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Immunoregulatory effects of very low density lipoprotein from healthy individuals and metabolic syndrome patients on glial cells

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ABSTRACT

Epidemiological studies have reported that elderly patients with metabolic syndrome (MetS) are significantly more likely to develop neuronal degenerative diseases than those without MetS. Our previous study showed that patients with MetS had significantly higher levels of negatively charged very low density lipoproteins (VLDLs) in the plasma than healthy controls. Highly electronegative VLDL is a key risk factor for endothelial dysfunction and atrial fibrillation. However, the impact of negatively charged VLDL in brain immunity remains unclear. In this study, VLDLs were isolated from normal healthy (nVLDL) individuals or patients with MetS (metVLDL). Primary astroglia and microglia mixed cell cultures as well as microglial-enriched cultures were used to test the effects of VLDLs. Microglia/astroglia activation as evidenced by their morphological changes and production of pro-inflammatory factors, such as tumor necrosis factor-α (TNF-α) and prostaglandin E2 (PGE2), were assessed by immunofluorescence staining and ELISA, respectively.

Our results showed that metVLDLs mainly act on the microglia, and not the astroglia, with low concentration (0.05–0.5 μg/mL) inducing cell morphological changes and decreased cellular processes in the microglia. However, nVLDL treatment at these concentrations had no effects on microglia and astroglia. Most importantly, TNF-α and PGE2 levels significantly increased in the microglia treated with metVLDL via a dose-dependent manner. Together, our data indicate that metVLDLs can contribute to MetS-associated brain disorders through microglia activation and neuroinflammation.

1. Introduction

Metabolic syndrome (MetS) is a cluster of conditions, including increased blood pressure, high blood sugar, excess body fat around the waist, and abnormal cholesterol or triglyceride levels (Mancia et al., 2010). Dyslipidemia, such as hypercholesterolaemia and hyperlipidemia, in patients with MetS represents a risk factor for cardiovascular system pathogenesis and may be highly associated with atherosclerosis (Fadaei et al., 2018) and heart diseases (Mancia et al., 2010). Moreover, it has also been highly associated with cognitive dysfunction or Alzheimer's disease (AD) (Altman and Rutledge, 2010; Vasantharekha et al., 2016). Our previous study demonstrated that patients with MetS have significantly higher levels of negatively charged VLDLs (V5) (15.2 mg/dL) in their plasma than healthy controls (V5, 5.5 mg/dL) (Chen et al., 2012a,b). Negatively charged V5 induced apoptosis in human vascular endothelial cell (Chen et al., 2012a,b). And the unfractionated VLDLs from patients with MetS (metVLDL, 10 μg/mL), but not the VLDL of healthy control (nVLDL), also induced the vascular

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endothelial cell apoptosis (Chen et al., 2012a,b). Chronic exposure to metVLDL (25 μg/mL) induced apoptosis in young adult mouse cardiomyocytes and increased the diameter of their left atrium (Lee et al., 2016). This finding indicates that higher levels of negatively charged VLDLs in patients with MetS can damage their vascular endothelium cells and cardiomyocytes as well as their heart function. However, the impact of negatively charged VLDLs on the CNS remains to be clarified.

In the CNS, the glial cells play important roles in the homeostasis of brain physiological function (Jha et al., 2014). In response to challenges, such as pathogen invasion, excess of cellular debris (Lewis and Kucenas, 2014), or a dyslipidemia microenvironment (Lakk et al., 2018), the glial cells are the first line immune effector cells in the brain. They protect the neurons against foreign insults via appropriate neuroinflammation and cytokine production (Lima Giacobbo et al., 2018). However, when uncontrolled neuroinflammation occurs (Ransohoff et al., 2015), the over-activated glial cells release pro-inflammatory factors, resulting in immune cell infiltration, gliosis, and neuronal cell death (Le Thuc et al., 2015; Dong et al., 2017; Spangenberg and Green, 2017). In rodents, the long-term intake of a high-fat diet causes a chronic neuroinflammatory response and glial cell activation (Buckman et al., 2014; Barron et al., 2016), which can trigger neuronal dysfunction and lead to cognitive and memory decline. However, whether negatively charged VLDLs play a role in dyslipidemia-associated brain inflammation remains unclear.

In this study, we hypothesize that negatively charged VLDL from patients with MetS are a risk factor for glial cell activation and increased pro-inflammatory factor production. Using primary mixed-glial and microglial-enriched cells, our results showed that negatively charged VLDLs from patients with MetS can function as an immune trigger to induce microglia activation and increase tumor necrosis factor-α (TNF-α) and Prostaglandin E2 (PGE2) production.

2. Material and methods

2.1. Animals

Time-pregnant adult female C57BL/6 mice were purchased from BioLASCO Taiwan CO., Ltd. All mice were given *ad libitum* access to food and water. Housing and breeding of the animals were performed in strict accordance with the guidelines of the National Health Research Institutes (NHRI). All procedures were approved by the NHRI Animal Care and Use Committee.

2.2. VLDLs isolation

Human VLDLs were isolated from the pooled blood of volunteers who did (MetS subjects, five males, average age 48 ± 5 years) (metVLDL) or did not (normal control subjects, two males and two females, average age 36 ± 8 years) (nVLDL) meet the criteria for MetS according to the National Cholesterol Education Program Adult Treatment Panel III guidelines (Expert Panel on Detection, E., Treatment of High Blood Cholesterol in, A et al., 2001). All participants gave informed consent. The study followed the Helsinki Declaration principles and was approved by the Kaohsiung Medical University Hospital Ethics Review Board. Whole blood was collected via venipuncture and collected by a blood donation bag which contains citrate phosphate dextrose adenine (CPDA1) anticoagulant. After sequential removal of blood cells and chylomicrons, Penicillin-Streptomycin mixture (Thermo Fisher Scientific, MA, USA) were added for antibacterial growth, and later plasma samples were ultracentrifuged at 40,000 rpm for 24 h. Total VLDL (density = 0.930–1.006 g/mL) was isolated as previously described (Chen et al., 2003). In short, the respective fractions were concentrated with Centriprep® filters (YM-30; EMD Millipore Corp., MA, USA), dialyzed against buffer salt, and passed through Minisart® 0.22-μm filters (Sartorius, Göttingen, Germany). The isolated fractions were quantified at their protein concentrations by the bicinchoninic acid method (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, MA, USA). VLDL samples were sealed with nitrogen and stored at 4 °C before use. VLDL isolated from healthy individuals served as a control for evaluating the effects of VLDL from patients with metabolic syndrome. All the procedures for handling VLDL samples were standardized and monitored by using HEK-Blue™ LPS Detection Kit 2 (InvivoGen, China) for possible bacterial contamination.

2.3. Primary cultures

2.3.1. Mixed-glial cultures

Primary mixed-glial cultures were prepared from whole brains of postnatal day 1 pups from C57BL/6 mice as previously described (Chen et al., 2013). The disassociated brain cells were seeded into poly-D-lysine-coated 24-well plates at 1.5×10^5 cells/well. The primary mixed cells were maintained in DMEM/F12 culture medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 U/mL penicillin, and 50 μg/mL streptomycin. The mixed-glial culture medium was refreshed every 3 days until treatment at 11–12 days after seeding, at which time, the cultures contained approximately 80% astrocytes and 20% microglia.

2.3.2. Microglial-enriched cultures

Primary microglial-enriched cultures were prepared from the whole brains of 1- or 2-day-old C57BL/6 mouse pups as previously described (Chen et al., 2013). The isolated cells (5×10^7) were seeded into 175 cm^2 culture flasks containing DMEM/F12 culture medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/mL penicillin, and 50 μg/mL streptomycin. The medium was changed every 3 days until the cells reached confluence (12–14 days). The microglia was separated from the astroglia by shaking the flask for 30 min at 180 rpm. Purity of the microglial-enriched cultures was > 98%.

2.4. Immunofluorescence staining

The cells were fixed with 4% paraformaldehyde before immunofluorescence staining. After blocking, the cells were incubated with primary antibodies for microglia (Iba1, Wako; 019-19741; 1: 1000 dilution) and astroglia (GFAP, Millipore; MAB360; 1: 500 dilution). After repeated washing in PBST, the cells were incubated with goat anti-mouse IgG Alexa 488 or goat anti-mouse IgG Alexa 594 secondary antibodies (Invitrogen; 1:2000 dilution). The stained cells were examined under a BX51 fluorescent microscope (Olympus) and analyzed using the ImageJ software analysis system.

2.5. TNF-α assay

Cell culture supernatants were collected 3 h after treating with PBS (control), nVLDL (0.05, 0.5, and 5 μg/mL), or metVLDL (0.05, 0.5, and 5 μg/mL). TNF-α levels were measured using the commercial mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems.

2.6. PGE2 assay

Cell culture supernatants were collected 24 h after treating with PBS (control), nVLDL (0.05, 0.5, and 5 μg/mL), or metVLDL (0.05, 0.5, and 5 μg/mL). PGE2 levels were measured using the commercial mouse PGE2 parameter assay kit from R&D Systems.

2.7. Statistical analysis

Data were presented as mean ± standard error of the mean. One-way analysis of variance (ANOVA) was used to analyze differences

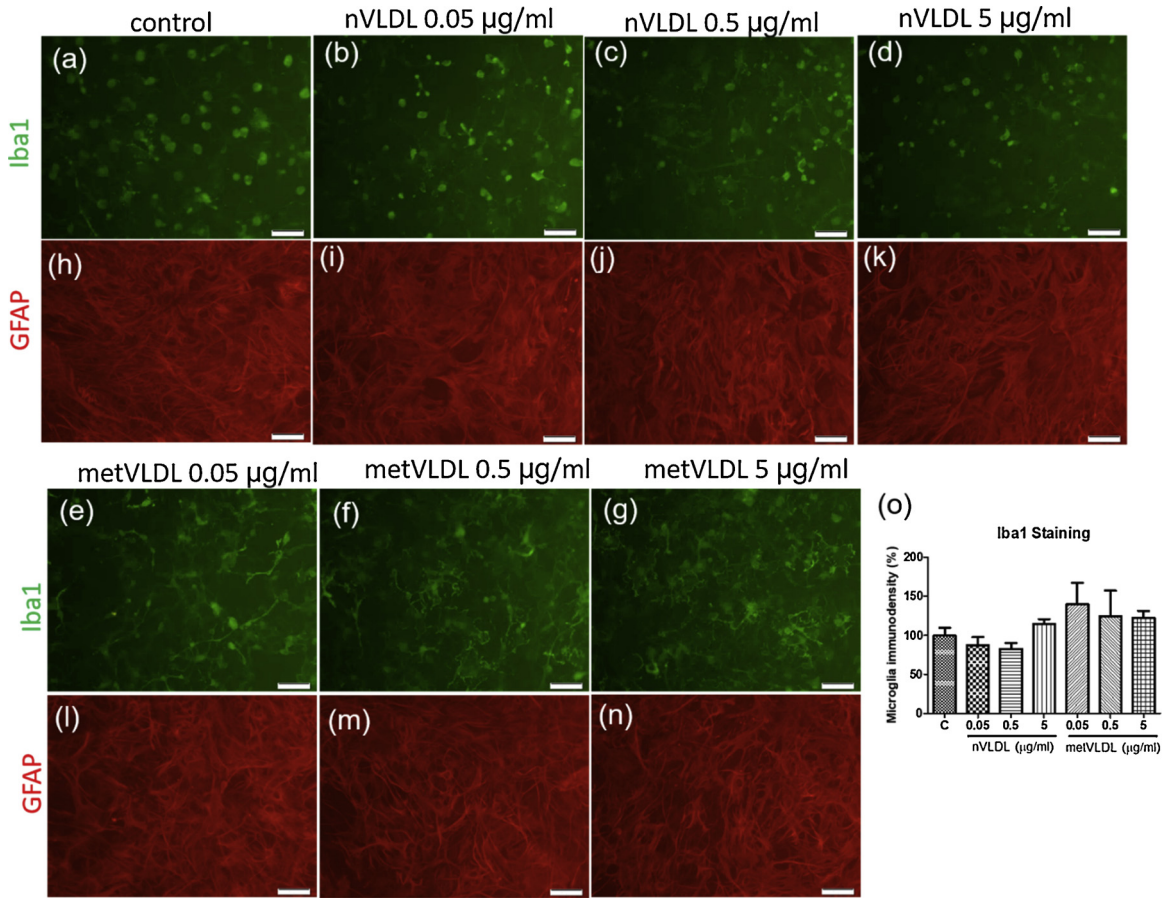

Fig. 1. MetVLDL treatment induces microglia activation in mixed-glial cultures. Representative immunofluorescence staining of Iba-1 (green, a–g) and GFAP (red, h–n) in mixed-glial cells 24 h after treatment with PBS (control), nVLDL (0.05–5 μg/mL), or metVLDL (0.05–5 μg/mL) (scale bar: 50 μm; magnification: 200×). Figure (o) represents the fluorescence intensity of the Iba-l-positive cells (ImageJ software). Data are expressed as a percentage of the control group (mean ± standard error of the mean). Three independent experiments were performed in duplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

among different groups (GraphPad Prism 5). Statistical significance among different groups was assessed using Bonferroni's correction. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Microglia are the metVLDL-targeted cells in mixed-glial cell cultures

To investigate the effects of nVLDL and metVLDL on the glial cells, we prepared primary mixed cultures of microglia and astrocytes from the brains of 1-day-old B6 mouse pups. Immunofluorescence staining of Iba-1, a protein marker for activated microglia, revealed that microglia treatment with a higher dose of nVLDL (5 μg/mL) (Fig. 1d) or metVLDL at doses of 0.05–5 μg/mL (Fig. 1e–g) displayed a rounded and extended morphology along with thick and less processes compared with the PBS treatment (control) group (Fig. 1a). However, the morphology of nVLDL-treated (Fig. 1i–k) and metVLDL-treated (Fig. 1l–n) astroglia was similar to the control group astroglia (Fig. 1h), as shown by GFAP immunofluorescence staining. Iba-1 protein expression was slightly increased in the presence of metVLDL but not in the presence of nVLDL (Fig. 1o).

3.2. metVLDL at low concentrations significantly alters microglial morphology

Primary microglial-enriched cells were prepared from the brains of 1-day-old B6 mouse pups to verify the effect of metVLDL on microglial morphology. Microglia cells incubated with metVLDL at concentrations of 0.05–5 μg/mL for 24 h (Fig. 2e–g & h) displayed a rounded morphology and enlarged cell bodies along with decreased cellular processes in comparison with control microglia cells incubated with PBS (Fig. 2a). By contrast, microglia treated with 0.05–0.5 μg/mL nVLDL (Fig. 2b–c) showed no morphological and cellular processes number changes (Fig. 2h), until its concentration up to 5 μg/mL nVLDL (Fig. 2d & h). There was no difference observed in microglia number after metVLDL and nVLDL treatments (Fig. 2i).

3.3. MetVLDL treatment induced TNF-α and PGE2 production in microglia

We determined whether metVLDL treatment can induce pro-inflammatory factor production in the mixed-glial and microglial cultures. ELISA assays revealed that mixed glia and microglia treated with metVLDL (0.05–5 μg/mL) increased TNF-α (Fig. 3) and PGE2 (Fig. 4) production in a dose-dependent manner. However, nVLDL (0.05–5 μg/mL) treatment failed to induce these pro-inflammatory factors (Figs. 3 and 4). Taken together, our data indicate that metVLDL treatment can trigger microglia activation that leads to increased TNF-α and PGE2 production.

4. Discussion

This study demonstrates that higher negatively charged metVLDL increases the supernatant level of pro-inflammatory factors, such as TNF-α and PGE2, in the mixed-glial and microglial cells. Therefore,

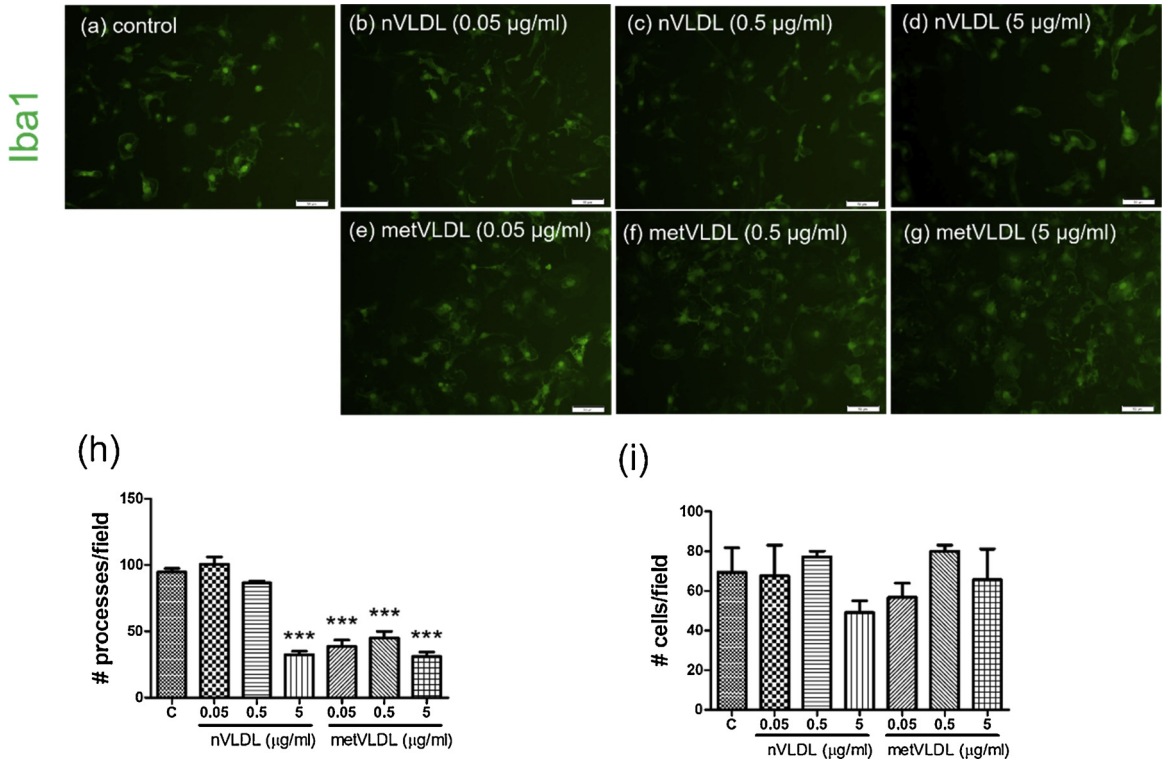

Fig. 2. MetVLDL treatment induces microglia activation in microglial-enriched cultures. Representative immunofluorescence staining of Iba-1 (green) expression in microglial-enriched cells 24 h after treatment with (a) PBS (control), (b–d) nVLDL (0.05–5 μg/mL), or (e–g) metVLDL (0.05–5 μg/mL) (scale bar 50 μm; 200× magnification). Cellular (h) processes and (i) cell numbers are calculated using ImageJ software. Data are expressed as mean ± standard error of the mean. ***$p < 0.001$ (one-way ANOVA). Three independent experiments were performed in duplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metVLDL targets on microglia and produced the inflammatory response. In contrast, VLDL from normal individuals has no effect on microglia activation and TNF-α and PGE2 production. To the best of our knowledge, this is the first report that compares the immunoregulatory effects of VLDLs from healthy individuals and patients with MetS on microglial activation and neuroinflammation.

VLDL dyslipidemia is strongly associated with cardiovascular-related diseases and type II diabetes (Adiels et al., 2008). Circular VLDL, a large size (30–80 nm diameter) triglyceride-rich lipoprotein, is metabolized by lipoprotein lipase to release free fatty acids, glycerol, and intermediate density lipoproteins (25–35 nm) (Mora et al., 2010). In classical, lipoprotein properties related to oxidative stress and lipotoxicity depend on their composition and size (Vaziri, 2010). Larger lipoproteins carry a higher risk for cardiovascular disease than smaller lipoproteins (Mora et al., 2010). However, our study demonstrates that surface charge is a novel important characteristic of lipoproteins to elicit brain inflammation. In the periphery, a physiological concentration of negatively charged metVLDL (10–25 μg/mL) not only induces vascular endothelial cell apoptosis (Chen et al., 2012a,b) but also damages the cardiomyocytes (Lee et al., 2016), resulting in lipid accumulation in the atria, susceptibility to atrial fibrillation (Lee et al., 2016), and modulation of gap junctions and cardiac conduction (Lee et al., 2017). We suggest that the peripheral immune response due to metabolic stress may decrease the integrity and function of the blood brain barrier (BBB). Consequently, negatively charged VLDLs penetrate into the brain via the leaky BBB to trigger neuroinflammation in patients with MetS. However, the composition of lipids or negatively charged VLDLs in the brain of patients with MetS still needs further investigation.

Microglia activation plays a critical role in chronic neuroinflammation-mediated brain disorders, particularly in neurodegenerative diseases (Smith et al., 2012). Consistent with the morphological

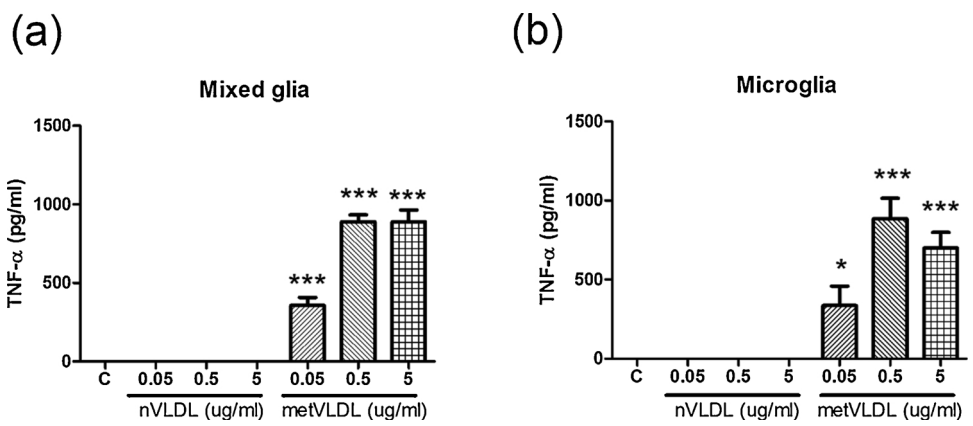

Fig. 3. MetVLDL treatment induces TNF-α production in mixed-glial and microglial-enriched cultures. TNF-α levels in supernatant of (a) mixed-glial and (b) microglial-enriched cultures 3 h after treatment with PBS (control, C), nVLDL (0.05, 0.5, and 5 μg/mL), or metVLDL (0.05, 0.5, and 5 μg/mL) (*$p < 0.05$, ***$p < 0.001$ vs. control group). Data are represented as mean ± standard error of the mean. Three independent experiments were performed in duplicate.

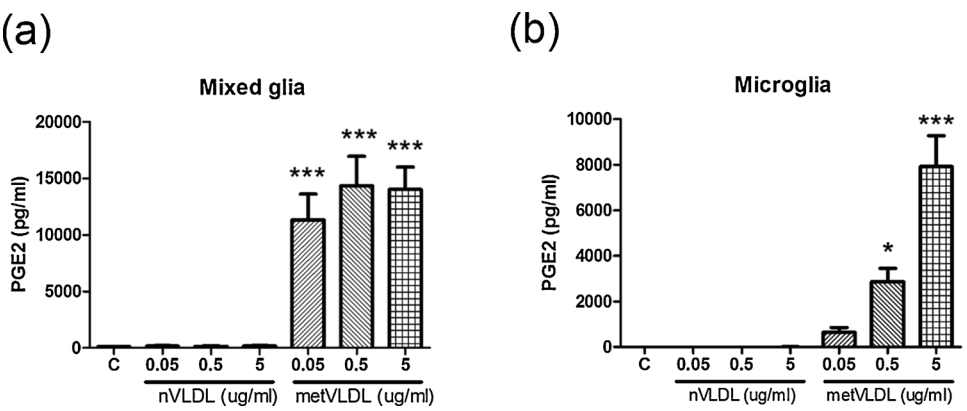

Fig. 4. MetVLDL treatment induces PGE2 production in mixed-glial and microglial-enriched cultures. PGE2 levels in supernatant of (a) mixed-glial and (b) microglial-enriched cultures 24 h after treatment with PBS (control, C), nVLDL (0.05, 0.5, and 5 μg/mL), or metVLDL (0.05, 0.5, and 5 μg/mL) (*$p < 0.05$, ***$p < 0.001$ vs. control group). Data are represented as mean ± standard error of the mean. Three independent experiments were performed in duplicate.

changes that occur in the activated microglia (Davis et al., 2017; Ghosh et al., 2016), our results showed that the microglia exposed to metVLDL display an altered morphology. In addition, our data also showed that metVLDL can be an immune trigger for production of pro-inflammatory factors, such as TNF-α and PGE2, in the microglia. While cardiomyocytes (Lee et al., 2016) and vascular endothelial cells (Chen et al., 2012a,b) are damaged due to 10–25 μg/mL metVLDL, the CNS microglial cells are activated at very low concentrations of metVLDL (0.05–5 μg/mL). These results indicate that negatively charged VLDL is more toxic to the CNS cells. Likewise, a study in microglial cultures demonstrated that VLDL (3.4 μg/mL) stimulated inflammation and increased nitrite levels at 48 h (Mohan and Ard, 1996). These pro-inflammatory factors can function as potent neurotoxic factors in the neurons (Smith et al., 2012). Regulatory mechanisms underlying neuroinflammatory cascades and neuron viability by negatively charged VLDLs are worth being investigated in the future.

In response to negatively charged VLDL, the VLDL receptor can be a high-affinity receptor on the microglia that binds to the negatively charged VLDL to produce pro-inflammatory factors. Interestingly, VLDL receptor (VLDLR), a brain-enriched protein, levels are increased in activated microglia that are associated with senile plaques (Christie et al., 1996). VLDLR activation triggers intercellular cascades, such as src-family kinase and Dab1 (Zhang et al., 2007), and participates in brain development regulation, adult synaptic plasticity (Lane-Donovan and Herz, 2017), and dendritic spine formation, all affecting learning and memory (DiBattista et al., 2015). Therefore, understanding the mechanism of VLDLR signaling by negatively charged VLDLs in microglia activation might provide new insights into a microglia-based therapy for MetS-associated brain disorders, such as AD (Altman and Rutledge, 2010; Vasantharekha et al., 2016).

5. Conclusion

In this study, we found that VLDLs from patients with MetS can cause microglial activation and increased TNF-α and PGE2 production in mixed-glial and microglial-enriched cultures. Our results indicate that surface charge is an important VLDL characteristic that can trigger a potent immune response in the CNS. Our data also suggest a novel detrimental role of the negatively charged VLDLs in MetS-associated brain disorders through microglia activation and neuroinflammation.

Author's contribution

"Shiou-Lan Chen: The author designed the study and wrote the manuscript", "Chia-Ling Li: The author completed the main experiment, analyzed the data and revised the manuscript". "Chun-Hsien Chu, The author helped the experiment and revised the manuscript". "Hsiang-Chun Lee, Mei-Chuan Chou, Ching-Kuan Liu, Chu-Huang Chen, Liang-Yin Ke: The 5 authors helped experiment".

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Declaration of Competing Interest

The authors declare no conflict of interest.

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