Gut Bacteria Products Prevent AKI Induced by Ischemia-Reperfusion


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ABSTRACT

Short-chain fatty acids (SCFAs) are fermentation end products produced by the intestinal microbiota and have anti-inflammatory and histone deacetylase–inhibiting properties. Recently, a dual relationship between the intestine and kidneys has been unraveled. Therefore, we evaluated the role of SCFA in an AKI model in which the inflammatory process has a detrimental role. We observed that therapy with the three main SCFAs (acetate, propionate, and butyrate) improved renal dysfunction caused by injury. This protection was associated with low levels of local and systemic inflammation, oxidative cellular stress, cell infiltration/activation, and apoptosis. However, it was also associated with an increase in autophagy. Moreover, SCFAs inhibited histone deacetylase activity and modulated the expression levels of enzymes involved in chromatin modification. In vitro analyses showed that SCFAs modulated the inflammatory process, decreasing the maturation of dendritic cells and inhibiting the capacity of these cells to induce CD4+ and CD8+ T cell proliferation. Furthermore, SCFAs ameliorated the effects of hypoxia in kidney epithelial cells by improving mitochondrial biogenesis. Notably, mice treated with acetate-producing bacteria also had better outcomes after AKI. Thus, we demonstrate that SCFAs improve organ function and viability after an injury through modulation of the inflammatory process, most likely via epigenetic modification.


AKI is an inflammatory process frequently observed in hospitalized patients. AKI is associated with the development of CKD and causes distress to the patient.1,2 AKI induced by ischemia and reperfusion injury (IRI) is closely linked to the activation of tubular epithelial and endothelial cells by endogenous danger signals released after cell stress and death due to enhanced production of reactive oxygen species (ROS), among other inducers.3 Additionally, IRI involves the migration and activation of innate and adaptive immune cells into the kidneys.

Increased ROS production disrupts the ratio of oxidant/antioxidant enzymes, leading to mitochondrial-mediated apoptotic cell death. Activation of endothelial

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cells enhances adhesion molecule expression, which recruits immune cells, contributing to ROS production. Similarly, kidney tubular epithelial cells (TECs) and resident antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), can produce cytokines and chemokines upon activation at the site of inflammation. Furthermore, APCs increase the expression of costimulatory molecules and migrate to the draining lymph node to activate CD4+ and CD8+ T lymphocytes, thereby contributing to tissue damage. Our group and others have demonstrated the role of different immune cell populations in kidney IRI.

Figure 1. SCFA ameliorates renal function. Mice (n=5) were subjected to kidney IRI and treated with acetate (Ac), propionate (Prop), and butyrate (But) at 0.5 hour before ischemia and at the moment of reperfusion (200 mg/kg each). Serum creatinine (A) and urea (B) were measured after 24 hours. (C) Quantification of necrosis in tubular epithelial cells. Ctl, control group.

Figure 2. Acetate treatment decreases cellular stress, cytokine and chemokine production, and cellular infiltrates. Mice (n=5) were subjected to kidney IRI and treated with acetate. (A) Glutathione reduced (GSS) and glutathione oxidized (GSSH) ratio. mRNA levels measured by real-time PCR (B) and protein levels measured by BioPlex (C) of proinflammatory cytokines and chemokines in kidney tissue and serum protein levels of the proinflammatory cytokines and chemokines (D). (E) mRNA levels of TLR4 and biglycan measured by real-time PCR in kidney tissue. (F) Right, Western blot analysis in kidney tissue for IκBα and β-actin; each band quantified (optical densitometry) for each protein in the group and normalized by quantification of β-actin for the respective group. (G) Myeloperoxidase (MPO) levels in kidney tissue. (H) Percentage of activated DCs (CD11c+/CD40+) and macrophages (CD11b+/F4/80+) in kidney tissue analyzed by flow cytometry. Ac, acetate; Ctl, control; KC, Cxcl1; MCP-1, monocyte chemotactic protein-1.
Recently, an intimate connection between the intestine and the kidneys has been proposed.\textsuperscript{10,11} Established data have already shown that modulation of microbiota composition could affect the outcome of glomerulopathies, and recent data indicate that renal inflammation is associated with the production of uremic toxins by the intestine and with augment intestinal permeability, leading to the onset of systemic inflammation.\textsuperscript{12,13} In this sense, molecules produced by local intestinal flora could have a direct effect on kidney injuries.

Short-chain fatty acids (SCFAs) are end products produced from the fermentation of complex carbohydrates by the intestinal microbiota, especially by anaerobic bacteria.\textsuperscript{14} The most abundant SCFAs are acetate, propionate, and butyrate. Locally, SCFAs are energy sources for colonocytes. However, they can reach the bloodstream.\textsuperscript{15} SCFA treatment has ameliorated colitis, airway disease, and metabolic syndrome in diet-induced obese mice.\textsuperscript{16–18}

So far, researchers suggested that SCFAs may operate in two manners: by binding G-protein membrane receptors (GPR41 and GPR43),\textsuperscript{19,20} or by entering cells directly through transporter channels in the cellular membrane and working as histone deacetylase (HDAC) inhibitors. Thus, they could act by modulating epigenetic processes.\textsuperscript{21}

Because AKI has an important inflammatory component yet SCFAs have anti-inflammatory properties, we investigate whether SCFAs treatment could protect mice from IRI. Furthermore, we investigated whether this protection could involve direct modulation of the inflammatory process and/or amelioration of the oxidative stress presented in this model.

RESULTS

SCFA Treatment Ameliorates Kidney Function after IRI

To investigate the role of SCFAs in IRI, mice were subjected to IRI and treated with SCFAs. SCFA treatment diminished levels of serum creatinine and urea after IRI, with acetate treatment...
resulting in the best protection (Figure 1, A and B). SCFA treatment did not interfere in kidney function or inflammatory parameters (Supplemental Figure 1, A–C). Hereafter, we consider in vivo analyses only for acetate treatment. Histopathologic analysis showed that acetate treatment preserved kidney structure as reflected in the reduced necrosis score in kidney tubular epithelial cells (Figure 1C).

**Acetate Treatment Reduces Cellular Stress and Local and Systemic Inflammation**

Acetate protection was associated with decreased ROS production (Figure 2A). Cytokines and chemokines were also diminished locally (Figure 2, B and C) and systemically (Figure 2D). Additionally, low levels of mRNA of toll-like receptor 4 and its endogenous ligand, biglycan (Figure 2E), and lesser activation of the NF-κB pathway were observed (Figure 2F). As a consequence, low levels of activated neutrophils and macrophages (myeloperoxidase in kidney tissue), a low frequency of infiltrating macrophages (CD11b+F4/80+), and a low frequency of activated DCs (CD11c+CD40+) (Figure 2, G and H) were observed in acetate-treated mice. Thus, acetate treatment diminished cellular stress and inflammation in kidney IRI.

**Acetate Treatment Reduces Apoptosis and Increases Autophagy and Tubular Proliferating Cells**

Apoptosis is a frequent event in kidney IRI. Acetate treatment diminished the number of apoptotic cells in kidney tissue (Figure 3A) but increased proliferation in kidney epithelial cells (Figure 3B) and BCL-2 gene expression (Figure 3C). Moreover, acetate treatment increased the levels of ATG-7 protein, which is involved in the autophagy pathway (Figure 3D). Supporting these findings was an increase in mitochondrial...
DNA in kidney tissue after acetate treatment compared with the IRI group (Figure 3E). Taken together, these results show that acetate treatment inhibits apoptosis and increases cell proliferation, potentially through autophagy activation.

SCFA Receptors and Epigenetic Modifications in Kidney Tissue

Gene expression analysis for SCFA receptors revealed no difference in GPR41 expression, whereas the levels of GPR43 increased after acetate treatment (Figure 4A). Additionally, the PCR-based array showed that acetate treatment modulated the expression of genes encoding enzymes involved in epigenetic modifications (Figure 4B). Another important function for these SCFAs is their ability to act as HDAC inhibitors. Acetate treatment inhibited the activity of HDACs (Figure 4C) in kidney IRI. Another important epigenetic process is DNA methylation. Global methylation status decreased in kidney tissue undergoing IRI, and acetate treatment reversed this process (Figure 4D).

Evaluation of SCFAs in DCs In Vitro

Having observed in vivo a lower frequency of activated DCs in kidney tissue after IRI, we sought to determine whether SCFAs may modulate these cells in vitro. SCFA treatment reduced the expression of the costimulatory molecules CD80 and CD40 in bone marrow DCs (BM-DCs) (Figure 5, A and B). These data corroborated the in vivo finding (Figure 2H). Furthermore, we evaluated whether this reduction is functional. We pretreated
APCs from RAGKO mice with LPS, with or without SCFAs, for 24 hours. Treatment of APCs with LPS plus SCFAs was sufficient to reduce the proliferation of CD8+ and CD4+ cells (Figure 5, C and D). Therefore, SCFA treatment modulates the activation and function of APCs.

Evaluation of SCFA Treatment in a Kidney Epithelial Cell Line
It is well known that TEC activation enhances inflammation in kidney tissue after IRI. To date, SCFA action in TECs has not been reported. Thus, we investigated whether SCFA treatment is effective in these cells. TECs stimulated with an 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) in the presence of acetate (Ac; 25 mM), propionate (Pr; 12 mM), and butyrate (But; 3.2 mM) for 24 hours and were evaluated by flow cytometry for (A) left: overlay histogram of NFκB activation measured through phosphorylation of the p65 subunit (percentage of p65+ cells) and right: percentage of the p65+ cells and (B) left: overlay histogram nitric oxide production (DAF-FM Diacetate) [4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate] and right: mean fluorescence intensity of the DAF-positive cells. *P<0.05.

Figure 6. SCFA treatment inhibits NFκB activation and nitric oxide production in epithelial kidney cell line. MM55.k kidney epithelial cells were 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) in the presence of acetate (Ac; 25 mM), propionate (Pr; 12 mM), and butyrate (But; 3.2 mM) for 24 hours and were evaluated by flow cytometry for (A) left: overlay histogram of NFκB activation measured through phosphorylation of the p65 subunit (percentage of p65+ cells) and right: percentage of the p65+ cells and (B) left: overlay histogram nitric oxide production (DAF-FM Diacetate) [4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate] and right: mean fluorescence intensity of the DAF-positive cells. *P<0.05.

DISCUSSION
Kidney IRI physiopathology has a complex network of events that involves a well known and important inflammatory process that includes tubular epithelial, endothelial, and resident immune cells. In addition to the high mortality rate in hospitalized patients, the extent of this event has been believed to be a risk factor associated with worse outcomes. Thus, its prevention is extremely important. In this study, we investigated whether SCFAs, which have anti-inflammatory properties, could ameliorate kidney IRI.

Treatment with SCFAs, especially acetate, reduced kidney damage after kidney IRI. To our knowledge, this is the first study demonstrating the protective role of SCFAs in kidney IRI. Other studies have observed a reduction in kidney injury in other models after SCFA treatment. The higher protection observed after acetate treatment might be due to the composition of acetate, favoring the rapid entry of the molecule into the metabolic pathway, whereas other types of SCFAs need to...
be converted into precursor molecules. The concentrations used in this study agree with the physiologic concentrations observed for these molecules in the intestine. SCFA treatment was also protective in colitis and lung injury models. SCFAs can reach the bloodstream even when produced and taken up by local cells in the intestine, but their presence in kidney cells has not been reported.

Inflammation and apoptosis are hallmarks of this model, and acetate treatment inhibited both processes. This is an expected result because SCFAs have previously been found to possess anti-inflammatory properties. The reduction of apoptosis levels could be due to inflammation reduction or enhancement of the autophagy activation pathway. Restricted deletion of ATG7 in TECs showed increased susceptibility to kidney injury in cisplatin-induced AKI. Additionally, in cancer cells, butyrate treatment induced autophagy as a defense strategy to retard mitochondrial apoptosis-induced cell death. We observed lower apoptosis levels and higher levels of the ATG-7 protein after acetate treatment, suggesting that acetate could induce autophagy and thereby retard apoptosis.

Two mechanisms have been proposed for the operation of SCFAs: as a ligand for GPR41 and GPR43 or as an HDAC inhibitor. The latter is believed to occur in a GPR-independent manner. In our study, GPR41 had similar mRNA levels with kidney IRI and acetate treatment, whereas GPR43 levels were increased. Considering the other proposed function of SCFAs, HDAC activity was decreased in kidney tissues and the expression of chromatin modification enzymes was modulated.

Among the differentially expressed genes, none of them encoded for an HDAC, which suggests that HDAC inhibition exerted by SCFAs may be only at the protein level. Other studies have observed modulation of HDAC activity by acetate. It is not fully understood whether HDAC inhibition by SCFAs is GPR41/43 dependent. Recently, HDAC inhibition was partially GPR43 dependent in colon tissue, but other researchers have observed HDAC inhibition in a GPR-independent manner. Although we observed high expression of GPR43 in our model, its expression has been reported as minimal or null in kidney tissue. Thus, we believe that acetate might be regulating the inflammatory process by regulating epigenetic modification in a GPR-independent manner.

Another interesting finding in our work is the increase of global methylation of DNA after acetate treatment. One limitation of this finding is that this assay is unspecific, barring speculation as to which regions and consequently target genes could be altered by modifications in methylation status. Because we do not know whether this effect is direct or indirect, future investigations are necessary to unravel the possible relationship between GPR activation, HDAC inhibition, and DNA methylation by SCFAs in the kidney.

To further investigate possible SCFA protection, we examined the role of SCFAs in immune cells and a kidney epithelial cell line. As observed in vivo, SCFA treatment reduced the expression of the costimulatory molecule CD40 and (to a lesser extent) CD80 in BM-DC. Other studies have also demonstrated a reduction in DC development and activation. Furthermore, we observed that SCFA-treated APC induced less proliferation of CD4+ and CD8+ T lymphocytes, demonstrating that the modulation of DC treated with SCFAs was functional. DC links innate immunity
with adaptive immunity, so lower activation of DC is important to reduce lymphocyte activation. Our group reported that Th1-lymphocyte factors, such as IFN-γ and IL-12, are important for AKI development in kidney IRI. Thus, it is plausible to speculate that low frequencies of activated DC and reduced lymphocyte activation occur in vivo.

Kidney ischemia is an important event that contributes to kidney damage because of ROS production and inflammation. SCFA treatment reduced NFκb activation and nitric oxide production in TECs. Moreover, lower production of ROS, lower HIF-1α translocation, and lower lactate production after hypoxia in the presence of SCFAs were observed. Lactate production and HIF-1α activation are directly associated. The expression of GPRs in kidney tissue is minimal, which suggests that SCFAs could operate via an epigenetic mechanism in these cells, as observed in vivo. Another possibility is that SCFAs ameliorate mitochondrial function after kidney ischemia, through SCFAs metabolism in these cells, providing energy. This idea is supported by the finding of increased mitochondrial DNA in kidney tissue undergoing IRI after acetate treatment (Figure 3E). Butyrate seems to have exerted a greater effect in vitro than in vivo. Not all SCFAs could bind to the same receptor, as shown recently for the receptor Olr78, which can be activated by acetate and propionate and is involved in kidney BP regulation after binding of SCFAs. This new finding raises the question of whether acetate could protect from kidney IRI through Olr78 activation. If so, this could explain, at least for butyrate treatment, the better results observed in vitro because Olr78 is not expressed in epithelial cells. Either way, SCFA treatment can modulate the pathways involved in ischemia injury and inflammation in TEC in vitro.

Finally, we treated animals with acetate-producing bacteria and observed an improvement of kidney function. B. longum, but not B. adolescentis, was recently reported to reduce death in a model of enterohemorrhagic

Figure 8. SFCA treatment inhibits HIF-1α translocation to the nucleus, lactate production, and VEGF expression under hypoxia. HK-2 human kidney epithelial cells were seeded on a cover slip and subjected to hypoxia for 24 hours in the presence of acetate (Ac; 25 mM), propionate (Pr; 12 mM), and butyrate (But, 3.2 mM). (A) Cells were labeled with anti-human HIF-1α followed by FITC-labeled secondary antibodies and analyzed with confocal microscopy. Scale bar, 10μm. (B) Left lactate levels after hypoxia were measured in culture supernatant. Right relative expression of VEGF in normoxia and hypoxia. *P<0.05; ***P<0.001.
Escherichia coli infection, which was associated with acetate production. Here, both types of bacteria treatment protected from kidney IRI. One explanation is that although B. adolescentis produces less acetate, the amount was enough to protect from kidney IRI. However, further investigation will be necessary to clarify this issue. It is important to mention that because these treatments were performed without previous antibiotic administration, and because we have found very low frequencies of these probiotics in intestine (none of them close to the mucus region of treated mice), these bacteria probably did not colonize the intestine. However, future studies are necessary to elucidate this issue. Germ-free mice are more susceptible to kidney IRI. In this study, we demonstrated that metabolites from microbiota are also important to control inflammatory processes in distant organs where SCFAs are not produced. Acetate administration can also protect from colitis in the absence of microbiota, whereas the administration of SCFAs in wild-type mice also protects from other diseases. Despite the importance of the presence of microbiota in maintaining intestinal homeostasis and preventing inflammation, it appears that an increase in the concentration of SCFAs could enhance this protection. It is already known that...
The gut microbiota can change composition rapidly through diet alteration. Augmented SCFA levels could be reached through changes of diet, and probiotics have been investigated as alternative therapeutic strategies in some contexts, strengthening the idea that acetate-producing bacteria could become a tool for management or prevention of inflammatory processes.

In conclusion, acetate treatment diminishes inflammation in kidney epithelial and immune cells and ameliorates kidney IRI, most likely through modulation of epigenetic processes.

**CONCISE METHODS**

**Mice**
Male C57BL/6 (H-2Ab) mice were purchased from Federal University of São Paulo, UNIFESP (n=5 per group). All animal procedures were performed in accordance with the Brazilian Committee for Experimental Animals, and the institutional ethics committee on animal use of the University of São Paulo approved the study (number 121/2011).

**AKI Model SCFA/Probiotics Treatment**
Bilateral kidney IRI was performed as previously reported. SCFAs (pH 7.4, diluted in PBS) were administered individually in two intraperitoneal dosages (200 mg/kg), 30 minutes before ischemia and at the moment of reperfusion.

**Renal Function**
Serum creatinine was measured by the Jaffe modified method, and serum urea was measured using a Labtest Kit (Minas Gerais, Brazil) according to the manufacturer’s instructions.

**Assessment of Apoptosis**
Apoptotic cells (terminal deoxynucleotidyl transferase–mediated digoxigenin–deoxyuridine nick-end labeling) were detected through the Cell Death Detection Kit TMR Red (Roche Diagnostics GmbH, Mannheim, Germany).

**Immunohistochemistry**
All steps to perform immunohistochemistry were performed as reported. Details are provided in the full methods section (see Supplemental Material).

**Flow Cytometry**
Kidney tissue was processed as recently published. See the full methods section (Supplemental Material).

**Detection of Myeloperoxidase in Renal Tissue**
Myeloperoxidase in renal tissue was estimated as previously described by Hillegass et al.

**BM-DC Generation/Maturation and Coculture**
BM-DCs were generated as recently described. See the full methods section (Supplemental Material).

**Global Methylation and Evaluation of Mitochondrial DNA**
Global methylation was performed according to the method of Sharma et al.  See the full methods section (Supplemental Material).

**Statistical Analyses**
The data are presented as mean and SD. A t test and ANOVA (with Bonferroni post-test) were used for comparisons between two and three or more groups, respectively. P < 0.05 was considered to indicate a statistically significant difference. All graphs and statistical analyses were performed using Graph-Pad PRISM (GraphPad Software Inc., La Jolla, CA).

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**DISCLOSURES**
None.

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