spike protein or HI-SARS induced significant alterations in the concentrations of diverse types of biomolecules in the mitochondria. First, the concentration of mitochondrial DNA was reduced almost 2-fold in the infected cells, from ~2.2 mg/mL in control cells to ~1.2 mg/mL in the cells treated with either viral agent (Figure 1), which indicates the decrease in mitochondrial DNA copy number. At the same time, the concentration of mtRNA was increased from ~2.25 mg/mL in the control to 2.8 mg/mL in HI-SARS-treated cells and ~4.0 mg/mL in cells treated with the spike protein; the latter difference was statistically significant. This increase in RNA is consistent with previous reports on mitochondrial genome upregulation in cells infected by SARS-CoV-2.[7] We also found a significant reduction in mitochondrial saccharides from ~1.5 mg/mL in the control to ~0.9 mg/mL in the HI-SARS-treated cells and ~0.7 mg/mL in the cells treated with the spike protein. The mitochondrial saccharide fraction includes glucose and pyruvate, and its reduction suggests a decrease of the respiratory function of mitochondria.

Furthermore, we detected a significant perturbation in the saturation of phospholipids populating the mitochondrial lipidome. The average number of unsaturated C=C bonds per phospholipid was significantly reduced from ~4.3 in the control to ~3.8 in the cells treated with the HI-SARS viral construct and ~3.7 in the cells treated with the spike protein. At the same time, we did not record any significant change in the total concentration of lipids in mitochondria (Figure 1). We thus concluded that the shift in lipidome saturation occurs as a result of biochemical processes inside the mitochondria and likely is not caused by trafficking of the saturated phospholipids to this organelle.

In parallel, we investigated the impact of HI-SARS on the major organelles involved in the metabolism of lipids such as GA. However, it appears that SARS-CoV-2 does not directly influence the lipid biogenesis. We found that all of the resolvable lipidome characteristics in the control and treated cells for these organelles were remarkably uniform. Similarly,
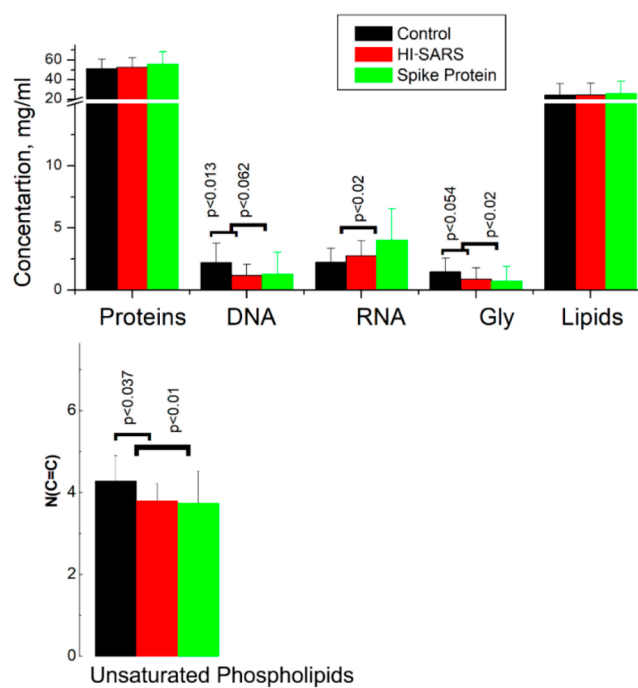

Figure 1. Comparative analysis of the molecular content in nontreated mitochondria (control) and mitochondria treated with either heat-inactivated SARS-CoV-2 or the SARS-CoV-2 spike protein (as indicated). The top chart shows absolute concentrations of proteins, DNA, RNA, saccharides (Gly), and lipids in live-cell mitochondria. The bottom chart shows a decrease in the number of C=C bonds in mitochondrial phospholipids (phospholipid unsaturation parameter) in both groups of treated cells. The statistically significant differences are indicated by horizontal brackets and p values.

the composition of LD in the treated cells remained largely unchanged. However, we found that HI-SARS induces an increase in the number of C=C bonds in the pool of unsaturated phospholipids stored in LD (Figures S5−S7).

In the interpretation of our data, we point to the fact that mitochondrial lipids are predominantly synthesized in the endoplasmic reticulum and then transported to the mitochondria through the GA. While these organelles show no differences in molecular composition between control and treated cells, the mitochondria demonstrate substantial differences not only in phospholipid saturation but also in the abundances of RNA, saccharides, and mtDNA (Figure 1). We propose that these changes originate in virus-induced ROS production, in part via oxidative damage to lipids and oxidation of respiratory chain proteins, affecting metabolism and protein import, which then induces DNA damage as reflected in a sharp decrease in the mtDNA level. Furthermore, the mechanistic link between lipid metabolism and inflammation is well-established, wherein lipids can directly activate inflammatory pathways.[22] Thus, significant changes in the composition and distribution of lipids within the brain are believed to contribute to neurocognitive decline.[23] Furthermore, SARS-CoV-2-induced oxidative stress impacts phospholipid membranes, causing additional perturbations of biological processes.[24] We propose that increased oxidative stress impacts the fluidity of phospholipid membranes, which can affect the interactions and activity of metabolic enzymes, resulting in membrane remodeling. The membrane fatty acid composition is thought to be altered in response to oxidative stress by a

decrease in the number of C=C bonds, which results in higher saturation of the organellar lipidome.[24,25] The physiological relevance of membrane remodeling remains unclear, but it may be an adaptive response to cellular stress. These data support our hypothesis that mitochondrial dysfunction, oxidative stress, and inflammation could lead to an increase in COVID-associated neurological dysfunction. In addition, our data support the premise that SARS-CoV-2 induces release of pathogen-associated molecular patterns (PAMPS) and danger-associated molecular patterns (DAMPS), ATP, oxidized lipids, and heat shock proteins, all of which are associated with apoptosis and autophagy.[26,27]

Overall, our study clarifies the role of mitochondrial dysfunction in SARS-CoV-2-induced neuropathology. Our data suggest that mitochondrial dysfunction is among the earliest and most prominent features of neurodegeneration. In addition, the absence of any significant changes in the lipidome of GA and LD indicate a targeted impact of SARS infection on mitochondria. Therefore, examining mitochondrial function or mitochondrial damage markers in the microglia cells in response to interactions with SARS-CoV-2 spike protein may help identify pathways of viral pathogenesis, unravel mechanisms of cellular vulnerability, and aid in the discovery of mitochondrial biomarkers relevant to SARS-CoV-2 neuro-inflammation and progression to neuropathogenesis. Furthermore, therapeutic strategies that modulate mitochondrial processes may be efficacious in treating patients with neuro-COVID. Our study calls for the development of mitochondria-targeted pharmaceutical drugs that can neutralize virus-induced ROS production in these cellular organelles.

■ ASSOCIATED CONTENT

ⓈⒾ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.1c00675.

Figures S1–S7 and Tables S1–S3 (PDF)

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Author Contributions

A.P., A.N.K., P.N.P., and S.D.M. conceived the project. A.P., A.N.K., and S.D.M. performed the experiments. All of the authors drafted and edited the manuscript.

Notes

The authors declare no competing financial interest.

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