Sodium butyrate activates NRF2 to ameliorate diabetic nephropathy possibly via inhibition of HDAC

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Abstract

Oxidative stress contributes to the pathogenesis of diabetic nephropathy (DN). Nuclear factor erythroid 2-related factor 2 (NRF2) plays a key role in cellular defense against oxidative stress. NRF2 activators have shown promising preventive effects on DN. Sodium butyrate (NaB) is a known activator of NRF2. However, it is unknown whether NRF2 is required for NaB protection against DN. Therefore, streptozotocin-induced diabetic C57BL/6 *Nrf2* knockout and their wild-type mice were treated in the presence or absence of NaB for 20 weeks. Diabetic mice, but not NaB-treated diabetic mice, developed significant renal oxidative damage, inflammation, apoptosis, fibrosis, pathological changes and albuminuria. NaB inhibited histone deacetylase (HDAC) activity and elevated the expression of *Nrf2* and its downstream targets heme oxygenase 1 and NAD(P)H dehydrogenase quinone 1. Notably, deletion of the *Nrf2* gene completely abolished NaB activation of NRF2 signaling and protection against diabetes-induced renal injury. Interestingly, the expression of Kelch-like ECH-associated protein 1, the negative regulator of NRF2, was not altered by NaB under both diabetic and non-diabetic conditions. Moreover, NRF2 nuclear translocation was not promoted by NaB. Therefore, the present study indicates, for the first time, that NRF2 plays a key role in NaB protection against DN. Other findings suggest that NaB may activate *Nrf2* at the transcriptional level, possibly by the inhibition of HDAC activity.

Key Words

- ▶ diabetes
- ▶ KEAP1
- ▶ NaB
- ▶ oxidative stress
- ▶ HDAC

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Introduction

As one of the long-term complications of diabetes, diabetic nephropathy (DN) is the main cause of end-stage renal disease (Dronavalli *et al.* 2008). Thus, end-stage

renal disease cannot be effectively prevented or treated. Therefore, there is an urgent need to develop effective methods to prevent or slow down the progression of DN.

Oxidative stress contributes to the pathogenesis and development of DN (Zhang *et al.* 2012, Keshari *et al.* 2014). Nuclear factor erythroid 2-related factor 2 (NRF2) has been identified as a governor of antioxidant and redox signaling. NRF2 activates the transcription of downstream antioxidant genes such as heme oxygenase-1 (*Ho1*) and NAD(P)H dehydrogenase quinone 1 (*Nqo1*) (Ruiz *et al.* 2013). The elevated antioxidants function as cellular scavengers for free radicals, thereby preventing the cells from oxidative damage. We and others have demonstrated the beneficial effects of NRF2 activation on the prevention of DN in mice (de Haan 2011, Zheng *et al.* 2011, Wu *et al.* 2015a). Administration of the NRF2 activator sulforaphane (SFN) prevented diabetic renal damage, whereas the deletion of the *Nrf2* gene completely abolished this action of SFN (Zheng *et al.* 2011, Wu *et al.* 2015a). These studies demonstrate that NRF2 is the key factor through which SFN ameliorates DN.

Sodium butyrate (NaB) is known as an activator of NRF2 (Yaku *et al.* 2012, 2013, Liu *et al.* 2015). NaB significantly increases the expression of *Nrf2* and its downstream antioxidant genes, glutathione S-transferase and *Nqo1*, in interstitial epithelial cells in a dose-dependent manner (Yaku *et al.* 2012, 2013). Moreover, a recent study by Khan and coworkers showed that NaB ameliorated

oxidative damage and fibrosis in the kidneys of diabetic rats along with a decrease in histone deacetylase (HDAC) activity (Khan & Jena 2014). However, the mechanism by which NaB attenuates oxidative damage in DN or how much NRF2 contributes to this process remain unclear. Given that HDAC inhibition may facilitate the transcription of multiple genes, it is important to investigate which gene may play a key role in the protection of NaB against DN.

Kelch-like ECH-associated protein 1 (KEAP1) sequesters NRF2 in the cytoplasm, restricting NRF2 from nuclear translocation and promoting its proteasomal degradation (Miyata *et al.* 2013). Several NRF2 activators such as SFN, curcumin and its analog, C66, enhance NRF2 function through the regulation of KEAP1 (Zhang & Hannink 2003, Hu *et al.* 2011, Esatbeyoglu *et al.* 2012, Wu *et al.* 2016). Thus, it is interesting to test the effect of NaB on the expression of *Keap1*, which might be a mechanism by which NaB enhances NRF2 function, in addition to the HDAC-inhibiting activity of NaB.

Therefore, this study aims to answer the following questions: (1) Is NRF2 required for NaB protection against DN in mice? (2) If required, how much does NRF2 contribute to the effect of NaB? (3) Is KEAP1 involved in NaB activation of NRF2 function in DN?

Figure 1

Deletion of the *Nrf2* gene completely abolished NaB protection against diabetes-induced renal dysfunction. 8-week-old male C57BL/6 wild-type and *Nrf2*-knockout mice were induced to diabetes by streptozotocin. Blood glucose levels in (A) wild-type mice and (B) *Nrf2*-knockout mice were monitored at 0, 4, 8, 12, 16 and 20 weeks after the onset of diabetes. Kidney weight, tibia length, urinary albumin and creatinine levels were determined with the ratios of (C) kidney weight to tibia length and (D) urinary albumin to creatinine (UACR) was calculated. Data are presented as means $\pm$ s.d. ($n = 7$). * $P < 0.05$ vs Ctrl; † $P < 0.05$ vs DM; ‡ $P < 0.05$ vs wild-type DM. Lines: black, control (Ctrl); red, control treated with sodium butyrate (Ctrl/NaB); blue, diabetes (DM); green, diabetes treated with NaB (DM/NaB). Bars: white, Ctrl; light grey, Ctrl/NaB; dark grey, DM; black, DM/NaB.

Accordingly, C57BL/6 male *Nrf2*-knockout mice and their wild-type controls were induced to diabetes through injection of streptozotocin (STZ). The mice were then treated in the presence or absence of NaB for 20 weeks.

Materials and methods

Animal treatment

C57BL/6 wild-type (*Nrf2*^{+/+}) and *Nrf2*-knockout (*Nrf2*^{-/-}) mice were obtained by the breeding of heterozygotes (*Nrf2*^{+/-}) (Zheng *et al.* 2011). All mice were housed in the Animal Center of Changchun University of Chinese Medicine at 22°C, on a 12:12-h light-darkness cycle with free access to rodent feed and tap water. The Institutional Animal Care and Use Committee at Changchun University of Chinese Medicine approved all experimental procedures for these animals. Consequently, this procedure was in accordance with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (2011, eighth edition).

Eight-week-old male mice received either sodium citrate or STZ (Sigma-Aldrich; 50 mg/kg daily, dissolved in 0.1 M sodium citrate, pH 4.5) through intraperitoneal injection for 5 consecutive days. One week after the last injection of STZ, fasting glucose levels (4-h fast) were measured. Mice with a fasting glucose level above 250 mg/dL were considered diabetic.

Diabetic and age-matched control mice were then given either a NaB diet or standard diet, as described in a previous publication (Gao *et al.* 2009). Briefly, NaB (PureOne Biotechnology, Shanghai, PRC) was blended into the diet using a food processor at 18 g at the proportion of 5%. The NaB-containing diet was pelleted and stored in a -20°C freezer until usage. On the supplemented diet, the mice received NaB at 5 g/kg/day (Gao *et al.* 2009) at the normal daily rate of caloric intake for a total period of 20 weeks.

Blood glucose levels were recorded on days 0, 28, 56, 84, 112 and 140 after the onset of diabetes. Urinary albumin and creatinine were recorded on day 140, after diabetes. The mice were then killed and their kidneys were harvested for analysis.

Figure 2

Deletion of the *Nrf2* gene completely prevented NaB protection against the increase in glomerular area and mesangial matrix expansion in diabetic mice. (A) Periodic acid-Schiff (PAS) staining was performed to evaluate renal morphological changes. (B) Glomerular area and (C) mesangial matrix expansion were calculated from PAS staining. Bar = 50 µm. Data are presented as means ± s.d. (n = 7). **P* < 0.05 vs Ctrl; †*P* < 0.05 vs DM. Bars: white, Ctrl; light grey, Ctrl/NaB; dark grey, DM; black, DM/NaB. Abbreviations are the same as Fig. 1. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0322>.

Analysis of kidney function

A mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) and a QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA) were used to determine urine albumin and creatinine levels on spot urine sample. Urinary albumin to creatinine ratio (UACR) was calculated.

Western blot analysis

Western blot analysis was performed using kidney cortex as described in our previous study (Cai *et al.* 2005). The primary antibodies used were anti-binding immunoglobulin protein (BIP, Cell Signaling, 1:1000),

anti-C/EBP homologous protein (CHOP, Cell Signaling, 1:1000), anti-connective tissue growth factor (CTGF, Santa Cruz Biotechnology, 1:500), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Santa Cruz Biotechnology, 1:3000), anti-Histone H3 (Santa Cruz Biotechnology, 1:1000), anti-HO1 (Santa Cruz Biotechnology, 1:1000), anti-intercellular adhesion molecule-1 (ICAM-1, Santa Cruz Biotechnology, 1:500), anti-inducible nitric oxide synthase (iNOS, Cell Signaling, 1:1000), anti-KEAP1 (Santa Cruz Biotechnology, 1:500), anti-NQO1 (Santa Cruz Biotechnology, 1:1000), anti-NRF2 (Santa Cruz Biotechnology, 1:1000), anti-PAI-1 (Santa Cruz Biotechnology, 1:2000), anti-transforming growth factor beta 1 (TGF- β 1, Cell Signaling, 1:1000), anti-tumor necrosis factor alpha (TNF- α , Cell Signaling,

Figure 3

NaB completely lost its action in preventing from diabetes-induced renal fibrosis in the absence of NRF2. Renal fibrosis was determined by calculating (A) Masson's positive area from Masson's trichrome staining (B). To further evaluate the upstream signaling of fibrosis, (C) transforming growth factor beta 1 (TGF- β 1), (D) connective tissue growth factor (CTGF) and (E) plasminogen activator inhibitor-1 (PAI-1) proteins were determined by Western blot. Bar = 50 μ m. Data are presented as means $\pm$ s.d. ($n=7$). * $P<0.05$ vs Ctrl; † $P<0.05$ vs DM; ‡ $P<0.05$ vs wild-type DM. Bars: white, Ctrl; light grey, Ctrl/NaB; dark grey, DM; black, DM/NaB. Abbreviations are the same as Fig. 1. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0322>.

1:500), anti-vascular cell adhesion molecule-1 (VCAM-1, Santa Cruz Biotechnology, 1:500) and anti-3-NT (Millipore, 1:1000).

Real-time PCR

Quantitative RT-PCR was performed as described in our previous study (Wu *et al.* 2014). Primers for *Nrf2*, *Ho1*, *Keap1*, *Gapdh* and *Nqo1* were all from Life Technologies.

Histologic staining

Kidney tissues were fixed immediately in 10% buffered formalin solution after harvesting, embedded in paraffin and sectioned into 5- μ m-thick sections onto glass slides. The slides containing the samples were then prepared using periodic acid-Schiff (PAS) and Masson's trichrome staining methods.

Isolation of nuclei

The renal nuclei were isolated using a nuclei isolation kit (Sigma-Aldrich), as described previously (Wu *et al.* 2016). Briefly, a kidney cortex (30mg) from each mouse was homogenized for 45 s in 150 μ L of cold lysis buffer containing 0.5 μ L of dithiothreitol (DTT) and 0.1% Triton X-100. *Post hoc*, 300 μ L of cold 1.8M Cushion solution (Sucrose Cushion solution:Sucrose Cushion buffer:DTT=900:100:1) was added to the lysis solution. The mixture was then transferred to a new tube preloaded with 150 μ L of 1.8M Sucrose Cushion solution followed

by centrifugation at 13,000rpm for 45 min. As a result of the centrifugation, the nuclei were visible as a thin pellet at the bottom of the tube. The supernatant fraction, containing cytosolic components, was kept for analysis of cytosolic NRF2 (c-NRF2).

Immunohistochemical staining

Immunohistochemical staining was performed, as previously described (Wu *et al.* 2014), using an antibody against NRF2 (Santa Cruz Biotechnology, 1:100).

Morphometric analyses

Morphometric analyses were quantified using Image-Pro Plus 6.0 software (Media Cybernetics Inc, Bethesda, MD, USA). Selection of areas to photograph and scoring was done by people blind to the identity of the samples.

Quantitative analysis of lipid peroxides

Renal malondialdehyde (MDA) concentration was calculated following the instructions by a lipid peroxidation assay kit purchased from Sigma-Aldrich.

HDAC activity assay

HDAC activity was determined using a HDAC assay kit (BioVision) following the manufacturer's protocol. Briefly, 25 μ g of renal nuclear extract from each mouse was diluted to 85 μ L of ddH₂O in each well. 10 μ L of

Figure 4

NaB protection against diabetes-induced renal inflammation was mediated by NRF2. Renal inflammation was evaluated by measuring protein levels of (A) tumor necrosis factor alpha (TNF- α), (B) vascular cell adhesion molecule 1 (VCAM-1) and (C) intercellular adhesion molecule 1 (ICAM-1) by Western blot. Data are presented as means $\pm$ s.d. (n = 7). **P* < 0.05 vs Ctrl; †*P* < 0.05 vs DM; ‡*P* < 0.05 vs wild-type DM. Bars: white, Ctrl; light grey, Ctrl/NaB; dark grey, DM; black, DM/NaB. Abbreviations are the same as Fig. 1.

Figure 5

NaB significantly reduced the expression of renal apoptotic genes, that was induced by diabetes. Renal apoptotic cell death was reflected by protein levels of (A) binding immunoglobulin protein (BIP) and (B) C/EBP homologous protein (CHOP). Data are presented as means $\pm$ s.d. ($n=7$). * $P<0.05$ vs Ctrl; † $P<0.05$ vs DM; ‡ $P<0.05$ vs wild-type DM. Bars: white, Ctrl; light grey, Ctrl/NaB; dark grey, DM; black, DM/NaB. Abbreviations are the same as Fig. 1.

the 10 $\times$ HDAC assay buffer was added to each well. 5 μ L of the HDAC fluorometric substrate was added to each well thereafter. After mixing thoroughly, plates were incubated at 37°C for 30 min. Reaction was then terminated by adding 10 μ L of lysine developer, followed by another 30-min incubation.

Statistical analysis

Seven mice per group were studied. The measurements for each group were summarized as means $\pm$ s.d. Image Quant 5.2 (GE Healthcare Bio-Sciences) was used to analyze Western blots. One-way ANOVA was performed for the comparisons among different groups. The statistical testing was then followed by *post hoc* pairwise comparisons using Tukey's test with Origin 8.6 data analysis and graphing software (OriginLab, Northampton, MA, USA). A test is considered significant if $P<0.05$.

Results

Deletion of the *Nrf2* gene completely abolished NaB protection against diabetes-induced renal dysfunction

Diabetic mice developed significantly higher blood glucose levels from 0 to 20 weeks after the onset of diabetes in both wild-type (Fig. 1A) and *Nrf2*-knockout (Fig. 1B) mice. NaB did not alter blood glucose levels under both diabetic and non-diabetic conditions in both types of mice (Fig. 1A and B). In wild-type mice, NaB significantly reduced the ratio of kidney weight to tibia length which was, in turn, increased by diabetes (Fig. 1C, left panel). However, NaB completely lost this action in *Nrf2*-knockout mice (Fig. 1C, right panel). UACR, which indicates renal function, was determined in all mice (Fig. 1D). Both wild-type and *Nrf2*-knockout diabetic mice had significantly higher UACRs as compared to their relative controls (Fig. 1D). Moreover, deletion of the *Nrf2* gene

Figure 6

NRF2 played a key role in NaB amelioration of diabetes-induced renal oxidative damage. Renal (A) malondialdehyde (MDA) concentration was calculated by a lipid peroxidation assay kit. (B) Inducible nitric oxide synthase (iNOS) and 3-Nitrotyrosine (3-NT) proteins were measured by Western blot. Data are presented as means $\pm$ s.d. ($n=7$). * $P<0.05$ vs Ctrl; † $P<0.05$ vs DM; ‡ $P<0.05$ vs wild-type DM. Bars: white, Ctrl; light grey, Ctrl/NaB; dark grey, DM; black, DM/NaB. Abbreviations are the same as Fig. 1.

Figure 7

NaB increased the expression of *Nrf2* downstream antioxidant genes. Heme oxygenase 1 (*Ho1*) (A) protein and (B) mRNA, as well as NAD(P)H dehydrogenase quinone (*Nqo1*) (C) protein and (D) mRNA were all determined to evaluate NRF2 function as a transcription factor for its downstream targets.

resulted in more severe diabetes-induced renal dysfunction compared with wild-type diabetic mice (Fig. 1D), which was in accordance with our previous studies (Wu *et al.* 2015b, 2016). Notably, NaB markedly decreased UACR that was elevated by diabetes in wild-type mice (Fig. 1D, left panel). However, the effect of NaB was completely abolished in the absence of *Nrf2* (Fig. 1D, right panel). These results indicate that NRF2 plays a key role in NaB protection against diabetes-induced renal dysfunction.

Deletion of the *Nrf2* gene completely prevented NaB protection against the increase in glomerular area and mesangial matrix expansion in diabetic mice

To test the effect of NaB on diabetes-induced renal pathological changes in wild-type and *Nrf2*-knockout mice, PAS staining was performed (Fig. 2A). Both wild-type and *Nrf2*-knockout diabetic mice had a significant increase in glomerular area and mesangial matrix expansion as compared to relative controls (Fig. 2B and C). Notably, NaB showed this efficacy in wild-type mice (Fig. 2B and C, left panels), but not in *Nrf2*-knockout mice (Fig. 2B and C, right panels). These results suggest that NRF2 is required for NaB protection against diabetes-induced increase in glomerular area and mesangial matrix expansion.

NaB completely lost its action in preventing from diabetes-induced renal fibrosis in the absence of NRF2

Next, we determined renal fibrosis in all mice. Thus, Masson's trichrome staining was performed (Fig. 3A) with Masson's positive area calculated (Fig. 3B). Protein levels of TGF- β 1 (Fig. 3C), CTGF (Fig. 3D) and PAI-1 (Fig. 3E) were determined by Western blot. NaB significantly prevented from diabetes-enhanced renal Masson's positive area, TGF- β 1, CTGF and PAI-1 in wild-type mice (Fig. 3B, C, D and E, left panels). However, deletion of the *Nrf2* gene completely blocked the actions of NaB (Fig. 3B, C, D and E, right panels). Therefore, NRF2 is the key factor through which NaB prevents diabetic renal fibrosis.

NaB prevention of diabetes-induced renal inflammation was mediated by NRF2

Renal inflammation was determined by Western blot analysis of TNF- α (Fig. 4A), VCAM-1 (Fig. 4B) and ICAM-1 (Fig. 4C). Diabetes produced renal inflammation in both types of mice (Fig. 4A, B and C). Wild-type mice, but not *Nrf2*-knockout mice, were prevented from diabetes-induced renal inflammation by NaB (Fig. 4A, B and C). Therefore, the results of the mechanism suggest that NaB prevention of diabetes-induced renal inflammation is mediated by NRF2.

NaB significantly reduced the expression of renal apoptotic genes that was induced by diabetes

Renal apoptotic gene expression was tested by measuring protein levels of BIP (Fig. 5A) and CHOP (Fig. 5B). Diabetic kidney exhibited increased BIP and CHOP proteins, and this effect was almost completely prevented by administration of NaB (Fig. 5A and B, left panels). In contrast, *Nrf2* gene deletion resulted in a complete abolishment of this protective effect of NaB (Fig. 5A and B, right panels). These findings indicate that NaB has the capacity to prevent from diabetes-induced expression of renal apoptotic genes.

NRF2 played a key role in NaB amelioration of diabetes-induced renal oxidative damage

Because NRF2 is the governor of the cellular antioxidant system (Kesic *et al.* 2011, Pendyala *et al.* 2011, Ruiz *et al.* 2013), it is important to test renal oxidative damage. MDA, iNOS and 3-NT, as indicators of oxidative damage (Sari *et al.* 2014), were determined in all mice (Fig. 6A, B and C). Diabetes significantly elevated renal MDA, iNOS and 3-NT in both types of mice (Fig. 6A, B and C). Furthermore, *Nrf2* gene deletion led to a significant increase in MDA, iNOS and 3-NT compared with wild-type mice (Fig. 6A, B and C). Moreover, NaB markedly

Figure 8

NaB upregulated *Nrf2* expression without increasing the proportion of NRF2 nuclear translocation. Western blot assays were performed to determine (A) nuclear NRF2 (n-NRF2), (B) cytosolic NRF2 (c-NRF2) and (C) total NRF2 (t-NRF2). To test the effect of NaB on *Nrf2* gene transcription, (D) NRF2 mRNA was determined by RT-PCR. To further investigate the effect of NaB on NRF2 translocation, (E) a comparison of the ratios of n-NRF2/Histone H3 to t-NRF2/GAPDH between the four groups was constructed. (F) Immunohistochemical staining was performed to observe the location of NRF2 expression in the kidneys of all mice. Data are presented as means $\pm$ s.d. ($n=7$). * $P<0.05$ vs Ctrl; † $P<0.05$ vs DM. Bars: white, Ctrl; light grey, Ctrl/NaB; dark grey, DM; black, DM/NaB. Abbreviations are the same as Fig. 1. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0322>.

decreased these levels in the kidneys of wild-type diabetic mice, but not *Nrf2*-knockout diabetic mice (Fig. 6A, B and C). These results, on one hand, confirmed the critical role of NRF2 in the prevention of diabetic renal oxidative damage and addressed the importance of NRF2 in NaB renal protective function in DN, on the other.

NaB increased the expression of *Nrf2* downstream antioxidant genes

To evaluate NRF2 function, the expression of *Ho1* and *Nqo1*, targets of NRF2 (Martin *et al.* 2004, He *et al.* 2009), was determined by Western blot and RT-PCR. In wild-type mice, NaB markedly elevated the expression of *Ho1* and *Nqo1* under both diabetic and non-diabetic conditions (Fig. 7A, B, C and D, left panels). However, the efficacies of NaB were completely blocked in *Nrf2*-knockout mice (Fig. 7A, B, C and D, right panels). Hence, NRF2 is the key factor through which NaB upregulates the expression of renal antioxidant genes.

NaB upregulated *Nrf2* expression without increasing the proportion of NRF2 nuclear translocation

As NRF2 is a transcription factor, it is important to measure the amount of NRF2 in the nucleus (nuclear NRF2, n-NRF2), which also reflects NRF2 function. NaB significantly increased n-NRF2 under both diabetic and non-diabetic conditions in wild-type mice (Fig. 8A, left panel). Similarly, NRF2 in the cytoplasm (c-NRF2), whole cell (total NRF2,

t-NRF2) and *Nrf2* mRNA were all increased by NaB (Fig. 8B, C and D, left panels). However, *Nrf2* protein and mRNA were not detectable in *Nrf2*-knockout mice (Fig. 8A, B, C and D, right panels), and this confirmed the deletion of the *Nrf2* gene. To investigate whether NaB promotes NRF2 nuclear translocation, we further constructed a comparison of the ratios of n-NRF2/Histone H3 to t-NRF2/GAPDH between the four groups (Fig. 8E). Hypothetically, this ratio could reflect the proportion of the nuclear translocated NRF2 in total NRF2. NaB did not alter the proportion of nuclear translocated NRF2 under either diabetic or non-diabetic conditions in wild-type mice (Fig. 8E, left panel). Hence, NaB may have no impact on NRF2 nuclear translocation. The fact that NaB increased NRF2 mRNA and did not promote NRF2 translocation to the nucleus sheds light on the regulation of NRF2 by NaB. Thus, the results indicate that this mechanism occurred predominantly at the transcriptional level. Immunohistochemical staining was performed to show the localization of NRF2 (Fig. 8F), which was observed preferably in the glomeruli of wild-type mice (Fig. 8F, upper panel). *Nrf2*-knockout kidney did not show a positive staining of NRF2 (Fig. 8F, lower panel).

KEAP1 was not involved in NaB regulation of NRF2 function

KEAP1 is known to sequester NRF2 in the cytoplasm, restricting NRF2 from nuclear translocation and facilitating NRF2 ubiquitination (Miyata *et al.* 2013). KEAP1 protein and mRNA were elevated in both

Figure 9

NaB did not alter renal Kelch-like ECH-associated protein 1 (*Keap1*) expression, while significantly inhibited the activity of histone deacetylase (HDAC). We further determined renal *Keap1* (A) protein and (B) mRNA, along with total HDAC activity in all mice. Data are presented as means $\pm$ s.d. ($n = 7$). * $P < 0.05$ vs Ctrl; † $P < 0.05$ vs DM. Bars: white, Ctrl; light grey, Ctrl/NaB; dark grey, DM; black, DM/NaB. Abbreviations are the same as Fig. 1.

wild-type and *Nrf2*-knockout diabetic mice (Fig. 9A and B). However, NaB showed no impact on *Keap1* expression in the two types of mice under both diabetic and non-diabetic conditions (Fig. 9A and B). Therefore, KEAP1 was not involved in NaB regulation of NRF2 expression and function. This result is in accordance with the finding that NaB did not promote nuclear translocation of NRF2 (Fig. 8E).

NaB inhibited HDAC activity in the kidneys of both wild-type and *Nrf2*-knockout mice

NaB is an inhibitor of HDAC activity. Therefore, the effect of HDAC inhibition by NaB may facilitate transcription factors to bind the promotor region of the *Nrf2* gene, which may consequently lead to *Nrf2* gene transcription. To further confirm the inhibitory effect of NaB on HDAC activity, we determined the total HDAC activity in all mice. As shown in Fig. 9C, HDAC activity was markedly inhibited by NaB in both wild-type and *Nrf2*-knockout mice, under both non-diabetic and diabetic conditions. Thus, the activation of *Nrf2* transcription (Fig. 8D) may be facilitated by NaB inhibition of HDAC activity.

Discussion

The present study explored the mechanism by which NaB ameliorates DN in mice. NaB showed a promising preventive effect on DN in C57BL/6 wild-type mice. By using *Nrf2*-knockout mice, we identified NRF2 to be the key factor through, which NaB ameliorated DN. Given that *Keap1* expression and NRF2 nuclear translocation were not altered by NaB, the present study indicates that NaB may upregulate *Nrf2* expression and function at transcriptional level. This action may be facilitated by the inhibitory effect of NaB on HDAC activity (Fig. 10).

Although NaB was found be beneficial to several diseases (He *et al.* 2016, Li *et al.* 2016, Salimi *et al.* 2016, Subramanian *et al.* 2016), little is known about its protection against DN. To the best of our knowledge, there has been only one study that reported NaB protection against DN (Khan & Jena 2014). The study by Khan and coworkers showed that NaB ameliorated oxidative damage and fibrosis in the kidneys of diabetic rats along with a decrease in HDAC activity (Khan & Jena 2014). However, given that HDAC inhibition could have a positive effect on transcription of a variety of genes, it is not certain which gene activation plays a key role

Figure 10

Possible mechanism by which NaB ameliorated DN. NaB inhibited the activity of HDAC, opening the structure of chromatin. This effect could facilitate transcription factor to bind the promoter region of *Nrf2* gene, followed by increased *Nrf2* gene expression. *Keap1* expression and NRF2 nuclear translocation were not altered by NaB, indicating that excessive cytosolic NRF2, in turn, might enter the nucleus and turned on the transcription of its downstream targets *Ho1* and *Nqo1*. As a result, antioxidants, such as HO1 and NQO1, were increased, and this blocked oxidative damage caused by diabetes, the effect of which ameliorated diabetic renal inflammation, fibrosis, apoptosis and eventually typical hallmarks of diabetic nephropathy. ↓, activation; ⊥, inhibition.

in the protective effect of NaB. In the present study, we observed the activation of *Nrf2* gene expression by NaB (Fig. 8A, B, C and D, left panels), along with increased expression of NRF2's downstream targets *Ho1* and *Nqo1* (Fig. 7A, B, C and D, left panels). Additionally, NaB ameliorated diabetes-induced renal oxidative damage, apoptosis, inflammation, fibrosis, pathological changes and renal dysfunction. Notably, these actions of NaB were completely abolished in the absence of *Nrf2*. Therefore, the results demonstrate that the activation of *Nrf2*, but not other genes, plays a key role in NaB protection against DN. The mechanism by which NaB-activated *Nrf2* transcription may be its inhibitory effect on HDAC activity (Fig. 9C). The result of the inhibition could, in turn, facilitate transcription factors to bind the promoter region of the *Nrf2* gene furthering its gene transcription (Fig. 10). Although NaB may facilitate the transcription of many genes by the inhibition of HDAC, the activation of *Nrf2* gene transcription provided the predominant benefit in NaB protection against DN. Supporting this notion, SFN, another HDAC inhibitor, completely lost its function in ameliorating DN in the absence of NRF2 (Wu *et al.* 2015b). This indicates that NRF2 is the key factor in both NaB and SFN protection against DN.

KEAP1 sequesters NRF2 in the cytoplasm, promoting its proteasomal degradation (Miyata *et al.* 2013). Thus, application of small molecules that can either modify the structure of KEAP1 to release NRF2 or reduce cytoplasmic KEAP1 can be used to activate NRF2 function. SFN modifies specific cysteine residues in KEAP1, enabling NRF2 to escape from KEAP1-mediated ubiquitination and degradation (Zhang & Hannink 2003). As a result, NRF2 translocates into the nucleus to activate transcription of downstream antioxidant enzymes (Zhang & Hannink 2003, Hu *et al.* 2011). Curcumin is also known as an activator of NRF2. Like SFN, curcumin disrupts the binding between KEAP1 and NRF2, leading to the release of NRF2 (Esatbeyoglu *et al.* 2012). However, the fact that curcumin downregulates *Keap1* expression (Soetikno *et al.* 2013) challenges the notion that curcumin only modifies the KEAP1-NRF2 complex. In our previous study, we demonstrated that the curcumin analog, C66, decreased *Keap1* mRNA and protein via the upregulation of microRNA-200a (Wu *et al.* 2016). Therefore, in addition to disruption of KEAP1-NRF2 complex, the decreased expression of *Keap1* may also account for NRF2 induction by curcumin (Wu *et al.* 2016). Nevertheless, unlike SFN, curcumin and C66, NaB had no impact on *Keap1* expression (Fig. 9A and B) and NRF2 nuclear translocation (Fig. 8E) under both diabetic and non-diabetic conditions.

The present study indicates a different regulatory mechanism of *Nrf2* expression by NaB from that by SFN, curcumin and C66. We, therefore, speculate that NaB treatment produced excessive NRF2 (Fig. 8A, B and C, left panels) that surpassed the neutralizing ability of KEAP1. Hence, NaB might solely regulate *Nrf2* expression at the transcriptional level, which may be facilitated by the inhibition of HDAC activity (Fig. 10).

Both NRF2 activators and NaB have been applied in clinical trials. Although bardoxolone methyl, a NRF2 activator, was terminated in a phase III study due to heart complications (Hall & Bhalla 2014), NRF2 remains a viable drug target as evidenced by the approval of BG-12 (dimethyl fumarate) in treatment of multiple sclerosis (Gold *et al.* 2012). NaB has also been used in clinical trials. NaB improved the efficacy of oral mesalazine in active ulcerative colitis (Vernia *et al.* 2000); reduced inflammation in stool of the patients with shigellosis, along with an improvement in rectal histopathology (Raqib *et al.* 2012); reduced the frequency of abdominal pain in patients with irritable bowel syndrome (Banasiewicz *et al.* 2013) and reduced the frequency of diverticulitis episodes (Krokowicz *et al.* 2014). Thus, there is a potential use of NaB, the NRF2 activator, in the prevention of DN. Although evidence shows that NaB functioned predominantly through the activation of NRF2 in the mouse model of this study, more animal studies are needed before the potential application of NaB in humans.

Taken together, the present study demonstrates, for the first time, that NRF2 plays a key role in NaB protection against DN. Furthermore, this study indicates that NaB regulates *Nrf2* expression at the transcriptional level, possibly through the inhibition of HDAC activity. Therefore, the present study may provide a basis for the future study or application of NaB.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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