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Original Contribution

Characterization of Nrf2 activation and *heme oxygenase-1* expression in NIH3T3 cells exposed to aqueous extracts of cigarette smoke

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Abstract

Cigarette smoke (CS) is a complex chemical mixture estimated to be composed of up to 5000 different chemicals, many of which are prooxidant. Here we show that, at least in vitro, the cellular response designed to combat oxidative stress resulting from CS exposure is primarily controlled by the transcription factor Nrf2, a principal inducer of antioxidant and phase II-related genes. The prominent role of Nrf2 in the cellular response to CS is substantiated by the following observations: In NIH3T3 cells exposed to aqueous extracts of CS (i) Nrf2 is strongly stabilized and becomes detectable in nuclear extracts. (ii) Nuclear localization of Nrf2 coincides with increased DNA binding of a putative Nrf2/MafK heterodimer to its cognate *cis*-regulatory site, i.e., the antioxidant-responsive element (ARE). (iii) Studies on the regulatory elements of the oxidative stress-inducible gene *heme oxygenase-1* (*hmox1*) using various *hmox1* promoter/luciferase reporter constructs revealed that the strong CS-dependent expression of this gene is primarily governed by the distal enhancers 1 ("E1") and 2 ("E2"), which both contain three canonical ARE-like stress-responsive elements (StREs). Notably, depletion of Nrf2 levels caused by RNA interference significantly compromised CS-induced *hmox1* promoter activation, based on the distinct Nrf2 sensitivity exhibited by E1 and E2. Finally, (iv) siRNA-dependent knock-down of Nrf2 completely abrogated CS-induced expression of phase II-related genes. Taken together, these results confirm the outstanding role of Nrf2 both in sensing (oxidant) stress and in orchestrating an efficient transcriptional response aimed at resolving the stressing conditions.

Keywords: Cigarette smoke; Nrf2; Heme oxygenase-1; Phase II; Oxidative stress; Free radical

Introduction

In situations of stress, i.e., chemical, physical, or oxidant, cells are primed to activate a defense program designed to efficiently destroy and counteract damage from harmful reactive species in order to avoid uncontrolled (necrotic) cell death, which may ultimately result in the pathogenesis of many chronic inflammatory diseases. In mechanistic terms, this protective program is initiated by an as yet only incompletely understood mechanism of stress sensing, signal generation, and transduction. This process eventually culminates in the alteration of the gene expression pattern leading to the stereotypic up-regulation of antioxidant genes and/or genes encoding detoxifying proteins belonging to the group of phase I and II drug-metabolizing enzymes (for review, see [1]). While

Abbreviations: AP-1, activated protein 1; ARE, antioxidant-responsive element; C/EBPβ, CAAT/enhancer-binding protein β; CNC/bZIP, "cap 'n' collar"/basic region-leucine zipper; CRE, cAMP-response element; CS, cigarette smoke; DMEM, Dulbecco's modified Eagle medium; EMSA, electrophoretic mobility-shift assay; FCS, fetal calf serum; GCLC, glutamate-cysteine ligase, catalytic subunit; hmox1, heme oxygenase-1 (gene); HO-1, heme oxygenase-1 (protein); HSF-1, heat-shock factor-1; MARE, Maf recognition element; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor κB; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, NF-E2-related factor 2; RT qPCR, reverse transcriptase real-time quantitative PCR; RNAi, RNA interference; sb PBS, smoke-bubbled phosphate-buffered saline; StRE, stress-responsive element; TRE, TPA-response element; wt, wild-type; γGCS, γ-glutamyl-cysteine synthetase.

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the phase I response is orchestrated by specific cytoplasmic receptors, as exemplified by the aryl hydrocarbon receptor (Ah-receptor) (for review, see [2]), recent investigations from different laboratories have clearly demonstrated that the "cap 'n' collar"/basic region-leucine zipper (CNC/bZIP) transcription factor NF-E2-related factor 2 (Nrf2), complexed with an appropriate dimerization partner, is central to the activation of the antioxidant and phase II response (for review, see [3–5]). Other potential addressees of stress signaling are the numerous bZIP proteins belonging to the Jun and Fos families, which may dimerize to build the transcription factor-activated protein-1 (AP-1) as well as proteins comprising the group of ATF/CREB transcription factors [6]. Notably, the functional relationship between AP-1, ATF/CREB, and Nrf2 becomes obvious by *cis*-regulatory elements, i.e., TPA-response element (TRE), cAMP-response element (CRE), and antioxidant-responsive element (ARE), which share high sequence homology. Finally, stressing conditions are also perceived by signaling pathways resulting in the activation of prosurvival/proinflammatory transcription factors nuclear factor-κB (NF-κB) and CAAT/enhancer-binding protein β (C/EBPβ).

Cigarette smoke (CS) is a known risk factor for cancer development and severe inflammation-related diseases, such as chronic obstructive pulmonary disease and cardiovascular diseases [7–9]. Although different facets of CS-induced stress have been studied in detail, we are just beginning to perceive the molecular and cellular mechanisms that underlie the appearance of clinically detectable symptoms consequent to CS exposure. Thus, several studies of our own and other laboratories have shown that aqueous extracts of CS (smoke-bubbled phosphate-buffered saline (smoke-bubbled PBS)) induce a distinct pattern of (oxidative) stress-related effects in exposed cells (e.g., [10–15]). By extending this experimental approach to DNA microarray techniques, we have demonstrated that CS—applied as aqueous extracts in vitro and mainstream smoke in vivo—has a strong impact on the gene expression profile of exposed cells in culture [16] or in tissues of the respiratory tract of CS-exposed rats [17]. Both gene expression profiling studies revealed the up-regulation of antioxidant and phase II-related genes, with CS-exposed tissues of the rat respiratory tract showing, in addition, a specific signature in the differential expression of phase I-related genes. Remarkably, significantly higher expression rates were observed for the antioxidant and phase II-related genes during in vivo inhalation in the nose when compared to the lung, indicating a deposition gradient of CS-dependent stressors from the upper to the lower respiratory tract, while, when comparing acute versus subchronic inhalation, considerably lower expression rates were observed for these genes after repeated CS exposure, thus pointing to an adaptive response [17].

In all of the studies noted above, the antioxidant response was hallmarked by the paramount expression of the gene encoding heme oxygenase-1 (HO-1), which catalyzes the initial and rate-limiting step in heme catabolism, leading to the formation of biliverdin along with the release of CO and "free" iron cations. In the presence of biliverdin reductase, biliverdin is converted to bilirubin. Since both biliverdin and bilirubin are efficient antioxidants [18], the "HO-1 pathway" represents a prime defense tool in protecting the cell from stress-dependent adverse effects induced by a broad spectrum of sources (e.g., [19–21]). Mechanistically, in the murine system, the expression of *heme oxygenase-1* (*hmox1*) is controlled by a complex system consisting of a promoter with a proximal enhancer ("E_p") and two distal enhancers ("E1" and "E2"), which are distributed over more than 10 kb upstream from the transcriptional start site (reviewed in [4,22]). This ternary system harbors a whole array of *cis*-regulatory motifs addressing numerous transcription factors known to be involved in oxidative stress and inflammatory responses, including NF-κB, AP-1, ATF/CREB, Nrf2, and heat-shock factor-1 (HSF-1).

Based on the obvious relevance of *hmox1* expression in CS exposure (summarized in [23]) and on the sensitivity of the *hmox1* promoter to different routes of stress signaling, we sought to evaluate how this system is controlled in cells exposed to aqueous extracts of CS (smoke-bubbled PBS). The results show that upon exposure of NIH3T3 cells to subcytotoxic doses of smoke-bubbled PBS, a major contribution to *hmox1* activation is accomplished by Nrf2, as indicated by stabilization, increased nuclear localization, and DNA binding of Nrf2. This process is paralleled by the induction of the *hmox1* promoter/enhancer system, mainly, but not exclusively, through the ARE-like stress-responsive elements (StRE) present in the E1 and E2 enhancer regions.

Material and methods

Chemicals and reagents

All chemicals, radiochemicals, and enzymes were obtained from Sigma (Taufkirchen, Germany), Amersham Biosciences (Freiburg, Germany), and Roche (Mannheim, Germany), respectively, at the highest purity available. Aqueous extracts of CS (smoke-bubbled PBS) were generated by bubbling 30 puffs of mainstream CS (1 puff = 33 cm^3 of mainstream CS, 1 cigarette = 10 puffs) generated from the University of Kentucky standard reference cigarette 2R1 (using a standard smoking procedure [24]) through 18 ml PBS. The resulting smoke-bubbled PBS solution contained the smoke of three cigarettes (~1.7 puffs/ml). To obtain the required concentrations for cell exposure, freshly prepared smoke-bubbled PBS was diluted in Dulbecco's modified Eagle medium (DMEM) containing 0.5% fetal calf serum (FCS).

Cell culture and treatment

NIH3T3 (ATCC CRL 1658) mouse fibroblast cells were cultured in high glucose DMEM supplemented with L-glutamine, 1% streptomycin/penicillin, and 10% FCS (Invitrogen, Karlsruhe, Germany).