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# Short-Chain Fatty Acids Inhibit Oxidative Stress and Inflammation in Mesangial Cells Induced by High Glucose and Lipopolysaccharide

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## ABSTRACT

Recently, an connection between Short-chain fatty acids (SCFAs) produced by intestinal microbiota and kidney has been revealed. The aim of this study was to explore whether SCFAs or their specific G protein-coupled receptors 43 (GPR43) agonist inhibit oxidative stress and inflammatory response in glomerular mesangial cells (GMCs) induced by high glucose and lipopolysaccharide (LPS). Our research showed that treatment with SCFAs, especially acetate and butyrate, or GPR43 agonist significantly inhibited GMCs proliferation induced by high glucose and LPS, and then reversed the production of reactive oxygen species (ROS) and malondialdehyde (MDA) but increased levels of antioxidant enzyme superoxide dismutase (SOD). Furthermore, SCFAs or GPR43 agonist obviously increased the protein expression of GPR43 induced by high glucose and LPS, but diminished the expression of adhesion molecule intercellular adhesion molecule-1 (ICAM-1), and then decreased the proinflammatory cytokine monocyte chemoattractant protein (MCP-1) and interleukin-1β (IL-1β) release from GMCs stimulated by the high glucose and LPS. These combined results support the hypothesis that SCFAs or GPR43 agonist can inhibit oxidative stress and inflammation of GMCs induced by high glucose and LPS, suggesting that SCFAs induced signaling pathway may act as new therapeutic targets of diabetic nephropathy (DN).

## Introduction

In recent years, gut microbiota have emerged as key players in the heightened risks of a systemic immuno-inflammatory response and in kidney failure progression, which has been coined the "Gut–kidney axis" [1]. One class of molecules that acts as a link between the microbiota and the inflammatory response are short-chain fatty acids (SCFAs), the main metabolic products by the bacterial fermentation of macro-fibrous material that escapes digestion in the upper gastrointestinal tract and enters the colon 2. 90~95% SCFAs in the colon are made up of acetate, propionate, and butyrate, with intraluminal concentrations of about 60% acetate, 25% propionate, and 15% butyrate [4]. Locally, SCFAs are energy sources for colonocytes and have been recognized as potential mediators involved in the effects of gut microbiota on intestinal immune function [5]. However, SCFAs can reach the bloodstream and are involved in inflammatory and immune responses by acting on their specific receptors GPR43, and increasing studies are shedding light on the roles of GPR43 in SCFAs-associated inflammatory diseases such as inflammatory bowel disease (IBD), asthma, arthritis and other inflammatory diseases [6, 7].

Systemic and local low-grade inflammation, generation of reactive oxygen species (ROS) and release of pro-inflammatory cytokines induced by the metabolism of hyperglycemia and dyslipidemia are implicated in the development and progression of type 2 diabetes (T2D) and diabetes nephropathy (DN) [8]. Recent, 3 studies [10–12] have attempted to therapeutically use SCFAs or their derivatives in systemic immunologic and inflammatory responses of kidney injury in both animal and cell models. Accordingly, therapies have been proposed to restore SCFAs production or to provide exogenous SCFAs in an attempt to reduce inflammation. However, it is currently unknown whether SCFAs or GPR43 agonist inhibit oxidative stress and inflammatory response in glomerular mesangialn cells (GMCs) induced by high glucose and LPS. Therefore, we observed the effects of the SCFAs or GPR43 agonist treatment on the protein expression of membrane receptor GPR43 and adhesion molecule ICAM-1, and detected the release of pro-inflammatory cytokine MCP-1 and IL-1β from GMCs stimulated by high glucose and LPS, and then analysised of the generation of oxidative stress relevant molecules (ROS, MDA, and antioxidant enzyme SOD), finally, discussed implications for DN pathogenesis.

## Materials and Methods

### Renal mesangial cell culture and stimulation

Mouse glomerular mesangialn cells (SV-40 MES 13, obtained from China Center for Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM, Glbco) containing 5.6 mmol/L glucose and 10 % fetal bovine serum (FBS, Glbco) at 37 °C and 5 % $CO_2$.

Initially, to determine proper concentrations of each SCFAs, GPR43 agonist and LPS, glomerular mesangialn cells were randomly divided and the following treatments were applied: (1) Lipopolysaccharide (LPS; Sigma-Aldrich) at 0.5, 1, 5, and 10 μg/ml concentrations; (2) Sodum acetate (Ac; Sigma-Aldrich) at 0.1, 1, 12.5, 25, and 50 mmol/L (mM) concentrations; (3) Sodium propionate (Pr; Sigma-Aldrich) at 0.1, 1, 12.5, 25, and 50 mM concentrations; (4) Sodium butyrate (But; Sigma-Aldrich) at 0.1, 1, 5, 10, and 20 mM concentrations; (5) A phenylacetamide compound (Merckmillipore) acts as an allosteric agonist of GPR43 at 0.1, 1, 10, and 20 μmol/L (μM) concentrations. All of the pH changes in media by high concentration of SCFAs were adjusted to PH 7.35 by $NaHCO_3$.

### Cell viability assay

GMCs were seeded in 96-well plates at concentrations of $1 \times 10^5$ cells/ml to 50 % confluence in DMEM complete growth medium, followed by treatments respectively supplemented with different concentrations of LPS, each SCFAs and GPR43 agonist as described above or mixed LPS and each SCFAs or GPR43 agonist for 24 h in full media. Cell viability was measured by quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 96-well plates. Absorbance at 570 nm was measured for the experimental groups using a microplate reader. MTT experiments were performed in 5 biological replicates. DMEM low glucose complete growth medium was used as a normal control (NC group). Cell viability was expressed as the ratio of the signal obtained from treated cultures and control cultures. According to MTT results and literatures review, 1 μg/ml LPS, 25 mM high glucose (HG), 25 mM Ac, 12.5 mM Pr, 5 mM But and 1 μM GPR43 agonist were used in the entire study.

### Detection of intracellular ROS level

GMCs ($1 \times 10^4$ per well) were seeded in 24-well plates and induced by 25 mmol/L high glucose and 1 μg/mL LPS, the specified concentration of SCFAs or GPR43 agonist was added to the wells and incubated for 24 h respectively. Intracellular production of ROS was measured using the ROS assay kit (Beyotime Institute of Biotechnology, China) with oxidation of 2',7'-dichlorofluorescin diacetate (DCFH-DA) to fluorescent 2',7'-dichlorofluorescin (DCF). The fluorescence images were taken with a fluorescence microscope (Leica, Germany) and measured in a plate reader with excitation at 488 nm and emission at 525 nm according to the manufacturer's instructions. The values were expressed as the mean absorbance normalized to the percentage of the normal control.

### Detection of malondialdehyde (MDA) and superoxide dismutase (SOD)

After incubation with different compounds for 24 h as described above, GMCs were harvested with 0.25 % trypsin, and washed twice with PBS. Then, the contents of MDA and total SOD were determined using the corresponding detection kits (Lipid Peroxidation MDA Assay Kit; Total Superoxide Dismutase Assay Kit with WST-8; Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions.

### Enzyme-linked immunosorbent assay (ELISA)

MCP-1 and IL-1β protein level in the culture supernatant was determined using commercially available ELISA MCP-1 and IL-1β kits (NeoBioscience, China) according to the manufacturer's protocols. MCP-1 and IL-1β protein levels were determined by comparing the samples to the standard curve generated by the kit.

### Protein extraction and western blotting

Total proteins were isolated from GMCs using a total protein extraction kit (Kaiji, Shanghai, China). The protein concentrations in the cell lysates were determined with a Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology, Rockford, USA). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Immunoblotting was performed using anti-GPR43 antibody (rabbit; dilution 1:800, Santa Cruz Biotechnology) and anti-β-actin antibody (mouse; dilution 1:2000, Beyotime, China).

### Immunofluorescence

GMCs were grown on coverslips in 6-well plates. After overnight adherence, cells were incubated with different compounds for 24 h as described above and then were fixed in 4 % paraformaldehyde and then blocked with 5 % rabbit serum. The cells were incubated overnight with the anti-ICAM-1 primary antibodies (goat; dilution 1:100; Santa Cruz Biotechnology) and incubated for 60 min with secondary antibody conjugated to the fluorescein isothiocyanate fluorescent dye (dilution 1:200; Boster, China). Images were taken with a fluorescence microscope (Leica, Germany) and the values of semiquantitative analysis for average intensity of ICAM-1 were assessed by Image-Pro Plus 6.0 software.

## Statistical analysis

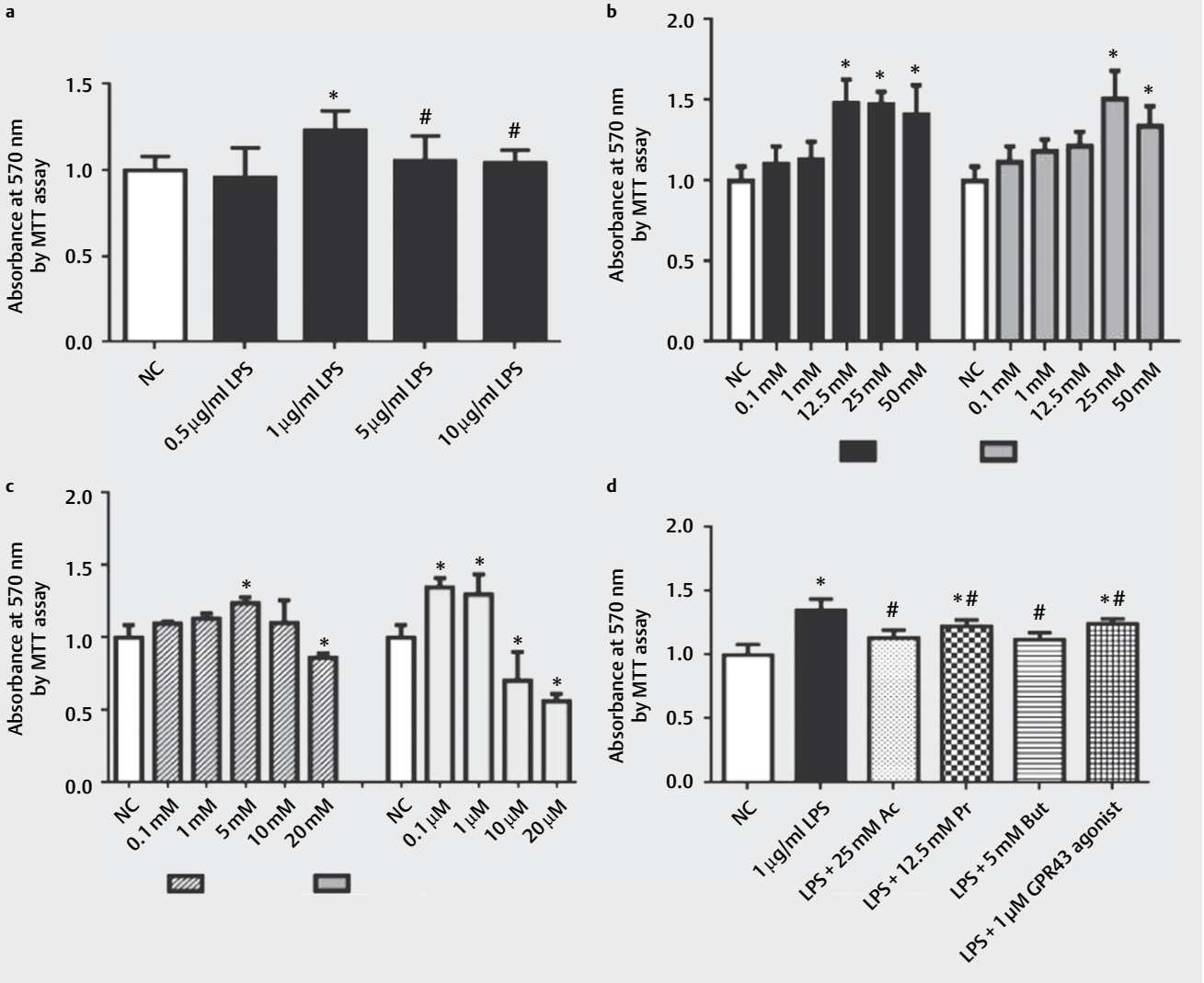
Each experiment was repeated at least 3 times using different batches of cells. The data are expressed as mean ± standard deviation (SD). Differences were statistically analyzed using one-way analysis of variance (ANOVA), followed by the Least Significant Difference post hoc test for multiple comparisons. A probability value of $p < 0.05$ was considered significant.

# Result

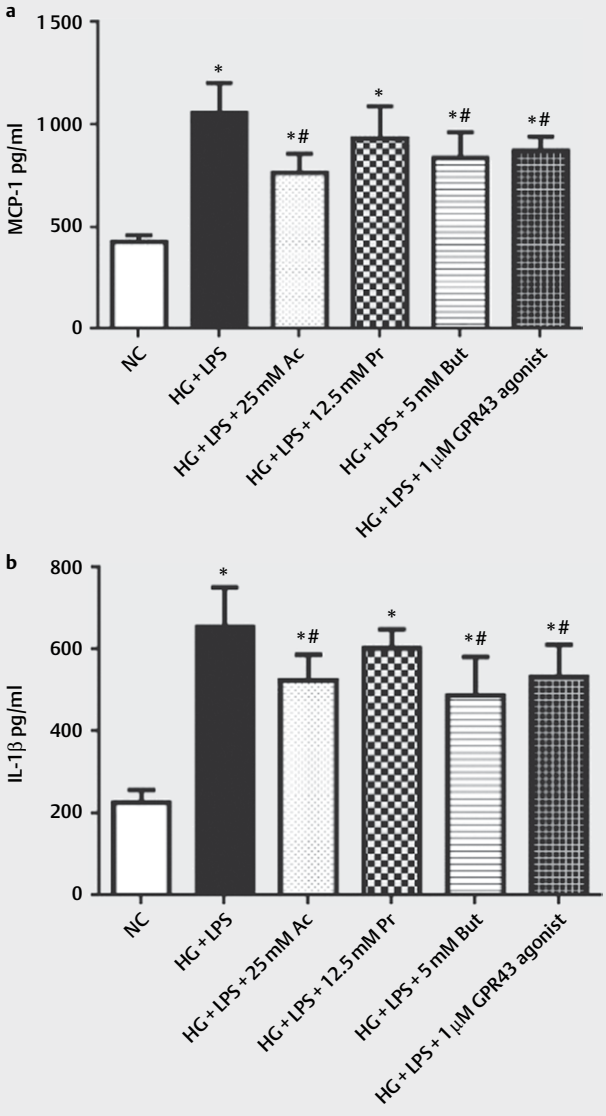
## The pharmacological concentrations of SCFAs or GPR43 agonist inhibied GMCs proliferation following LPS treatment

The MTT results showed that 1 μg/ml LPS could induce proliferation of GMCs compared with NC group ($p < 0.05$), while the higher concentration (5, 10 μg/ml) of LPS group did not promote the cells proliferation compared with 1 μg/ml LPS group ($p < 0.05$), indicating a severe inflammatory state would lead to the inhibition of cell growth and stimulation of apoptosis (▶ **Fig. 1a**).

The effect of different concentrations of these SCFAs or GPR43 agonist on GMCs' viability was also assessed respectively by MTT assay. We observed that 0.1–50 mM concentration range of Ac ($p < 0.05$), 0.1–25 mM Pr ($p < 0.05$), 0.1–5 mM But ($p < 0.05$) or 0.1–1 μM GPR43 agonist ($p < 0.05$) promoted the cells proliferation in a dose-dependent manner; however, higher concentrations of SCFAs (≧10 mM But) or GPR43 agonist (≧10 μM) may inhibit cell viability ($p < 0.05$) (▶ **Fig. 1b, c**). Considering the cytotoxicity detected by MTT and the pharmacological concentrations of these SCFAs or GPR43 agonist reported in the literature, we chose the 25 mM Ac, 12.5 mM Pr, 5 mM But and 1 μM GPR43 agonist as the intervention reagent. Thereafter, the results showed that the intervention effects of these SCFAs or GPR43 agonist on cell prolifer-




▶ **Fig. 1** The effects of SCFAs or GPR43 agonist on GMCs proliferation following LPS treatment were analyzed by MTT assay. **a** GMCs were incubated with indicated concentrations (0.5, 1, 5, 10 μg/ml) of LPS for 24 h. **b** and **c** GMCs were incubated with different concentration range of acetate (Ac), propionate (Pr), and butyrate (But) or GPR43 agonist for 24 h. **d** GMCs were treated with 1 μg/ml LPS alone, or were intervened by specific concentration of these SCFAs or GPR43 agonist following 1 μg/mL LPS treatment. Data were normalized with respect to normal control and are expressed as mean ± SD (n = 5), * $P < 0.05$ compared with the NC group; # $P < 0.05$ compared with the 1 μg/ml LPS group.




▶ **Fig. 2** The effects of SCFAs or GPR43 agonist on MCP-1 and IL-1β release from GMCs following high glucose and LPS stimulation were detected by ELISA. Incubation of specific concentration of these SCFAs or GPR43 agonist for 24 h with GMCs cultured in 25 mM glucose and 1 μg/ml LPS (HG + LPS) condition, MCP-1**a** and IL-1β **b** protein level in the cell culture supernatant were determined by ELISA-based quantification. Data are expressed as mean ± SD (n = 5), * $P < 0.05$ compared with the NC group; #$P < 0.05$ compared with the HG + LPS group.

ation following 1 μg/ml LPS treatment was significantly lower than that of LPS stimulation solitar group ($p < 0.05$) (▶ **Fig. 1d**).

### The pharmacological concentrations of SCFAs or GPR43 agonist diminished high glucose and LPS induced MCP-1 and IL-1β release from GMCs

As depicted in ▶ **Fig. 2a**, after 24 h of stimulation, pro-inflammatory cytokines MCP-1 release from GMCs was obviously increased in the 25 mM glucose and 1 μg/ml LPS synergy treatment group (HG + LPS group) compared with exposure to normal-glucose (5.6 mM, NC group) ($p < 0.05$), this result suggested that synergy between high glucose and LPS could induce inflammatory response in vitro. However, 25 mM Ac, 5 mM But or 1 μM GPR43 agonist reversed high glucose and LPS induced MCP-1 release ($p < 0.05$). In accordance with MCP-1, similar profiles of IL-1β released from GMCs were found, incubation of 25 mM Ac, 5 mM But or 1 μM GPR43 agonist with GMCs cultured in high glucose and LPS condition resulted in a significant decrease of IL-1β protein concentration level ($p < 0.05$) (▶ **Fig. 2b**).

### SCFAs or GPR43 agonist increased the protein expression of GPR43, but inhibited ICAM-1 in GMCs induced by high glucose and LPS

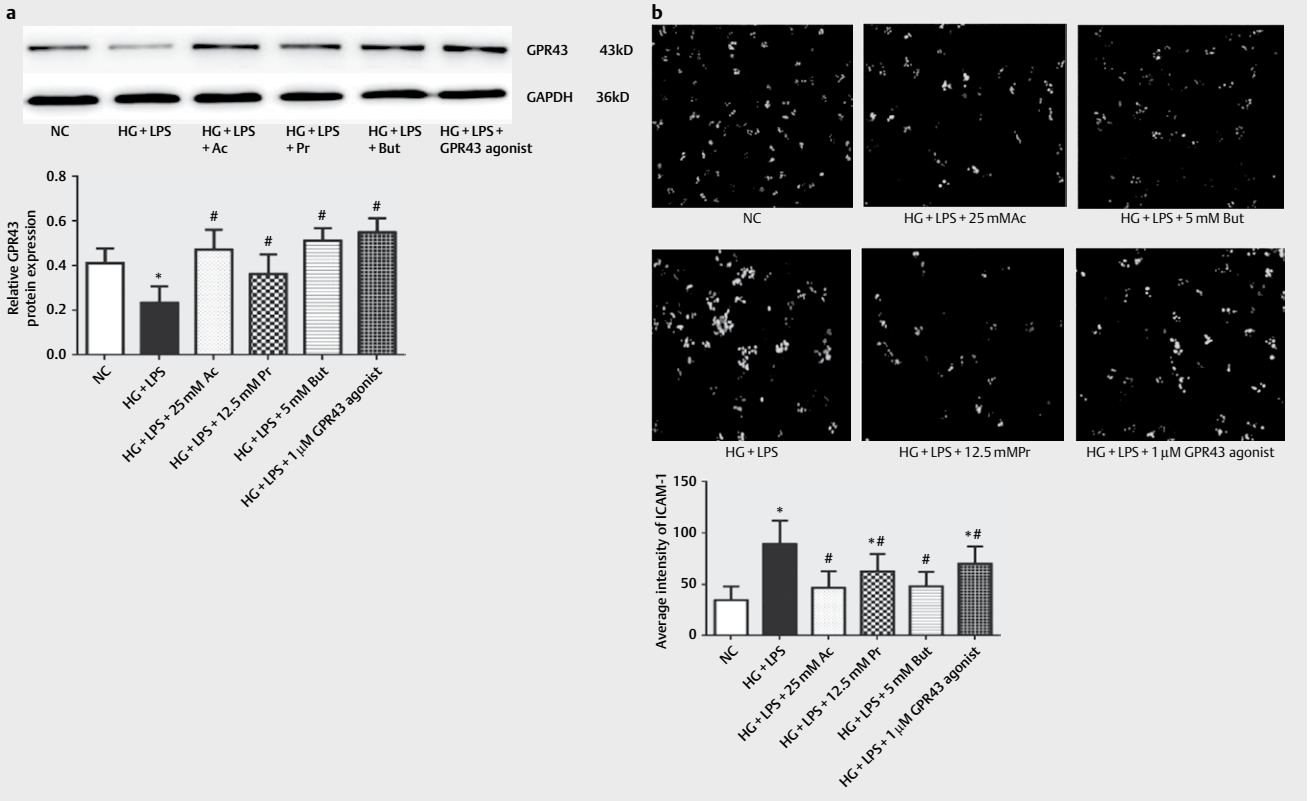
Compared with NC group, the protein expression of GPR43 in lysates of GMCs was inhibited by high glucose and LPS stimulation for 24 h ($p < 0.05$), but downregulation of GPR43 was reversed by 25 mM Ac, 12.5 mM Pr, 5 mM But, or 1 μM GPR43 agonist ($p < 0.05$) (▶ **Fig. 3a**). Moreover, high glucose and LPS stimulation for 24 h markedly increased the ICAM-1 protein level in cytoplasm compared with NC group by immunofluorescence ($p < 0.05$); this trend was reversed by 25 mM Ac, 12.5 mM Pr, 5 mM But, or 1 μM GPR43 agonist ($p < 0.05$), especially 25 mM Ac and 5 mM But (▶ **Fig. 3b**).

### SCFAs or GPR43 agonist inhibited the intracellular production of ROS and MDA, and reversed decrease of SOD in high glucose and LPS treated GMCs

The fluorescence images for ROS generation and quantitative assay showed that intracellular ROS (▶ **Fig. 4a**) and MDA (▶ **Fig. 4b**) in the HG + LPS group were obviously higher than in the NC group ($p < 0.05$), while the levels of SOD (▶ **Fig. 4c**) were significantly decreased ($p < 0.05$), indicating that high glucose and LPS may induce excessive oxidative stress in GMCs. However, the pharmacological concentrations of these SCFAs or GPR43 agonist inhibited the generation of ROS and MDA and reversed decrease of SOD induced by high glucose and LPS ($p < 0.05$). In addition, the ratio of SOD/MDA (▶ **Fig. 4d**), which reflect the overall anti-oxidative stress effect of these SCFAs or GPR43 agonist, were calculated to confirme these trends ($p < 0.05$).

## Discussion

Taking into account the fact that SCFAs are important energetic substrates for epithelial cells, the confirmation that they are regulators of their proliferation have already been expected [5, 13]; but the effect of SCFAs on the proliferation of GMCs is still unknown. It must be pointed out that the physiological concentrations of SCFAs in colon look quite different from peripheral circulation, peripheral concentrations of SCFAs in particular propionate and butyrate are lower than 1 mM because of SCFAs metabolism in colonocytes and liver [4]. Most of the anti-inflammatory mechanistic studies of these SCFAs that are discussed at present have been performed with mM concentrations in vitro, gut or kidney [6, 7, 14]. In this study, we initially determined the viability and cell toxicity effects of SCFAs at physiological and pharmacological concentrations and molar ratios 4, 15. Our results showed that the pharmacological concentration range of SCFAs (0.1–50 mM Ac, 0.1–12.5 mM Pr, or 0.1–5 mM But), although may be higher than physiological con-

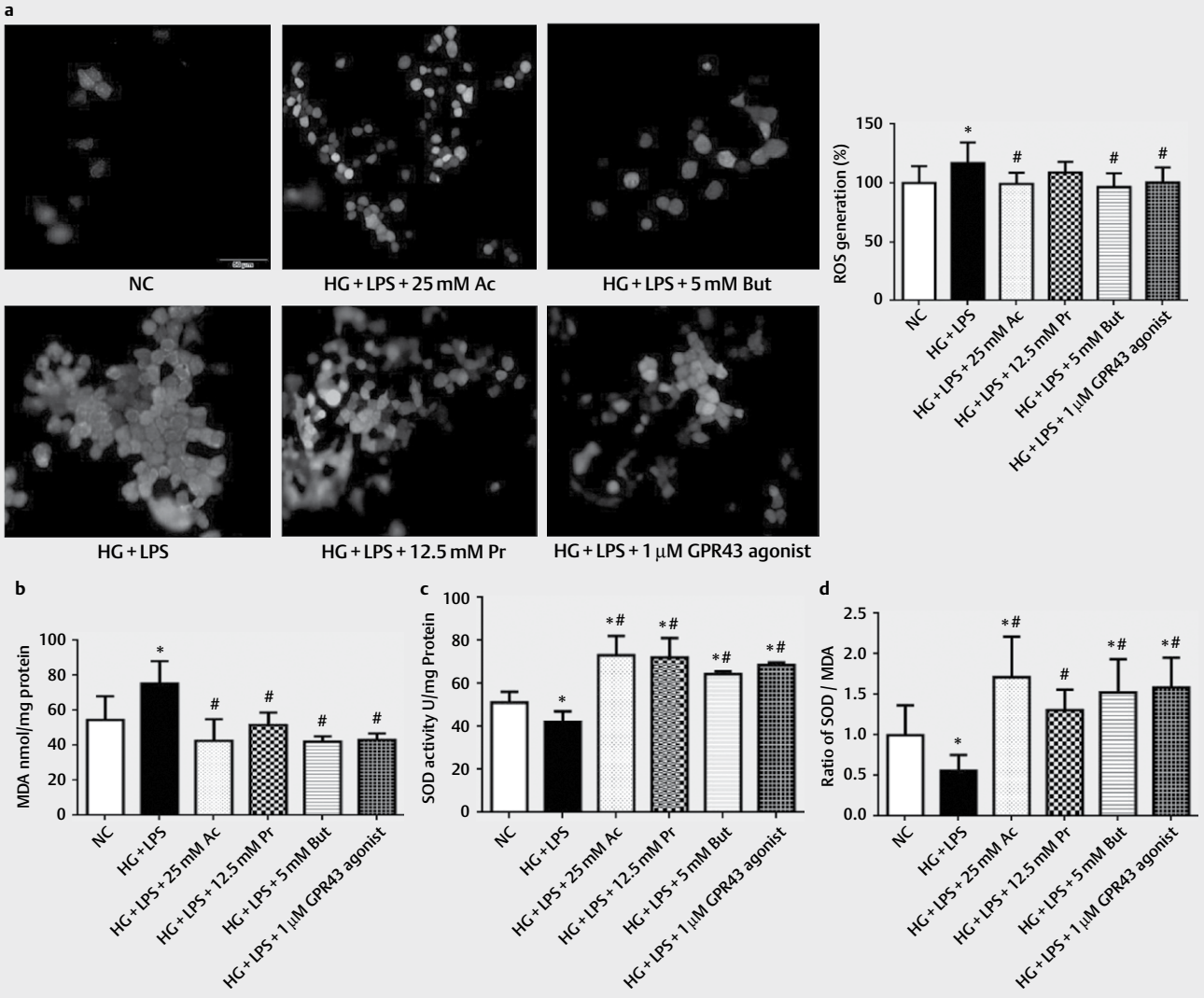



▶ **Fig. 3** The effects of SCFAs or GPR43 agonist on the expression of GPR43 and ICAM-1 in GMCs following high glucose and LPS stimulation. **a** Incubation of specific concentration of these SCFAs or GPR43 agonist for 24 h with GMCs cultured in high glucose and LPS condition, the protein expression of GPR43 in cell lysates were detected by western blotting. Data were normalized with respect to GAPDH and the gray graphs confirmed these trends. **b** The expressions of ICAM-1 were detected as green fluorescence in the cytoplasm of GMCs by immunofluorescence and fluorescence microscope (200×). The values of semiquantitative analysis for average intensity of ICAM-1 were assessed and the gray graphs confirmed these trends. Data are expressed as mean ± SD (n = 3), $^{*}P < 0.05$ compared with the NC group; $^{\#}P < 0.05$ compared with the HG + LPS group.

centrations in plasm, improved the growth of GMCs, which likely due to the provision of energy source by SCFAs [5]. However, the higher concentrations of SCFAs (≧10 mM But) may inhibit GMCs viability, which may be attributed to high concentration related cytotoxicity and apoptosis. Previous research has also shown that oral administration of all major SCFAs, when chronically increased higher than physiological levels in vivo, induce tissue inflammation in ureteral tissues, leading to kidney hydronephrosis in mice [16]. Taken together, these evidences indicates that SCFAs may have a dual role in GMCs proliferation depending on the stimulus concentration, but the mechanism is still needed to be further studied. Therefore, an exploration of the larger concentration range of SCFAs in various kidney diseases, such as DN, the leading cause of ESRD, is necessary. This knowledge may resolve some of these controversies by allowing us to identify the pharmacological concentration and genuine functions of SCFAs in kidney tissue, be it pro- or anti-inflammatory.

GMCs proliferation and hypertrophy, ECM accumulation, as well as consequent renal fibrosis induced by high glucose, AGEs, or LPS have been recognized as major pathogenic events in the progression of renal failure in DN [17]. In this research, the MTT results showed that LPS (1 μg/ml) could induce proliferation of GMCs, however, the pharmacological concentrations of SCFAs (25 mM Ac, 12.5 mM Pr, or 5 mM But) or GPR43 agonist (1 μM) intervention inhibited GMCs proliferation following LPS treatment. There is a contrary to the trend of these SCFAs or GPR43 agonist at the choosed concentration and LPS (1 μg/ml). Interestingly, SCFAs, mainly butyrate, present different effects on the growth of normal and tumoral colonocytes. Butyrate inhibits the growth of cancerous colonic cells, but not of normal colonocytes and, depending on the concentration, it actually increases the proliferation of normal colonocytes [13]. The mechanism proposed for this difference is not clear, may be involved in the cross-talk of related signaling pathways, such as GPCRs and the inhibition of histone acetylation (HDACs) [13, 19]. Similar concentrations of SCFAs (25 mM acetate, 12 mM propionate, or 3.2 mM butyrate) were used in bone marrow DCs (BM-DCs) stimulated with LPS (20 ng/ml), MM55. k kidney epithelial cells stimulated with inflammatory cocktail (LPS, 10 μg/ml; zymosan, 10 μg/ml; IL-6, 50 ng/ml; IL-1β, 50 ng/ml, and TNF-α, 100 ng/ml), and HK-2 human kidney epithelial cells after hypoxia, these findings indicated that SCFAs treatment diminished hypoxia and inflammation in these cells [10]. Therefore, the pharmacological concentrations of these SCFAs or GPR43 agonist as mentioned above were used in the entire study [7, 10, 14].

SCFAs are well known for their anti-inflammatory functions by modulating immune cell adhesion molecule, chemotaxis as well as




▶ **Fig. 4** The effects of SCFAs or GPR43 agonist on the intracellular production of ROS, MDA and SOD in high glucose and LPS treated GMCs. The fluorescence images (100×) and quantitative assay for ROS **a**, the contents of MDA **b**, and SOD **c** were determined, respectively by detection kits after incubation for 24 h with specific concentration of these SCFAs or GPR43 agonist. The ratio of SOD/MDA **d** were calculated to confirme these trends. Data are expressed as mean ± SD (n = 5), $^{*}P < 0.05$ compared with the NC group; $^{\#}P < 0.05$ compared with the HG + LPS group.

cytokine release, finally resulting in inhibition of leukocyte migration to inflammatory sites. Previous research reported that SCFAs were able to reduce immune cell infiltration to adipose tissue by preventing inflammatory cell adhesion and chemotaxis [20]. Our results demonstrated that incubation of SCFAs, especially acetate and butyrate, or GPR43 agonist with GMCs cultured in high glucose and LPS condition for 24 h resulted in a significant decrease of MCP-1, IL-1β, and ICAM-1, suggesting that these SCFAs or GPR43 agonist treatment, diminished inflammation in kidney tissues and cells.

Oxidative stress and inflammation are inseparably linked as each causes and intensifies the other, which could cause glomerulosclerosis, tubular atrophy, and fibrosis [21]. Our previous research showed high glucose and LPS prime the NLRP3 inflammasome and NF-κB inflammatory signaling in GMCs via ROS/TXNIP pathway [22]. Up to present, several studies have looked into the effect of stress and inflammatory signaling pathways as effector mechanisms of SCFAs, the anti-inflammatory activity of SCFAs or GPR43 agonist were shown to inhibit the production of ROS and oxidative stress in this and other studies. Previous research showed that activation of GPR43 decreased inflammatory markers, which has been associated with diet-induced obesity and T2D [24]. Our data shown that the pharmacological concentration of SCFAs or GPR43 agonist reversed the high glucose and LPS – induced inhibition of GRP43 protein expression, further more, these SCFAs or GPR43 agonist also inhibited the intracellular production of ROS and MDA, and reversed decrease of SOD in GMCs induced by high glucose and LPS. Similar results are demonstrated that the gene expression levels of GPR43 were increased after acetate treatment in kidney tissue induced by ischemia-reperfusion injury, and the these SCFAs treatment inhibited ROS production in HK-2 human kidney epithelial cells after hypoxia [10]. By contrast, acetate was shown to promote the release of ROS when added on mouse neutrophils by activat-

ing GPR43 [26]. As ROS and oxidative stress have been reported be involved in a wide array of inflammatory conditions, including DN; and GPR43 signaling are positive regulators of high glucose and (or) LPS-induced inflammatory signaling pathways, including NF-κB and MAPK, both of which are associated with inflammatory response and kidney injury [10, 11, 28]; these results suggested that the activation of GPR43 may be related to the molecular mechanisms through which SCFAs exert their anti-inflammatory effects in kidney tissue. However, many questions regarding the function of these SCFAs and GPR43 receptor remain unanswered and controversial on account of lack of specific GPCRs blockers, therefore, potent and selective tools will be required for future studies on SCFAs and their receptors [29].

In conclusion, the present study found that SCFAs, especially acetate and butyrate, and GPR43 agonist reversed the high glucose and LPS induced mesangial cells proliferation, ROS and MDA generation, and inflammatory cytokine MCP-1, IL-1β and ICAM-1 release. These combined results support the hypothesis that SCFAs inhibit oxidative stress and inflammation in GMCs induced by high glucose and LPS, suggesting that SCFAs or GPR43 signaling pathway may act as potential therapeutic targets for DN. However, our studies by no means rule out other potential mechanisms by which SCFAs may take part in inflammatory response, such as inhibition of HDAC, in view of the fact that the regulatory mechanisms of SCFAs are extremely complex [13, 30]. Future studies will focus on the interaction among SCFAs-associated molecular patterns and innate immunity in order to clarify the molecular mechanisms behind kidney injury in the pathogenesis of DN.

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## Conflict of interest

The authors declare no conflict of interests.

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