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The Nrf2 Activator, tBHQ, Differentially Affects Early Events Following Stimulation of Jurkat Cells

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Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that is activated by cellular stresses, such as oxidative compounds. After activation, Nrf2 induces transcription of its target genes, many of which have cytoprotective functions. Previously, we have shown that activation of Nrf2 by *tert*-butylhydroquinone (tBHQ) skews murine CD4⁺ T-cell differentiation. Although the role of Nrf2 in murine T cells is somewhat characterized, it is largely uncharacterized in human T cells. Therefore, the aim of the current studies was to characterize the effects of the Nrf2 activator, tBHQ, on the early events of human CD4⁺ T-cell activation. Pretreatment of Jurkat T cells with tBHQ, prior to activation with anti-CD3/anti-CD28, diminished the production of interleukin-2 (IL-2) at both the transcript and protein levels. Similarly, the expression of CD25 also diminished, albeit to a lesser degree than IL-2, after pretreatment with tBHQ. The decrease in IL-2 production was not due to decreased nuclear translocation of c-fos or c-jun. Although tBHQ caused both a delay and a decrease in Ca^{2+} influx in activated Jurkat cells, no decrease in nuclear factor of activated T cells (NFAT) DNA binding or transcriptional activity was observed. In contrast to NFAT, tBHQ significantly decreased NFκB transcriptional activity. Collectively, our studies show that the Nrf2 activator, tBHQ, inhibits IL-2 and CD25 expression, which correlates with decreased NFκB transcriptional activity in activated Jurkat cells. Overall, our studies suggest that Nrf2 represents a novel mechanism for the regulation of both human and mouse T cell function.

Key Words: **Nrf2; IL-2; calcium; NFκB; tBHQ; T cell.**

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that is activated by cellular stresses, such as oxidative stimuli (Chan *et al.*, 1996; Dinkova-Kostova *et al.*, 2002). When inactive, Nrf2 is tethered to the actin cytoskeleton within the cytosol by its repressor protein, Kelch ECH associating protein 1 (Keap1). Interaction of Nrf2 with Keap1 causes its continual ubiquitination and degradation (Itoh *et al.*, 1999; McMahon *et al.*, 2003). When activated by cellular stresses, Nrf2 translocates into the nucleus and binds to the promoters of genes containing the antioxidant response element, inducing their transcription (Itoh *et al.*, 1997; Kensler *et al.*, 2007; Venugopal and Jaiswal, 1996). Genes regulated by this pathway are involved in detoxification, antioxidant response, and glutathione homeostasis among other cytoprotective functions (Lee *et al.*, 2005; Li *et al.*, 2004; Maher *et al.*, 2007). In mice, it has been shown that Nrf2 is inducible in leukocytes and upregulates both Heme oxygenase-1 (Hmox-1) and NAD(P)H quinone oxidoreductase 1 (Nqo1) (Kim and Nel, 2005; Thimmulappa *et al.*, 2006b). Accordingly, Nrf2 activity can be assessed by measuring induction of Hmox-1 and Nqo1 mRNA expression in addition to nuclear translocation of Nrf2.

Widely used as a preservative in food, *tert*-butylhydroquinone (tBHQ) is a well-characterized activator of Nrf2. It has been previously shown that tBHQ activates Nrf2 by interacting with thiol groups of cysteine molecules on the Keap1 protein, which interferes with the ability of Keap1 to repress Nrf2 (Li and Kong, 2009). In industry, tBHQ functions as a food preservative by preventing the rancification of lipids (Shahidi, 2000). Previous studies have shown serum concentrations of tBHQ reaching the high micromolar range in healthy volunteers who ingested 100–150 mg of tBHQ (WHO, 1975).

Although a key player in xenobiotic detoxification and metabolism, a growing number of studies show Nrf2 also plays a role in immune cell function and in various inflammatory diseases, including autoimmune disease and allergy (Innamorato *et al.*, 2008; Johnson *et al.*, 2010; Rangasamy *et al.*, 2005). Our previous studies demonstrate that Nrf2 activation in primary mouse splenocytes modulates murine CD4⁺ T-cell differentiation (Rockwell *et al.*, 2012). Studies by other groups have demonstrated that female Nrf2-knockout mice are susceptible to an autoimmune disease resembling systemic lupus erythematosus, which is characterized by the presence of antibodies targeting

host dsDNA (Ma *et al.*, 2006; Yoh *et al.*, 2001). Importantly, the majority of studies investigating the role of Nrf2 in immune cells have used murine models, and thus, the role of Nrf2 in human immune cells remains largely uncharacterized.

Activation of $CD4^+$ T cells is integral to initiating a healthy immune response to a pathogen. T cells are typically in a resting, quiescent state. In order for activation to occur, the cell requires two stimuli. First, the T-cell receptor (CD3) must encounter a major histocompatibility complex class II molecule, which is loaded with antigen. A costimulatory signal is also required and can be provided by numerous costimulatory receptors and ligands (Cantrell, 1996). Experimentally, T cells can be activated with antibodies directed against CD3 and CD28, which is a costimulatory receptor. These interactions lead to a cascade of signaling events, bringing about T-cell activation. One of the earliest events of T-cell activation is the influx of Ca^{2+} through the calcium release–activated channels (Cantrell, 1996). This sudden change in the intracellular Ca^{2+} concentration activates the calcium-sensitive transcription factor, nuclear factor of activated T cells (NFAT), which translocates to the nucleus and binds to the promoters of target genes (Cantrell, 1996). T-cell activation also initiates several kinase cascades, including RAC-JNK, PKC-SEK1-JNK, and RAS-RAF-ERK (Kim *et al.*, 2006). The result of this signal transduction is the translocation of the transcription factors NFκB, c-fos, and c-jun, which subsequently induce target genes, such as interleukin-2 (IL-2) (Cantrell, 1996). In addition to induction of IL-2, T-cell activation also upregulates expression of the canonical T-cell activation markers, CD25 and CD69. CD25 is the high affinity chain of the IL-2 receptor, and CD69 is a lectin-type receptor; both become highly expressed after activation. These early events of T-cell activation are vital in initiating an effective immune response.

Our previous studies demonstrated that activation of Nrf2 modulates murine $CD4^+$ T-cell differentiation; however, the role of Nrf2 in T-cell activation itself was not investigated (Rockwell *et al.*, 2012). The purpose of the present studies was not only to determine the effect of the Nrf2 activator, tBHQ, on the early events of T-cell activation but to do so in a human T-cell model.

MATERIALS AND METHODS

Materials. tBHQ and all other reagents were purchased from Sigma–Aldrich (St Louis, MO), unless otherwise specified.

Cell culture. Human Jurkat E6-1 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were then cultured in RPMI 1640 media, with the addition of 10% fetal bovine serum (Biowest LLC, Kansas City, MO), 25mM HEPES, 1mM sodium pyruvate, 10mM nonessential amino acids, 100 µ/ml penicillin, and 100 µ/ml streptomycin. All treatments and activation of Jurkat T cells are discussed in figure legends.

mRNA quantification by real-time PCR. Total RNA was isolated from 2×10^6 Jurkat T cells using TRIzol Reagent per the manufacturer's protocol (Life Technologies, Grand Island, NY). After isolation, reverse transcription was performed prior to Sybr green real-time PCR analysis. Relative mRNA expression was calculated by DDCt, normalized to ribosomal protein L13a, using Ct values quantified by Life Technologies/Applied Biosystems Sequence Detection System 7500 (Grand Island, NY). All primer sequences were retrieved from qPrimerDepot (http://primerdepot.nci.nih.gov/) and synthesized by Integrated DNA Technologies (Coralville, IA). The primer sequences are as follows: RPL13A forward primer, 5′GTTGATGCCTTCACAGCGTA-3′ and RPL13A reverse primer, 5′-AGATGGCGGAGGTGCAG-3′; Nrf2 forward primer, 5′-TCTTGCCTCCAAAGTATGTCAA-3′ and Nrf2 reverse primer, 5′-CACGGTCCACAGCTCATC-3′; NQO1 forward primer, 5′-TCCTTTCTT CAAAGCCG-3′ and NQO1 reverse primer, 5′-GGACTGCACCAGAGCCAT-3′; HMOX-1 forward primer, 5′-GGCTTCCCTCTGGGAGTCT-3′ and HMO X-1 reverse primer, 5′-AGCTGCTGACCCATGACAC-3′; IL-2 forward primer, 5′-GCACTTCCTCCAGAGGTTTG-3′ and IL-2 reverse primer, 5′-TCACCAG GATGCTCACATTT-3′; CD69 forward primer, 5′-ACAGGAACTTGGAAG GACCC-3′ and CD69 reverse primer, 5′-AGAACAGCTCTTTGCATCCG-3′; CD25 forward primer, 5′-TAGGCCATGGCTTTGAATGT-3′ and CD25 reverse primer, 5′-ATACCTGCTGATGTGGGGAC-3′.

IL-2 quantification by ELISA. Jurkat T cells were cultured in 96-well plates (1×10^5 cells/well) and treated as described, and cell supernatants were harvested 24 h after activation with anti-CD3/anti-CD28. IL-2 was quantified from cell supernatants by the use of a commercially available kit per the manufacturer's protocol (BioLegend, San Diego, CA). Relative light intensity was quantified at 450 nm by Bio-Tek µQuant microplate reader (Highland Park, VT). The IL-2 concentrations of each sample were then calculated using a linear standard curve on Microsoft Excel (Microsoft, Redmond, WA).

Measurement of extracellular markers by flow cytometry. Jurkat T cells were labeled with anti-CD69/PECy7 (BioLegend) and anti-CD25/APC (eBioscience, San Diego, CA). The cells were then washed, resuspended in FACS buffer, and subsequently analyzed by flow cytometry using the BD Accuri C6 (BD Accuri, San Jose, CA). Fluorescence was quantified using CFlow software (BD Accuri).

Measurement of Ca^{2+} influx by flow cytometry. Jurkat T cells were labeled, at a density of 1×10^6 cells per ml, with Fluo-4, AM (Life Technologies) as per the manufacturer's protocol. The cells were then washed and analyzed by flow cytometry using a BD Accuri C6 (BD Accuri). Fluorescence was quantified using CFlow software (BD Accuri).

Protein isolation. Nuclei were extracted from 1×10^7 Jurkat T cells, 3 h after activation by anti-CD3/anti-CD28. Cells were lysed using a solution containing 10mM HEPES, 100mM KCl, 1.5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 0.5% NP-40 substitute, and 1× Halt protease inhibitor cocktail (Thermo Scientific, Waltham, MA). The cells were then spun down and resuspended in nuclear extraction buffer containing 10mM HEPES, 420mM NaCl, 1.5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 5% glycerol, and 1× Halt protease inhibitor cocktail. Samples were kept in nuclear extraction buffer for 1 h, vortexing intermittently. After incubation, samples were spun down, and supernatants containing the nuclear fraction of the protein extract were collected. Nuclear protein was quantified via Bradford assay (Bio-Rad, Hercules, CA).

Western blotting. After quantification, nuclear protein was diluted in Laemmli Sample Buffer (Bio-Rad) supplemented with 2-mercaptoethanol so that each well contained 10 µg of nuclear protein. Samples were subjected to SDS-PAGE and subsequently transferred to a PVDF membrane. Membranes that were probed for histone H3 were blocked with 5% chicken albumin, from egg white (Sigma–Aldrich), in PBS containing 0.05% Tween 20 (PBST). Blots targeting c-fos, c-jun, and Nrf2 were blocked with 5% nonfat dry milk (NFDM) in 0.05% PBST. Histone H3 primary antibody (FL-136) was diluted 1:100 in 0.05% PBST containing 2% chicken albumin. Primary antibodies for Nrf2 (H-300), c-Jun (D), and c-Fos (H-125) were diluted 1:1000 in 0.05% PBST containing 2% NFDM. All primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was HRP linked and was diluted 1:2000 in 0.05% PBST containing 2% of the blocking compound. The anti-rabbit IgG was obtained

from Cell Signaling (Danvers, MA). All blots were developed via ECL Western Blotting Substrate (Thermo Scientific) as per the manufacturer's protocol. The bands were visualized by the LI-COR Odyssey FC infrared imaging system (Lincoln, NE).

ELISA-based DNA binding assay. One hour after activation by anti-CD3/anti-CD28, nuclear protein was extracted from 1×10^7 Jurkat T cells, using a commercially available kit (Active Motif, Carlsbad, CA). After extraction, nuclear protein was quantified via the Bradford assay (Bio-Rad). One microgram of nuclear protein was used to quantify NFAT DNA binding using a commercially available ELISA-based DNA binding assay (Active Motif). Assays were done as per the manufacturer's protocol.

Transient transfection and luciferase assay. Human Jurkat T cells were reverse transfected with 1.5 µg of plasmid and 3 µl of Lipofectamine 2000 (Life Technologies) per 5×10^5 cells for 12 h in complete media. After 12 h, the cells were washed and resuspended at a concentration of 5×10^5 cells/ml in complete media. After resuspension, the cells were seeded in a 96-well plate at a volume of 200 µl per well. The cells were then treated as described in the figure legends and incubated for 12 h. After incubation, the luciferase activity was measured via a commercially available kit using the manufacturer's protocol (Promega, Madison, WI). Luciferase luminescence was quantified by the Tecan Infinite M1000 Pro Microplate Reader (Tecan, San Jose, CA).

Plasmids. The NFAT luciferase reporter plasmid was obtained from Signosis, Inc. (Sunnyvale, CA). The AP-1 and NFκB luciferase reporters were purchased from Promega (Madison, WI).

Statistical analysis. For each treatment group, the mean ± the standard error was calculated. One-way ANOVA followed by the Dunnett's *post hoc* test was used to determine statistical significance at $p < 0.05$ compared with the vehicle control. All statistical analyses were calculated by SigmaPlot 12.3 (Systat Software Inc., San Jose, CA).

RESULTS

Nrf2 Activator, tBHQ, Inhibits Production of IL-2

We have previously demonstrated that activation of Nrf2 by the food preservative, tBHQ, modulates T-cell differentiation in primary murine CD4+ T cells (Rockwell *et al.*, 2012). However, the role of Nrf2 and the effect of tBHQ in human CD4+ T cells remain largely uncharacterized. Therefore, the purpose of our current studies was to investigate the effects of tBHQ on the early events of T-cell activation in human Jurkat T cells. The Nrf2 activator, tBHQ, markedly decreased IL-2 production by Jurkat T cells activated with anti-CD3 and anti-CD28 (Fig. 1). The decrease in IL-2 by tBHQ was concentration dependent and was observed at both the mRNA and protein levels. Decreased IL-2 protein production by tBHQ occurred at concentrations as low as 0.1µM. No decrease in cellular viability was seen at the concentrations used in this study (data not shown). Consistent with our previous findings in murine T cells, these data suggest that tBHQ modulates cytokine production in human T cells as well.

Activation of Nrf2 by tBHQ in Jurkat T Cells

To determine whether activation of Nrf2 may play a role in inhibition of IL-2 by tBHQ, we investigated whether Nrf2 is activated by tBHQ in Jurkat cells. tBHQ caused an increase in

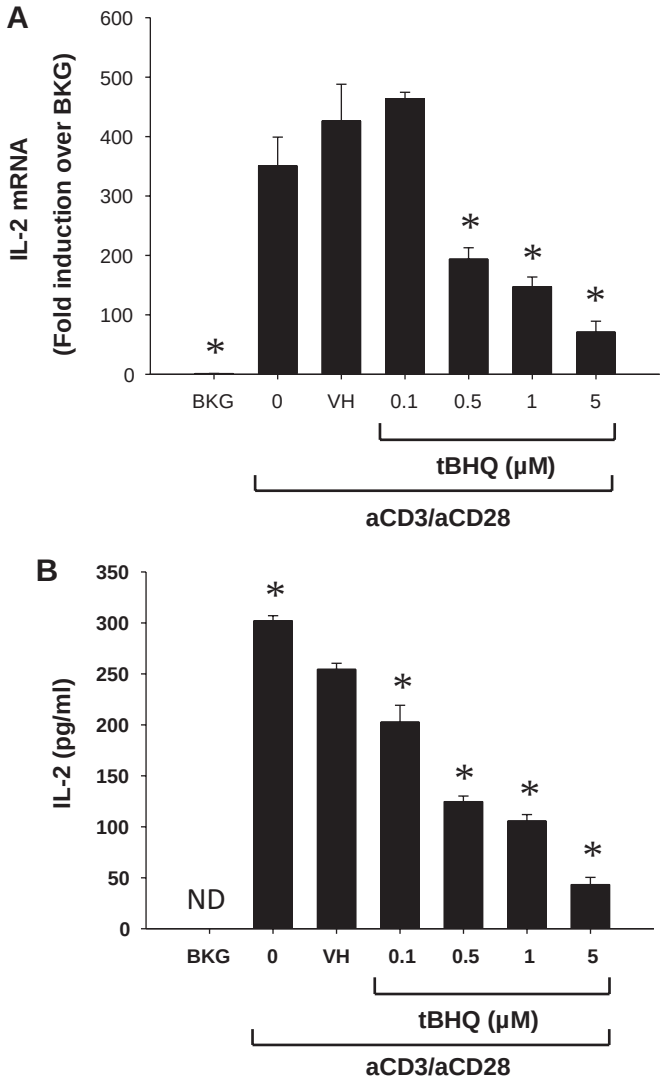

FIG. 1. The Nrf2 activator, tBHQ, inhibits both mRNA and protein production of IL-2 in human Jurkat T cells. Jurkat T cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1, or 5µM) or the vehicle (VH) control (0.01% ethanol). Pretreatment of cells with both VH and tBHQ occurred 30 min prior to activation of the cells by anti-CD3/anti-CD28. (A) Six hours post-activation, cells were harvested and mRNA was collected. Quantification of IL-2 mRNA was then performed by real-time PCR. (B) Twenty-four hours after activation with anti-CD3/anti-CD28, cell supernatants were collected and IL-2 protein was quantified by ELISA. * represents $p < 0.05$ versus VH.

Nrf2 in nuclear protein extracts as determined by Western blot analysis, which is consistent with increased nuclear translocation (Fig. 2a). Although tBHQ tended to increase Nrf2 mRNA levels, this effect was not statistically significant (Fig. 2b). In addition, mRNA levels of the Nrf2 target genes NQO1 and HMOX-1 were substantially increased by tBHQ at concentrations as low as 0.1µM (Figs. 2c and d). Overall, these data strongly suggest that tBHQ activates Nrf2 in Jurkat T cells, as has been observed in other cell types.

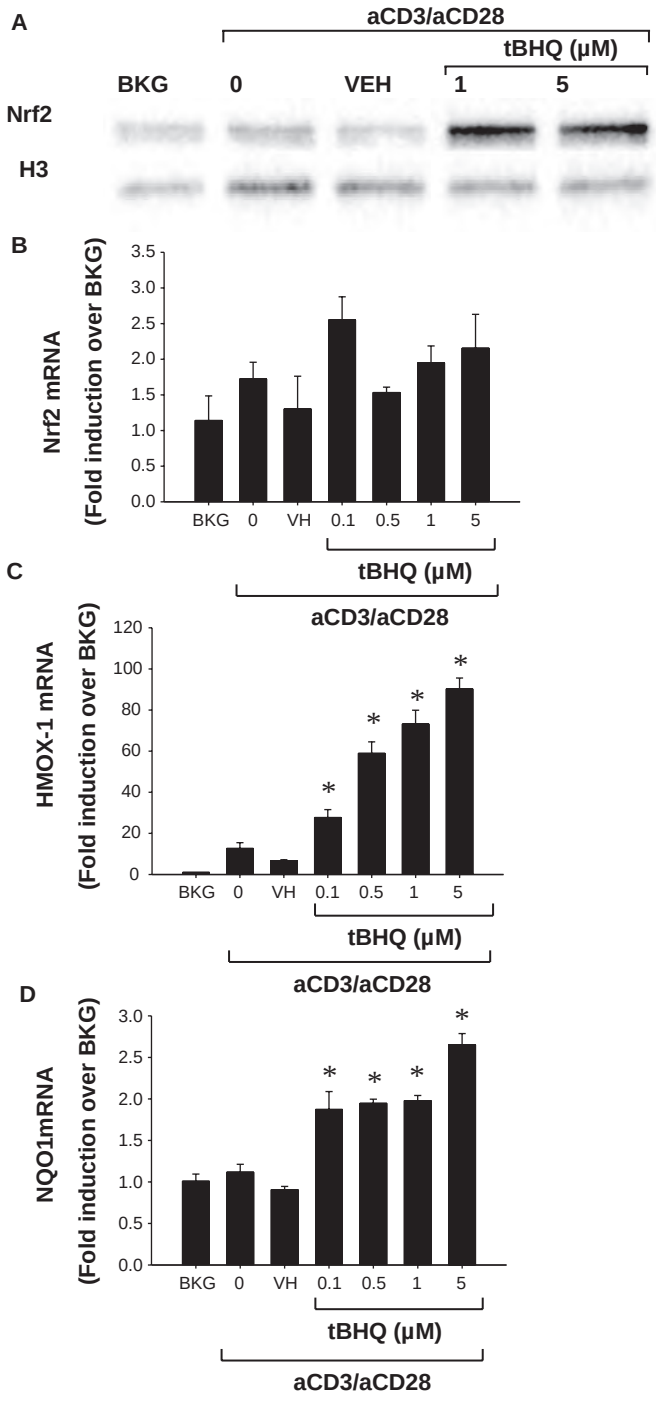

FIG. 2. tBHQ causes nuclear translocation of Nrf2 and induction of Nrf2 target genes in Jurkat T cells. Jurkat T cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1, or 5μM) or the vehicle (VH) control (0.01% ethanol). Pretreatment of cells with both VH and tBHQ occurred 30 min prior to activation of the cells by anti-CD3/anti-CD28. (A) Three hours after activation with anti-CD3/anti-CD28, cells were harvested and nuclear protein was extracted. Western analysis was performed, and the resulting blot was probed for Nrf2. Histone H3 was used as the loading control. Six hours post-activation, cells were harvested and mRNA was collected. Quantification of (B) Nrf2, (C) HMOX-1, or (D) NQO1 mRNA was performed by real-time PCR. * represents $p < 0.05$ versus VH.

Differential Effects of tBHQ on CD25 and CD69 Expression in Jurkat Cells

Because tBHQ markedly suppressed IL-2 production by activated Jurkat cells (Fig. 1), we next investigated the effects of tBHQ on other early events of T-cell activation. The high affinity chain of the IL-2 receptor (CD25) is an early activation marker in $CD4^+$ T cells. Although treatment of Jurkat T cells with tBHQ caused a statistically relevant decrease in CD25 expression at both the protein and mRNA levels, the effect of tBHQ on CD25 was more modest than that observed in IL-2 production (Fig. 3). In addition to CD25, CD69, a lectin-type receptor, is also a marker of early T-cell activation. Although tBHQ caused a modest decrease in CD69 protein expression, this was not observed at the mRNA level. Taken together, the data suggest that Nrf2 activation may moderately decrease CD25 expression.

Effect of tBHQ on Nuclear Translocation of the Transcription Factors, c-fos and c-jun

To determine the mechanism by which tBHQ suppresses IL-2 production in Jurkat cells, we next examined the effect of tBHQ on the nuclear translocation of c-fos and c-jun, known regulators of IL-2 transcription. As expected, activation of Jurkat T cells caused robust nuclear translocation of both c-fos and c-jun (Fig. 4). Pretreatment with tBHQ did not decrease nuclear translocation of c-fos or c-jun and, in fact, appears to cause a modest increase at the highest concentration (5μM). These data suggest that inhibition of IL-2 by tBHQ is not due to decreased nuclear translocation of c-fos/c-jun.

tBHQ Inhibits Calcium Influx in Activated Jurkat T Cells

Calcium influx is a critical event in the activation of T cells. Calcium is integral in many cellular processes, including the activation of the transcription factor, NFAT (Cantrell, 1996). NFAT is a calcium-sensitive transcription factor that has multiple binding sites on the promoter of the IL-2 gene. The lack of effect of tBHQ on either c-fos or c-jun suggests that Nrf2 is inhibiting the expression of this gene by another means, such as inhibition of calcium influx. Thus, calcium influx in Jurkat T cells was measured over time following cell activation. After measurement for 1.5 min to establish baseline, anti-CD3/anti-CD28 was added to the suspension, which caused a subsequent spike in calcium influx a couple of minutes later (Fig. 5a). Cells that were pretreated with tBHQ displayed significant reduction in both fluorescent intensity and number of cells that were positive for calcium influx at both 1 and 5μM tBHQ (Figs. 5b–d). In addition, tBHQ caused a delay in calcium influx (Fig. 5a). Overall, these data demonstrate that tBHQ causes both a delay and a decrease in calcium influx in activated Jurkat T cells.

Activity of the Transcription Factor NFAT Is Unaffected by the Nrf2 Activator, tBHQ

The decrease in calcium influx by tBHQ prompted us to investigate DNA binding and transcriptional activity of the

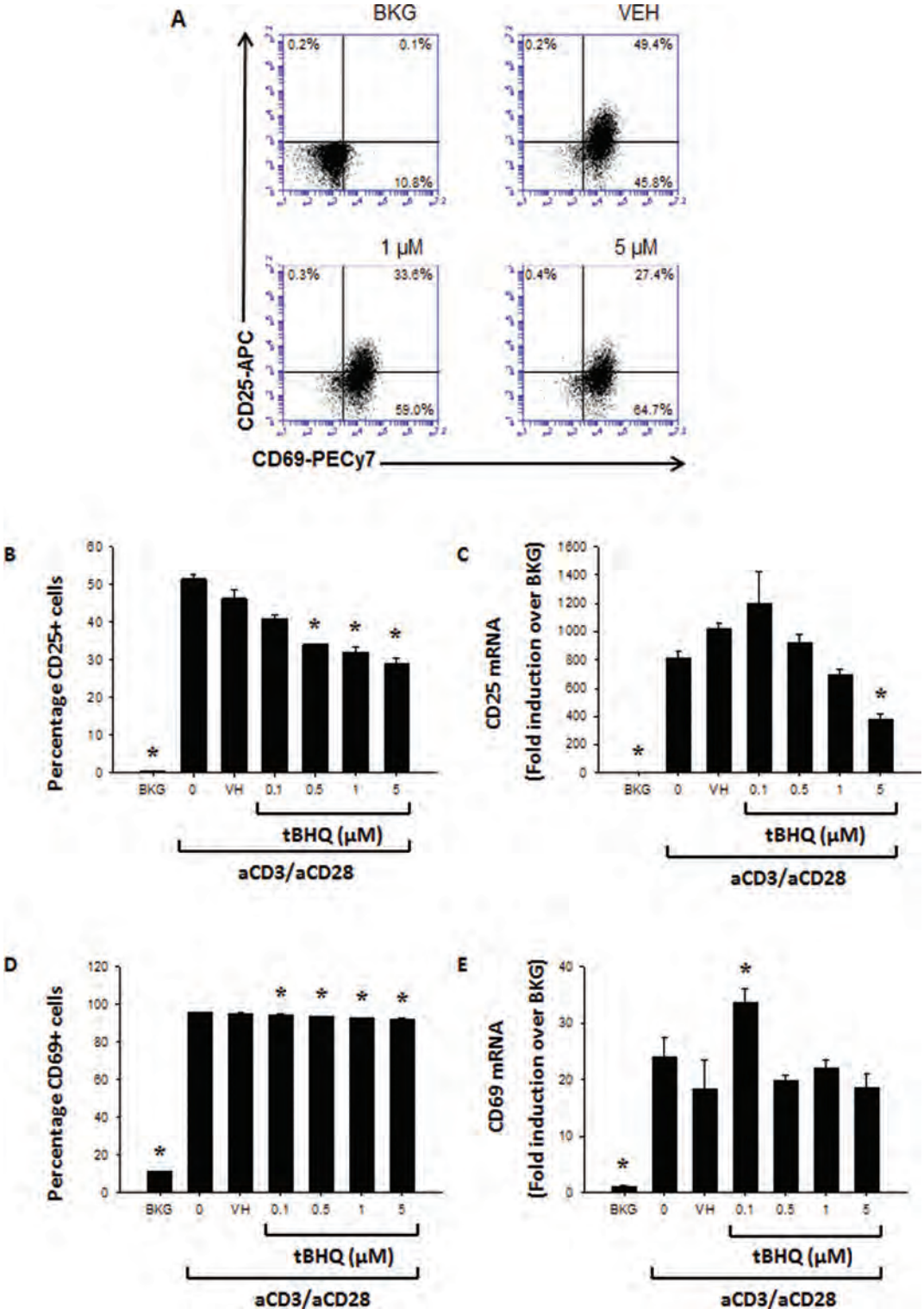

FIG. 3. The Nrf2 activator, tBHQ, causes a modest reduction in CD25 expression in activated Jurkat cells. Jurkat T cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1, or 5μM) or the vehicle (VH) control (0.01% ethanol). Pretreatment of cells with both VH and tBHQ occurred 30 min prior to activation by anti-CD3/anti-CD28. Twenty-four hours post-activation with anti-CD3/anti-CD28, Jurkat T cells were isolated and labeled with anti-CD25/APC and anti-CD69/PECy7. CD25 and CD69 expression was analyzed by flow cytometry. For mRNA analysis, cells were harvested 6 h after activation with anti-CD3/anti-CD28 for RNA isolation. CD25 and CD69 mRNAs were measured by real-time PCR. (A) Representative dot plots from flow cytometry. (B) Graphical representation of CD25-positive cells by flow cytometry. (C) Quantification of CD25 mRNA. (D) Graphical representation of CD69-positive cells by flow cytometry. (E) Quantification of CD69 mRNA. * represents $p < 0.05$ versus VH.

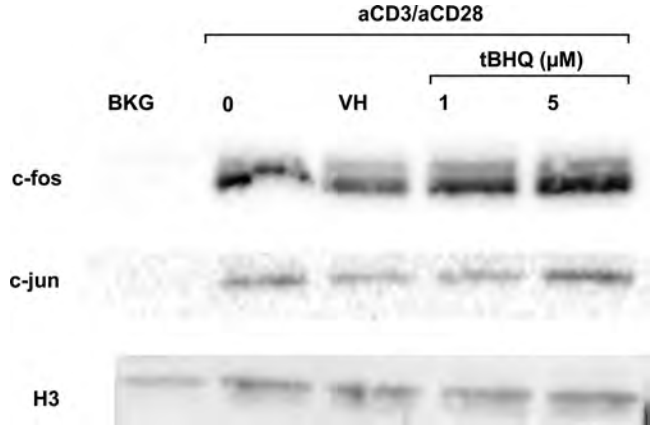

FIG. 4. The Nrf2 activator, tBHQ, has a modest effect on the nuclear translocation of c-fos and c-jun. Jurkat T cells were left untreated (BKG) or were pretreated with nothing (0), tBHQ (1 or 5μM) or the vehicle (VH) control (0.01% ethanol), 30 min prior to activation with anti-CD3/anti-CD28. Three hours after activation with anti-CD3/anti-CD28, cells were harvested and nuclear protein was extracted. Nuclear translocation of c-fos and c-jun was determined by western analysis. Histone H3 was used as the loading control. Visualization of the resulting Western blots was performed by the LI-COR Odyssey FC infrared imaging system.

calcium-sensitive transcription factor, NFAT. Toward this end, we quantified NFAT DNA binding with an ELISA-based assay and transcriptional activity with an NFAT-luciferase reporter assay. Unexpectedly, NFAT DNA binding and transcriptional activity were largely unaffected by tBHQ (Figs. 6a–b). Collectively, these data indicate that tBHQ does not impair NFAT activity.

Nrf2 Activator, tBHQ, Modulates AP-1 and NFκB Activity

The lack of effect of tBHQ on NFAT activity was an unexpected finding and prompted us to investigate the activity of other transcription factors that regulate IL-2 transcription. Thus, the transcriptional activity of AP-1 (c-fos/c-jun) and NFκB was investigated using luciferase reporter genes. At the highest concentration (5μM), tBHQ markedly increased AP-1 luciferase activity (Fig. 7a). Conversely, tBHQ caused a concentration-dependent decrease in NFκB luciferase activity (Fig. 7b). Taken together, these data show that the Nrf2 activator, tBHQ, causes an increase in AP-1 transcriptional activity and a concurrent decrease in NFκB transcriptional activity in activated Jurkat T cells.

DISCUSSION

The purpose of the current studies was to characterize the effect of the Nrf2 activator, tBHQ, on the early events of T-cell activation in a human T-cell model. The data from these studies suggest that Nrf2 is activated by tBHQ in human Jurkat T cells, as evidenced by nuclear translocation of Nrf2 and upregulation of the Nrf2 target genes, HMOX-1 and NQO1. The current studies also indicate that the Nrf2 activator, tBHQ, inhibits IL-2 secretion and the expression of CD25 on the cell surface, but only a modest reduction was observed in the expression of CD69. The decrease in IL-2 by tBHQ is consistent with what has recently been observed with benzo(a)pyrene, which is also a Nrf2 activator (Murugaiyan *et al.*, 2013). Although tBHQ decreased calcium influx in activated Jurkat cells, NFAT DNA binding and transcriptional activity were not diminished, which was an unexpected finding. In contrast, tBHQ increased AP-1 transcriptional activity at 5μM and dose dependently decreased NFκB activity. Collectively, these data suggest that the inhibition of IL-2 secretion by tBHQ is due to decreased NFκB transcriptional activity.

Nrf2 is an important player in modulating the immune response in several models of inflammation and has been shown to be a potential therapeutic target in the treatment of inflammatory diseases, such as sepsis and brain inflammation (Innamorato *et al.*, 2008; Kataoka *et al.*, 2001; Thimmulappa *et al.*, 2006b). Additionally, our lab has shown that activation of Nrf2 skews $CD4^+$ T-cell differentiation in primary murine $CD4^+$ T cells (Rockwell *et al.*, 2012). Although the role of Nrf2 in the regulation of murine T cell function has been somewhat characterized, the role of Nrf2 in human T cells remains largely unexplored. Similar to our previous studies in murine T cells, this study suggests that Nrf2 also plays a role in the regulation of human T cell function.

This study demonstrates that the Nrf2 activator tBHQ has an inhibitory effect on some of the early events of T-cell activation, including calcium influx. In contrast to this study, a previous study showed the Nrf2 activator, bis (tri-n-butyltin) oxide (TBTO), increased calcium influx in Jurkat cells (Katika *et al.*, 2011). There are many differences between the two studies, however, including the kinetics of calcium influx and the Nrf2 activators used. In addition to effects on T-cell activation, treatment of Jurkat cells by tBHQ has also been shown to regulate chromatin remodeling in a mechanism that involves the tumor suppressor, phosphatase and tensin homolog (PTEN) (Sakamoto *et al.*, 2009).

Additives to food, such as tBHQ, are widely used today to increase shelf life and decrease spoilage (Shahidi, 2000). The presence of the preservative, tBHQ, can be seen in products ranging from vegetable oil to crackers and cereal. Previously, the primary concern in using tBHQ and similar compounds was carcinogenesis, and thus, early toxicological analyses focused on genotoxicity. In contrast, there are few published studies on the potential immunotoxic effects of tBHQ. This study shows decreased IL-2 protein production at concentrations of tBHQ as low as 0.1μM and a decrease in CD25 protein expression at concentrations as low as 0.5μM. A previous study reported that treatment of human male subjects with 100–150 mg of tBHQ produces plasma concentrations of tBHQ in the high micromolar range, suggesting that the treatment range in this study (0.1–5μM) is relevant to humans (WHO, 1975).

Within T cells, an increase in intracellular calcium is essential for full activation. Upon an increase in intracellular

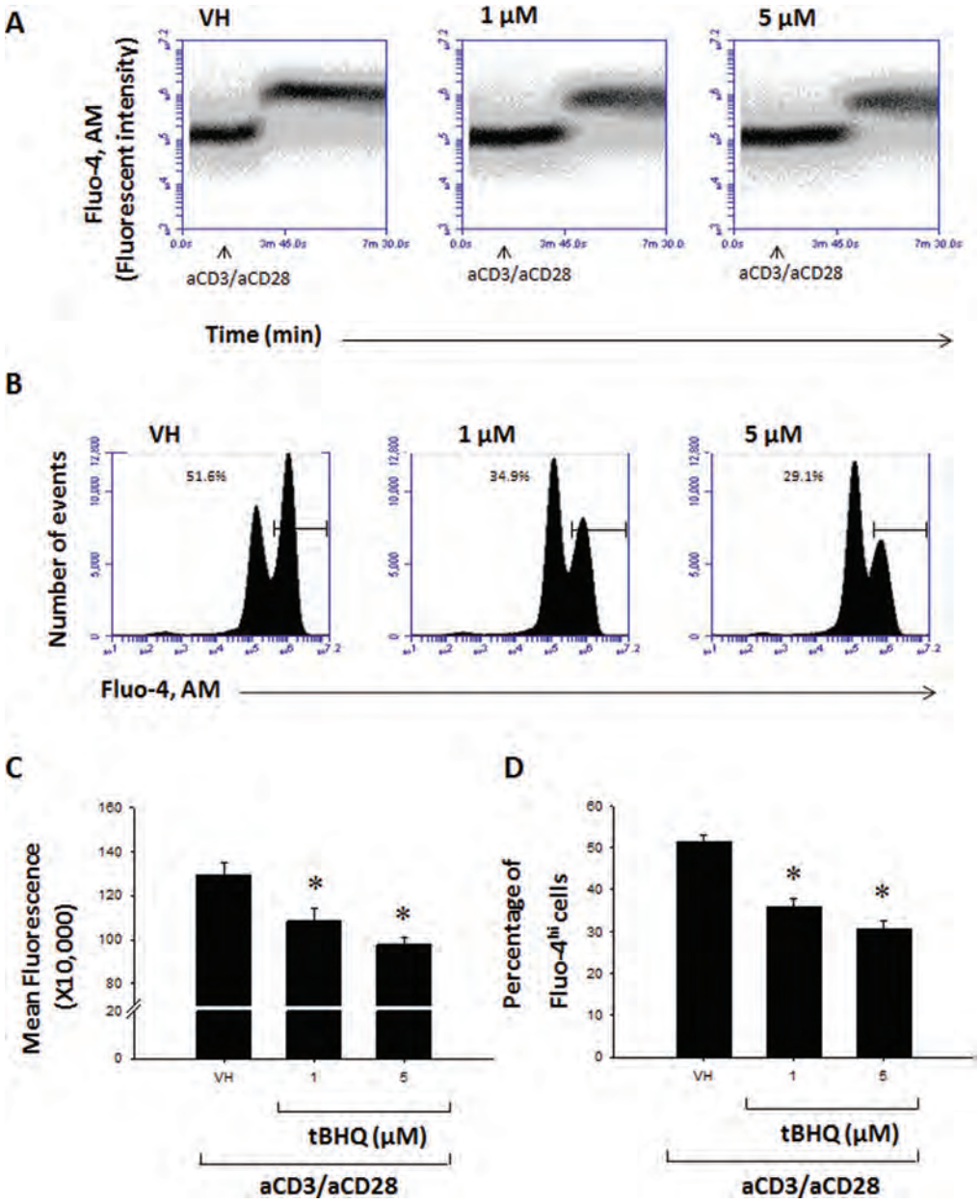

FIG. 5. The Nrf2 activator, tBHQ, delays and decreases the magnitude of calcium influx in activated Jurkat T cells. Jurkat T cells were pretreated with either tBHQ (1 or 5μM) or the vehicle (VH) control (0.01% ethanol) and loaded with Fluo-4/AM prior to activation with anti-CD3/anti-CD28. Calcium influx was determined by an increase in Fluo-4 fluorescence and measured over time by flow cytometry; 1.5 minutes after beginning the analysis, anti-CD3/anti-CD28 was added to each sample and measurements continued for another 6 min. (A) Fluo-4 fluorescence as measured over time by flow cytometry. (B) Histograms of Fluo-4 fluorescence. Graphical representation of (C) MFI or (D) percentage Fluo-4^{hi} cells. * represents $p < 0.05$ versus VH.

calcium, the transcription factor, NFAT, translocates to the nucleus and binds to the promoters of its target genes, such as IL-2 (Cantrell, 1996). Jurkat T cells treated with tBHQ prior to activation with anti-CD3/anti-CD28 demonstrated a decrease in number of cells positive for calcium influx, a delay in influx, and a decrease in the magnitude of calcium influx. The lack of effect of tBHQ on the binding and activity of the calcium-sensitive transcription factor, NFAT, was unexpected and suggested that the decrease in calcium influx by tBHQ was not great enough to diminish NFAT activation. Alternatively, the decreased calcium influx by tBHQ may rebound at some later time point, ultimately allowing for full NFAT activation. These observations also suggested that the decrease in IL-2 production by tBHQ was not due to impaired NFAT activity.

Along with the influx of Ca^{2+} into the cell upon ligation of the TCR and coreceptor, one of the earliest events of T-cell activation is the initiation and propagation of kinase cascades. These signal transduction cascades result in the activation of

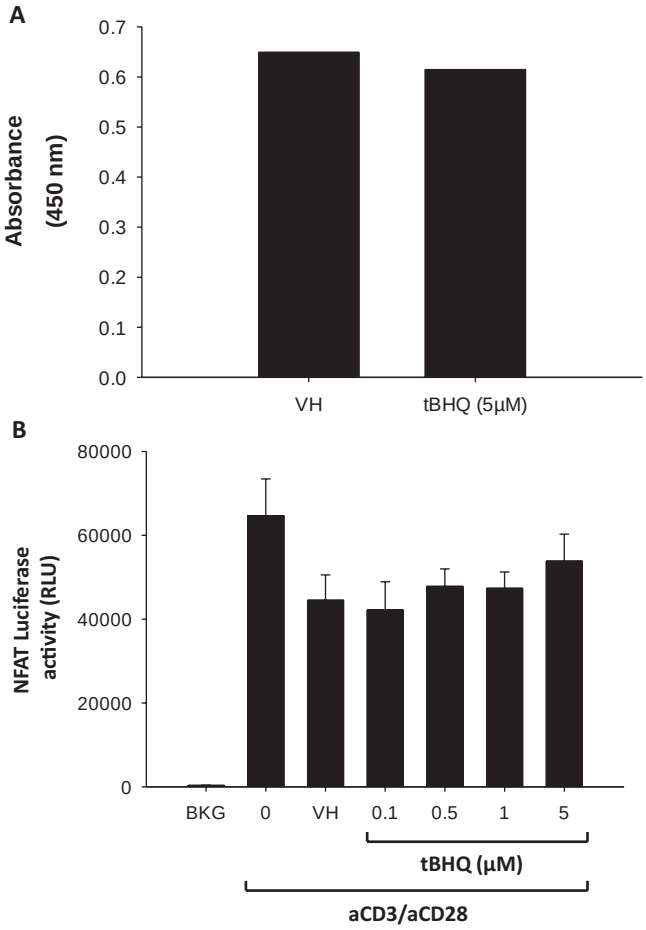

FIG. 6. The Nrf2 activator, tBHQ, does not affect NFAT DNA binding or transcriptional activity in activated Jurkat cells. (A) Jurkat T cells were pretreated with 5μM tBHQ or vehicle (VH) control (0.01% ethanol), 30 min prior to activation with anti-CD3/anti-CD28. One hour post-activation, nuclear proteins were extracted. NFAT DNA binding was quantified using an ELISA-based assay. (B) Jurkat T cells were transiently transfected with an NFAT luciferase reporter plasmid. After transfection, the cells received activator alone (0), were left untreated (BKG), or were pretreated with either tBHQ (0.1, 0.5, 1, or 5μM) or vehicle (VH) control (0.01% ethanol), 30 min prior to activation with anti-CD3/anti-CD28. Twelve hours post-activation, cells were harvested and luciferase luminescence was measured. None of the groups were statistically different from the VH control.

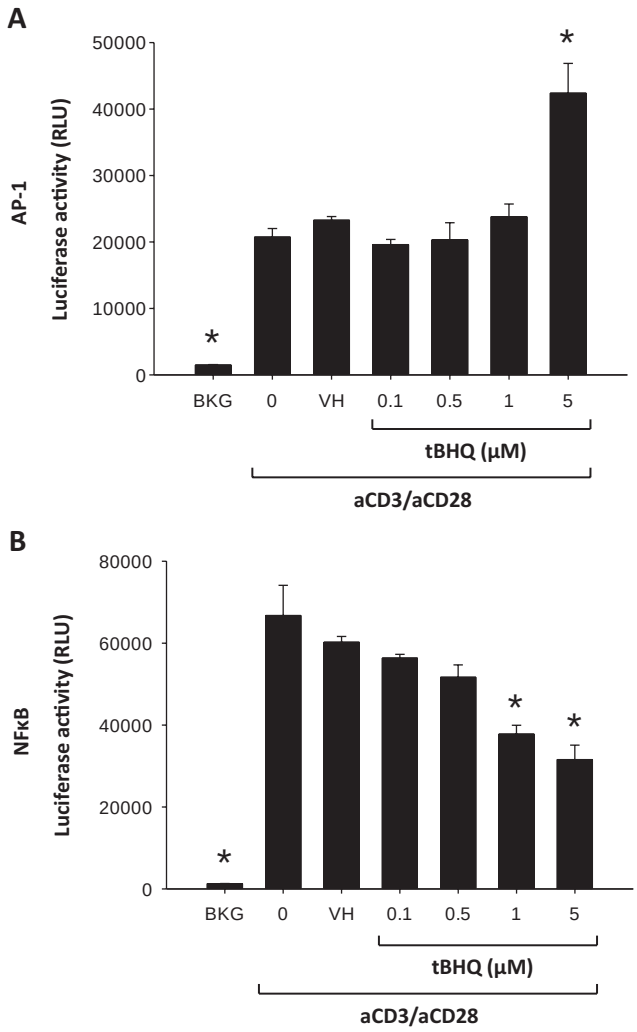

FIG. 7. The Nrf2 activator, tBHQ, modulates AP-1 and NFκB transcriptional activity in activated Jurkat cells. Jurkat T cells were transiently transfected with either (A) AP-1 luciferase reporter or (B) NFκB luciferase reporter plasmid. After transfection, the cells were left untreated (BKG), received activator alone (0), or were pretreated with either vehicle (VH) control (0.01% ethanol) or tBHQ (0.1, 0.5, 1, or 5μM), 30 min prior to activation with anti-CD3/anti-CD28. Twelve hours post-activation, cells were harvested and luciferase luminescence was measured. *$p < 0.05$ compared with the VH control.

transcription factors that regulate IL-2 transcription, such as AP-1 (c-fos/c-jun) and NFκB. This study shows that treatment of cells with 5μM tBHQ causes a significant increase in AP-1 activity. It has been shown previously that Nrf2 can heterodimerize with AP-1 and increase the transcription of AP-1 target genes. In contrast, tBHQ caused a marked concentration-dependent decrease in NFκB activity. Previously published data have shown that Nrf2 has the ability to inhibit the activity of NFκB by inhibition of IKK, the kinase responsible for phosphorylation and activation of NFκB (Kwon *et al.*, 2007; Thimmulappa *et al.*, 2006a). Activation of NFκB is necessary for IL-2 transcription; thus, it seems likely that the decrease in IL-2 production by tBHQ in activated Jurkat T cells is due to the decrease in NFκB transcriptional activity.

Although well known for its cytoprotective functions and detoxification activity, the role of Nrf2 in the immune system remains unclear. Numerous studies indicate an anti-inflammatory role for Nrf2 in models of inflammatory disease, but the specific cellular and molecular mechanisms are not known. Our previous study demonstrated that activation of Nrf2 modulates murine $CD4^+$ T-cell differentiation, and our current studies suggest suppression of several key events of early T-cell activation by tBHQ in human Jurkat T cells. Taken together, our studies in human and murine T cells suggest that Nrf2 represents a novel

mechanism for the regulation of T cell function and may provide insight into the mechanism of some disease states, such as SLE. These studies suggest that activation of Nrf2 by tBHQ may inhibit IL-2 production and CD25 expression, but further studies will be needed to clarify the role of Nrf2 more conclusively. In addition, further studies will be needed to determine the immunomodulatory effects of tBHQ in humans.

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