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Prohibitin (PHB) is a novel regulator of antioxidant response that attenuates colonic inflammation in mice

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Abstract

Background & Aims—Increased free radicals and/or impaired antioxidant defenses have been shown to play a pathogenetic role in human and animal models of inflammatory bowel disease. Our previous studies showed that prohibitin (PHB) levels are decreased during colitis and that cultured intestinal epithelial cells overexpressing PHB are protected from oxidative stress. This study investigated the effect of intestinal epithelial cell-specific PHB overexpression on oxidative stress associated with experimental colitis and the potential mechanism by which PHB functions as an antioxidant using PHB transgenic mice.

Methods—Colitis was induced using two established mouse models (*S. typhimurium* and dextran sodium sulfate) in PHB transgenic mice and wild-type littermates. Oxidative stress was determined by measuring glutathione and protein carbonyl levels in the cecum or colon. Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional regulator of oxidant responses, expression and activation was assessed in colon mucosa and cultured intestinal epithelial cells overexpressing PHB.

Results—Cells overexpressing PHB showed sustained Nrf2 nuclear accumulation and DNA binding during oxidant stress. PHB transgenic mice exhibited decreased oxidative stress and colitis and increased Nrf2 mRNA expression, nuclear protein translocation and DNA binding compared to wild-type littermates during colitis.

Conclusions—These results demonstrate that PHB is a regulator of Nrf2 expression in intestinal epithelial cells during oxidative conditions and prevents inflammation-associated oxidative stress and injury through sustained activation of Nrf2. Our data demonstrate that PHB is a novel antioxidant and suggest that restoration of PHB levels represents a potential therapeutic approach in inflammatory bowel disease.

Introduction

The two main forms of IBD, Crohn's disease and ulcerative colitis, are characterized by episodic inflammation of the gastrointestinal tract presenting with abdominal pain, diarrhea,

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and mucosal damage. The etiopathogenesis of IBD remains unknown but is thought to involve both environmental and genetic factors that regulate mucosal immune response, mucosal barrier function, and response to microbial factors.¹ Upon stimulation, mucosal cells and infiltrating leukocytes release reactive oxygen species (ROS) that contribute to the onset and progression of tissue damage. Multiple studies have shown that there is an imbalance of ROS to antioxidant response in IBD mucosa and in animal models of experimental colitis.^{2–4} Antioxidant enzymes in the mucosa can become overwhelmed with excess ROS levels during times of inflammation and leukocyte infiltration leading to disrupted mucosal barrier function of intestinal epithelial cells characterized by increased permeability and impaired wound healing.⁵ The importance of antioxidants in the pathogenesis of intestinal inflammation is evident from several studies showing that antioxidant therapy improves colitis in multiple experimental models.^{6–9}

Prohibitin (PHB) is a ubiquitously expressed and highly conserved protein that has multiple functions in the cell including regulation of proliferation and apoptosis,^{10, 11} regulation of transcription,^{12, 13} and mitochondrial protein folding.¹⁴ PHB can localize to the cell membrane, mitochondrial membrane, or the nucleus depending on cell type and situation.^{12, 15, 16} Little is known regarding PHB in the intestine. We recently demonstrated that PHB is primarily expressed in epithelial cells in the colon.¹⁶ Our data also demonstrated that PHB expression is decreased during oxidative stress (induced by H₂O₂) in cultured intestinal epithelial cells, inflammatory bowel disease (IBD) and in experimental models of colitis.¹⁶ Further, overexpression of PHB in intestinal epithelial cells restored glutathione (GSH) levels during oxidative stress and protected from oxidant-induced epithelial barrier dysfunction. Our studies also demonstrated that PHB induced mRNA and protein levels of glutathione S-transferase-pi (GST-pi), the most abundant GST isoform expressed in the colon.¹⁶ Together our findings suggested that PHB functions as an antioxidant in intestinal epithelial cells and repletion of PHB may protect from oxidant damage in diseases such as IBD. This study investigated the potential mechanism by which PHB functions as an antioxidant and examined the effect of intestinal epithelial cell-specific PHB overexpression on *Salmonella typhimurium*- or dextran sodium sulfate (DSS)-induced colitis using PHB transgenic mice specifically overexpressing PHB in intestinal epithelial cells (PHB TG).

Methods

Cell culture

See supplementary material online.

Microarray analysis

See supplementary material online.

Plasmid and transgene construction

The human PHB gene was amplified by PCR using the PHB/pcDNA4-HisMax vector (Invitrogen, Carlsbad, CA) previously cloned from Caco2-BBE cells¹⁶ as a template and using the following primers: 5'-TAATGGTACCTCACTGGGGCAGCTGGA-3' (underlined nucleotides indicate a *KpnI* site); 5'-TAATACCCGGGATGGCTGCCAAAGTG-3' (underlined nucleotides indicate a *XmaI* site). The 818 bp human PHB PCR product was confirmed by sequence analysis. The 12.4 kb villin promoter fragment cloned into the pBluescript II SK+ (pBSII SK+) plasmid (Stratagene, La Jolla, CA) was kindly provided by Dr. Deborah L. Gumucio, The University of Michigan, Ann Arbor, Michigan, USA. The ATG and first 18 amino acids of the villin gene were removed from this promoter fragment (pBSII-12.4kbVill/ΔATG) to generate a more useful tool for expression of any cDNA.¹⁷

Human PHB was cloned into pBSII-12.4kb4kbVill/ Δ ATG using *KpnI* and *XmaI* restriction sites to create the pBSII-12.4kbVillPHB plasmid.

Generation of transgenic mice

pBSII-12.4kbVillPHB plasmids were digested with *EcoRI* and *KpnI* to release villin-PHB DNA fragments. The transgene DNA was purified and injected into the pronuclei of fertilized ova (C57BL/6J) by Emory University School of Medicine Transgenic Mouse and Gene Targeting Core Facility. Founder mice were identified by PCR amplification of tail genomic DNA with primers specific for the transgene: 5'-CAAGCCTGGCTCGACGGCC -3' and 5'-GCCTTGGAGTCGCCCTCAGC-3'. Founders were mated to C57BL/6J mice to obtain F1 progeny, which were analyzed for transgene expression. PHB transgenic mice (PHB TG) were identified by genotyping by PCR using genomic DNA isolated from a small piece of tail and the primers from above. Overexpression of PHB in intestinal epithelial cells was confirmed by quantitative real-time PCR and Western immunoblot using colon mucosa as described below. All mice were group-housed in standard cages under a controlled temperature (25°C) and photoperiod (12:12 h light/dark cycle) and were allowed standard chow and tap water *ad libitum*. All procedures using mice were in accordance with the Emory University Institutional Animal Care.

Characterization of mice with intestinal epithelial cell-specific overexpression of PHB (PHB TG)

To determine the level of PHB overexpression in intestinal epithelial cells of PHB TG mice, mucosa from colon was isolated and used to assay human PHB (hPHB) mRNA and total PHB (mouse and human) protein expression. hPHB mRNA was increased approximately 150-fold in PHB TG mucosa compared to WT mucosa; PHB TG mucosa showed a 2-fold increase in PHB protein expression versus WT mucosa (see Supplemental Fig. 1 online). PHB TG mice showed no overt phenotype upon observation of the colon, including no change in colon length, colon weight, or histology of the colon mucosa as assessed by H&E-stained sections. Immunohistochemical staining demonstrated specific PHB overexpression in the colon epithelium of TG mice (see Supplemental Fig. 1 online). See supplementary material online for methods.

Salmonella typhimurium infection

See supplementary material online.

Induction of Dextran Sodium Sulfate Colitis

See supplementary material online.

Clinical Activity Score

See supplementary material online.

Histological Assessment of Colitis

See supplementary material online.

Myeloperoxidase activity

See supplementary material online.

RNA isolation and quantitative real-time PCR analysis

See supplementary material online.

Cytokine measurements

See supplementary material online.

SDS-PAGE and Western immunoblot analysis

See supplementary material online.

In vivo permeability assay

See supplementary material online.

Glutathione (GSH) assay

See supplementary material online.

Protein carbonyl content

See supplementary material online.

Measuring apoptosis

See supplementary material online.

Electrophoretic mobility shift assay (EMSA) and Nrf2 supershift

See supplementary material online.

Statistical Analysis

Values are presented as mean $\pm$ SEM. Statistical analysis was performed with GraphPad InStat 3 software (San Diego, CA). Groups were compared by 2-way analysis of variance (ANOVA) with subsequent Bonferroni post-hoc test for pair-wise comparisons between two particular groups. *P* values < 0.05 were considered statistically significant in all analyses.

Results

Nrf2 activation is increased in Caco2-BBE cells overexpressing PHB during oxidant stress

To elucidate the function of PHB in intestinal epithelial cells, gene microarray analysis was performed on Caco2-BBE cells overexpressing PHB or empty vector. PHB-transfected cells demonstrated an upregulation of a number of antioxidant genes compared to vector-transfected cells (see Supplemental Table 1 online). A common mechanism of regulation for a number of antioxidant genes is the presence of the antioxidant response element (ARE) in the promoter region. Our previous studies demonstrated that PHB overexpression in intestinal epithelial cells increased mRNA and protein levels of GST-pi and protected cells from H₂O₂-induced depletion of GSH;¹⁶ GST-pi and enzymes involved in *de novo* GSH synthesis are regulated by ARE activation which occurs predominantly through the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). Therefore, we sought to determine the effect of PHB on Nrf2 activation during oxidative stress. Caco2-BBE cells, a model colonic epithelial cell line, transfected with PHB or vector were treated with H₂O₂ as an *in vitro* model of oxidant stress. Nuclear Nrf2 expression did not change in PHB-transfected cells compared to vector-transfected cells at baseline (Figure 1A). Vector-transfected cells showed a time dependent decrease in nuclear Nrf2 expression with H₂O₂ treatment compared to baseline with a significant decrease at 60 min of treatment, whereas cells overexpressing PHB showed no significant change (Figure 1A).

We next assessed Nrf2 binding to the ARE consensus binding site in Caco2-BBE cells overexpressing PHB or vector and treated with or without H₂O₂. Nrf2 binding did not change

in PHB-transfected cells compared to vector-transfected cells at baseline or 30 min of H₂O₂ treatment (Figure 1B). At 60 min of H₂O₂ treatment, vector-transfected cells showed a significant decrease in Nrf2/DNA binding compared to baseline. Cells overexpressing PHB showed decreased Nrf2 binding compared to baseline, but binding is significantly higher than H₂O₂-treated vector-transfected cells (Figure 1B). Binding was competed by unlabeled ARE oligonucleotides (cold). The binding complex was supershifted when a specific Nrf2 antibody was included, indicating that Nrf2 is involved in the binding complex. The lower band is non-specific binding and is not competed by unlabeled oligonucleotides. These results suggest that PHB overexpression in Caco2-BBE cells sustains Nrf2 nuclear expression and DNA binding which is reduced under oxidative stress.

Characterization of mice with intestinal epithelial cell-specific overexpression of PHB (PHB TG)

To investigate whether PHB functions as an antioxidant *in vivo*, we generated transgenic mice with intestinal epithelial cell-specific PHB overexpression (PHB TG). PHB TG mice showed a 2-fold increase in PHB expression in colon mucosa compared to WT mice (see Supplemental Fig. 1 online). Immunohistochemical staining for PHB expression in colon sections from WT mice showed PHB staining in the muscularis (not shown), muscularis mucosa, and epithelial layers. TG mice showed increased PHB expression in the epithelium compared to WT mice, but showed similar expression in the muscularis and muscularis mucosa layers (see Supplemental Fig. 1 online). PHB TG mice were found to be healthy; body weight, breeding and general appearance were normal. PHB TG mice showed no overt phenotype upon observation of the intestine, including no change in colon length, colon weight, or histology of the colon mucosa as assessed by H&E-stained sections (see Supplemental Fig. 1 online).

Oxidative stress is ameliorated in PHB TG mice during *Salmonella typhimurium*- and dextran sodium sulfate-induced colitis

Oral infection with *S. typhimurium* after pretreatment of mice with streptomycin induces clinical and histological features of enterocolitis involving the cecum that recapitulate some aspects of human infection, as well as acute flares of IBD wherein enteric bacteria is thought to play an important role in pathogenesis.¹⁸ Histological characteristics of *S. typhimurium*-induced enterocolitis are neutrophil infiltration into the intestinal mucosa, epithelial ulceration and edema particularly in the cecum.¹⁸ Mice were pretreated with streptomycin and then administered *S. typhimurium* as described in the Methods section. As an alternate model of colitis, we used the DSS model that demonstrates a number of features of human inflammatory bowel disease.¹⁹ Acute colitis was induced in PHB TG and WT littermates using 3.0% DSS for 7 days.

GSH is a prevalent antioxidant in mammalian cells where it eliminates peroxides and maintains the thiol/disulfide redox state of proteins with its conversion to GSSG (GSH oxidized disulfide form).²⁰ GSH and GSSG levels were decreased in WT mice given DSS (Table 1) or infected with *S. typhimurium* (Table 2) compared to control mice. GSH levels were not decreased in DSS-treated or *S. typhimurium*-infected PHB TG mice compared to control mice and were significantly higher than DSS-treated WT mice. The E_h (the redox state) of the cellular GSH pool did not change across the DSS treatment groups (Table 1), but was more negative in PHB TG mice infected with *S. typhimurium*, indicating more reducing potential (Table 2). This variation could be due to the different mechanisms driving disease across the two models of colitis. Regardless, these results suggest a net increase of total GSH (GSH and GSSG) in PHB TG mice during intestinal inflammation.

Protein carbonyl content, the most general and well-characterized biomarker of severe oxidative protein damage, was measured in distal colon (DSS-treated mice) or cecum (*S.*

typhimurium-infected mice) of WT and PHB TG mice. Protein carbonyl content was increased in WT mice during colitis (Tables 1 and 2). PHB TG mice showed no increase in protein carbonyl content during DSS- (Table 1) or *S. typhimurium*-induced (Table 2) colitis, suggesting that overexpression of PHB in intestinal epithelial cells protects mice from inflammation-induced oxidative stress.

Overexpression of PHB in intestinal epithelial cells protects from *Salmonella typhimurium* induced colitis

The ceca of WT mice infected with *S. typhimurium* appeared shriveled in size compared to control mice given streptomycin alone and PHB TG mice infected with *S. typhimurium* (Figure 2A). Myeloperoxidase (MPO) activity, which is an index of granulocyte infiltration into the injured tissue, in the cecum reflected the macroscopic findings; MPO activity was increased approximately 3-fold in WT mice infected with *S. typhimurium*, whereas there was no increase in MPO activity in PHB TG mice infected with *S. typhimurium* (Figure 2B). Leukocyte infiltration and histological score were increased in WT mice infected with *S. typhimurium* compared to PHB TG mice infected with *S. typhimurium* (Figure 2C, D, and E). Together these data demonstrate that epithelial cell-specific PHB overexpression protects against *S. typhimurium*-induced colitis.

PHB TG mice exhibit reduced severity of DSS-induced colitis compared to WT mice

PHB TG and WT mice given water as a control showed no clinical signs of intestinal inflammation, as indicated by measurements of body weight (Figure 3A) and colon length. DSS-treated WT mice showed significant weight loss starting on day 5 of DSS treatment and reached -7.6% on day 7 of treatment (Figure 3A). The percent change in body weight of DSS-treated WT mice started to vary from the other treatment groups on day 3 of DSS treatment. DSS-treated PHB TG mice showed a delay in body weight loss compared to WT mice given DSS; weight loss reached -3.5% on day 7 of treatment which was significantly less than WT mice given DSS, and began to vary significantly from water controls as late as day 6 of treatment. Colon length is a macroscopic indicator of the severity of colitis in this model. DSS-treated WT mice showed a reduction in colon length compared to water controls (WT water = 6.91 ± 0.04 cm, TG water = 6.68 ± 0.04 cm vs. WT DSS = 5.02 ± 0.05 cm; $P < 0.01$). The colons of PHB TG mice given DSS showed shortening compared to water controls but they were significantly longer than DSS-treated WT mice (WT DSS = 5.02 ± 0.05 cm vs. TG DSS = 5.69 ± 0.05 cm; $P < 0.05$). A clinical score was assigned on day 7 of DSS treatment which is a well-established tool for assessing severity of disease in this animal model. DSS-treated WT mice showed a significantly higher clinical score compared to DSS-treated PHB TG mice (Figure 3B). Distal colonic MPO activity was increased in WT mice given DSS compared to water controls (Figure 3C). PHB TG mice given DSS showed significantly less MPO activity compared to WT mice given DSS. Quantitative real-time PCR experiments showed that DSS treatment increased mRNA levels of the proinflammatory cytokines $\text{TNF-}\alpha$, IFN- γ , and IL-1 β in WT mouse colon (Figure 3D). DSS-treated PHB TG mice showed decreased expression of these cytokines compared to DSS-treated WT mice.

To further assess the effect of PHB overexpression in intestinal epithelial cells on colitis, intestinal permeability was measured using translocation of 4 kDa FITC-dextran into serum in PHB TG and WT mice. Barrier dysfunction is one of the earliest events in DSS-induced colitis that precedes evident inflammation or mucosal damage. Therefore, we measured intestinal permeability after 3 days of DSS treatment which was when DSS-treated WT mice began to vary from PHB TG mice in terms of body weight loss. WT mice given DSS showed an increase in FITC-dextran translocation compared to DSS-treated PHB TG mice (Figure 3E). These results suggest that PHB TG mice are protected against DSS-induced increased intestinal epithelial cell permeability.

For recovery experiments, WT and PHB TG mice were administered 3% DSS for 7 days at which time they were switched to water and followed for additional 7 or 14 days (recovery). Both WT and TG mice recovered by 14 days and the mortality rate for both WT and PHB TG mice was 42%, with death occurring on days 9 and 10 of recovery for both groups. There was no significant difference in the percent change in body weight between WT and PHB TG mice after 7 (day 14) or 14 (day 21) days recovery from DSS colitis (Figure 3F). These results suggest that overexpression of PHB in epithelial cells does not play a role in recovery from inflammation, but rather protects against the acute inflammatory response.

DSS-induced colitis is histopathologically characterized by infiltration of inflammatory cells into the mucosa and submucosa, epithelial ulceration, and crypt damage, most severely affecting the distal colon. H&E-stained sections of distal colon of water control mice did not show inflammation (Figure 4A). Distal colon sections of DSS-treated WT mice showed severe inflammatory infiltration, complete crypt loss in some sections, and increased ulceration. In contrast, DSS-treated PHB TG mice showed moderate inflammatory infiltration, less crypt loss, and less ulceration compared to DSS-treated WT mice (Figure 4A). Semiquantitative histological scoring of inflammation revealed that DSS-treated WT mice exhibited a significantly higher total score (severity of inflammatory infiltration, ulceration and crypt damage) compared to DSS-treated PHB TG mice (Figure 4B). Histological score correlated well with MPO activity and macroscopic clinical signs of inflammation, including colon length and clinical score.

Our previous study showed that PHB expression is decreased in colon biopsies of human inflammatory bowel disease compared to normal colon as well as in mouse models of colitis including DSS-induced colitis.¹⁶ To determine whether this is replicated in this study and whether PHB TG mice maintain PHB levels during colitis, we assayed PHB levels in WT mice compared to PHB TG mice. To distinguish between endogenous mouse PHB expression versus exogenous human PHB expression, total RNA was isolated from colon mucosa from WT and PHB TG mice treated with DSS for 7 days and analyzed by quantitative real-time PCR. In DSS-treated WT mice, mouse PHB mRNA was decreased compared to control mice, whereas in DSS-treated PHB TG mice, mouse PHB mRNA levels did not significantly change compared to water control mice (Figure 5A). Human PHB mRNA was highly expressed in PHB TG mucosa only and there was no change in exogenous PHB expression during DSS-induced colitis (Figure 5B).

Nrf2 activation is sustained in PHB TG mice during DSS compared to WT mice

Nrf2 mRNA expression, Nrf2 nuclear protein accumulation, and Nrf2/DNA binding was measured in colonic mucosa of PHB TG and WT mice treated with DSS for 3 days, at which time barrier dysfunction is evident and DSS-treated WT mice begin to vary from PHB TG mice in terms of body weight loss. Nrf2 mRNA expression was similar in colon mucosa from control WT and PHB TG mice (Figure 6A). Mucosa from PHB TG mice showed increased Nrf2 mRNA expression during DSS-induced colitis compared to control mice, whereas DSS-treated WT mice showed no change in Nrf2 mRNA expression (Figure 6A). These results indicate that PHB is a regulator of Nrf2 expression in intestinal epithelial cells during oxidative conditions. Nuclear Nrf2 protein accumulation was similar in WT and PHB TG control mice (Figure 6B). Nuclear Nrf2 protein accumulation was decreased in WT mice treated with DSS compared to control, whereas PHB TG mice treated with DSS showed no significant change (Figure 6B). Nuclear protein extracts from PHB TG mice showed similar levels of Nrf2 binding to the ARE consensus binding site as WT (Figure 6C). DSS-treated WT mucosa showed decreased binding compared to control mice. DSS-treated PHB TG mucosa showed a slight decrease in binding compared to control mice but this did not reach significance and was more than DSS-treated WT mice (Figure 6C). Binding was competed by unlabeled ARE oligonucleotides (data not

shown). The binding complex was shifted when the Nrf2 antibody was included, but not with an IgG control antibody, indicating that Nrf2 is involved in the binding complex. These results suggest that PHB overexpression in intestinal epithelial cells sustains Nrf2 nuclear translocation and DNA binding which is normally reduced during intestinal inflammation. The non-specific band present in the EMSA from Caco2-BBE cells (Figure 1B) is not present in this EMSA using protein from colon mucosa, possibly due to the difference between *in vivo* versus *in vitro* extracts.

Discussion

It is well established that increased oxidative stress is associated with inflammatory bowel disease and animal models of colitis.^{2–4, 21} In inflammatory bowel disease, ROS have been demonstrated to play a pathophysiologic role in barrier dysfunction, apoptosis, and wound healing.^{5, 22–25} The importance of antioxidants in the pathogenesis of intestinal inflammation is evident from studies showing that antioxidant therapy improves colitis in multiple experimental models.^{6–9, 26} Despite these studies, antioxidants have not proven to be effective in human disease.²⁷ In addition, targeting single pathways may not be efficient in combating the complex nature of oxidative stress in IBD. In contrast, we demonstrate that PHB is a regulator of Nrf2 which activates multiple antioxidant pathways, and therefore targeting PHB could be more effective as a potential therapeutic strategy in IBD.

PHB TG mice infected with *S. typhimurium* or administered DSS exhibited a net increase of total GSH as well as less severe oxidative protein damage during colitis compared to WT mice. Furthermore, compared to WT mice, PHB TG mice showed less severe weight loss, diarrhea, colon/cecum shrinkage, inflammatory infiltration into the tissue, proinflammatory cytokine expression, crypt damage and increased intestinal permeability, one of the earliest events in DSS-induced colitis that precedes evident inflammation or mucosal damage. These results suggest that overexpression of PHB in intestinal epithelial cells protects from oxidative stress and acute intestinal inflammation. However, recovery from DSS-induced colitis was not altered in PHB TG mice as indicated by similar mortality rates and percent change in body weight. These results suggest that overexpression of PHB in epithelial cells does not play a role in recovery from inflammation, but rather protects against the acute inflammatory response.

Combined with previously published data demonstrating that PHB protects against oxidative stress *in vitro*, our data demonstrate that PHB protects against colonic inflammation by reducing oxidative stress through Nrf2 activation. Nrf2 is the principal transcription factor that binds to the ARE and recruits the general transcriptional machinery for expression of ARE-regulated genes.^{28, 30} ARE-regulated genes include NAD(P)H:quinone oxidoreductase (NQO1), GSTs, heme oxygenase-1 (HO-1), as well as genes associated with the GSH homeostasis such as γ -glutamylcysteine synthetase, glutathione peroxidase and glutathione reductase.²⁸ The activation of Nrf2 is thus considered as a master transcriptional regulator of antioxidant response and provides enzymes that directly inactivate oxidants, increases levels of GSH synthesis and regeneration, and enhances the recognition, repair, and removal of damaged proteins. Consistent with its regulatory role in antioxidant defense and anti-inflammatory pathways, recent data show that Nrf2^{-/-} mice show increased susceptibility to colitis.³¹ Our results in Caco2-BBE cells showing that Nrf2 nuclear protein accumulation and Nrf2/DNA binding were maintained in cells overexpressing PHB compared to vector transfected cells suggest that PHB sustains Nrf2 activation during H₂O₂ treatment. These results were corroborated *in vivo*. No previous studies have shown the effect of colitis on Nrf2 nuclear protein accumulation or DNA binding in WT mice. We show here that both Nrf2 nuclear protein accumulation and binding to a consensus ARE binding site were decreased in DSS-treated WT mice but not in DSS-treated PHB TG mice. Nrf2 levels can be increased by enhancing transcription of Nrf2 mRNA or by interfering with degradation of existing Nrf2

protein.²⁹ Further, mucosa from PHB TG mice showed increased Nrf2 mRNA expression during DSS-induced colitis compared to WT mice, suggesting that increased Nrf2 mRNA transcription contributes to increased Nrf2 levels in PHB TG during oxidative conditions. These results suggest that restoration of PHB via exogenous expression in intestinal epithelial cells increases Nrf2 mRNA expression, sustains Nrf2 nuclear accumulation and Nrf2/DNA binding which are reduced during intestinal inflammation. It remains unknown why DSS treatment decreases Nrf2 activation but this likely contributes to the imbalance of oxidants to antioxidants noted during intestinal inflammation, promoting tissue damage.

Studies in Nrf2^{-/-} mice challenged with inflammatory models suggest that Nrf2 acts to quench inflammatory responses since these mice are more sensitive to inflammation.²⁸ In our study, sustained activation of Nrf2 by maintaining PHB levels in intestinal epithelial cells resulted in less intestinal inflammation and oxidative stress *in vivo*. The mechanism by which PHB activates Nrf2 is not known. Possible mechanisms include increasing Nrf2 gene expression by PHB or sustaining Nrf2 mRNA or protein levels that are decreased during oxidative conditions as indicated by Nrf2 mRNA levels in DSS-treated PHB TG mice versus WT mice. Alternatively, PHB may act to enhance degradation of Kelch ECH associating protein (Keap1) during inflammation, thereby allowing Nrf2 translocation into the nucleus. Under quiescent conditions, Nrf2 is bound by Keap1, a cytosolic repressor protein that retains Nrf2 in the cytoplasm and promotes its proteasomal degradation, thus maintaining low basal expression of Nrf2-regulated genes. Upon oxidation or modification of cysteine residues on Keap1, Nrf2 is released from Keap1, escapes proteasomal degradation, translocates to the nucleus, and activates the expression of several cytoprotective genes.²⁸ This coordinated response is regulated through the ARE located in the promoter region of target genes. Given that Nrf2 deficiency increases susceptibility to oxidative stress and inflammation, our studies suggest that sustained Nrf-2 activation by PHB during colitis mediates its protective effects.

Collectively, these results indicate that maintaining PHB expression in intestinal epithelial cells during colitis protects against inflammation-induced oxidative stress in mice. During both *in vivo* (colitis) and *in vitro* (hydrogen peroxide treatment) situations of oxidative stress overexpression of PHB in intestinal epithelial cells increases Nrf2 activity. Reduced levels of PHB during intestinal inflammation may be one underlying factor that contributes to oxidant-induced mucosal barrier dysfunction. Restoring or increasing PHB expression in the intestinal epithelium during inflammatory bowel disease is a potential therapeutic strategy to prevent mucosal barrier disruption and tissue damage by increased oxidants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ARE	antioxidant response element
DSS	dextran sodium sulfate
GSH	glutathione
IBD	inflammatory bowel disease
Nrf2	nuclear factor erythroid 2-related factor 2
PHB	prohibitin
ROS	reactive oxygen species

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Figure 1. Nrf2 activation is increased in Caco2-BBE cells overexpressing PHB during oxidant stress
 Caco2-BBE cells were transfected with vector (V) or PHB and treated with 0.1 mM H₂O₂ to induce oxidative stress. (A) Representative Western immunoblots of Nrf2 and Histone H3 (loading control) in nuclear protein extracts. Colon mucosa protein extracts isolated from Nrf2^{-/-} mice were included as a negative control. Histograms show mean band densitometry $\pm$ SEM. ^a*P* < 0.05 vs. control; *n* = 4 per group. (B) Electrophoretic mobility shift assay (EMSA) showing binding of nuclear proteins to the consensus ARE binding site. Addition of Nrf2 antibody to the binding reactions results in a supershift of the complexes. Extracts incubated with an isotypic control antibody instead of the Nrf2 antibody as a negative control did not supershift (IgG Ab). Binding was competed by unlabeled ARE oligonucleotides (cold).

Histograms show mean band densitometry $\pm$ SEM. ^a $P < 0.05$ vs. control, ^b $P < 0.05$ vs. vector 60 min H₂O₂; n = 3 per group.

Figure 2. Overexpression of PHB in intestinal epithelial cells inhibits *Salmonella typhimurium* induced enterocolitis

Mice were randomized into 4 groups: WT vehicle or *S. typhimurium* (S. t.) and PHB TG vehicle or *S. typhimurium*. Mice were pretreated with streptomycin before the administration of *S. typhimurium*, as described in the Methods section. Cecum was harvested 48 h after the administration of *S. typhimurium*, photographed (A) and processed for MPO activity (B). MPO values are represented as mean $\pm$ SEM. ^a*P* < 0.001 vs. all other groups; n = 5 per treatment. (C) Number of leukocytes were counted per 300 μm² area. Results are expressed as mean $\pm$ SEM of 6 counts per animal. ^a*P* < 0.01 vs. control; n $\geq$ 5 per treatment. (D) Histological score. Values are represented as mean $\pm$ SEM. ^a*P* < 0.01 vs. control; n $\geq$ 5 per treatment. (E)

Representative photomicrographs of paraffin-embedded, H&E-stained sections of cecum.
Original magnification 40×.

Figure 3. PHB TG mice have less severe DSS-induced colitis compared to WT mice

Mice were administered 3.0% DSS dissolved in water for 7 days. Control mice received water alone. (A) Percent change in body weight. ^a $P < 0.01$ vs. control, ^b $P < 0.01$ vs. WT DSS; $n \geq 13$ per treatment. (B) Changes in clinical score. ^a $P < 0.01$ vs. WT DSS. (C) Colonic myeloperoxidase (MPO) activity. ^a $P < 0.01$ vs. other groups; $n \geq 10$ per treatment. (D) Quantitative real-time PCR was used to quantify mRNA levels of the cytokines TNFα, IL-1β, and IFN-γ. Values represent means $\pm$ SEM of 2 determinations per animal. ^a $P < 0.05$ vs. control; ^b $P < 0.05$ vs. WT DSS. $n = 4$ per treatment group. (E) On day 3 of DSS treatment, mice were gavaged with 4 kDa FITC-dextran. Translocation of fluorescent FITC dextran across the intestinal epithelium was measured in serum collected 4 h after gavage. ^a $P < 0.01$ vs. other groups; $n = 3$ for water groups, $n = 7$ for DSS groups. (F) Percent change in body weight in animals administered 3% DSS for 7 days (DSS) at which time they were switched to water and followed for additional 7 or 14 days (recovery). Histograms show mean $\pm$ SEM. ^a $P < 0.05$ vs. WT DSS; $n \geq 3$ per group.

Figure 4. PHB TG mice have reduced histological damage induced by DSS compared to WT mice (A) Representative photomicrographs of paraffin-embedded, H&E-stained sections of the distal colon. Original magnification 10×(left panels), 40×(right panels). (B) Histological score of crypt damage, severity of inflammatory infiltrate, and extent of inflammation in the distal colon. Sections were scored in a blinded fashion as described in the Methods section. ^a $P < 0.001$ vs. control, ^b $P < 0.05$ vs. WT DSS; $n = 9$ for water groups, $n = 11$ for DSS groups.

Figure 5. Levels of PHB mRNA decreased by DSS colitis in WT mice are restored in PHB TG mice
 Total RNA from colon mucosa from WT and PHB TG mice was subjected to reverse transcription followed by quantitative real-time PCR amplification using mouse PHB- (A) or human PHB-specific primers (B). Histograms show mean $\pm$ SEM. ^a $P < 0.01$ vs. control; $n = 3$ per group, performed in duplicate.

Figure 6. Nrf2 activation is increased in PHB TG mice during DSS compared to WT mice
 (A) Total RNA isolated from colon mucosa after 3 days of DSS treatment was subjected to reverse transcription followed by quantitative real-time PCR amplification using mouse Nrf2-specific primers. $n = 5$ per group, performed in duplicate. (B) Representative Western immunoblot of Nrf2 protein expression in nuclear extracts isolated from colon mucosa. Nup98, a nucleoporin protein, was used as a loading control. $n = 6$ per group. (C) Electrophoretic mobility shift assay (EMSA) showing binding of nuclear proteins isolated from colon mucosa after 3 days of DSS treatment to the consensus ARE binding site. Addition of Nrf2 antibody to the binding reactions results in a supershift of the binding complexes. Extracts incubated with an isotypic control antibody instead of the Nrf2 antibody as a negative control did not supershift (IgG Ab). All histograms show mean band densitometry $\pm$ SEM. ^a $P < 0.01$ vs. other treatments; $n = 6$ per group.

Table 1

Effect of DSS-induced colitis on glutathione (GSH), glutathione disulfide (GSSG), and protein carbonyl content in WT and PHB TG colon.

	WT control	PHB TG control	WT DSS	PHB TG DSS
GSH, μM	1429 $\pm$ 106	1446 $\pm$ 68	1158 $\pm$ 53 ^a	1614 $\pm$ 81
GSSG, μM	48 $\pm$ 6	36 $\pm$ 2	27 $\pm$ 2 ^a	57 $\pm$ 7
E _h GSH/GSSG, mV	-223 $\pm$ 1	-226.8 $\pm$ 1	-224.7 $\pm$ 1	-224.6 $\pm$ 1
Carbonyl content, nmol/mg protein	1.1 $\pm$ 0.4	1.1 $\pm$ 0.2	2.5 $\pm$ 0.4 ^a	1.6 $\pm$ 0.1

Data are mean $\pm$ SEM, n $\geq$ 11.

^a $P < 0.05$ vs. other treatments. E_h, redox state.

Table 2

Effect of *S. typhimurium*-induced colitis of glutathione (GSH), glutathione disulfide (GSSG), and protein carbonyl content in WT and PHB TG cecum.

	WT control	PHB TG control	WT S.t.	PHB TG S.t.
GSH, μM	783 ± 66	639 ± 78	641 ± 39^a	$1013 \pm 132^{a,b}$
GSSG, μM	204 ± 47	232 ± 65	118 ± 15^a	136 ± 9^a
E_h GSH/GSSG, mV	-183 ± 6	-182 ± 6	-188 ± 1	$-197 \pm 3^{a,b}$
Carbonyl content, nmol/mg protein	1.5 ± 0.2	1.1 ± 0.2	2.4 ± 0.2^a	1.4 ± 0.3

Data are mean $\pm$ SEM, $n \geq 5$.

^a $P < 0.05$ vs. control,

^b $P < 0.05$ vs. WT DSS. E_h , redox state.