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Nrf3-deficient mice are not protected against acute lung and adipose tissue damages induced by butylated hydroxytoluene

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ABSTRACT

We found that both wild type and Nrf3 (NF-E2-related factor 3) deficient mice are sensitive to BHT single administration exhibiting respiratory distress and considerably lose body weight following treatment. At time of sacrifice, the BHT-treated Nrf3$^{-/-}$ mice had lost significantly more body weight than their WT counterparts. In the lung, transcript levels of the transcription factors Nrf1, Nrf2 and Nrf3 were differentially regulated by BHT treatment. In addition, genes implicated in adipogenesis were repressed following BHT exposure in the white adipose tissue. Together, our data provide the first evidence that BHT exposure not only affects lung function but also leads to impaired adipogenesis in adipocytes.

1. Introduction

Acute lung injury is associated with a high mortality rate and characterized by diffuse alveolar damage, inflammation, fibrosis and hypoxemia [1]. Butylated hydroxytoluene (BHT), a phenolic antioxidant used as food additive and preservative, has been shown to mimic acute lung injury after a single administration in a mouse model [2]. The family of the forkhead box transcription factors has been shown to play an important role under acute lung injury conditions [3,4]. Targeted gene disruption of the transcription factor Nrf2 showed that this protein is required to protect mice against BHT-induced hyperoxic lung injury [5]. Nrf2 is a member of the "cap 'n' collar" (CNC) basic leucine zipper family which also includes Nrf1 and Nrf3. CNC transcription factors heterodimerize with small Maf (musculoaponeurotic fibrosarcoma) proteins and the resulting complexes recognize MARE (Maf recognition element) or ARE (antioxidant response element) type DNA binding sites [6]. We and others have previously identified Nrf3 as a dimerization partner of the small Mafs [7,8] and as a novel endoplasmic reticulum-associated protein that is Asn-glycosylated [9,10]. We have also generated mice lacking the *Nrf3* gene and showed that these mice do not show any obvious abnormalities under non-challenging conditions [11]. Nrf2, a close homolog of Nrf3, has been shown to protect mice against acute lung injury induced by BHT [5]. Thus, we investigated the in vivo role of Nrf3 in this pulmonary injury model by using mice deficient for this transcription factor.

Abbreviations: ARE, antioxidant response element; BHT, butylated hydroxytoluene; Cebpb, CCAAT enhancer binding protein beta; CNC, cap 'n' collar; Fas, fatty acid synthase; Foxf1, forkhead box f1; Gclc, catalytic subunit of glutamate cysteine ligase; Gclm, modifier subunit of glutamate cysteine ligase; Hmox1, heme oxygenase 1; Maf, musculoaponeurotic fibrosarcoma; MARE, Maf recognition element; Nrf, NF-E2-related factor; Pparg, peroxisome proliferator-activated receptor gamma; Ptgs, prostaglandin-endoperoxide synthase; Srebp1, sterol regulatory element binding protein 1; WAT, white adipose tissue; WT, wild type

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2. Materials and methods

2.1. Animals and treatments

Generation of the Nrf3$^{-/-}$ mice has been reported elsewhere [11]. Female WT and Nrf3 deficient mice (129S6/SvEvTac background, 10 weeks old) were treated once by gavage (100 μl) with

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BHT (Sigma–Aldrich) at a dose of 400 mg per kg body weight dispersed into corn oil as vehicle. Mice were weighed daily, monitored for any signs of distress (rapid breathing rate, ruffled fur, hunched postures, impaired ambulation) and sacrificed 4 days after BHT administration. Upon necropsy, tissues were excised, weighed immediately and properly stored for further analysis. Procedures involving animals and their care were conducted according to McGill University guidelines, which are set by the Canadian Council on Animal Care. Mice were kept at 22 °C with equal periods of darkness. Water and food were available ad libitum.

2.2. Histology

Hematoxylin–eosin (H&E) staining of the lung slides was performed according to standard procedures. The slides were examined by a board certified veterinary pathologist (MP).

2.3. RNA isolation, reverse-transcription, and real-time quantitative PCR

All procedures concerning isolation of total RNA, reverse-transcription and real-time quantitative PCR were carried out according to the manufacturer's instructions. Primer sets are listed in Table 1 (Supplementary data).

2.4. Preparation of lung homogenates for catalase activity assay

Catalase activity was determined as previously described [12] with minor modifications. Catalase activity was calculated from the decrease in absorbance at 240 nm using a molar extinction coefficient of 39.4 $M^{-1} \times cm^{-1}$ for H_2O_2 [12].

2.5. Statistical analysis

Data are expressed as the means ± standard error of the mean (S.E.M.). Statistical analysis was performed using Graph Pad Prism (Graph Pad software) and Student's *t* test. A *P* value of <0.05 was considered statistically different ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results and discussion

3.1. Severe physiopathological changes following BHT exposure

Female wild type (WT) and Nrf3-deficient mice (10 weeks old) were treated with a single dose of BHT (400 mg/kg). By the third day post-BHT treatment, we observed that all treated mice displayed signs of respiratory distress, morbidity and moribundity. These observations were accompanied by a dramatic loss of body weight for all BHT-treated mice starting from day 3 post-administration (Fig. 1). Interestingly, Nrf3-deficient mice treated with BHT lost significantly more body weight than their WT counterparts indicating that mice lacking the Nrf3 gene are more susceptible to BHT-induced body weight loss. In accordance with the body weight loss, we observed upon necropsy a reduction of periepididymal white adipose tissue content in both WT and Nrf3-deficient mice treated with BHT (Fig. 2A). Exposure to BHT also resulted in a 2.6-fold increase of lung-to-body weight ratio compared to control mice in both genotypes (Fig. 2B). Hematoxylin–eosin stained lung sections revealed that BHT led in WT and Nrf3$^{-/-}$ mice to severe alveolar damage with interstitial pneumonia and alveolar hemorrhages multifocally (Fig. 3). The lung lesions were characterized by damages to the alveolar epithelial cells, protein exudation in the alveolar space with type II pneumocyte hyperplasia, foamy alveolar macrophages, alveolar hemorrhages and perivascular infiltration of inflammatory cells. Similar morphological changes were observed when a single dose of 600 mg/kg of BHT was used (data not shown).

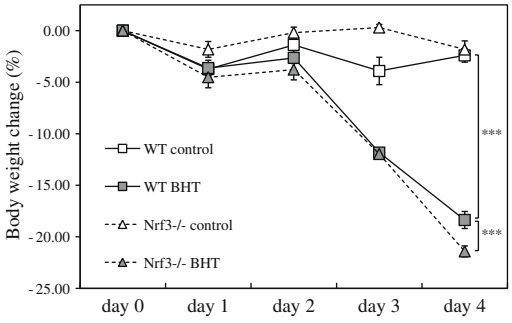

Fig. 1. Body weight change percentage in wt and Nrf3$^{-/-}$ mice following treatment with BHT. WT ($n = 12$) and Nrf3$^{-/-}$ mice ($n = 8$) were treated with a single dose of 400 mg/kg BHT.

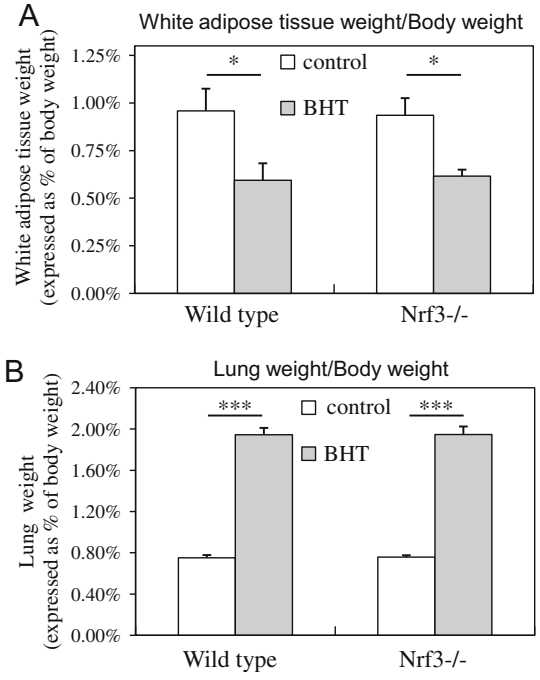

Fig. 2. (A) Periepididymal white adipose tissue weight in wt ($n = 12$) and Nrf3$^{-/-}$ ($n = 8$) mice following treatment with BHT. (B) Lung weight in wt and Nrf3$^{-/-}$ mice following treatment with BHT.

3.2. BHT modulates pulmonary expression of specific genes including CNC transcription factors

Catalase is a peroxisomal enzyme which metabolizes the reduction of hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals [13]. A significant reduction of 48% of catalase activity was observed in lung of WT mice treated with BHT. In Nrf3$^{-/-}$ mice, the reduction of the catalase activity was significantly less pronounced (35%) (Fig. 4). We further noticed that basal catalase activity was diminished by 11% in mice deficient in Nrf3 compared to WT mice hinting at a possible role of Nrf3 in the basal expression of catalase.

We next performed an extensive analysis of gene transcript levels by real-time PCR of lung tissue from mice exposed to BHT (Fig. 5 and Fig. S1). As previously shown [4], we observed a significant reduction of *Foxf1* mRNA levels in the lung of WT mice treated with

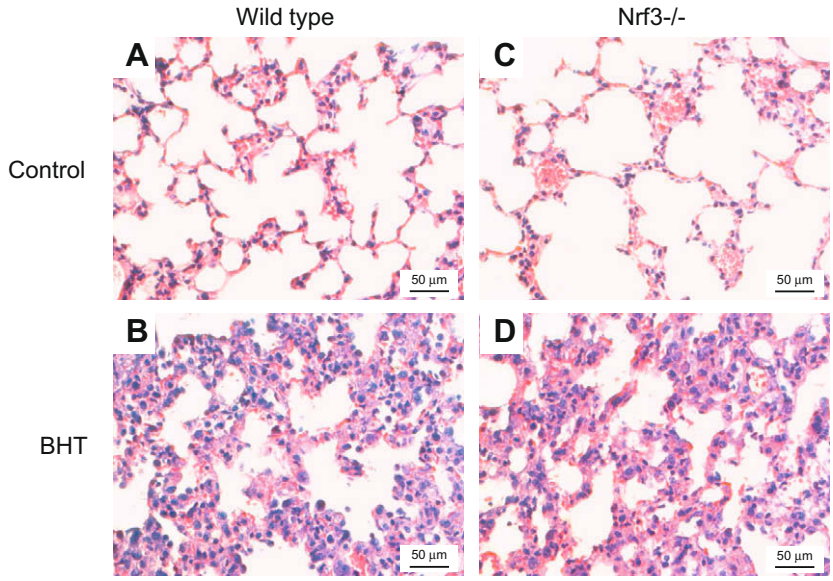

Fig. 3. Photomicrographs of lungs of wild type (A and B) and Nrf3-deficient mice (C and D) treated with 400 mg/kg BHT (B and D). Paraffin-embedded left lung tissue sections were processed for histological analyses and hematoxylin and eosin staining was performed. Presented photomicrographs are representative of all mice from a same group (bars = 50 µm).

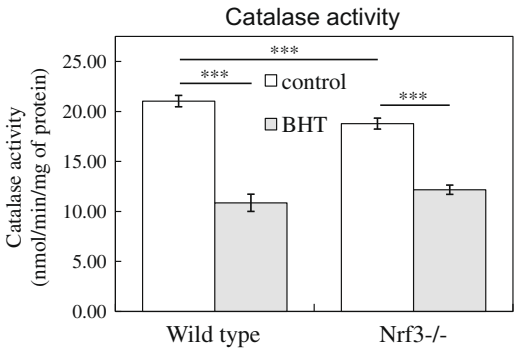

Fig. 4. Catalase activity was measured as described in the Section 2. Protein extracts were prepared from lung of wt ($n = 10$) and Nrf3$^{-/-}$ ($n = 9$) mice treated with a single dose of BHT (400 mg/kg).

BHT (Fig. 5A). Analysis of *Nrf2* mRNA levels indicated a 1.6-fold increase following BHT exposure in lung of wild type mice (Fig. 5B). Interestingly, *Nrf2* expression remained unchanged in the lung of BHT-treated Nrf3-deficient mice suggesting that Nrf3 may regulate BHT-induced expression of the *Nrf2* gene. In agreement with this hypothesis, a functional ARE-like sequence in the proximal region of the *Nrf2* promoter has been previously identified [14]. Nrf3 may thus regulate *Nrf2* gene expression through binding to this ARE-like sequence. Furthermore, we showed that typical target genes of Nrf2 including *Gclc* and *Gclm* are not regulated by BHT in the lung of mice (Fig. S1A and S1B) suggesting that the phase II detoxification pathway is not regulated upon BHT exposure.

Contrary to the induction of *Nrf2* transcripts levels, BHT treatment led to a 3.3-fold decrease in *Nrf3* transcripts in the lung of WT mice (Fig. 5C). These data provide the first evidence of regulation of *Nrf3* expression in vivo. Analysis of *Nrf1*, a third member of the CNC family, revealed a decrease of its transcript levels upon BHT exposure in both WT and Nrf3$^{-/-}$ mice (Fig. 5D). The fact that Nrf1 and Nrf3 are both downregulated by BHT suggests that they may control complementary pathways. This is supported by previous biochemical data showing that Nrf1 and Nrf3 are closely related factors that are both glycosylated proteins and they both have forms located in the endoplasmic reticulum [7,9,10,15]. However, the significance of the downregulation of *Nrf1* and *Nrf3* gene expression by BHT remains unknown, but we hypothesize that Nrf1 and Nrf3 exhibit distinct functions to Nrf2 in response to BHT.

Considering that mice treated with BHT display extensive alveolar damages and hemorrhages characterized by perivascular infiltration of inflammatory cells (Fig. 3), we next analyzed expression of genes involved in inflammatory response such as prostaglandin-endoperoxide synthase 1 and 2 (*Ptgs1* and *Ptgs2*). In response to BHT, *Ptgs1* and *Ptgs2* mRNA levels were induced in the lung of WT mice 2.0 and 1.8-fold, respectively (Fig. 5E and F). In addition, we showed that expression of *Ptgs1* gene was also induced 2.4-fold by BHT further supporting the recent notion that Ptgs1 is not invariably expressed as a housekeeping gene but might be regulated under certain pathological conditions [16]. In contrast, no induction of *Ptgs2* expression with BHT was observed in the lung of Nrf3$^{-/-}$ mice suggesting that BHT may regulate *Ptgs2* gene expression through binding of *Nrf3* to its promoter. In accordance with this hypothesis, an ARE-like motif has been identified in the proximal region of *Ptgs2* promoter [17]. Thus, although an inflammatory response occurs following BHT treatment in both WT and Nrf3$^{-/-}$ mice, distinct regulatory mechanisms might be involved in the two genotypes. Transcript levels of the stress protein heme oxygenase 1 (*Hmox1*) were increased 2.3-fold following BHT treatment in the lung of WT mice (Fig. 5G). This induction appears to be independent of Nrf3 gene expression. In conclusion, our current study as well as previous data [5] suggest that BHT toxicity in lung is caused by both an inflammatory response and an inability to correctly metabolize BHT and/or its metabolites as evidenced by the absence of regulation of the phase II detoxifying enzymes.

3.3. BHT treatment leads in adipose tissue to inhibition of the adipogenesis

Many studies focused on the effects of BHT in lungs of mice and rats [2,5,18]. In contrast, limited information is available on the impact of BHT in the adipose tissue of mice even though BHT is known to accumulate in adipose tissue both in humans and rodents [19]. Thus, we investigated the regulation of a series of genes in adipose tissue (Fig. 6 and Fig. S2). We first analysed the genes

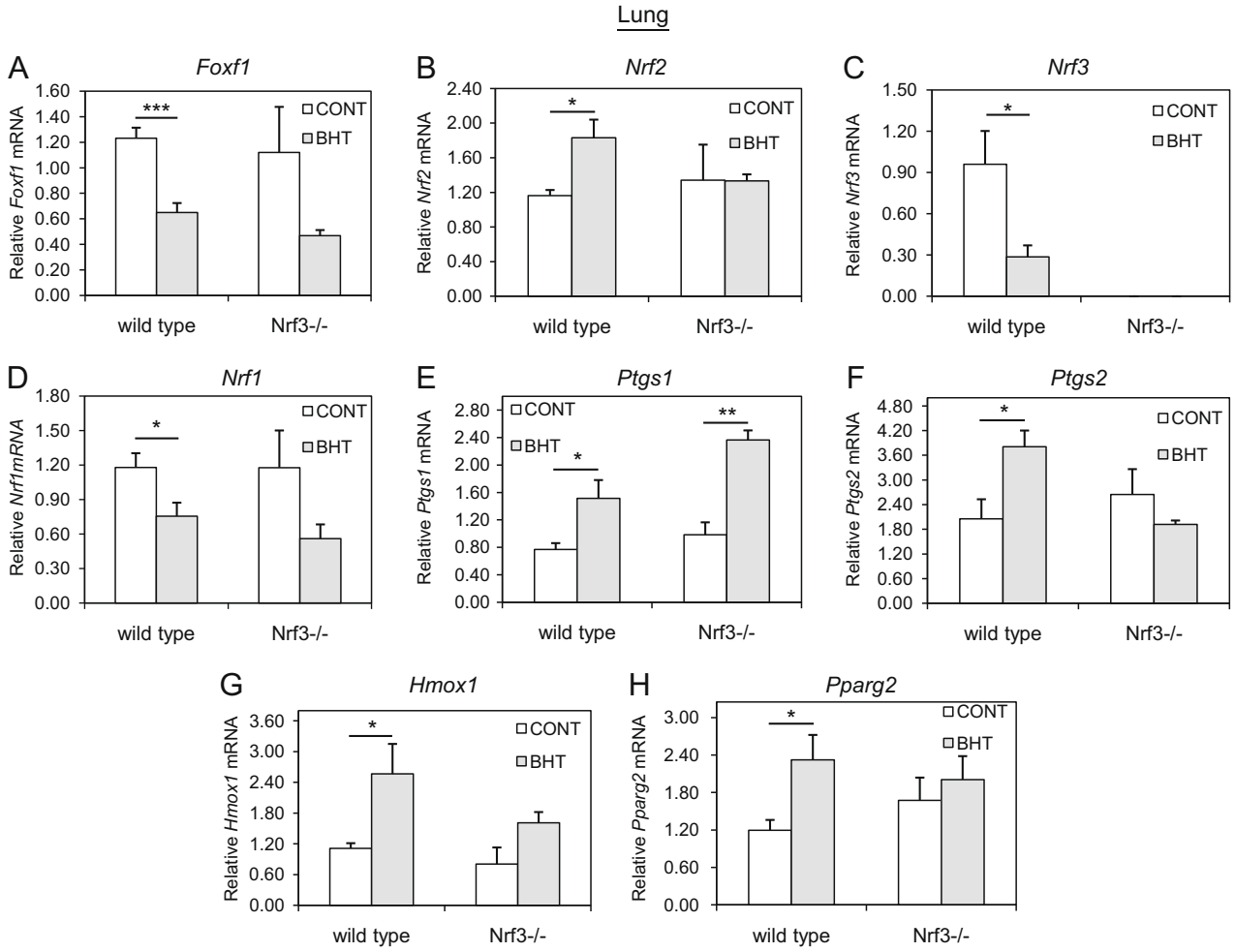

Fig. 5. Relative expression of mouse *Foxf1*, *Nrf2*, *Nrf3*, *Nrf1*, *Ptgs1*, *Ptgs2*, *Hmox1* and *Pparg2* in the lung of mice treated with BHT as determined by quantitative real-time PCR. Data are presented as means ± S.E.M. from at least three mice per group performed in duplicate.

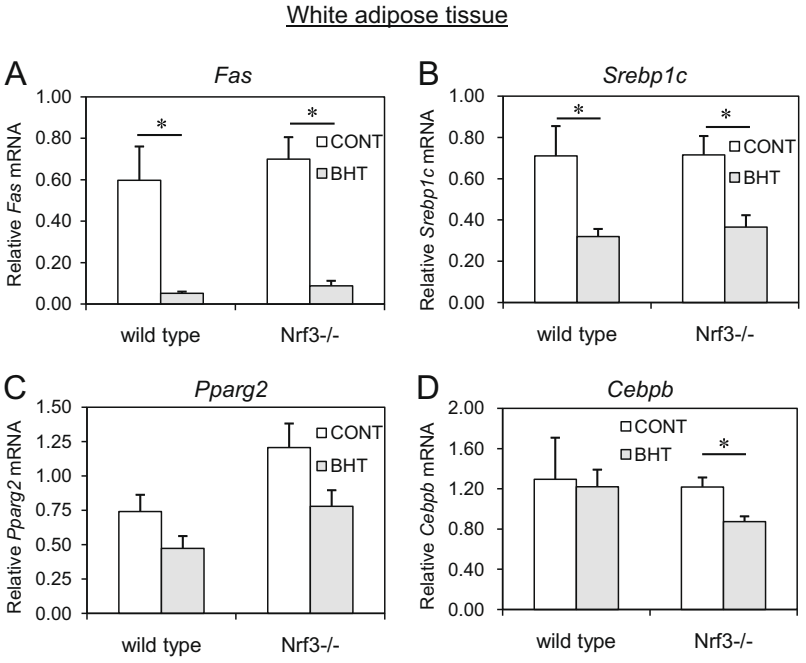

Fig. 6. Relative expression of mouse *Fas*, *Srebp1*, *Pparg2* and *Cebpb* in periepididymal white adipose tissue of mice treated with BHT as determined by quantitative real-time PCR. Data are presented as means ± S.E.M. from at least three mice per group performed in duplicate.

which we identified as regulated by BHT in the lung (Fig. 5). In contrast to lung, BHT does not or minimally affects *Nrf3*, *Nrf2*, *Nrf1*, *Foxf1*, *Ptgs1* nor *Ptgs2* mRNA levels in adipose tissue of both WT and Nrf3-deficient mice (Fig. S2).

We also examined the regulation of several critical factors involved in lipogenesis. Fatty acid synthase (*Fas*) gene encodes an enzyme involved in the de novo synthesis of fatty acids. Upon BHT treatment, we observed a drastic decrease of *Fas* gene expression both in adipose tissue of WT and Nrf3-deficient mice (11.5 and 8-fold decrease, respectively) (Fig. 6A). The *Fas* gene is known to be transcriptionally regulated by the sterol regulatory element binding protein 1c (*srebp1c*) [20]. Concomitantly, we found a significant reduction of *Srebp1c* gene expression by BHT in adipose tissue of WT (2.2-fold) and Nrf3$^{-/-}$ mice (2.0-fold) (Fig. 6B). Protein levels of Pparg (peroxisome proliferator-activated receptor gamma), a master regulator of adipocyte differentiation [21] have been shown to be downregulated in the adipose tissue of mice following endotoxin-induced acute lung injury [22]. Consistent with these findings, we observed a decrease of *Pparg2* transcript levels in WAT of wild type and Nrf3$^{-/-}$ mice exposed to BHT (Fig. 6C). In addition, we found that basal expression of *Pparg2* in adipose tissue was increased in mice deficient in Nrf3 suggesting a role for Nrf3 in the transcriptional regulation of *Pparg2*. Interestingly, sequence inspection of the promoter region of *Pparg2* gene revealed the presence of a putative binding site for Nrf3 (data not shown) supporting the notion that Nrf3 may physically bind to the *Pparg2* promoter. Pparg is also expressed in the lung of rodents [23] and is involved in acute lung injury, most likely due to its anti-inflammatory properties [24]. In our mouse model, *Pparg2* mRNA levels were increased 2.0-fold following BHT treatment in the lung of WT mice, whereas no effect is observed in Nrf3$^{-/-}$ mice (Fig. 5H). In conclusion, regulation of *Pparg* gene expression by BHT both in the lung and the adipose tissue of mice is an important finding since Pparg agonists may provide a possible tool to treat lung inflammatory diseases [25].

Analysis of the expression of the adipocyte differentiation-inducing transcription factor CCAAT enhancer binding protein beta (Cebpb) indicated that BHT does not affect its expression in WAT of WT mice but a significant 1.4-fold decrease is observed in WAT of Nrf3$^{-/-}$ mice (Fig. 6D). Together, our data demonstrate that BHT treatment leads to a decrease of the transcript levels of the key factors *Srebp1c*, *Pparg2* and *Fas* involved in adipogenesis further supporting the notion that lipogenesis is impaired in the adipose tissue of BHT-treated mice. However, one could not exclude that the effects observed in white adipose tissue is an indirect effect due to a loss of appetite in mice exposed to BHT (data not shown). In this case, the mice would need to be considered in a caloric restriction state and lipogenesis in adipose tissue would thus be reduced allowing the use of fat as a source of energy.

In summary, our studies provide new insights into the regulation of BHT-induced acute lung injury. We showed that in lung BHT induces a subset of specific genes mainly involved in inflammation. Moreover, we showed that BHT treatment differently modulates the expression levels of the CNC transcription factors Nrf1, Nrf2 and Nrf3 in the lung of mice. In adipocytes, BHT prevents lipogenesis by repressing critical regulators including *Srebp1*, *Pparg* and their target gene *Fas*. The molecular mechanisms linking Nrf3-mediated transcription, inflammatory response and lipid metabolism are currently being investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.01.028.

References

[1] Ben Salem, C., Hmouda, H. and Bouraoui, K. (2007) Acute lung injury and acute respiratory distress syndrome. Lancet 370, 383. author reply 384–385.
[2] Witschi, H., Malkinson, A.M. and Thompson, J.A. (1989) Metabolism and pulmonary toxicity of butylated hydroxytoluene (BHT). Pharmacol. Ther. 42, 89–113.
[3] Kalin, T., Meliton, L., Meliton, A., Zhu, X., Whitsett, J. and Kalinichenko, V. (2008) Pulmonary mastocytosis and enhanced lung inflammation in mice heterozygous null for the Foxf1 gene. Am. J. Respir. Cell Mol. Biol. 39, 390–399.
[4] Kalinichenko, V.V., Zhou, Y., Shin, B., Stolz, D.B., Watkins, S.C., Whitsett, J.A. and Costa, R.H. (2002) Wild-type levels of the mouse forkhead box f1 gene are essential for lung repair. Am. J. Physiol. Lung Cell. Mol. Physiol. 282, L1253–L1265.
[5] Chan, K. and Kan, Y.W. (1999) Nrf2 is essential for protection against acute pulmonary injury in mice. Proc. Natl. Acad. Sci. USA 96, 12731–12736.
[6] Blank, V., Hirsch, E., Challis, J.R., Romero, R. and Lye, S.J. (2008) Cytokine signaling, inflammation, innate immunity and preterm labour – a workshop report. Placenta 29 (Suppl A), S102–S104.
[7] Chenais, B., Derjuga, A., Massrieh, W., Red-Horse, K., Bellingard, V., Fisher, S.J. and Blank, V. (2005) Functional and placental expression analysis of the human NRF3 transcription factor. Mol. Endocrinol. 19, 125–137.
[8] Massrieh, W., Derjuga, A., Doualla-Bell, F., Ku, C.Y., Sanborn, B.M. and Blank, V. (2006) Regulation of the MAFF transcription factor by proinflammatory cytokines in myometrial cells. Biol. Reprod. 74, 699–705.
[9] Nouhi, Z., Chevillard, G., Derjuga, A. and Blank, V. (2007) Endoplasmic reticulum association and N-linked glycosylation of the human Nrf3 transcription factor. FEBS Lett. 581, 5401–5406.
[10] Zhang, Y., Kobayashi, A., Yamamoto, M. and Hayes, J. (2009) The Nrf3 transcription factor is a membrane-bound glycoprotein targeted to the endoplasmic reticulum through its N-terminal homology box 1 sequence. J. Biol. Chem. 284, 3195–3210.
[11] Derjuga, A., Gourley, T.S., Holm, T.M., Heng, H.H., Shivdasani, R.A., Ahmed, R., Andrews, N.C. and Blank, V. (2004) Complexity of CNC transcription factors as revealed by gene targeting of the Nrf3 locus. Mol. Cell Biol. 24, 3286–3294.
[12] Aebi, H. (1984) Catalase in vitro. Methods Enzymol. 105, 121–126.
[13] Schrader, M. and Fahimi, H.D. (2006) Peroxisomes and oxidative stress. Biochim. Biophys. Acta 1763, 1755–1766.
[14] Kwak, M., Itoh, K., Yamamoto, M. and Kensler, T. (2002) Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. Mol. Cell Biol. 22, 2883–2892.
[15] Zhang, Y., Lucocq, J.M., Yamamoto, M. and Hayes, J.D. (2007) The N-terminal homology box 1 (NHB1) sequence in transcription factor Nrf1 is required to anchor it to the endoplasmic reticulum and also to enable its Asn-glycosylation. Biochem. J..
[16] Erovic, B.M., Woegerbauer, M., Pammer, J., Selzer, E., Grasl, M. and Thurnher, D. (2008) Strong evidence for up-regulation of cyclooxygenase-1 in head and neck cancer. Eur. J. Clin. Invest. 38, 61–66.
[17] Sherratt, P.J., McLellan, L.I. and Hayes, J.D. (2003) Positive and negative regulation of prostaglandin E2 biosynthesis in human colorectal carcinoma cells by cancer chemopreventive agents. Biochem. Pharmacol. 66, 51–61.
[18] Lin, H.M., Yen, F.L., Ng, L.T. and Lin, C.C. (2007) Protective effects of *Ligustrum lucidum* fruit extract on acute butylated hydroxytoluene-induced oxidative stress in rats. J. Ethnopharmacol. 111, 129–136.
[19] Conacher, H., Iverson, F., Lau, P. and Page, B. (1986) Levels of BHA and BHT in human and animal adipose tissue: interspecies extrapolation. Food Chem. Toxicol. 24, 1159–1162.
[20] Latasa, M.J., Griffin, M.J., Moon, Y.S., Kang, C. and Sul, H.S. (2003) Occupancy and function of the −150 sterol regulatory element and −65 E-box in nutritional regulation of the fatty acid synthase gene in living animals. Mol. Cell Biol. 23, 5896–5907.
[21] Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I. and Spiegelman, B.M. (1994) MPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. 8, 1224–1234.

[22] Hill, M., Young, M., McCurdy, C. and Gimble, J. (1997) Decreased expression of murine PPARgamma in adipose tissue during endotoxemia. Endocrinology 138, 3073–3076.
[23] Liu, D., Zeng, B. and Shang, Y. (2006) Decreased expression of peroxisome proliferator-activated receptor gamma in endotoxin-induced acute lung injury. Physiol. Res. 55, 291–299.
[24] Paola, R. and Cuzzocrea, S. (2007) Peroxisome proliferator-activated receptors and acute lung injury. PPAR Res. 2007, 63745.
[25] Becker, J., Delayre-Orthez, C., Frossard, N. and Pons, F. (2006) Regulation of inflammation by PPARs: a future approach to treat lung inflammatory diseases? Fundam. Clin. Pharmacol. 20, 429–447.