Original Article
The protective effect of beta-hydroxybutyric acid on renal glomerular epithelial cells in adriamycin-induced injury

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Abstract: Beta-hydroxybutyric acid (BHB) exerts a protective effect in experimental of kidney disease models. However, the mechanisms underlying this activity are not well defined. BHB stands out for its ability to inhibit the Nε-lysine acetylation of histone and non-histone proteins, which may affect cellular processes and protein functions. In adriamycin-injured murine glomerular podocytes, BHB ameliorates podocyte damage and preserves actin cytoskeleton integrity, reminiscent of the effect of MS275, a highly selective inhibitor of lysine deacetylase. Further research found that adriamycin causes the reduced acetylation of nephrin, WT-1, and GSK3β. This process is abrogated by the lysine deacetylase inhibitor or BHB, suggesting that the acetylation of these molecules regulates their activity. In contrast, anacardic acid, a selective inhibitor of acetyltransferase, decreases the acetylation of nephrin, WT-1, and GSK3β and mitigates the podocyte protective effects of BHB. Taken together, BHB attenuates adriamycin-elicited glomerular epithelial cell injury, at least in part, by inhibiting the deacetylation of the key molecules implicated in glomerular injury.

Keywords: Beta-hydroxybutyric acid, acetylation, kidney disease, podocyte, Adriamycin

Introduction
Glomerular disease is the third leading cause of end-stage kidney disease (ESRD) and causes approximately 25% of chronic kidney disease (CKD) worldwide. Glomerular disease is a well-recognized risk factor for cardiovascular, metabolic, infectious, and other diseases, even if it does not progress to the late stage of CKD. However, the current treatment is still based on glucocorticoids or cytotoxic drugs. Progress in the treatment of glomerular diseases has been very limited, and there is an urgent need to develop new therapeutic targets or modalities. Microscopically, the foot processes (FPs) of glomerular podocytes are closely connected, forming the final barrier to prevent urinary protein loss. The dysfunction or reduction of podocytes leads to progressive sclerosis of the glomeruli. Therefore, podocyte loss is regarded as a principal cause of the progression of glomerular disease [1]. Podocytes exhibit a complex cellular morphology, which includes sophisticated cell polarity and an extensive network of protrusions. FPs are essential for increasing the cell surface, which relies on a diverse cytoskeletal repertoire to maintain and mediate the effective attachment of podocytes to the glomerular basement membrane. The retraction and simplification of the FP network (also known as FP effacement) is the initial manifestation of podocyte damage [2]. The actin cytoskeleton constantly undergoes polymerization and severing under physiological conditions, which prominently accumulates in parallel to the glomerular basement membrane when FP effacement occurs. This remodeling of the actin cytoskeleton is believed to provide increased adhesion forces [3]. In vitro, the transition of podocytes to a motile state has been considered a pathological manifestation of the effects of FP effacement. Indeed, the injury of podocytes from Adriamycin (ADR) or other nephrotoxic drugs results in increased motility [2]. Therefore, changes in the actin cytoskeleton architecture might represent the potential
molecular pattern of this tremendous morphogenetic transformation, pointing to a promising therapeutic target. Consistent with this, a recent study demonstrated that stabilizing the actin cytoskeleton ameliorates CKD in diverse animal models [4]. As one of the ketone bodies, beta-hydroxybutyric acid (BHB) is a small lipid-derived molecule that is mainly produced by the liver. When the body is undergoing exercise or calorie restriction, the ketone bodies can circulate to other parts of the blood to serve as a vital alternative metabolic fuel [5]. Then, the level of circulating BHB starts to increase to 1-2 mmol/l and becomes even higher (6-8 mmol/l) with the passing of time. Clinical and experimental evidence suggests that ketone bodies have beneficial effects other than serving as energy fuels. In this regard, low concentrations of BHB (1-2 mmol/l), either endogenous or exogenous, have antioxidant and anti-inflammatory effects and thereby play a significant role in maintaining physiological homeostasis [6, 7]. Among the ketone bodies, BHB stands out for its ability to regulate the host biological responses, possibly via two major mechanisms. The first involves signaling through G protein-coupled receptors. This important homeostatic function is mediated by the hydroxy-carboxylic acid receptor 2 (HCA2, GPR109A) expressed by several cells or tissues, including the adipocytes, brain, kidney, neutrophils, and macrophages. In addition, GPR109A is required for the beneficial effect of BHB, as this effect is lost in Gpr109a−/− mice. Furthermore, a strong protective role of GPR109A-agonists has been reported in the renal ischemia-reperfusion injury model. Recently, a study found high expressions of GPR109A on podocytes, which may explain the beneficial response in the podocyte injury model. A second mechanism involves the direct inhibition of histone deacetylases (HDACs), which have been renamed as lysine deacetylases (KDACs), since non-histone deacetylases were found. HDACs belong to a family of enzymes that remove acetyl groups from proteins, in which non-histone protein acetylation has been implicated in all major biological processes [8]. Butyrate, a product of bacterial anaerobic fermentation that is closely related to BHB, is also a small-molecule inhibitor of class I and class II HDACs, thereby affecting gene transcription and also signals through GPR109A. A recent study found that butyrate attenuates proteinuria by protecting podocytes and improved glomerulosclerosis in mouse models of focal segmental glomerulosclerosis [9]. This protective effect is associated with the preservation of podocyte-specific proteins, including forms of acetylation and methylation. Additionally, the beneficial effect of a butyrate diet suggests an important molecular connection between the gut microbiota and kidney disease [9].

Nephrin is known to be the main body of the extracellular portion of the slit diaphragm, a cell-cell junction of podocytes. Nephrin was identified as a product of a mutated gene in patients with congenital nephrotic syndrome of the Finnish type [10]. Multiple studies have confirmed that the antibody against nephrin is capable of inducing massive proteinuria [11-13]. Moreover, the reduction of nephrin has also been observed in several laboratory nephropathy models and clinical cases, such as minimal change disease [14], membranous nephropathy, membranous proliferative glomerulonephritis, IgA nephropathy, lupus nephritis [15], and diabetic nephropathy [16]. Emerging evidence indicates that post-translational modifications of nephrin, including phosphorylation [16], ubiquitination [17], or acetylation [18], play an important role in the various physiological or pathological conditions of podocytes. Wilms’ tumor 1 (WT-1), a zinc finger protein, is a complex transcription factor expressed by podocytes. Therefore, WT-1 can directly regulate several genes, including podocalyxin [19] and nephrin [20]. The loss of WT-1 or its gene mutation is associated with several forms of focal glomerulosclerosis [21, 22]. Lysine acetylation is involved in the transcriptional regulation of WT-1 [23]. In mice, the expression of WT-1 is dependent on intact HDAC activity and WT-1 is sensitive to the HDAC inhibitor (HDACi) [23]. Glycogen synthase kinase 3β (GSK3β) is a widely-expressed and constitutively active proline-directed serine/threonine kinase that plays a role in a variety of pathophysiological processes, including kidney injury [24]. The blockade of GSK-3β in podocytes can attenuate podocyte apoptosis and preserves the actin cytoskeleton upon injury, suggesting a beneficial effect of GSK3β inhibition [25]. The activity of GSK3 is dynamically regulated by the phosphorylation of key residues. Recent evidence points to acetylation regulating the activity of GSK-3β [26-28].
In this study, we tested the effect of BHB on Adriamycin (ADR)-induced injury in podocytes and determined the potential mechanisms underlying its action. Our data indicate that BHB inhibits ADR-induced actin derangement and apoptosis by inhibiting the deacetylation of nephrin, WT-1, and GSK3β in podocytes.

**Materials and methods**

**Cell culture and treatment**

Immortalized mouse podocytes were cultured at 33°C in an RPMI-1640 medium with 10% fetal bovine serum and interferon-γ (IFN-γ; Invitrogen) in a humidified incubator with 5% CO₂. After that, the podocytes were induced to differentiate at 37°C in the absence of IFN-γ. Both the primary and immortalized podocytes were serum-starved for 16 hours before treatment with ADR (0.25 mg/ml, catalogue # D1515, Sigma-Aldrich), entinostat (MS275, 10 μM, catalogue # EPS002, Sigma-Aldrich), anacardic acid (AA, 10 μM, catalogue # SMB00129, Sigma-Aldrich), or beta-hydroxybutyric acid (BHB, 10 mM, catalogue # 54925, Sigma-Aldrich).

**Western blotting**

Cultured cells were lysed and processed for an immunoblot analysis. The antibodies against acetylated lysine (Aclys), cleaved caspase-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz).

**Immunoprecipitation**

Immunoprecipitation (IP) was carried out as described elsewhere [26]. Briefly, cells were lysed on ice for 30 min. After pre-clearing, the protein was immunoprecipitated using protein A/G agarose (Santa Cruz Biotechnology) and an ac-lysine antibody (Santa Cruz Biotechnology). The precipitated complexes were collected, washed, and separated on SDS-polyacrylamide gels and blotted with various antibodies as indicated.

**Immunofluorescence staining**

Podocytes were fixed with 4% paraformaldehyde, permeabilized, blocked, and stained with rhodamine-phalloidin (Invitrogen). Then, the samples were mounted with ProLong Gold antifade reagent with DAPI.

**Cell viability and apoptosis assay**

The viability was assessed using MTT viability assays as previously described [29]. The apoptosis was quantified using cell apoptosis detection kits (Roche Applied Science, Indianapolis, IN, USA).

**High-content image analyses (HCA)**

Morphometric analyses were carried out as described previously [30, 31]. Imaging and morphometric analyses of F-actin (phalloidin; Invitrogen) were performed to characterize the actin cytoskeleton. The actin cytoskeletal integrity was scored by a blinded expert using phalloidin images. The image analysis and quantification were performed using the ImageJ and CellProfiler suites. The Hoechst 33342 channel and the phalloidin channel were used to identify the unclear and cell boundaries, respectively. Also, the measurements were exported directly to CSV files and subsequently analyzed using MATLAB to generate histograms and distribution plots.

**Statistical analysis**

The statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc.). The results are depicted as the median ± SD. For the imaging and morphometric analyses, normality was determined using D’Agostino and Pearson omnibus normality tests at $P \geq 0.05$. For data sets with a non-normal distribution, Mann-Whitney rank sum tests with a non-parametric 95% confidence interval were used. If the distribution was normal, a two-tailed t-test was performed with a 95% confidence interval. For all the other experiments, we used Student’s unpaired two-tailed t-tests. A $P$ value less than 0.05 was considered statistically significant.

**Results**

**Beta-hydroxybutyric acid (BHB) treatment preserves viability and attenuates apoptosis in ADR-injured podocytes**

To assess the optimal concentration and the temporal effect of BHB, we performed cell viability assays using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays after different treatments. A previous study reported that BHB inhibits HDACs in a dose-dependent manner [32]. Accordingly, we
found that low concentrations (0-10 mM) of BHB exerted beneficial effects on the cell viability. However, at higher concentrations, the BHB treatment ended up with poor cell viability. These data suggest that BHB may have a biphasic effect on cellular viability. Treatment with 10 mM BHB significantly increased cell viability in podocytes injured with ADR (Figure 1A). The impairment of podocyte viability by ADR is known to be attributable to podocyte death, including apoptosis. This effect was significantly attenuated by BHB co-treatment, as assessed by TUNEL staining and by measuring the expression of cleaved caspase 3 using an immunoblot analysis (Figure 1B-E).

**BHB protects podocyte cytoskeletal integrity against ADR injury**

Evidence suggests that the formation and distribution of actin is a dynamic process closely related to podocyte function [33]. We estimated the changes using immunofluorescence staining for F-actin. Normal podocytes show a stretched cellular shape and evident stress fibers. ADR injury remarkably diminishes ventral stress fibers, resulting in an aster-like cell shape (Figure 2A). ADR-treated cells display significantly smaller spreading areas (Figure 2B) and lower levels of F-actin intensity per cell (Figure 2C). In contrast, BHB treatment largely preserves the integrity of the actin cytoskeleton and restores the cellular shape (Figure 2A-C).

**BHB induces enhanced acetylation of multiple key molecules in podocytes**

Since BHB can protect podocytes and is also a deacetylation inhibitor, we tested the degree of acetylation of podocyte homogenate. The experiment found that BHB can increase the degree of acetylation at multiple molecular levels (Figure 3A). Therefore, three key molecules (nephrin, WT-1, and GSK3β) related to podocyte function were investigated separately. At the level of molecular acetylation, after calibration, we found that ADR decreased the expressions of acetylated nephrin, WT-1, and GSK3β, while BHB increased their acetylated expres-
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Figure 2. BHB ameliorates podocytes’ actin cytoskeleton disorganization after ADR injury. A. The cells were fixed and subjected to staining for cytoskeletal F-actin after stimulation with different drugs for 24 h. The ADR-treated cells were disorganized and there was a reduced actin cytoskeleton. At the same time, the cells were star-shaped and the cells’ surface areas were reduced. However, the BHB treatment prevented the effect of ADR. Scale bar, 10 µm for original magnification of × 400. B, C. Shown are scatter plots of the individual cell morphometrics of podocytes. Sample sizes are n\textsubscript{PBS} = 328, n\textsubscript{ADR} = 364, n\textsubscript{ADR+BHB} = 253, which were captured at 200 × magnification, ***P < 0.001.

Histone deacetylase inhibitor (HDACi) ameliorates podocyte injury, mimicking the effect of BHB

To further explore the acetylation signaling underlying the beneficial effect of BHB, we
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Figure 3. BHB induced the enhanced acetylation of multiple key molecules in the podocytes. A. After different treatments for 24 h, cell lysates were prepared for immunoblot analysis for acetylated lysine (Aclys). GAPDH served as a loading control. B. BHB treatment increased the nephrin, WT-1, and GSK3β immunoprecipitation as determined using anti-acetyl lysine. Rabbit IgG was used as a negative control. C. Densitometric analyses of the expression of acetylated nephrin, WT-1, and GSK3β. The data are given as the means ± SD. n = 4. *P < 0.05 versus all other groups, #P ≥ 0.05 versus PBS group.

compared BHB with another HDACI (MS275) in regulating ADR-induced podocyte injury. We tested the cell viability, which showed effects similar to those of BHB and MS275 (Figure 4A).
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TUNEL staining also demonstrated a similar effect on cell survival (Figure 4B, 4C). ADR-treated podocytes showed actin derangement and lower F-actin intensity per cell. However, the BHB and MS275 treatment was associated with the integrity of the actin cytoskeleton and...
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Comparably abundant F-actin densities (Figure 4D, 4E). At the level of molecular acetylation, after calibration, BHB and MS275 increased the expressions of acetylated WT-1, NP, and GSK3β with comparable magnitude, suggesting similar effects of BHB and MS275 (Figure 4F, 4G).

The podocyte protective effect of BHB is abolished by AA, a histone acetyltransferase inhibitor.

Since BHB decreases deacetylation and AA decreases acetylation, we used both drugs concurrently to prove that the acetylation of nephrin, WT-1, and GSK3β is a protective factor for podocytes. As we showed previously, BHB enhances the acetylation of nephrin, WT-1, and GSK3β and protects podocytes from ADR injury. However, AA diminishes the effect of BHB, followed by corresponding decreases in cell activity and cell survival (Figure 5A-C). BHB treatment protects the integrity of the actin cytoskeleton, and after adding AA, the actin cytoskeleton was disorganized as in the group treated with ADR alone, as shown by the F-actin intensity per cell (Figure 5D, 5E). Similar findings were also identified in the expressions of acetylated WT-1, NP, and GSK3β, which also showed that AA abrogated this podocyte protective effect of BHB (Figure 5F, 5G).

Discussion

This study investigated the effects of BHB on ADR-injured podocytes and found that BHB has strong podocyte protective activity. The possible mechanism behind this is that BHB protects against ADR-induced actin derangement and apoptosis by inhibiting the deacetylation of nephrin, WT-1, and GSK3β. To the best of our knowledge, this is the first study to reveal the signaling pathway by which BHB protects podocytes from injury (Figure 6).

Currently, the main laboratory indicators of CKD progress are proteinuria and the glomerular filtration rate (GFR). However, there may be no proteinuria or GFR elevation in the early stages of CKD [1]. The weakness of proteinuria as a CKD biomarker is that sometimes severe CKD histopathological changes are observed but, in fact, there is no proteinuria. Therefore, it is not recommended to rely solely on proteinuria and GFR to monitor disease activity or determine the prognosis of CKD. The histopathological features of percutaneous renal biopsy can provide more sufficient information, but the indications for this procedure are controversial in the early stages of CKD, and the biopsy is invasive and carries a risk of complications. Currently, there is no alternative biomarker for the disease. Nevertheless, growing evidence of the main role of podocytes (glomerular epithelial cells) in the pathogenesis of kidney disease has drawn attention to these cells as alternative biomarkers. The implementation of the earliest diagnosis and intensive treatment of this entity may prevent or greatly delay the onset of ESRD. As a major component of endogenous ketone body, BHB is one of the body’s energy substrates. However, emerging evidence suggests that BHB not only serves as energy fuel but also has a number of signaling functions that can affect gene expression and cell metabolism and function [7, 36, 37]. Here we provide evidence that the appropriate concentration of BHB can protect podocytes from ADR, suggesting its protective effect occurs through other mechanisms independent of energy donors. Previous studies have also shown that butyrate (an HDAC inhibitor) treatment maintains normal metabolism in high-fat diet mice, with reduced glucose and insulin levels, amended glucose tolerance, diminished weight gain, and improved respiratory efficiency [7]. Butyrate also provides beneficial effects in proteinuric kidney disease by reducing the podocyte detachment and ameliorating proteinuria. A decrease in glomerulosclerosis was also seen in pathophysiology, as well as improved glomerular function [9]. Furthermore, BHB treatment is able to attenuate renal ischemia-reperfusion injury both on histology and renal function, identified by an improved serum creatinine level, brush border loss, vacuolization, and the desquamation of epithelial cells in renal tubules [32]. Elevated BHB levels may lower the severity of AKI and the following CKD progression. Moreover, BHB therapy increases cell phagocytic, polarization, and ramification capacity in microglial cells, indicating that BHB has a certain ability to influence the cytoskeleton [38]. Additionally, podocytes are similar to neurons and become terminally differentiated shortly after birth. Both their ability to re-enter the cell cycle and to proliferate are significantly restricted [39]. Importantly,
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BHB was also identified as a novel therapy to protect the nervous system and not necessarily by supplying extra energy in a rat model of neonatal hypoxia ischemia [40].

Figure 5. The beneficial effect of BHB was abrogated by the acetyltransferase inhibitors. A. Cell viability assays of the podocytes treated with different drugs for 24 h. B. The cells were fixed and subjected to TUNEL staining. Scale bar, 30 µm for original magnification of × 200. C. Absolute count of TUNEL-positive podocytes expressed as a percentage of the total number of podocyte nuclei per high-power field. D. The cells were fixed and subjected to staining for cytoskeletal F-actin with rhodamine-phalloidin. Scale bar, 30 µm for original magnification of × 200. E. Shown are scatter plots of individual cell morphometrics of podocytes. Sample sizes are n
ADR = 364, n
ADR+BHB = 253, n
ADR+BHB+AA = 232, which were captured at 200 × magnification. F. The BHB treatment increased the nephrin, WT-1, and GSK3β immunoprecipitation as determined using anti-acetyl lysine, which was diminished by the AA. Rabbit IgG was used as a negative control. G. Densitometric analyses of the expressions of acetylated nephrin, WT-1, and GSK3β. The data are given as the means ± SD. n = 4. ***P < 0.001, *P < 0.05 versus all other groups, #P ≥0.05 versus ADR.
Acetylation is an important post-translational protein modification process, and it plays an important role in regulating protein structure, activity, and function. We used different post-translational modification databases (http://141.61.102.18/phosida/index.aspx and http://bioinfo.bjmu.edu.cn/huac/) to predict the potential acetylation sites, both of which showed highly consistent HDAC1/2/3 class acetylation sites for nephrin, WT-1, and GSK3β. Consistently, the deacetylase Sirt1 suppressed the Lys205 acetylation of GSK3β, with increased GSK3β activity, suggesting that GSK3β deacetylation leads to GSK3β activation [26]. A recent study also reported that Sirt2 deacetylase improves the activity of GSK3β with Lys183 deacetylation [28]. Moreover, Sirt3 deacetylase has been described to deacetylate residue Lys15, which increases GSK3β activity [27]. This evidence suggests that GSK3β acetylation may be regulated by multiple acetyltransferases and acetylation sites. WT-1 is extremely sensitive to HDACi, and its expression was determined to be down-regulated 2 hours after HDACi treatment [23]. Another study found that histone acetyltransferases (HATs) can regulate the gene expression of WT-1 and pointed out that a variety of class II HDACs can inhibit the regulation of HATs, and the reversible acetylation-mediated regulation mode of the WT1 gene in mice [34]. Moreover, in large domestic animals like pigs, Wen and his colleagues also found the same pattern, namely that butyrate-induced histone hyperacetylation up-regulates the WT-1 expression [41]. In addition, diabetes significantly decreases the levels of nephrin and acetylated nephrin, while pentraxin 3 significantly increases the levels of nephrin and acetylated nephrin [42]. This trend implies that the acetylation of nephrin regulates its expression. In addition, studies have found that some microRNAs (miRs), such as miR-155 [18] and miR-29a [35], can regulate the expression of nephrin through acetylation and protect the kidneys.

BHB, an endogenous HDAC inhibitor, has been reported to promote stress resistance in the kidneys by changing the protein (FOXO3A and Mt2) acetylation and the gene expression [6, 32]. Our results showed that MS275, a HDACi, shares similar effects with BHB, both of which increase the acetylation of nephrin, WT-1, and GSK3β. In order to test the acetylation signaling in the beneficial effect of BHB, we adopted the lysine acetyltransferase inhibitor AA. AA has been shown to inhibit histone and non-histone acetylation, which is the opposite of the effect of BHB [43]. The present study found that when using AA and BHB at the same time, the two functions offset each other, indicating that acetylation and deacetylation have reached a new balance. Not only is the inhibition of podocyte deacetylation beneficial for the treatment of a variety of kidney diseases, but it is also beneficial for the treatment of many other important diseases, including type 2 diabetes, bipolar disorder, Alzheimer’s disease, cardiovascular disease, and several cancers [44-47]. Therefore, a new type of regulation mechanism, such as acetylation regulation, may bring a new balance of signaling pathways and may serve as a new therapeutic target.

In summary, our data indicated that BHB mitigated the ADR-induced injury of glomerular epithelial cells by decreasing the deacetylation of
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nephrin, WT-1, and GSK3β. BHB attenuates the ADR-induced actin disorganization, protecting against ADR-induced damage. However, further studies are required to determine the specific acetylation sites and the downstream signaling cascades responsible for the podocyte protection. Our findings may provide new insights into the molecular mechanisms underlying the action of BHB in glomerular disease and may help design new therapies for intervention in podocytopathy.

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Disclosure of conflict of interest

None.

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