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The Nrf2 activator tBHQ inhibits T cell activation of primary human CD4 T cells

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ABSTRACT

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) regulates a battery of antioxidant, detoxification, and cell stress genes. It is activated by oxidative stress and a number of exogenous compounds, one of which is *tert*-butylhydroquinone (tBHQ), a widely used food preservative. Nrf2 modulates immune responses in numerous rodent models of inflammation, but its effects on human immune cells are not well characterized. The purpose of these studies was to evaluate the effects of the Nrf2 activator tBHQ on early events of T cell activation in primary human cells. Treatment with tBHQ induced mRNA expression of the Nrf2 target genes HMOX-1, GCLC, and NQO1, and also increased NRF2 mRNA expression, albeit to a lesser extent than the other target genes. tBHQ decreased production of the cytokines IL-2 and IFN-γ at both the protein and mRNA levels after stimulation with anti-CD3/anti-CD28 in human peripheral blood mononuclear cells and to an even greater extent in isolated CD4 T cells. Likewise, tBHQ decreased induction of CD25 and CD69 in peripheral blood mononuclear cells (PBMCs) and this decrease was even more marked in isolated CD4 T cells. In addition, tBHQ inhibited induction of NFκB DNA binding in anti-CD3/anti-CD28-activated PBMCs. Collectively, these data suggest that tBHQ inhibits activation of primary human CD4 T cells, which correlates with activation of Nrf2 and inhibition of NFκB DNA binding. Although these studies suggest the food additive tBHQ negatively impacts T cell activation, further studies will be needed to fully elucidate the effect of tBHQ on human immune responses.

1. Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that acts as a sensor for oxidative stress. Under basal conditions, Nrf2 is tethered in the cytosol to its repressor protein, Kelch-like ECH-associated protein 1 (Keap1), which facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 [1]. After stimulation by reactive oxygen species or electrophilic stimuli, Nrf2 ubiquitination is disrupted so that Nrf2 translocates to the nucleus. Upon heterodimerizing with small Maf proteins or other binding partners, Nrf2 binds to antioxidant response elements to regulate the transcription of a number of detoxification, antioxidant, and cell stress-related genes [2,3]. One activator of Nrf2 is *tert*-butylhydroquinone (tBHQ), a commonly used food additive found in a number of processed foods [4–6].

Nrf2 activation has been shown to have anti-inflammatory effects, and conversely, Nrf2 deletion has been shown to have pro-inflammatory effects [7–9]. Nrf2-null mice develop a lupus-like autoimmune disease, and have increased sensitivity to inflammation and infection in models such as sepsis and lung injury [10–14]. Recently, our laboratory demonstrated that Nrf2 modulates T cell responses in primary mouse CD4 T cells and Jurkat T cells [15,16]. Collectively, this indicates that Nrf2 modulates immune responses in a variety of different models and cell types. However, the role of Nrf2 in the activation of primary human T cells remains unclear.

T cells are a critical part of the adaptive immune response. Helper (CD4) T cells direct the immune response to different pathogens; thus T cell activation is critical for an effective adaptive immune response. T cell activation is characterized by a number of early events which have important downstream effects, including production of early cytokines, such as IL-2 and IFNγ, and upregulation of CD25 and CD69. IL-2 acts in an autocrine/paracrine fashion to help drive the proliferation and clonal expansion of naïve T cells, among other functions [17]. IFN-γ is the signature cytokine produced by Th1 cells and is important in driving cell-mediated

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immunity [18]. CD25 is the high affinity subunit of the IL-2 receptor, whereas CD69 is a C-type lectin protein. Both CD25 and CD69 are considered cell surface markers of T cell activation [19]. Several transcription factors have been shown to be important in T cell activation, including NFAT, NFκB, and AP-1 [17,20]. Our previous studies demonstrated that the Nrf2 activator tBHQ inhibits IL-2 secretion, CD25 expression, and NFκB induction in activated Jurkat cells [16]. The purpose of the present studies was to determine the effects of the Nrf2 activator tBHQ on the early events following T cell activation in primary human cells.

2. Methods

2.1. Peripheral blood mononuclear cell (PBMC) and CD4 T cell isolation

Whole human blood was purchased from Innovative Research (Novi, MI). PBMCs were isolated using Lymphocyte Separation Medium following the manufacturer's protocol (MP Biomedicals, Santa Ana, CA). CD4 T cells were isolated from PBMCs by positive selection using commercially available magnetic bead separation (Miltenyi Biotec, Auburn, CA). Cell treatments are described in figure legends. T cells were activated with purified hamster anti-human CD3ε (clone UCHT1, 1.5 μg/ml), purified hamster anti-human CD28 (clone CD28.2, 1.5 μg/ml), and an $F(ab')_2$ fragment specific for anti-Syrian hamster IgG that was used to cross-link CD3 and CD28. Anti-CD3 and anti-CD28 were purchased from Affymetrix/E-Bioscience (San Diego, CA), and the $F(ab')_2$ cross-linker from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2. Cytokine analysis

Concentrations of IL-2 or IFN-γ were quantified in cell supernatants using commercially available human IL-2 or IFN-γ ELISA kits following the manufacturer's protocol (Biolegend, San Diego, CA). Absorbance was quantified on a Bio-Tek μQuant microplate reader (Highland Park, VT).

2.3. mRNA quantification

Total RNA was isolated using TRIzol (Ambion, Life Technologies, Grand Island, NY) extraction according to the manufacturer's protocol. RNA was reverse transcribed to cDNA after which quantitative-real time PCR was performed using Sybr green analysis (Applied Biosystems, Life Technologies). Fluorescence was detected by a Life Technologies/Applied Biosystems Sequence Detection System 7500, and relative transcript levels were quantified using the ΔΔCT method, comparing the target genes to ribosomal protein L13a. Primer sequences were acquired from qPrimerDepot (http://primerdepot.nci.nih.gov/) and are as follows: RPL13A forward primer, 5′GTTGATGCCTTCACAGCGTA-3′ and reverse primer, 5′-AGATGGCGGAGGTGCAG-3′; IL-2 forward primer, 5′-GCACTTCCTCCAGAGGTTTG-3′ and reverse primer 5′-TCACC AGGATGCTCACATTT-3′; IFN-γ forward primer 5′-TCAGCCATCACT TGGATGAG-3′ and reverse primer 5′-CGAGATGACTTCGAAAAGCT G-3′; CD69 forward primer, 5′-ACAGGAACTTGGAAGGACCC-3′ and reverse primer, 5′-AGAACAGCTCTTTGCATCCG-3′; CD25 forward primer, 5′-TAGGCCATGGCTTTGAATGT-3′ and reverse primer, 5′-A TACCTGCTGATGTGGGGAC-3′; NRF2 forward primer, 5′-TCTTGCCT CCAAAGTATGTCAA-3′ and NRF2 reverse primer, 5′-CACGGTCCA CAGCTCATC-3′; NQO1 forward primer, 5′-TCCTTTCTTCAAAGCCG-3′ and NQO1 reverse primer, 5′-GGACTGCACCAGAGCCAT-3′; HMOX-1 forward primer, 5′-GGCTTCCCTCTGGGAGTCT-3′ and HMOX-1 reverse primer, 5′-AGCTGCTGACCCATGACAC-3′; GCLC forward primer, 5′-CTTTCTCCCCAGACAGGACC-3′ and GCLC reverse primer 5′-CAAGGACGTTCTCAAGTGGG-3′ All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

2.4. Flow cytometry

PBMCs or isolated CD4 cells were labeled with CD4-FITC (Affymetrix/E-Bioscience), CD69-PE/Cy7 (Biolegend), and CD25-APC (Affymetrix/E-Bioscience) for 30 min in the presence of an FcR blocking reagent (Miltenyi Biotec, Auburn, CA) and then washed. Fluorescence was detected by a C6 BD Accuri flow cytometer (BD Accuri, San Jose, CA). Fluorescence was quantified using CFlow software (BD Accuri).

2.5. ELISA-based DNA binding assay

PBMCs were treated with tBHQ (1 μM) or vehicle, then activated with anti-CD3/anti-CD28 30 min later. Three hours after activation, nuclear protein was extracted from 1×10^7 cells using a commercially available kit (Active Motif, Carlsbad, CA). After extraction, nuclear protein was quantified via the Bradford assay (BioRad). NFκB DNA binding was quantified from 10 μg of nuclear protein, by a commercially available ELISA-based DNA binding assay (Active Motif). Assays were performed per manufacturer's protocol.

2.6. Statistical analysis

All data were analyzed using SigmaPlot 12.3 (Systat, Chicago, IL). Data were analyzed by one-way ANOVA followed by a Dunnett's two-tailed post-hoc test. Data are expressed as mean ± standard error. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. The food additive tBHQ induces expression of the Nrf2 target genes HMOX-1, NQO1, and GCLC in human peripheral blood mononuclear cells (PBMCs)

tBHQ is a known activator of Nrf2, and accordingly we investigated the ability of our tBHQ treatment (0.1–5 μM) to induce expression of Nrf2 target genes. Consistent with other cell types, tBHQ treatment increased expression of the Nrf2 target genes, heme oxygenase 1 (HMOX-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutamate–cysteine ligase, catalytic subunit (GCLC), in human peripheral blood mononuclear cells (Fig. 1) [15,16]. In addition, tBHQ caused a modest induction of NRF2 mRNA expression itself, which has also been observed previously in primary mouse T cells and suggests that Nrf2 upregulates itself. Taken together, these data suggest that the concentrations of tBHQ used in this study activate Nrf2 in primary human PBMCs.

3.2. The Nrf2 activator tBHQ inhibits production of IL-2 in human PBMCs and isolated CD4 T cells activated with anti-CD3/anti-CD28

Our previous studies showed the Nrf2 activator tBHQ inhibited IL-2 production in activated Jurkat T cells, but the effect of tBHQ on primary human T cells has not been fully characterized [16]. Therefore, we determined the effect of tBHQ treatment on IL-2 production by PBMCs activated with anti-CD3/anti-CD28, a T cell specific activator. IL-2 protein and mRNA levels were significantly decreased by tBHQ in PBMCs activated with anti-CD3/anti-CD28 (Fig. 2A and B). Interestingly, tBHQ caused a more marked inhibition of IL-2 production in isolated CD4 T cells activated with anti-CD3/anti-CD28, suggesting that isolated T cells are more sensitive