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Bromodomain and Extraterminal Proteins Suppress NF-E2–Related Factor 2–Mediated Antioxidant Gene Expression

Charalambos Michaeloudes,1 Nicolas Mercado,1 Colin Clarke, Pankaj K. Bhavsar, Ian M. Adcock, Peter J. Barnes, and Kian Fan Chung

Oxidative stress, a pathogenic factor in many conditions, including chronic obstructive pulmonary disease, arises due to accumulation of reactive oxygen species and defective antioxidant defenses in the lungs. The latter is due, at least in part, to impaired activation of NF-E2–related factor 2 (Nrf2), a transcription factor involved in the activation of antioxidant and cytoprotective genes. The bromodomain and extraterminal (BET) proteins, Brd2, Brd3, Brd4, and BrdT, bind to acetylated lysine residues on histone or nonhistone proteins recruiting transcriptional regulators and thus activating or repressing gene transcription. We investigated whether BET proteins modulate the regulation of Nrf2-dependent gene expression in primary human airway smooth muscle cells and the human monocytic cell line, THP-1. Inhibition of BET protein bromodomains using the inhibitor JQ1+ or attenuation of Brd2 and Brd4 expression using small interfering RNA led to activation of Nrf2-dependent transcription and expression of the antioxidant proteins heme oxygenase-1, NADPH quinone oxidoreductase 1, and glutamate-cysteine ligase catalytic subunit. Also, JQ1+ prevented H2O2-induced intracellular reactive oxygen species production. By coimmunoprecipitation, BET proteins were found to be complexed with Nrf2, whereas chromatin–immunoprecipitation studies indicated recruitment of Brd2 and Brd4 expression using small interfering RNA led to activation of Nrf2-dependent transcription and expression of the antioxidant proteins heme oxygenase-1, NADPH quinone oxidoreductase 1, and glutamate-cysteine ligase catalytic subunit. Also, JQ1+ prevented H2O2-induced intracellular reactive oxygen species production. By coimmunoprecipitation, BET proteins were found to be complexed with Nrf2, whereas chromatin–immunoprecipitation studies indicated recruitment of Brd2 and Brd4 to Nrf2-binding sites on the promoters of heme oxygenase-1 and NADPH quinone oxidoreductase 1. BET proteins, particularly Brd2 and Brd4, may play a key role in the regulation of Nrf2-dependent antioxidant gene transcription and are hence an important target for augmenting antioxidant responses in oxidative stress–mediated diseases. The Journal of Immunology, 2014, 192: 4913–4920.

Cellular oxidative stress that results from an imbalance between the production of reactive oxygen species (ROS) and their removal by antioxidant defense mechanisms is pivotal in the pathogenesis of a number of diseases, including chronic obstructive pulmonary disease (COPD). In COPD, cigarette smoke inhalation and the resulting chronic inflammatory response lead to release of ROS in the airways. Defective antioxidant mechanisms in COPD lungs could lead to accumulation of ROS, resulting in activation of redox-sensitive transcription factors, epigenetic changes, and oxidative damage in immune and structural cells (1). NADPH oxidase–mediated ROS trigger airway smooth muscle cell (ASM) proliferation and hypertrophy (2). Exogenous oxidative stress stimuli reduce histone deacetylase 2 activity in alveolar macrophages, leading to increased expression of inflammatory genes (3). Moreover, cigarette smoke–mediated ROS reduce sirtuin 1 activity, leading to upregulation of matrix metalloproteinase-9 in monocytes and sputum macrophages (4). As a result, there is amplification of the inflammatory response and induction of pathogenic processes, such as lung parenchymal destruction and small airway remodeling (5, 6).

Cells are normally protected from ROS by the concerted action of a large array of endogenous antioxidant proteins. The transcription factor NF-E2–related factor 2 (Nrf2) plays a pivotal role in cellular antioxidant defenses by activating a wide range of antioxidant genes, including heme oxygenase (HO)-1, NADPH quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase catalytic subunit (GCLC) (7). Under normal conditions, Nrf2 is found in a complex with its inhibitor Kelch-like ECH-associated protein (Keap1) and the ubiquitin-ligase Cul3, where it is constantly targeted for proteosomal degradation. In the presence of oxidative stress, Nrf2 dissociates from the complex, leading to an increase in its protein levels and subsequent activation of antioxidant genes by binding to common regulatory elements, termed antioxidant response elements (AREs), in their promoter regions (8). Impaired Nrf2 function has been linked to defective antioxidant defenses in several age-related diseases, including COPD (9–12). Nrf2 expression has been shown to be reduced in lung tissue and alveolar macrophages of patients with COPD (9, 13, 14). Moreover, we have reported that reduced histone deacetylation activity in monocyte-derived macrophages leads to decreased Nrf2 protein stability through increased acetylation, implicating epigenetic mechanisms in impaired Nrf2 activation in COPD (15). Identification of molecular targets, particularly epigenetic
effectors, for augmenting Nrf2 activity would therefore be crucial for the development of new antioxidant therapies for COPD and other oxidative stress–dependent diseases.

The bromodomain and extraterminal (BET) proteins act as readers of protein acetylation by binding to acetylated lysine residues through two highly conserved N-terminal bromodomain modules to regulate gene expression (16). Specifically, BET proteins interact with transcription factors and chromatin remodeling complexes via their extraterminal (ET) and C-terminal domains, and recruiting them to the gene promoter to either activate or repress transcription of genes involved in inflammation, cell cycle, and differentiation (17–20). The human BET protein family comprises Brd2, Brd3, Brd4, and Brd7, the latter being solely expressed in the testis (21, 22). Small molecule bromodomain inhibitors, developed in recent years, are important tools for the study of BET proteins. The prototype compound, JQ1, is a thieno-triazolo-1,4-diazepine that specifically interferes with the binding of BET bromodomains to acetylated lysines (23). BET proteins have been shown to be involved in diseases in which oxidative stress and compromised antioxidant protection play an important pathogenic role, such as acute myeloid leukemia (24), heart failure (25), and obesity (26, 27). However, it is currently unknown whether BET proteins are directly involved in the regulation of antioxidant gene expression and hence the protection against ROS-mediated disease pathology. We hypothesized that BET proteins are involved in the regulation of antioxidant genes in both structural and immune cells and are thus important targets for augmenting antioxidant defenses in COPD. We therefore examined the effect of pharmacological and molecular inhibitors of BET protein activity on antioxidant gene expression in primary human ASMCs and the human monocytic cell line, THP-1. Furthermore, we investigated the effect of BET protein inhibition on the Nrf2 pathway, the interaction of BET proteins with Nrf2, and their recruitment to antioxidant gene promoters.

**Materials and Methods**

**Reagents**

The active JQ1 enantiomer (+)– JQ1 (JQ1+) and the inactive enantiomer (−)– JQ1 (JQ1−) were purchased from Cayman (Cambridge, U.K.). MG132 was purchased from Sigma–Aldrich (Poole, U.K.).

**Cell culture and stimulation**

THP-1 cells (human monocytic cell line) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Cayman) containing 10% heat-inactivated FBS and 2 mM l-glutamine at 37°C, 5% CO2, and humidified atmosphere. For stimulation, THP-1 cells were seeded (0.3 × 10^6 cells/ml) in starvation medium containing RPMI 1640 supplemented with 1% FBS and 15 mM l-glutamine. ASMCs were dissected from tracheas or bronchi of transplant donor lungs and were cultured in DMEM supplemented with 4 mM l-glutamine, 20 U/L penicillin, 20 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 10% FBS. Presence of ASMCs was confirmed by identifying the characteristic “hill and valley” morphology using light microscopy. Cell stocks were kept in 150-cm² flasks at 37°C, 5% CO2, and humidified atmosphere. Cells between passages 3 and 7 were used for experiments. Before treatment, cells were incubated in serum-free medium containing phenol-free DMEM supplemented with 1 mM sodium pyruvate, 4 mM l-glutamine, nonessential amino acids, 1% insulin-transferrin-selenium-X supplement, 0.1% BSA, and antibiotics, as described above.

PBMCs were isolated from the peripheral blood of three healthy volunteers, using AccuSPIN columns (Sigma–Aldrich). Monocytes were isolated by adherence to tissue culture plates for 90 min. This study was approved by the local ethics committee of Royal Brompton and Harefield National Health Service Trust, and written informed consent was obtained from each volunteer. PBMCs were cultured in RPMI 1640 medium containing 10% FBS and 2 mM l-glutamine at 37°C, 5% CO2, and humidified atmosphere.

**Small interfering RNA transfection**

THP-1 cells were transfected with 300 nM ON-TARGETplus SMARTpool small interfering (siRNA) against Nrf2 (catalogue L-003755-00-0005), Brd2 (catalogue L-004935-00-0005), Brd3 (catalogue L-004936-00-0005), or Brd4 (catalogue L-004937-00-0005) (Thermo Scientific, Epsom, U.K.), or a random oligonucleotide control siRNA (Qiagen, Crawley, U.K.) for 48 h using HiPerfect transfection reagent (Qiagen) following the manufacturer’s instructions. ASMCs were transfected with the same siRNA sequences (300 nM) for 48 h using Amaxa nucleofection (Lonza AG, Cologne, Germany), following the manufacturer’s instructions.

**ARE reporter assay**

ASMCs were transfected, using Amaxa nucleofection, with DNA plasmid constructs (2.5 μg) expressing ARE-inducible firefly luciferase and constitutively active Renilla luciferase (SABiosciences, Frederick, MD) for 24 h, and then incubated with the required treatments for an additional 24 h. At the end of the incubation time, cells were lysed and firefly and Renilla luciferase activities were determined by measuring luminescence.

ARE-inducible luciferase activity was normalized to Renilla luciferase activity. The Nrf2 inducer sulforaphane (4 μM) was used as a positive control in all experiments.

**Western blotting**

Protein extracts were prepared using modified radioimmunoprecipitation assay buffer (50 mM Tris HCL [pH 7.4], 0.5% Nonidet P-40, 0.5% w/v Na deoxycholate, 150 mM NaCl containing protease inhibitors) (Roche, Welwyn Garden City, U.K.). Protein extracts were fractionated by SDS-PAGE on 3–8% Tris-acetate or 4–12% Bis-Tris precast polyacrylamide gels (Invitrogen, Paisley, U.K.) and transferred to a nitrocellulose membrane (Invitrogen). Proteins were detected using anti-NQO1 (Sigma Aldrich); Nrf2 and HO-1 (Santa Cruz Biotechnology, Santa Cruz, CA); GCLC and β-actin (Abcam, Cambridge, U.K.); Brd2, Brd3, and Brd4 (Bethyl Laboratories, Cambridge, U.K.); and Keap1 (Origene, Rockville, MD). Bands were visualized by chemiluminescence (ECL Plus; GE Healthcare, Hatfield, U.K.), and protein expression levels were normalized to β-actin expression.

**Real-time PCR**

Total RNA was isolated using RNasey Mini Kit (Qiagen) and reverse transcribed using random primers and avian myeloblastosis virus reverse transcriptase (Promega, Southampton, U.K.). mRNA was quantified by real-time PCR (Rotor Gene 3000; Qiagen) using SYBR Green PCR Master Reagent and QuantiTect primer assays (Qiagen) for HO-1 (catalogue QT0002645), NQO1 (catalogue QT0009281), GCLC (catalogue QT00037110), Nrf2 (catalogue QT00027384), Keap1 (catalogue QT0008220), MnSOD (catalogue QT00186963), and catalase (catalogue QT00079674), and normalized to 18S rRNA or GNB2L1 expression. GNB2L1 expression was also determined by a QuantiTect primer assay (catalogue QT01156610). For 18S rRNA, the following primer sequences were used: 5′-CTTGGAGGCAACTG TGCCG-3′ and 5′-ACGCCTGAGCACTCAGTGA-3′.

**Immunoprecipitation**

Nrf2 was immunoprecipitated from 300 to 1000 μg cell lysate by incubating with 5 μg anti-Nrf2 Ab (Santa Cruz Biotechnology) overnight at 4°C. Immunoprecipitates were captured with rabbit TrueBlot IP beads (eBio science, Hatfield, U.K.). After extensive washing, bound proteins were released by boiling in SDS-PAGE sample buffer (Invitrogen). Immunoprecipitates were fractionated by SDS-PAGE, and the presence of Brd2, Brd3, and Brd4 was determined by Western blot analysis, as described above, and normalized to the amount of immunoprecipitated Nrf2. Cells were treated with the protease inhibitor MG132 (2.5 μg/ml) for 1.5 h before immunoprecipitation to maximize the amount of Nrf2 protein immunoprecipitated.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChiP) assays were performed with the Magna ChIP A/G kit (Millipore, Temecula, CA). Cells were fixed in 1% formaldehyde for 10 min, and DNA was fragmented by means of sonication (five 10-s pulses). Samples were diluted in ChiP dilution buffer and incubated with 4 μg Brd2 or Brd4 Ab (Bethyl Laboratories) and protein A/G magnetic beads overnight. Ab/DNA complexes were captured, washed, eluted, and reverse cross-linked. DNA was purified by means of spin columns containing activated silica membrane filters. The precipitated DNA was resuspended, and real-time PCR was performed using primers spanning ARE sites on the HO-1 and NQO1 gene promoters. The primers used were as follows: NQO1 forward, 5′-CATTGCATGCACCCACCAG GGA-A-3′, and reverse, 5′-GCTATCCCGCCCTTATAGCCTGGCA-3′; and HO-1 forward, 5′-CTGCCAAAACACTCTTGGT-3′, and reverse, 5′-ATAAGAGGCGCCTCGGTTGA-3′ (29). DNA expression was normalized to input DNA for each sample.
Cigarette smoke extract preparation

Cigarette smoke extract (CSE) was prepared by filtering one cigarette through a 50-ml syringe and passing the smoke through 10 ml starvation media. The concentration of CSE used was calculated as percentage v/v of this extract with respect to the total volume of media.

Detection of ROS using dihydroethidium staining

THP-1 cells were seeded (0.3×10^6 cells/ml) in 96-well tissue culture plates in starvation medium and then incubated with the indicated treatments. Cells were then incubated with dihydroethidium (40 μM) for 30 min at 37°C and 5% CO2, washed using HBSS, and reseeded in 96-well tissue culture plates at a density of 0.3×10^6 cells/ml. Fluorescence was measured at excitation/emission wavelengths of 520/610 nm using a fluorescence plate reader (Synergy HT Biotek).

Statistical analysis

Data are expressed as mean ± SEM. Results were analyzed using one-way ANOVA for repeated measures, followed by Dunnet post hoc test. Statistical analysis was performed using the GraphPad Prism 4 software (Prism, San Diego, CA). A p value <0.05 was considered statistically significant.

Results

JQ1+ increases antioxidant gene expression

In THP-1 cells, the BET bromodomain inhibitor JQ1+ (30–1000 nM) augmented the mRNA expression of Nrf2-dependent genes HO-1 (maximum of ~10-fold), NQO1 (maximum of ~3-fold), and GCLC (maximum of ~2-fold) in a concentration-dependent manner, 24 h posttreatment (Fig. 1A–C). In contrast, its inactive enantiomer, JQ1−, did not modulate gene expression. JQ1+ (300 nM) increased the mRNA of HO-1 (~8-fold), NQO1 (~2.5-fold), and GCLC (~4-fold) 8–24 h posttreatment (Fig. 1D–F). These changes were accompanied by increased HO-1 (Fig. 1G, 1H), NQO1 (Fig. 1I, 1J), and GCLC (Fig. 1G, 1J) protein levels 24–32 h posttreatment. Consistent with the increase in antioxidant protein expression, we observed that pretreatment of THP-1 cells with JQ1+ for 32 h reduced both baseline and H2O2 (100 μM)-induced intracellular ROS production, whereas JQ1− had no effect (Fig. 1K).

FIGURE 1. Effect of JQ1+ and JQ1− on HO-1, NQO1, and GCLC expression in THP-1 cells. (A–C) mRNA expression of HO-1 (A), NQO1 (B), and GCLC (C) was determined in THP-1 cells after treatment with vehicle, JQ1− or JQ1+ (30–1000 nM), for 24 h and was normalized to GNB2L1 mRNA levels. Data are represented as fold changes with respect to vehicle control. (D–F) The mRNA expression of HO-1 (D), NQO1 (E), and GCLC (F) was determined after treatment with vehicle, JQ1− or JQ1+ (300 nM), for 4, 8, and 24 h and was normalized to GNB2L1 mRNA levels. Data are represented as fold change with respect to vehicle control at each time point (dotted line). (G–J) HO-1, NQO1, and GCLC protein expression was determined in whole-cell protein extracts after treatment with vehicle, JQ1− or JQ1+ (300 nM), for 8, 24, and 32 h and normalized to β-actin expression (G). Fold changes in HO-1 (H), NQO1 (I), and GCLC (J) protein were determined with respect to vehicle control (dotted line) at each time point. (K) THP-1 cells were pre-treated with JQ1+ for 32 h and then stimulated with H2O2 (100 μM) for 15 min. Intracellular ROS levels were determined by staining with dihydroethidium and measuring fluorescence at 520/610 nm using a fluorescence plate reader. Data are represented as fold change with respect to vehicle control. Results are representative of mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 versus vehicle.
We also assessed the effect of JQ1+ on antioxidant protein expression in THP-1 cells in the presence or absence of CSE, a potent source of oxidative stress. CSE increased Nrf2, HO-1, and NQO1 protein expression. JQ1+ increased CSE-induced HO-1 and NQO1 protein levels further, without affecting Nrf2 protein expression, suggesting that BET bromodomain inhibition can augment antioxidant gene expression even under conditions of oxidative stress (Supplemental Fig. 1D).

The effect of JQ1+ on Nrf2 target genes was also investigated in primary blood monocytes from three healthy volunteers. In line with our findings in THP-1 cells, JQ1+ (300 nM) increased HO-1 and GCLC mRNA levels in the monocytes from all volunteers, 4 h posttreatment (Supplemental Fig. 1E, 1F). However, JQ1+ increased NQO1 mRNA in the monocytes from only one volunteer, suggesting patient-specific differences in the regulation of NQO1 gene expression (Supplemental Fig. 1G).

In ASMCs, JQ1+ also increased HO-1 (maximum of ∼5-fold) and NQO1 (maximum of ∼6-fold) mRNA expression in a concentration-dependent manner, 24 h posttreatment (Fig. 2A, 2B). JQ1+ (300 nM) also increased HO-1 (∼4-fold), NQO1 (∼4-fold), and GCLC (∼2-fold) mRNA 8–24 h posttreatment (Fig. 2C–E). These changes were accompanied by increased HO-1 (Fig. 2F, 2G), NQO1 (Fig. 2H, 2I), and GCLC (Fig. 2J, 2K) protein expression. Interestingly, the antioxidant genes MnSOD and catalase, which are not classical Nrf2 targets, were not modulated by JQ1+ in ASMCs (Supplemental Fig. 1A, 1B). These data suggest an involvement of BET bromodomains in the inhibition of Nrf2-dependent antioxidant genes in both structural and immune cells.

**FIGURE 2.** Effect of JQ1+ and JQ1− on HO-1, NQO1, and GCLC expression in ASMCs. (A and B) The mRNA expression of HO-1 (A) and NQO1 (B) was determined in ASMCs after treatment with vehicle, JQ1− or JQ1+ (30–1000 nM), for 24 h and was normalized to 18S rRNA expression. (C–E) The mRNA expression of HO-1 (C), NQO1 (D), and GCLC (E) was determined after treatment with vehicle, JQ1− or JQ1+ (300 nM), for 4, 8, and 24 h and was normalized to 18S rRNA expression. Data are represented as fold change with respect to vehicle control (dotted line) at each time point. (F–K) HO-1 (F and G), NQO1 (H and I), and GCLC (J and K) protein expression was determined in whole-cell protein extracts, after treatment with vehicle, JQ1− or JQ1+ (300 nM), for 8, 24, and 32 h and was normalized to β-actin expression. Data are represented as fold change with respect to vehicle control (dotted line) at each time point. Results are representative of mean ± SEM of three ASMC (A and B) and four to five ASMC donors (C–K). *p < 0.05, **p < 0.01, and ***p < 0.001 versus vehicle.

**siRNA-mediated inhibition of BET protein expression leads to increased antioxidant expression**

The role of BET proteins in the regulation of antioxidant genes was confirmed by specific inhibition of Brd2, Brd3, and Brd4 expression using siRNA-mediated gene knockdown. In THP-1 and ASMCs cells, a strong reduction of Brd2, Brd3, and Brd4 protein levels was observed 48 h after transfection with specific siRNA sequences (300 nM) (Fig. 3A–D, 3G–J). In THP-1 cells, HO-1 expression was increased by ∼6-fold in response to Brd4 siRNA and by ∼3-fold in response to Brd2 siRNA, whereas Brd3 siRNA had no effect (Fig. 3E) 48 h after transfection. In contrast, NQO1 expression was increased only by Brd2 siRNA (∼3-fold) (Fig. 3F). In ASMCs, HO-1 (∼4-fold) and NQO1 (∼3-fold) protein expression were increased in response to Brd2 siRNA, whereas it was not affected by Brd3 or Brd4 siRNA (Fig. 3K, 3L). Thus, BET proteins are directly involved in the suppression of Nrf2-dependent antioxidant genes in both cell types.

**Effect of BET proteins on Nrf2 signaling**

To determine whether the inhibitory effect of BET proteins on antioxidant gene expression occurs through suppression of Nrf2 signaling, we determined the effect of BET bromodomain inhibition on Nrf2 transcriptional activity using an ARE-driven luciferase reporter gene vector in ASMCs. JQ1+ (300–1000 nM) increased luciferase activity 24 h posttreatment (maximum of ∼2-fold), whereas JQ1− did not have any effect (Fig. 4A). Intriguingly, the effect of JQ1+ was comparable to the effect of the Nrf2 inducer sulforaphane at 4 μM (Supplemental Fig. 1C). In line with these findings, knockdown of Nrf2 gene expression using siRNA
abrogated the upregulation of HO-1 and NQO1 protein by JQ1+ in both ASMCs (Fig. 4B) and THP-1 cells (Fig. 4C), confirming that the inhibitory effect of BET proteins on antioxidant genes occurs through inhibition of Nrf2 signaling.

To better understand the mechanism behind the inhibition of Nrf2-mediated transcription by BET proteins, we determined the effect of JQ1 on the expression of Nrf2 and Keap1. In ASMCs, JQ1+ (300 nM) increased Nrf2 protein (Supplemental Fig. 2A, 2B) and mRNA (Supplemental Fig. 2C), 8–32 h posttreatment. At the same time, JQ1+ reduced Keap1 protein levels 24–48 h posttreatment (Supplemental Fig. 2A, 2D), without significantly affecting Keap1 mRNA (Supplemental Fig. 2E). This change in the balance between Nrf2 and Keap1 occurs concurrently with the increase in antioxidant genes, suggesting that it is not the mechanism behind the activation of Nrf2 but probably a secondary effect that leads to sustained Nrf2 activation in response to BET protein inhibition. Intriguingly, although a reduction in Keap1 protein was also observed in JQ1+ (300 nM)-treated THP-1 cells (Supplemental Fig. 2F, 2H), JQ1+ did not significantly affect the recruitment of Brd2 or Brd4 to the antioxidant gene promoters compared with vehicle control (Supplemental Fig. 2F, 2G). These findings suggest cell-specific differences in Nrf2 homeostasis.

Study of in vitro BET protein–Nrf2 interaction

To determine whether the suppression of Nrf2 activity by BET proteins is a result of direct protein–protein interaction, we performed coimmunoprecipitation of Nrf2 with BET proteins in THP-1 cells. Immunoprecipitation of Nrf2, followed by detection of Brd2, Brd3, and Brd4 protein by Western blot analysis, revealed interaction of Nrf2 with all three BET proteins. Treatment with JQ1+ (300 nM) did not attenuate the association between Nrf2 and BET proteins, suggesting that the interaction is bromodomain independent (Fig. 5A).

Recruitment of BET proteins to ARE elements of NQO1 and HO-1 gene promoters

We investigated the presence of Brd2 and Brd4 at ARE sites on the promoters of HO-1 and NQO1 genes by ChIP in THP-1 cells. We observed increased recruitment of Brd2 (~4-fold) and Brd4 (~3-fold) to the NQO1 promoter (Fig. 5B, 5C). There was also increased recruitment of Brd2 (~3-fold) and Brd4 (~2-fold) to the HO-1 promoter (Fig. 5D, 5E), suggesting that BET proteins are constitutively associated with the promoters of these genes. Baseline recruitment of Nrf2 to these sites was also observed (data not shown). Unexpectedly, the inactive enantiomer JQ1− led to an increase in the recruitment of Brd2 and Brd4 to both promoters compared with vehicle control (Fig. 5B–E). This is possibly a nonspecific effect of the compound that does not have a functional consequence, as JQ1− did not modulate Nrf2-dependent transcription. Moreover, JQ1+ did not significantly affect the recruitment of Brd2 or Brd4 to the antioxidant gene promoters compared with JQ1−, indicating that recruitment of BET proteins is also bromodomain independent.
transcription and increased expression of the antioxidant genes Nrf2, NQO1, and GCLC. Our findings demonstrate that BET proteins act as inhibitors of Nrf2 activity and are thus putative molecular targets for enhancing endogenous antioxidant defenses and thus protection from oxidative stress.

BET proteins are primarily known as activators of gene transcription. Brd2 and Brd4 in particular are involved in cell cycle progression by recruiting coactivators, such as E2F transcription factor 1 and the positive transcription elongation factor complex, to acetylated histone at the promoters of proliferative genes (21, 22, 30). Brd4 has also been shown to directly interact with acetylated NF-κB, leading to activation of inflammatory gene transcription (18). Intriguingly, BET proteins can also act as transcriptional repressors. Brd2 has been shown to interact with peroxisome proliferator-activated receptor (PPAR)-γ, leading to suppression of its transcriptional activity (27). We show that inhibition of BET bromodomain using the inhibitor JQ1+ or siRNA-mediated knockdown of Brd2 and Brd4 genes leads to activation of Nrf2-dependent antioxidant genes in ASMCs and THP-1 cells, indicating an inhibitory effect of BET proteins on Nrf2 activity. In line with these findings, JQ1+ had a protective effect against oxidative stress–induced intracellular ROS production, highlighting the potential of BET proteins as therapeutic targets for augmenting antioxidant responses. This protective effect of BET protein inhibition was further corroborated by our findings that JQ1+ can upregulate Nrf2-dependent antioxidant proteins even in cells exposed to CSE, an important instigator of oxidative stress in COPD (31). Moreover, we show that this mechanism also occurs in primary monocytes that are a major source of ROS in the airways (32). Our study identifies Nrf2 as a new target of the inhibitory effect of BET proteins, providing a novel insight into the mechanisms regulating Nrf2 activity and thus antioxidant protection.

Nrf2 activity is primarily regulated through changes in its protein stability as a result of its association or dissociation from the Keap1/Cul3 complex (8). Nrf2 nuclear translocation and DNA binding can also be modulated by posttranslational modifications, whereas its transcriptional activity is regulated through interaction with coactivators or repressors (33–36). We show that JQ1+ did not modulate Nrf2 or Keap1 levels at early time points, suggesting that BET proteins do not affect Nrf2 protein stability (data not shown). In ASMCs JQ1+ led to a delayed increase, 8 h post-treatment, in Nrf2 mRNA and protein expression, possibly a result of positive feedback activation of the Nrf2 gene itself, which is known to contain an ARE in its promoter (37). Intriguingly, this effect was not observed in THP-1 cells, suggesting cell-specific differences in this mechanism. Moreover, JQ1+ reduced Keap1 protein expression in both ASMCs and THP-1 cells. However, as this occurs concurrently with the activation of the antioxidant genes, it is possibly not related to the mechanism of Nrf2 activation by JQ1+, but may constitute a feedback-loop activation mechanism leading to prolonged Nrf2 activation.

Our data indicate that BET proteins interact with Nrf2 and are found at the ARE sites of antioxidant gene promoters under baseline conditions, suggesting that BET proteins may be constitutively present in the Nrf2 transcriptional complex. Depletion of BET proteins, particularly Brd2 and Brd4, using siRNA leads to upregulation of Nrf2 target proteins, indicating that the presence of BET proteins in the complex leads to repression of Nrf2-mediated transcription. Treatment with JQ1+ also leads to activation of Nrf2-dependent antioxidant genes and an ARE-driven luciferase gene, indicating that the bromodomains are directly involved in the inhibition of Nrf2-mediated transcription by BET proteins. In contrast, the interaction of BET proteins with Nrf2 and their recruitment to the antioxidant gene promoters are not

Discussion

BET proteins act as readers of protein acetylation by binding to acetylated lysine residues and providing a scaffold for transcriptional activators or repressors (16). To our knowledge, we demonstrate for the first time that BET proteins are constitutively associated with Nrf2 on ARE sites of antioxidant gene promoters. Reduction of BET protein expression using siRNA or inhibition of their binding to acetylated lysine residues, using the pharmacological inhibitor JQ1+, leads to activation of Nrf2-mediated transcription and increased expression of the antioxidant genes HO-1, NQO1, and GCLC. Our findings demonstrate that BET proteins act as inhibitors of Nrf2 activity and are thus putative molecular targets for enhancing endogenous antioxidant defenses and thus protection from oxidative stress.

BET proteins were transfected with an ARE-driven luciferase reporter vector (2.5 µg) for 24 h and then treated with vehicle, JQ1− or JQ1+ (300 and 1000 nM), for 24 h. ARE-driven transcriptional activity was determined by measuring luciferase activity and normalizing to Renilla luciferase activity. Data are expressed as fold change with respect to vehicle control. Results are representative of mean ± SEM of five ASMC donors. **p < 0.01 and ***p < 0.001 versus vehicle. (B and C) ASMCs (B) and THP-1 cells (C) were transfected with random oligonucleotide control (Ct) or Nrf2 siRNA (300 nM) for 24 h and then treated with vehicle, JQ1− or JQ1+ (300 nM), for 32 h. Nrf2, NQO1, HO-1, and β-actin protein expression was determined by Western blotting. Blots are representative of one experiment from each cell type.

FIGURE 4. Effect of JQ1+ and JQ1− on Nrf2-dependent transcription. (A) ASMCs were transfected with an ARE-driven luciferase reporter vector (2.5 µg) for 24 h and then treated with vehicle, JQ1− or JQ1+ (300 and 1000 nM), for 24 h. ARE-driven transcriptional activity was determined by measuring luciferase activity and normalizing to Renilla luciferase activity. Data are expressed as fold change with respect to vehicle control. Results are representative of mean ± SEM of five ASMC donors. **p < 0.01 and ***p < 0.001 versus vehicle. (B and C) ASMCs (B) and THP-1 cells (C) were transfected with random oligonucleotide control (Ct) or Nrf2 siRNA (300 nM) for 24 h and then treated with vehicle, JQ1− or JQ1+ (300 nM), for 32 h. Nrf2, NQO1, HO-1, and β-actin protein expression was determined by Western blotting. Blots are representative of one experiment from each cell type.

Discussion

BET proteins act as readers of protein acetylation by binding to acetylated lysine residues and providing a scaffold for transcriptional activators or repressors (16). To our knowledge, we demonstrate for the first time that BET proteins are constitutively associated with Nrf2 on ARE sites of antioxidant gene promoters. Reduction of BET protein expression using siRNA or inhibition of their binding to acetylated lysine residues, using the pharmacological inhibitor JQ1+, leads to activation of Nrf2-mediated transcription and increased expression of the antioxidant genes HO-1, NQO1, and GCLC. Our findings demonstrate that BET proteins act as inhibitors of Nrf2 activity and are thus putative molecular targets for enhancing endogenous antioxidant defenses and thus protection from oxidative stress.

BET proteins are primarily known as activators of gene transcription. Brd2 and Brd4 in particular are involved in cell cycle progression by recruiting coactivators, such as E2F transcription factor 1 and the positive transcription elongation factor complex, to acetylated histone at the promoters of proliferative genes (21, 22, 30). Brd4 has also been shown to directly interact with acetylated NF-κB, leading to activation of inflammatory gene transcription (18). Intriguingly, BET proteins can also act as transcriptional repressors. Brd2 has been shown to interact with peroxisome proliferator-activated receptor (PPAR)-γ, leading to suppression of its transcriptional activity (27). We show that inhibition of BET bromodomain using the inhibitor JQ1+ or siRNA-mediated knockdown of Brd2 and Brd4 genes leads to activation of Nrf2-dependent antioxidant genes in ASMCs and THP-1 cells, indicating an inhibitory effect of BET proteins on Nrf2 activity. In line with these findings, JQ1+ had a protective effect against oxidative stress–induced intracellular ROS production, highlighting the potential of BET proteins as therapeutic targets for augmenting antioxidant responses. This protective effect of BET protein inhibition was further corroborated by our findings that JQ1+ can upregulate Nrf2-dependent antioxidant proteins even in cells exposed to CSE, an important instigator of oxidative stress in COPD (31). Moreover, we show that this mechanism also occurs in primary monocytes that are a major source of ROS in the airways (32). Our study identifies Nrf2 as a new target of the inhibitory effect of BET proteins, providing a novel insight into the mechanisms regulating Nrf2 activity and thus antioxidant protection.

Nrf2 activity is primarily regulated through changes in its protein stability as a result of its association or dissociation from the Keap1/Cul3 complex (8). Nrf2 nuclear translocation and DNA binding can also be modulated by posttranslational modifications, whereas its transcriptional activity is regulated through interaction with coactivators or repressors (33–36). We show that JQ1+ did not modulate Nrf2 or Keap1 levels at early time points, suggesting that BET proteins do not affect Nrf2 protein stability (data not shown). In ASMCs JQ1+ led to a delayed increase, 8 h post-treatment, in Nrf2 mRNA and protein expression, possibly a result of positive feedback activation of the Nrf2 gene itself, which is known to contain an ARE in its promoter (37). Intriguingly, this effect was not observed in THP-1 cells, suggesting cell-specific differences in this mechanism. Moreover, JQ1+ reduced Keap1 protein expression in both ASMCs and THP-1 cells. However, as this occurs concurrently with the activation of the antioxidant genes, it is possibly not related to the mechanism of Nrf2 activation by JQ1+, but may constitute a feedback-loop activation mechanism leading to prolonged Nrf2 activation.

Our data indicate that BET proteins interact with Nrf2 and are found at the ARE sites of antioxidant gene promoters under baseline conditions, suggesting that BET proteins may be constitutively present in the Nrf2 transcriptional complex. Depletion of BET proteins, particularly Brd2 and Brd4, using siRNA leads to upregulation of Nrf2 target proteins, indicating that the presence of BET proteins in the complex leads to repression of Nrf2-mediated transcription. Treatment with JQ1+ also leads to activation of Nrf2-dependent antioxidant genes and an ARE-driven luciferase gene, indicating that the bromodomains are directly involved in the inhibition of Nrf2-mediated transcription by BET proteins. In contrast, the interaction of BET proteins with Nrf2 and their recruitment to the antioxidant gene promoters are not
prevented by JQ1+, suggesting that BET proteins do not directly bind to acetylated lysine residues on either histones or Nrf2. The interaction of BET proteins with Nrf2 thus possibly occurs through their ET or C-terminal domains either directly or indirectly through other proteins or protein complexes (21, 38, 39). These interactions need to be investigated in more depth in the future.

Inhibition of Nrf2-dependent transcription by BET proteins may occur through the recruitment of a repressive complex to the Nrf2-regulated gene promoter. Belkina and Denis (40) suggest that Brd2 inhibits PPAR-γ–mediated transcription by recruiting a repressor complex containing the silencing mediator for retinoid and thyroid hormone receptor (SMRT), through association with retinoic X receptor (RXR), which is found in a heterodimer with PPAR-γ. Both RXR and SMRT have also been shown to interact with Nrf2 and inhibit its transcriptional activity (36, 41). Thus, a mechanism involving the recruitment of RXR and SMRT could also be implicated in the inhibition of Nrf2 by BET proteins; this will be explored in future studies.

Another interesting observation arising from our study is that BET family members show cell-type and gene specificity. Although all three BET proteins were found to interact with Nrf2, the siRNA knockdown experiments revealed that Brd2 and Brd4 were predominantly involved in the inhibition of antioxidant gene expression. Furthermore, in THP-1 cells, Brd2 was found to be more important in the inhibition of NQO1, whereas Brd4 in the inhibition of HO-1. In contrast, in ASMCs, Brd2 is the main isoform involved in the inhibition of both HO-1 and NQO1. These data suggest that, although all isoforms can interact with Nrf2, they are likely to recruit different proteins due to differences in their protein–protein interaction motifs. Indeed, although the bromodomains and ET domains are highly similar between BET family members (>80% similarity), their C-terminal domains show different lengths and structures, which may account for the differences in the proteins with which they interact (38, 40). Furthermore, the differences in gene specificity may be a result of differences in the local environment of gene promoters in each cell type. This disparity between BET protein isoform specificities is reflected by the divergence observed in the phenotypes of Brd2 and Brd4 knockout mice (42). Further work looking at other Nrf2-dependent cytoprotective genes should shed more light on the roles of Brd2, Brd3, and Brd4.

**Nrf2** is an important mechanism through which cells are protected from oxidative stress, and thus the molecular mechanisms regulating its activity are of great interest, especially in diseases involving oxidative stress like COPD (7, 9, 13). Our study suggests that BET proteins may be a key player in the regulation of Nrf2 activity and thus important targets for augmenting antioxidant defenses. Moreover, it raises the possibility that perturbed BET protein signaling in disease could result in gene-specific repression of Nrf2-dependent genes, contributing to defective antioxidant defense. Our findings highlight an important role of BET protein inhibitors as novel antioxidant treatments.

**Disclosures**

The authors have no financial conflicts of interest.
References


Figure S1. Effect of JQ1+ and JQ1- on MnSOD and catalase mRNA expression and effect of sulforaphane on Nrf2 transcriptional activity. (A-B) MnSOD (A) and catalase (B) mRNA expression was determined in ASMCs after treatment with vehicle, JQ1- or JQ1+ (300 nM) for 4-24 hrs. Data are expressed as fold-change with respect to vehicle control (dotted line). Results are representative of mean ± SEM of 4 ASMC donors (C) ASMCs were transfected with an ARE-driven luciferase reporter vector (2.5 µg) and then treated with vehicle or sulforaphane (4 µM) for 24 hrs. ARE-driven transcriptional activity was determined by measuring firefly luciferase activity and normalising to Renilla luciferase activity. Results are representative of mean ± SEM of 5 ASMC donors. (D) THP-1 cells were treated with vehicle, JQ1- or JQ1+ (300 nM) in the presence or absence of CSE (3% v/v) for 24 hrs. Nrf2, HO-1 and NQO1 protein expression was determined in whole cells extracts by western blotting. (E-G) Primary blood monocytes were treated with vehicle, JQ1- or JQ1+ (300 nM) and HO-1 (E), GCLC (F) and NQO1 (G) mRNA expression was determined 4 hrs post-treatment. Data are expressed as fold change with respect to vehicle control. Results are representative of mean ± SEM of 3 donors. * p<0.05.
Figure S2. Effect of JQ1+ and JQ1- on Nrf2 and Keap1 expression. Nrf2 and Keap1 mRNA and protein expression was determined in ASMCs (A-E) and THP-1 cells (F-J) after treatment with vehicle, JQ1- or JQ1+ (300 nM) for different times over a 48 hr period. Data are expressed as fold-change with respect to vehicle control (dotted line). Results are representative of mean ± SEM of 3 ASMC donors and 3 independent experiments for THP-1 cells. * p<0.05, ** p<0.01 and *** p<0.001 vs vehicle.