Published in final edited form as:

Food Chem Toxicol. 2020 November ; 145: 111595. doi:10.1016/j.fct.2020.111595.

Nrf2-dependent and -independent effects of tBHQ in activated murine B cells

Jenna K. Bursley, Cheryl E. Rockwell

Department of Pharmacology & Toxicology, Institute for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, U.S.A.

Abstract

Nrf2 is a transcription factor that regulates cytoprotective cellular responses to oxidative and electrophilic stress. Nrf2 is potentially activated by the synthetic food additive, tert-butylhydroquinone (tBHQ), which is widely used as a preservative in oils and processed foods. Previously published studies have established that tBHQ has numerous effects on T cell function. The purpose of this study was to determine the effect of tBHQ on B cell function and the role of Nrf2 in these effects. Specifically, we investigated T cell-independent B cell activation, differentiation, and IgM antibody production. Murine wild-type and Nrf2-null splenocytes were isolated, treated with tBHQ (0.25-2.5 μ m), and activated by lipopolysaccharide (LPS), a T cell-independent B cell activator. Our findings indicate that tBHQ significantly enhanced IgM production in activated wild-type, but not Nrf2-null, B cells, suggesting this effect is Nrf2-dependent. In contrast, tBHQ significantly decreased the induction of CD69, CD25, CD22, and CD138 in both wild-type and Nrf2-null splenocytes. These findings indicate that the tBHQ-mediated increase in IgM is Nrf2-dependent, whereas the inhibition of CD69, CD25, CD22 and CD138 is Nrf2-independent. Overall, this study demonstrates that in addition to its effects on T cells, tBHQ also has potent effects on T cell-independent B cell function.

Keywords

B cell; Nrf2; tBHQ; CD25; CD138; IgM

Corresponding Author Cheryl E. Rockwell: rockwelc@msu.edu, Office: 517-353-1251, Address: Michigan State University, Life Science Building, 1355 Bogue Street, Room B346, East Lansing, MI 48824.

Credit Author Statement

Jenna K. Bursley: Investigation, Formal Analysis, Writing- Original draft preparation; Cheryl E. Rockwell: Formal Analysis, Writing—Reviewing and Editing, Supervision

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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1. Introduction

Nrf2 is a member of the cap 'n' collar subfamily of bZIP transcription factors that are characterized structurally by a basic leucine zipper domain as well as a conserved Cnc domain and include Nrf1, Nrf2, Nrf3, Bach1 and Bach2 (1-3). Nrf2 is activated by cell stress, including oxidative and electrophilic stresses (4,5). In the absence of cell stress, Nrf2 is bound to its repressor protein, Keap1, which is an adaptor protein in a complex with a ubiquitin 3 ligase that polyubiquitinates Nrf2, targeting it for proteasomal destruction under homeostatic conditions (6). In the presence of cell stress, Keap1 undergoes a conformational change which renders it unable to facilitate the ubiquitination and proteasomal degradation of Nrf2 (7). Upon activation, Nrf2 translocates to the nucleus where it drives the upregulation of a battery of genes, many of which are cytoprotective in nature (8-11).

Nrf2 is activated by a wide variety of structurally-disparate xenobiotic activators and thus is often referred to as a xenobiotic sensor. For example, Nrf2 is activated by numerous environmental contaminants, including heavy metals, such as cadmium, phenols and various arsenic compounds (2,4). In addition to environmental contaminants, Nrf2 is also potently activated by food preservatives, such as tert-butylhydroquinone (tBHQ) (8). As a phenolic antioxidant, tBHQ is a robust activator of Nrf2 and induces the expression of a number of Nrf2 target genes, including Hmox1, Nqo1, Gclc, Gelm and many phase II metabolic enzymes (4,8,9). In the food industry, tBHQ is a useful preservative which extends the shelf life of oils and lipids (12). In particular, tBHQ is used to stabilize vegetable and other plant-based oils, margarine, frozen fish and many other foods. Thus, humans are regularly exposed to tBHQ through a variety of processed foods.

Nrf2 mediates a number of effects in the immune system. Specifically, Nrf2 plays an anti-inflammatory role in a number of different animal models of inflammation, including autoimmune hepatitis, endotoxemia and sepsis, acute lung injury and experimental autoimmune encephalitis, a mouse model of multiple sclerosis (13-20). In addition, in vitro studies have established that Nrf2 regulates the activation of macrophages and T cells. In macrophages, Nrf2 suppresses the inflammatory response to LPS (15). Likewise, treatment of activated NK cells with a Nrf2 activator inhibits effector function (21). In activated T cells, Nrf2 affects both the early cytokine response as well as late cytokine expression by polarized T cells. With respect to the early cytokine response, Nrf2 promotes IL-2 induction, while inhibiting the upregulation of IFN γ , whereas at later timepoints Nrf2 impacts T cell differentiation (22). Specifically, our previous studies demonstrate that activation of Nrf2 by tBHQ promotes Th2 differentiation, while suppressing Th1 differentiation (23). While both Th1 and Th2 cells regulate B cell antibody class switching, Th2 cytokines are considered stronger promoters of B cell activation.

As discussed above, previous studies from our lab and others have shown that activation of Nrf2 modulates T cell function, however the effect of Nrf2 activation on B cell function has remained largely uncharacterized (23-26). Thus, the purpose of the present studies was to elucidate the effect of Nrf2 activation on B cell stimulation in response to LPS, a T cell-independent B cell activator. In particular, we investigated the impact of tBHQ, which is a common food additive and a potent Nrf2 activator in immune cells. The B cells in this study

are activated with lipopolysaccharide (LPS), which is a T cell-independent mode of B cell activation. The experimental strategy here was to avoid effects of tBHQ on T cell help because our previously published studies have established that tBHQ has numerous effects on T cell function.

2. Materials and Methods

2.1 Materials:

Tissue culture reagents were obtained from Gibco/ThermoFisher (Waltham, MA) unless otherwise noted. tBHQ was purchased from Sigma-Aldrich (St. Louis, MO), along with all other non-culture reagents, unless otherwise stated.

2.2 Nrf2-null mice:

Nrf2-null mice on a mixed C57BL/6 and AKR background were generated by and received from Dr. Jefferson Chan (1) The mice were backcrossed 8 generations onto the C57BL/6 background and are 99% congenic (confirmed by Jackson Laboratories, Bar Harbor, ME). The female progeny from the backcross were used in these studies. Age-matched female wild-type (C57BL/6) control mice were purchased from Charles River Laboratories (Wilmington, MA). Food and water were provided *ad libitum*. All animal studies were conducted in accordance with the *Guide for Care and Use of Animals* as adopted by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee (IUCAC) at Michigan State University.

2.3 Cell Culture:

Splenocytes from C57BL/6 (wild-type) mice and Nrf2-null mice were isolated and cultured at 1×10^6 cells/mL in DMEM media supplemented with 10% fetal bovine serum (FBS) (Biowest LLC, Kansas City, MO), 25 mM HEPES, 50 μ M 2-mercaptoethanol, nonessential amino acids (1X final concentration from 100X stock solution), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were left untreated or treated with vehicle (0.01 % EtOH) or tBHQ (0.25 – 2.5 μ M) for 30 min. Samples were subsequently activated with LPS (20 μ g/ml) or left unactivated (BKG) for 48 h. The concentrations of tBHQ were selected based upon concentrations to which humans may be exposed and additionally because our previous studies have shown that higher concentrations can cause cell stress and off-target effects (27).

2.4 Flow Cytometry:

Cells were labeled with various antibodies against cell-surface markers (Table 1). After washing and resuspending the cells in FACS buffer, fluorescence was detected by a BD Accuri C6 flow cytometer and quantified by C-Flow software (BD Accuri, San Jose, CA).

2.5 IgM ELISA:

The method for quantifying IgM by ELISA has been published previously which we implemented with minor modifications (28). Briefly, the plates were blocked with 3% BSA for 30 min followed by washing and incubation with the samples for 1 h at ambient

temperature after which the plates were washed again and incubated with detection antibody for 1 h at ambient temperature. Recombinant mouse IgM was used to make standards for absolute quantification (Cat. # M3795, Sigma-Aldrich, St. Louis, MO). Capture and HRP-conjugated detection antibodies were obtained from Sigma-Aldrich (Cat. # M8644 and A8786, St. Louis, MO). Kinetic absorbances were quantified by a Tecan microplate reader over the course of 1 h (Tecan, Männedorf, Switzerland). Mean optical density slopes were collected and used in the standard curve calculations to determine the total concentration of IgM in the sample.

2.6 Statistical Analysis:

The mean $\pm$ SE was determined for each treatment group in individual experiments. Normality was determined by Shapiro-Wilk analysis and equal variance by Levene's mean test (SigmaPlot 12.3, Systat Software, San Jose, CA). The data were subsequently analyzed by two-way parametric ANOVA. When significant differences were observed, the Holm-Sidak post hoc test was used to compare treatment groups using SigmaPlot 12.3 (Systat Software, San Jose, CA).

3. Results

3.1 Activation of Nrf2 does not affect the percentage of CD19+ B cells

To determine the impact of tBHQ on the B cell population, we used flow cytometry analysis to identify cells expressing CD19, a B cell marker. Activation of splenocytes with LPS for 48 h caused a modest increase in the percentage of CD19+ B cells, which is likely due to an increase in proliferation (Fig. 1). There is a modest, albeit statistically significant, genotype difference in the CD19+ population where the percentage of CD19+ B cells in the spleen was 5 – 10% greater in the Nrf2-null mice, as compared to wild-type, however this trend is not reproducible. The Nrf2 activator tBHQ had no effect on the percentage of CD19+ cells in either wild-type or Nrf2-null splenocytes (Fig. 1).

3.2 tBHQ inhibits CD69 and CD25 induction in LPS-stimulated B cells in a Nrf2-independent manner

We next sought to determine the role of Nrf2 in the induction of CD69 and CD25 in LPS-activated B cells. CD69 is a C-type lectin receptor with an unknown function that is rapidly upregulated following B cell activation. CD25 is the high-affinity subunit of the IL-2 receptor and is also rapidly upregulated upon B cell activation. Both are useful early markers of B cell activation. tBHQ caused a significant decrease in the induction of both CD69 and CD25 at the 2.5 μ M concentration (Figs. 2 and 3). There was no genotype difference in the induction of either CD69 or CD25.

3.3 Nrf2 promotes IgM production by LPS-activated B cells

Upon activation, B cells secrete antibodies, which serve a number of functions including neutralizing pathogens and toxins, tagging pathogens for phagocytosis (opsonization), activation of complement and many other functions. There are numerous classes of antibody, including IgD, IgM, IgG, IgA and IgE. IgM is the first antibody to be produced following B cell activation. We found that tBHQ caused a significant increase in IgM by activated B cells

from wild-type, but not Nrf2-null, B cells (Fig. 4). The 20% increase in IgM by tBHQ is in addition to an already robust response to LPS and thus is notable. In addition, B cells from Nrf2-null mice produced significantly less IgM in response to LPS as compared to wild-type B cells.

3.4 tBHQ inhibits CD22 expression on B cells in a Nrf2-independent manner

Because our results showed that activation of Nrf2 by tBHQ promotes IgM production, we next investigated the effect of tBHQ on the expression of CD22, a B cell-restricted inhibitory receptor that suppresses IgM production. We found that tBHQ (2.5 μ M) inhibited CD22 expression, which would be expected to correlate with an increase in IgM expression (Fig. 5). However, in contrast to IgM, this effect occurred in both wild-type and Nrf2-null B cells.

3.5 Suppression of CD138 expression by tBHQ in wild-type and Nrf2-null B cells

Following activation, B cells may differentiate into plasma cells, which produce enormous amounts of antibody. To understand the effect of tBHQ on plasma cell differentiation, we quantified the percentage of cells expressing CD138, a marker of plasma cell differentiation. tBHQ at 2.5 μ M caused a decrease in the percentage of CD138+ cells in spleen (Fig. 6). This decrease was observed in splenocytes from both wild-type and Nrf2-null mice.

4. Discussion

Our previously published studies have demonstrated that Nrf2 modulates activation, differentiation and function of T cells (22-25,29). In the present study we sought to determine the role of Nrf2 in B cell activation and function. These studies reveal that neither tBHQ nor Nrf2 affect the percentage of B cells in spleen. In contrast, tBHQ (at 2.5 μ M) inhibits the induction of CD69 and CD25 by B cells activated with LPS, a T cell-independent activator. The inhibition of CD69 and CD25 by tBHQ occurs independently of Nrf2. We also found that tBHQ caused a 20% increase in the secretion of IgM by LPS-activated B cells and that this effect was dependent on Nrf2. The tBHQ-mediated increase in IgM production is notable given that LPS-activated B cells already produce a substantial amount of IgM. Likewise, LPS-activated B cells from Nrf2-null mice produced significantly less IgM in response to LPS as compared to wild-type B cells. We also observed that tBHQ caused a decrease in CD22, an inhibitory receptor expressed by B cells that suppresses IgM production. The decrease in CD22 by tBHQ was observed in both wild-type and Nrf2-null B cells. In addition, we found that tBHQ suppresses the expression of CD138 in a Nrf2-independent manner, suggesting that tBHQ suppresses plasma cell differentiation. Overall, this study shows that the food preservative tBHQ has marked effects on the activation and differentiation of primary murine B cells activated with LPS. These data also indicate that Nrf2 promotes IgM production by LPS-activated B cells.

Activation of B cells by LPS is a T cell-independent mode of activation that requires toll like receptor (TLR) signaling. Published studies suggest that B cells express at least 5 different TLRs, including TLR1, TLR2, TLR3, TLR4, TLR6, TLR7 and TLR9 (30-32). Of these receptors, TLR4, and possibly TLR2, can be activated by LPS. LPS stimulation of B cells has been shown to cause proliferation and antibody production (33-35). LPS-stimulated B

cells can also produce cytokines, such IL-6 and IL-10 (36,37). Activation of B cells by LPS has been shown to be TLR4-dependent as evidenced by hyporesponsiveness of B cells from TLR4^{-/-} mice and C3H mice, a strain known to have a mutated TLR4 receptor (38-40). In addition, signaling through the TLR4 receptor has been shown to be important in B cell maturation and these effects are suppressed by TLR2 signaling (41). Signaling through the TLR4 receptor results in activation of transcription factors, such as NF κ B and AP-1 (42-47). NF κ B, in particular, has been shown to be important in B cell activation through TLR4, however PI3 kinase has also been shown to contribute (36,48). The current studies show that tBHQ inhibits LPS-mediated induction of the B cell activation markers, CD69 and CD25. This could occur in multiple ways as our previous studies have shown that tBHQ can inhibit NF κ B activation and calcium influx—both of which are necessary for B cell activation (24). We have also found that tBHQ inhibits Blimp-1, an important transcription factor for B cell function (unpublished observation).

The regulation of IgM induction in activated B cells is fairly complex and multifactorial. The Nrf2-dependent increase in IgM production by tBHQ was unexpected given the inhibitory effect of tBHQ on the B cell activation markers, CD69 and CD25. Although the regulation of IgM production is complex, there are a number of transcription factors that are known to be important in the initial induction of IgM following B cell activation, including NF κ B, AP-1 and NFAT (49-52). Of relevance, our previous studies showed that tBHQ induces AP-1 activation in anti-CD3/anti-CD28-stimulated Jurkat T cells, which could be a potential mechanism by which Nrf2 could promote IgM production (24). In contrast to IgM, the B cell inhibitory receptor, CD22, was decreased by tBHQ. However, this effect occurred in a Nrf2-independent manner and thus does not fully account for the Nrf2-dependent increase in IgM production by tBHQ.

The findings in this study are also significant in that tBHQ is a commonly used food additive to which many humans are regularly exposed. Although tBHQ has multiple uses in the food industry, it is often used to stabilize oils to prevent rancidification. In particular, tBHQ is a useful preservative for vegetable oil. Although there are not many studies that have quantified tBHQ levels in humans, plasma tBHQ concentrations in the high micromolar range have been reported in humans (27). Thus, the concentrations of tBHQ used in the current study are likely within the range of human exposure.

Overall, the current study demonstrates that the synthetic food additive tBHQ has a number of effects on the function of LPS-activated B cells, including inhibition of expression of CD25, CD69 and CD22 and induction of IgM production. Importantly, the concentrations of tBHQ used in this study are low—in the nanomolar to low micromolar range, which are concentrations at which tBHQ has been found in human blood (27). In addition, the data also indicate that the stress-activated transcription factor Nrf2 may be an important regulator of B cell antibody production.

Acknowledgements

The authors would like to acknowledge assistance from Dr. Daniel Barnett, Supawadee Umthong, Dr. Joe W. Zagorski, Dr. Alex E. Turley, Heather Dover, Taylor Dunivin and Lucas M. Kaiser for support in implementing these studies and with data analysis.

Funding

This research was supported by NIH grants R01 ES024966 and R03 ES030766.

Abbreviations:

AP-1	activator protein 1
Bach	BRCA-1-associated carboxy-terminal helicase
DMEM	Dulbecco's modified Eagle medium
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
Gclc	glutamate-cysteine ligase catalytic subunit
Gclm	glutamate-cysteine ligase modifier subunit
IFN	interferon
Hmox1	heme oxygenase 1
IL	interleukin
Keap1	Kelch ECH associating protein 1
LPS	lipopolysaccharide
NFAT	Nuclear factor of activated T cells
NFκB	nuclear factor kappa-light-chain-enhancer
Nqo1	NAD(P)H quinone dehydrogenase 1
Nrf2	Nuclear factor erythroid 2-related factor
Nfe2l2	Nuclear factor erythroid-derived 2-like 2
tBHQ	tert-butylhydroquinone
TLR	toll like receptor

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Highlights:

- The food additive tBHQ inhibits induction of CD25, CD69 and CD22 at low concentrations in LPS-stimulated B cells.
- tBHQ inhibits plasma cell differentiation as evidenced by decreased expression of the plasma cell marker, CD138.
- In contrast to its effects on activation and differentiation, tBHQ induces a Nrf2-dependent increase in IgM secretion by LPS-stimulated B cells.

Fig. 1.

The percentage of CD19⁺ B cells is not affected by tBHQ treatment or Nrf2 expression. Freshly isolated splenocytes derived from wild-type or Nrf2-null mice were treated with tBHQ (0.25 – 2.5 μ M) for 30 min prior to activation with LPS (20 μ g/ml). 48 h after activation, the cells were collected, labeled with CD19-PE and analyzed by flow cytometry. A.) Representative histogram plots. B.) A graph representing averaged values from a single experiment. The bars represent mean $\pm$ SE. N = 3. * depicts $p < 0.05$ as compared to wild-type VH. † depicts $p < 0.05$ as compared to Nrf2-null VH. ‡ depicts $p < 0.05$ as compared to the wild-type control of the same treatment group.

Fig. 2.

The Nrf2 activator tBHQ inhibits the induction of CD69 in both wild-type and Nrf2-null splenic B cells activated with LPS. Freshly isolated splenocytes derived from wild-type or Nrf2-null mice were treated with tBHQ (0.25 – 2.5 μ M) for 30 min prior to activation with LPS (20 μ g/ml). 48 h after activation, the cells were collected, labeled with CD19-PE and CD69-PE/Cy7 and analyzed by flow cytometry. A.) Representative dot plots. B.) A graph representing averaged values of percentage CD19+ CD69+ cells within the splenocyte population from a single experiment. The bars represent mean $\pm$ SE. N = 3. * depicts $p < 0.05$ as compared to wild-type VH. † depicts $p < 0.05$ as compared to Nrf2-null VH. ‡ depicts $p < 0.05$ as compared to the wild-type control of the same treatment group.

Fig. 3.

The food preservative tBHQ suppresses CD25 induction by LPS-activated B cells in a Nrf2-independent manner. Freshly isolated splenocytes derived from wild-type or Nrf2-null mice were treated with tBHQ (0.25 – 2.5 μ M) for 30 min prior to activation with LPS (20 μ g/ml). 48 h after activation, the cells were collected, labeled with CD19-PE and CD25-APC and analyzed by flow cytometry. A.) Representative dot plots. B.) A graph representing averaged values of percentage CD19⁺ CD25⁺ cells within the splenocyte population from a single experiment. The bars represent mean $\pm$ SE. N = 3. * depicts $p < 0.05$ as compared to wild-type VH. † depicts $p < 0.05$ as compared to Nrf2-null VH. ‡ depicts $p < 0.05$ as compared to the wild-type control of the same treatment group.

Fig. 4. Nrf2 promotes IgM production by LPS-activated B cells. Freshly isolated splenocytes derived from wild-type or Nrf2-null mice were treated with tBHQ (0.25 – 2.5 μ M) for 30 min prior to activation with LPS (20 μ g/ml). 48 h after activation, the supernatants were harvested and total IgM levels were quantified by ELISA. The bars represent mean $\pm$ SE. N = 3. * depicts $p < 0.05$ as compared to wild-type VH. † depicts $p < 0.05$ as compared to Nrf2-null VH. ‡ depicts $p < 0.05$ as compared to the wild-type control of the same treatment group.

Fig. 5.

Suppression of the inhibitory receptor CD22 by tBHQ occurs in a Nrf2-independent manner.

Freshly isolated splenocytes derived from wild-type or Nrf2-null mice were treated with tBHQ (0.25 – 2.5 μ M) for 30 min prior to activation with LPS (20 μ g/ml). 48 h after activation, the cells were collected, labeled with CD22-APC and analyzed by flow cytometry. A.) Representative histogram plots. B.) A graph representing averaged values from a single experiment. The bars represent mean $\pm$ SE. N = 3. * depicts $p < 0.05$ as compared to wild-type VH. † depicts $p < 0.05$ as compared to Nrf2-null VH. ‡ depicts $p < 0.05$ as compared to the wild-type control of the same treatment group.

Fig. 6.

tBHQ inhibits plasma cell differentiation of B cells from both wild-type and Nrf2-null mice. Freshly isolated splenocytes derived from wild-type or Nrf2-null mice were treated with tBHQ (0.25 – 2.5 μ M) for 30 min prior to activation with LPS (20 μ g/ml). 48 h after activation, the cells were collected, labeled with CD138-PE and analyzed by flow cytometry. A.) Representative histogram plots. B.) A graph representing averaged values from a single experiment. The bars represent mean $\pm$ SE. N = 3. * depicts $p < 0.05$ as compared to wild-type VH. † depicts $p < 0.05$ as compared to Nrf2-null VH. ‡ depicts $p < 0.05$ as compared to the wild-type control of the same treatment group.

Table 1.

Antibodies used for flow cytometry

Marker	Indicates	Fluorochrome	Dilution Factor	Source
CD19	B cells	PE	1:100	eBioscience
CD69	Activation	PE/Cy7	1:100	Biolegend
CD25	Activation	APC	1:100	Biolegend
CD138	Plasma Cells	PE	1:200	Biolegend
CD22	Activation	APC	1:200	Biolegend