Short chain fatty acids reduces OPCs loss by inhibiting activation of astrocytes via SGK1/IL-6 signaling pathway

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Abstract

Short chain fatty acids (SCFAs) are known to be actively involved in neurological disease, but their roles in hypoxic-ischemic brain injury (HIBI) is unclear. This study established a rat model of HIBI, then measured the changes in IL-6 and NLRP3, proliferation and apoptosis indicators of oligodendrocyte precursor cells (OPCs). The mechanism of SCFAs on astrocytes were also investigated. To further explore the mechanism, astrocytes were treated with hypoxia in vitro and OPCs were treated with IL-6. The results showed that SCFAs significantly alleviated the activation of astrocyte and loss of OPCs induced by HIBI. SCFAs pretreatment was associated with the following: (1) downregulation of NLRP3, IL-6, CCL2, and IP-10; (2) was no effect on proliferation of OPCs; (3) amelioration of the abnormal expression of Bax and Bcl-2; and (4) regulation of IL-6 expression via SGK1 related pathway in astrocytes. Our findings revealed that SCFAs alleviated loss of OPCs through regulating the astrocytes activation by SGK1/IL-6 signaling pathway.

Introduction

Stroke can lead to poor neurological prognosis and ischemic hypoxic brain injury is the main mechanism [1]. Axonal demyelination and neuroinflammation are important components of HIBI [2–4], The adult brain contains a large population of oligodendrocyte progenitor cells (OPCs) which affects the final myelination and astrocytes which affects the neuroinflammation, our previous study showed that HIBI result in axonal demyelination[3, 5–7], but the relationship between astrocytes and OPCs is unclear. Activation of astrocytes may be involved in OPCs injury.

Recent studies have shown that astrocyte activation includes the secretion of inflammatory factors and chemokines which are involved in the regulation of neuroinflammation,controlling astrocyte overactivation is a promising strategy in HIBI [8, 9]. Recent studies have highlighted the important effects of natural products on brain diseases. More and more evidence shows that short chain fatty acids (SCFA) are closely related to a variety of brain diseases. SCFA, including acetate, propionate and butyrate, is produced by the metabolism of dietary fiber by intestinal flora. SCFA can enter the brain from the intestine, play a variety of roles and regulate the function of the central nervous system [3, 10]. But the mechanism of SCFAs in regulating neuroinflammation and OPCs loss remains unclear, astrocyte activation may be involved. Serum and glucocorticoid-induced protein kinase 1 (SGK1) is a member of protein kinases, it is involved in cell function metabolism[11], the role of SGK1 in astrocyte activation is not clear. Whether SCFAs improves activation of astrocytes in HIBI through the regulation of the SGK1 signaling pathway is unclear. The present study aims to explore the molecular mechanism of astrocyte activation after treatment with SCFAs in HIBI, and to provide scientific theoretical basis for the treatment of HIBI.

Materials And Methods

Animals and study design

The animals are purchased from the company (Jihui Experimental Animal Breeding Co., Ltd, Shanghai, China). A total of 100 male SD rats ranging from 200 g to 250g were selected. Pentobarbital 30mg/kg was injected intraperitoneally and the induction time was 20min. In this study, grouped as follows :(1) Sham operation group; (2) Bilateral common carotid artery ligation (BCCAO) was performed in the experimental group, and data collection was prevented if the animals died prematurely. (3) SCFAs (acetate: propionate: butyrate at a ratio of 3: 1: 1, 500mg/kg, intragastrically, for 7days before BCCAO) +BCCAO. The rats were fed under normal oxygen condition for 1, 3 and 7 days before death. Animals were killed for cervical dislocation. Animal treatment and experiments shall be approved by the Shanghai Municipal Commission for Animal Protection and Use.

To observe the effect of SCFAs on astrocyte activation via SGK1 (lentivirus transfection,caudal vein). The rats were divided into sham operation group; BCCAO group; BCCAO+SCFAs group; BCCAO+SCFAs+overexpression SGK1 group; BCCAO+SCFAs+si-SGK1 group; SCFAs group. The rats were fed under normal oxygen condition for 1 day and then killed.

Primary Culture of Oligodendrocytes

Concrete details were in reference [3]. To explore the IL-6 on oligodendrocytes. Grouped as follows: the control group; 20ng/ml IL-6 group; 20ng/ml IL-6 + 20ng/ml IL-6Ra (Sarilumab, anti-IL-6Rα) group; 20ng/ml IL-6Ra group.

Primary Cultured Astrocytes

Briefly, the mixed cells were isolated from 1 days old SD rats. The mixed culture method was shown in literature [3]. Mixed cells for 10 d, and then oscillated at 180 RPM and 36.5℃ for 1h to remove OPCs and microglias. Standing under normal conditions for 24h, the cells were treated differently. In this study, astrocytes with a purity of more than 90% were cultured.

Intervention of Astrocytes

To see if SCFAs had an effect on NLRP3 ,IL-6, CCL2, and IP-10 in astrocytes, the astrocytes were grouped as follows: the control group; oxygen glucose deprivation (OGD) group; OGD+20ng/ml SCFAs group; SCFAs group.

To examine whether SCFAs affect SGK1 in astrocytes. Grouped as follows: the control group; OGD group; OGD+SCFAs group; OGD+SCFAs+SGK1 group; OGD+SCFAs+si-SGK1 group (The transfection method was shown in Ref. [6]).

To examine whether SCFAs affect IL-6 via SGK1 signaling pathway. Grouped as follows: the control group; OGD group; OGD+SCFAs group; OGD+SCFAs+SGK1 group; OGD+SCFAs+si-SGK1 group; SCFAs group.

Western Blot

IL-6 protein levels were determined by western blotting, concrete details were in reference [3]. The primary antibodies used were as follows: IL-6 (1:1,000,Bosterm,China), NLRP3 (1:1,000, CST; USA), IL-6 receptor (1:1,000, Santa Cruz, USA), Bax (1:1,000, Santa Cruz,USA); Bcl-2 (1:1,000), GFAP (1:1,000), CCL2 (1:1,000), IP-10 (1:1,000) β-actin (1:1,000) [all from Cell Signaling Technology]. After washing with TBST for three times, the cell membrane was treated with HRP were incubated for 1 h. Then immunoluminescence was performed.

Double Immunofluorescence

Each group was incubated with the following antibodies, against IL-6 (1:100) and GFAP (1:100). anti-IL-6R (1:100). To verify the apoptosis and proliferation of OPCs: anti-cleaved caspase-3 (1:100), anti-BrdU (1:100, concrete details were in reference [6]), and anti-NG2 or anti-PDGFR-α (1:100) were utilized. 4°C overnight and then incubate with secondary antibody and observe the slice under the fluoroscope.

Electron Microscopy

sections were gavaged in 0.1M phosphate buffer with a mixture of 2% paraformaldehyde and 3% glutaraldehyde. then, the brain was removed and coronal sections (about 1mm thick) were cut. They were then fixed in 1% osmium tetroxide for 2 hours, dehydrated, and subsequent processing. The ultrathin sections were cut and observed on electron microscope (FEI Corporation, Hillsboro, OR).

Statistical Analysis

All data were evaluated using SPSS13.0. Distribution values are expressed as mean ± standard deviation. Four groups of univariate data were analyzed by univariate analysis of variance under homogeneity of variance. In addition, Welch analysis of variance was used for analysis. If the data were homogenous of variance, the least significant difference (LSD) method was used for multiple comparisons. $P<0.05$ was statistically significant.

Results

Inflammatory Cytokines expression levels in vivo

In the corpus callosum (CC), the IL-6 expression levels were detected (Figs. 1A–1I). The IL-6 immunoreactivities were induced in astrocytes after BCCAO (Figs. 1D–1F) compared to their controls (Figs. 1A–1C). But SCFAs improved expression of IL-6 (Figs. 1G–1I). The NLRP3 and IL-6 protein levels trend of were the same as that of double immunofluorescence (Figs. 1J-1L).

Apoptosis of OPCs levels in the CC

After BCCAO, a increase in the Cleaved Caspase-3 were observed in the rats (Figs. 2A-2J). Compared to the control group (2A-2C), Cleaved Caspase-3 increased in the corpus callosum at 1 d after BCCAO (2D-2F). This changes were reversed by SCFAs treatment (Figs. 2G-2J). The immunoreactive band of Bax

protein levels trend of were the same as that of double immunofluorescence (Figs. 2K-2L), the expression of bcl-2 is opposite to Bax (Figs. 2K-2M).

Proliferation of OPCs levels in CC

Compared to their controls (Figs. 3A–3C), the number of PDGFR-α + OPCs was significantly declined (Figs. 3D–3F). But SCFAs had no effect on BrdU+ OPCs (Figs. 3G–3J). The expression of PCNA and Ki-67 protein trend were the same as that of double immunofluorescence, SCFAs had no effect on them (Figs. 3K–3M). The experimental data show that SCFAs had no effect on proliferation of OPCs. SCFAs may affect the number of OPCs in other ways.

SCFAs affects the expression of SGK1 and GFAP

Compared to controls (Figs. 4A), the SGK1 immunoreactivities decresed in CC after BCCAO (Figs. 4B). This changes were reversed by SCFAs treatment (Figs. 4C). The expression of GFAP and SGK1 trend were the same as that of double immunofluorescence, SCFAs have no effect on GFAP (Figs. 4D–4F). We used electron microscopy to observe the nuclear fragmentation in OPCs. Nuclear breakage occurred in BCCAO when compared with to controls (Figs. 4G-4I). SCFAs alleviates SGK1 and reduce OPCs loss, in vitro experiments showed that SCFAs had no effect on the apoptosis of OPCs by itself (Figs. 4J-4L).It is suggested that SCFAs affects the loss of OPCs through other ways, and astrocytes-derived IL-6 may be involved.

Expression of IL-6 receptor in OPCs in vivo

Expression of IL-6R was localized OPCs as showing colocalization with NG2 (Fig. 5A-5L). Compared to their controls (Figs. 5A–5C), the IL-6R immunoreactivities were induced in OPCs after BCCAO (Figs. 5D–5F). SCFAs have no effect on IL-6Ra (Figs. 5G–5I). The IL-6R protein level trend of was the same as that of double immunofluorescence(Figs. 5J–5K).

IL-6 regulates apoptosis of OPCs in vitro

Compared to the controls, a higher expression of Cleaved Caspase-3 was observed after insulted by IL-6. IL-6R antagonist (IL-6Ra) could decrease the expression of Cleaved Caspase-3 (Figs. 6A-6J). Bax increased and Bcl-2 decreased significantly at 1d after IL-6 compared to their controls. However, IL-6Ra could improve the OPCs apoptosis (Figs. 6K-6M), suggesting that astrocytes affect the survival of OPCs through IL-6.

SCFAs could inhibit the IL-6 in vitro

Compared to the controls (Figs. 7A–7C, GFAP-labeled, green, IL-6-labeled, red), the expression level of IL-6 increased in the OGD group (Figs. 7D–7F) and alleviated in OGD+SCFAs group (Figs. 7G–7I). Compared to the control group, NLRP3 and IL-6 in the OGD group was obviously increased at 1 h (Figs. 7J-7L),

However, SCFAs improved the upregulation of IL-6 and NLRP3. SCFAs could alleviated the IL-6 expression in vitro.

SCFAs regulates CCL2 and CCL10 in astrocytes

Compared to the controls, upregulated CCL2 was induced after insulted by OGD at 1 h. This changes were reversed by SCFAs treatment (Figs. 8A-8I). The levels of CCL2 and CCL10 were increased after OGD at 1 h when compared to controls (Figs. 8J-8L). SCFAs could alleviated the over expression of CCL2 and CCL10 proteins. The above results show that SCFAs regulates astrocyte activation in vitro, the mechanism is unclear .

SCFAs regulates IL-6 via SGK1 in vivo and in vitro

We hypothesized that SCFAs regulates IL-6 via SGK1 singaling pathway. Then, the SGK1 was remarkably downregulated after OGD compared to controls at 1h. SCFAs could increase the SGK1 protein, the effect was weakened by the addition of siRNA-SGK1 (SGK1 knockdown gene), if overexpression-SGK1 (SGK1 overexpression gene) was added, the opposite result is obtained (Figs. 9A-9B). The protein expression level of IL-6 was remarkably upregulated after OGD compared to controls. Overexpression-SGK1 gene could improve the overexpression of IL-6 and CCL2 protein. If si-SGK1 was added, the opposite result is obtained (Figs. 9C-9E). In order to further verify the regulation of IL-6 expression by SGK1, in vivo experiments show that the effection of SCFAs was weakened after adding additional si-SGK1 gene, if overexpression-SGK1 was added, the opposite result is obtained (Figs. 9F and 9G). Therefore, in vivo and in vitro data confirm the effectiveness of clematine reduces astrocyte activation via SGK1/IL-6 signaling pathway.

Discussion

Excessive inflammatory factor release is a sign of astrocyte activation [12]. SCFAs are known to be actively involved in neurological disease, but their roles in HIBI is unclear [10]. The results showed up-regulation of NLRP3, and IL-6 in rat after BCCAO, astrocyte activation was the source of IL-6. Our results showed that the expression levels of IL-6 receptor was increased by BCCAO, expression of IL-6 receptor on OPCs, OPCs may be the target cells of IL-6. OPCs are NG2 or PDGFR-α bipolar cells and sequentially develop to Oligodendrocytes (OLs) [13–15], OLs are the myelinating cells of the central nervous system throughout adulthood, OPCs are the foundation of OLs, loss of OPCs results in neuroconduction disorders [16–18]. The results show significant increase of cleaved-caspase-3 or bax, and decrease of bcl-2 after HIBI, It was suggested that apoptosis of OPCs occurs after HIBI, SCFAs could improve apoptosis. Our results showed that SCFAs did not affect the proliferation of OPCs at 1d. GFAP is a marker of astrocytes, SCFAs did not affect the GFAP, SCFAs may affect the the function of astrocytes, mechanism is unknown. Serum and glucocorticoid-induced protein kinase 1 (SGK1) is a member of the ‘AGC’ subfamily of protein kinases, it is involved in cell function metabolism [21]. Our results showed that SCFAs improved the expression of SGK1, indicating that it may improve astrocyte overactivation via

SGK1, IL-6 may be involved in this process. To verify this, in vitro experiments were carried out. The results preliminarily showed that SCFAs could regulate IL-6 and NLRP3 in vitro. NG2+cells also thought mainly to be OPCs [22], apoptosis of OPCs is recognized as the main mechanism of OPCs loss [23]. In vitro results showed that the expression levels of NG2+/Cleaved Caspase-3 cells were upregulated after IL-6 treatment. IL-6Ra could imptove the OPCs loss, indicating that IL-6 can promote apoptosis of OPCs. Results showed that IL-6 can promote the apoptosis of OPCs. How to control the secretion of IL-6 is the key to reduce the loss of OPCs. Overexpression of chemokines is one of the markers of astrocyte activation [24–26]. Results showed that SCFAs can inhibit the overexpression of cytokines. We hypothesized that SCFAs inhibit astrocyte activation by promotiting SGK1 signaling pathway. This study found that SGK1 expression levels decreased after OGD injury, SCFAs improve SGK1 after OGD. IL-6 and CCL2 expression levels increased after OGD injury, SCFAs improve the expression of them. In addition, When siRNA-SGK1 was added, the effect was reduced, indicating that SCFAs regulates expression of IL-6 and CCL2 through SGK1. However, when overexpression of SGK1 was added, the effect of SCFAs was strengthened, suggesting that SCFAs inhibit IL-6 via SGK1. In order to further determine this mechanism, we performed in vivo validation and obtained the same results. Therefore, SCFAs alleviated activation of astrocytes by regulating SGK1/IL-6 signaling pathway.

In summary, Activated astrocytes in CC produce excess IL-6 which induced neuroinflammation and OPCs loss. However, SCFAs improved IL-6. Further analysis revealed that SCFAs reduces IL-6 by regulating SGK1 signaling pathway in astrocytes. Learn more about this process will help to design effective therapeutic strategies to improve neuroinflammation.

Abbreviations

HIBI
Hypoxic-ischemic brain injury
BCCAO
Bilateral common carotid artery occlusion
OLs
Oligodendrocytes
OPCs
oligodendrocyte progenitor cells
SCFAs
Short chain fatty acids
OGD
Oxygen glucose deprivation
PBS
Phosphate-buffer saline
FBS
Fetal bovine serum

Declarations

Ethics approval and consent to participate

Approval of the Laboratory Animal Ethical and Welfare Committee Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine: XHEC-F-2018-038

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' contributions

HJ. CJG. and SMP. conceived and designed the experiments. YMG. DX. and YW. performed the experiments. YMG. DX. analyzed the data. CJG. and HJ. contributed reagents/materials/analysis tools. YMG. and DX. wrote the article.

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Not applicable

Data Availability Statement

Data can be provided if necessary

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Figures

Figure 1

(A–L) Double immunofluorescence staining showing the distribution of GFAP- (green) and IL-6- (red) labeled immunoreactive astrocytes in vivo. (C, F, and I) Colocalized expression of GFAP and IL-6 in astrocytes. (J) NLRP3, IL-6 , and β-actin bands. (K and L) Compared to control group, NLRP3 and IL-6 increased significantly after BCCAO. However, SCFAs can downregulate the expression of NLRP3 and IL-6 at 1 day. N=5. * $P < 0.05$. Scale bars: A–I 100 μm.

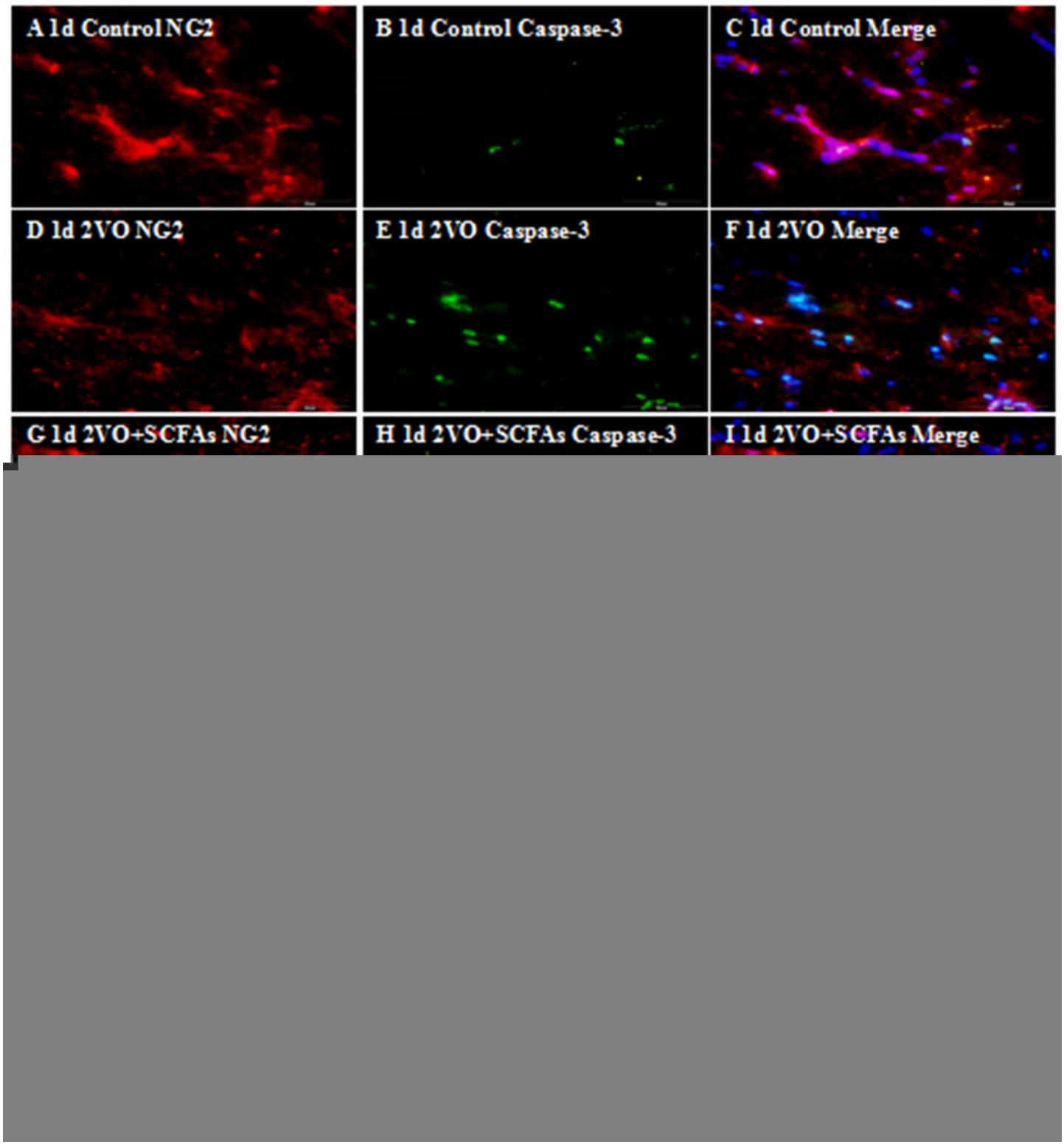

Figure 2

Immunofluorescence staining showing cleaved-caspase-3 immunoreactive cells (green) in the CC of rats (A-I). Bar graph in J shows a increase in the CC at 1 day after the BCCAO; SCFAs could improve the apoptosis. Panel K-M shows Bax and Bcl-2 immunoreactive bands. N =5. * $P < 0.05$. Scale bars: A–I 50 μm.

Figure 3

A–J show PDGFR-α and BrdU positive cells expression level in vivo at 1 days. PDGFR-α (green) and BrdU (red). Panels K-M show bar graphs depicting significant decreases in PCNA and Ki-67 after BCCAO when compared to controls. SCFAs could improvement this. N = 5. *P < 0.05. Scale bars: A–I 50 μm.

Figure 4

SCFAs improves expression of SGK1 protein in CC. SGK1 (red) immunoreactive in CC at 3d(A-C) . Bar graph D-F depicting significant decrease expression of GFAP and SGK1 expression. H showed nuclear fragmentation in OPCs compared to controls (G), SCFAs could improvement this (I). SCFAs had no effect on the apoptosis of OPCs by itself (J-L), N=5. *P < 0.05. Scale bars: A–C 50μm.EM 1μm.

Figure 5

IL-6 receptor α (IL-6Rα) expression in the corpus callosum. NG2- (green) and IL-6Rα- (red) labeled immunoreactive oligodendrocyte precursor cells (OPCs) in corpus callosum. Panel J shows IL-6Rα bands. Panel K show bar graph depicting the expression of IL-6Rα following the BCCAO when compared to their controls. .N = 5. *P < 0.05. Scale bars: A–I 50 μm.

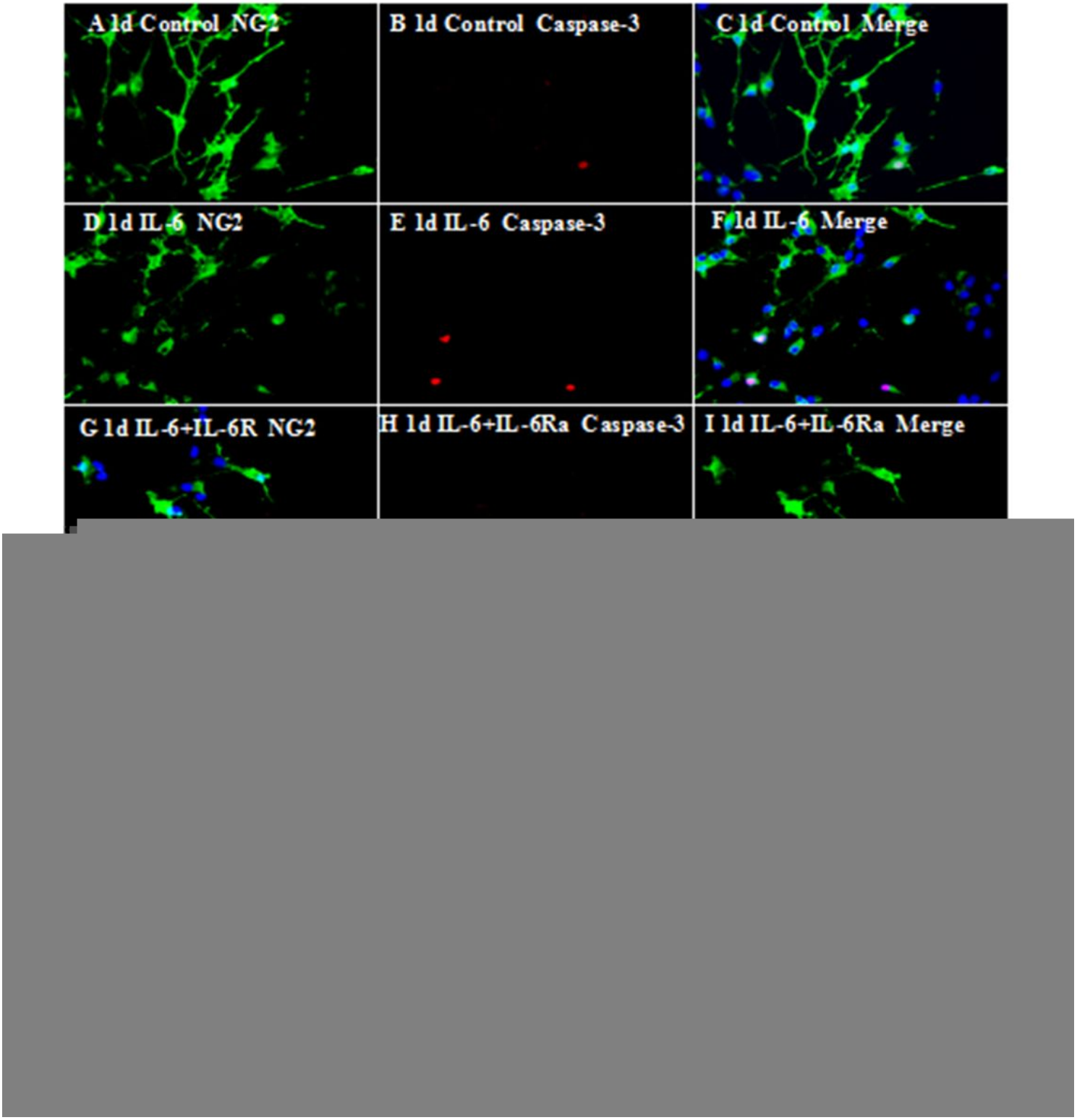

Figure 6

IL-6 promotes the apoptosis of OPCs in vitro. NG2 (green) and cleaved-caspase-3 (red) labeled immunoreactive OPCs in vitro at 1 d after IL-6 and IL-6+IL-6Ra and their corresponding control group. (C, F, I and J). Bax, Bcl-2, and β-actin immunoreactive bands. (K-M) . IL-6Ra can improve abnormal expression of Bax and Bcl-2. N=5. *P < 0.05. Scale bars: A–I 50 μm.

Figure 7

SCFAs inhibited the expression of IL-6 and NLRP3 in astrocytes after OGD in vitro. GFAP- (green) and IL-6- (red) labeled immunoreactive astrocytes in vitro at 1 h in each group (C, F, and I) Colocalized expression of GFAP and IL-6 in astrocytes. NLRP3, IL-6, and β-actin immunoreactive bands. (J-L) Similar results to immunofluorescence. N=5. *P < 0.05. Scale bars: A–I 50 μm.

Figure 8

SCFAs regulates endoplasmic reticulum stress in astrocytes in vitro. GFAP (green) and CCL2 (red) labeled immunoreactive in vitro at 1 h in each group. (C, F, and I). CCL2, IP-10, and β-actin immunoreactive bands. (J-L). SCFAs can improve abnormal expression of CCL2 and IP-10. N=5. *P < 0.05. Scale bars: A–I 50 μm.

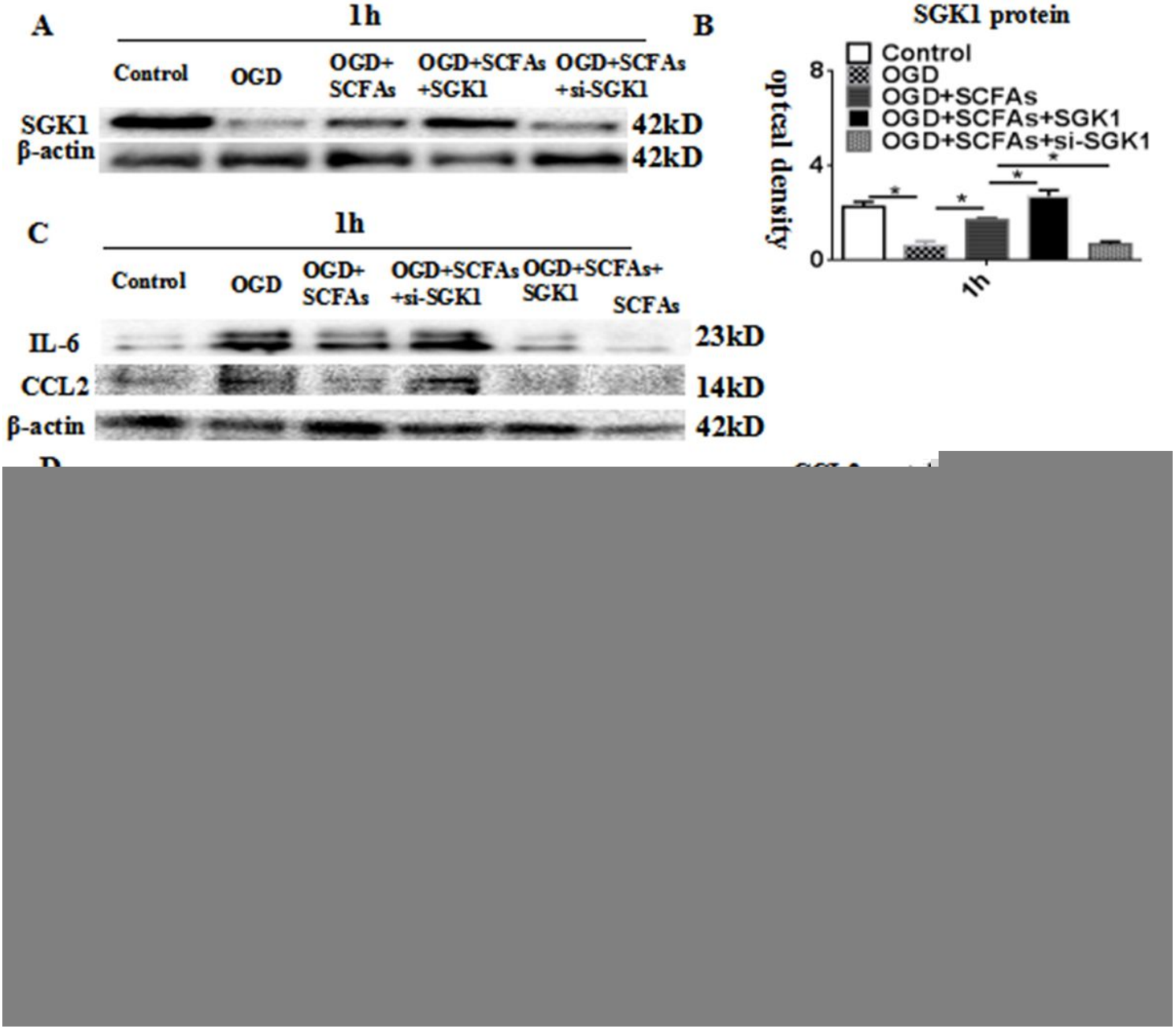

Figure 9

SCFAs regulates IL-6 via SGK1 in astrocyte. (A) Western blot of protein expression levels of SGK1 in astrocytes at 1 h (42 kDa). (B) Significantly downregulated protein expression level of SGK1 at after OGD. (C) IL-6 and CCL2 levels in astrocytes at 1 h. (D–E) Significantly upregulated protein level of IL-6 and CCL2 at 1 h after treatment with OGD compared to control group. overexpression-SGK1 can improve IL-6 and CCL2 proteins. The effect weakens after adding si-SGK1. (G and H) Significantly downregulated protein expression level of IL-6 after treatment with SGK1 gene overexpression after OGD+SCFAs compared with that of the OGD+SCFAs. N=5 *P < 0.05.