ΔNp63α/IRF6 interplay activates NOS2 transcription and induces autophagy upon tobacco exposure

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Tobacco-induced oxidative stress leads to chronic inflammation and is implicated in the development of many human epithelial cancers, including head and neck cancer. Cigarette smoke exposure was shown to induce the expression of the ΔNp63α and nitric oxide synthase (NOS)-2 in head and neck squamous cell carcinoma cells and immortalized oral keratinocytes. The NOS2 promoter was found to contain various cognate sequences for several transcription factors including interferon regulatory factor (IRF)-6 and p63, which were shown in vivo binding to the NOS2 promoter in response to smoke exposure. Small interfering (si)-RNAs against both ΔNp63α and IRF6 decreased the induction of NOS2 promoter-driven reporter luciferase activity and were shown to inhibit NOS2 activity. Furthermore, both mainstream (MSE) and sidestream (SSE) smoking extracts induced changes in expression of autophagic marker, LC3B, while siRNA against ΔNp63α, IRF6 and NOS2 modulated these autophagic changes. Overall, these data support the notion that ΔNp63α/IRF6 interplay regulates NOS2 transcription, thereby underlying the autophagic-related cancer cell response to tobacco exposure.

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Introduction

Primary and second-hand smoking is one of the causative risk factors for various diseases, including chronic obstructive pulmonary disease, asthma, cardiovascular disease and cancer [1,2]. Studies show that cigarette smoking increases nitric oxide (NO) synthesis by activating inducible NO synthase (NOS2) in many cell types, including endothelial cells, alveolar macrophages, and epithelial cells of lung [3]. NO is increased in the lung periphery of patients with chronic obstructive pulmonary disease (COPD) [4,5]. More than 30% of asthmatic patients, whose conditions were induced by primary smoking or SHS, showed elevated levels of NOS2 expression and therefore higher levels of NO in the epithelium and smooth muscle cells [6–8]. Also, levels of NOS2 or nitrosyrosine were shown to be much higher in the alveolar macrophages and alveolar epithelium of cigarette smokers than in non-smokers [9].

Carcinogen exposure induces a chronic inflammation, which is implicated in the development of many human epithelial cancers, including head and neck cancer [10–13]. Studies of genetic mechanisms underlying head and neck cancer demonstrated that tobacco exposure is causing inactivation of tumor suppressor genes via genetic/epigenetic changes therefore, affecting many cellular processes, including cell survival (proliferation) or cell death (apoptosis and autophagy) [9,12,13]. Smoking has been linked to human epithelial cancers overexpressing proteins implicated in inflammatory signaling pathways including NF-kB, NOS2, COX-2, etc., [9,10,12–14]. Because of the strong link of NF-kB with different stress signals including smoking exposure, it has been called a “smoke-sensor” of the body [14]. Benzo[a]pyrene (B[a]P), a polycyclic hydrocarbon carcinogen, found in tobacco smoke was shown to induce cancer in multiple organ sites in animals and may be a causative agent for certain human cancers, such as esophageal cancer [15]. B[a]P was shown to induce NOS2 through the ERK/NF-kB dependent pathway [15].

This is the first evidence that cigarette smoke treatment (mainstream smoke extract (MSE) and sidestream smoke extract (SSE)
of head and neck squamous cell carcinoma (HNSCC) cells and minimally transformed oral keratinocytes (OKF6/TERT2) activated a novel interplay between \( \Delta Np63 \alpha \) and IRF6 leading to the NOS2 transcriptional activation in both HNSCC and OKF6/TERT2 cells, therefore promoting the tobacco-induced autophagy.

Materials and methods

Preparation of cigarette smoke extracts

Mainstream smoke extract (MSE) and sidestream smoke extract (SSE) were prepared from tobacco that contained nicotine (0.85 mg/cigarette and tar: 9.70 mg/cigarette), as previously described [16]. The concentration of SSE was monitored by the absorbance of 1 (100%) at 230 nm, while MSE preparation was measured by the absorbance of 1 (100%) at 220 nm as described elsewhere [16,17], which then was diluted with culture media supplemented with 10% FBS immediately before use. Control medium was prepared by bubbling air through 10 ml serum-free media, adjusting pH to 7.4, and sterile-filtered [17]. MSE and SSE were used to imitate cigarette primary smoking and second-hand smoking, respectively [16].

Cell cultures and transfections

Human head and neck squamous cell carcinoma (HNSCC) cell line (wild type p53 expression, predominant \( \Delta Np63 \alpha \) expression, no p53 or p63 mutations, p63 is amplified, Refs. [18–22]) was generated from the primary tumor tissue sample at the Head and Neck Cancer Research Division [Department of Otolaryngology/Head and Surgery, Johns Hopkins University School Medicine (JHMI)]. OKF6/TERT6 cells (wild type p53 and p63), which represent normal oral mucosal epithelium cells immortalized by forced expression of telomerase via retroviral transduction were also used and maintained as previously described [23–25]. Cell lines were characterized, tested and provided by Dr. Joseph A. Califano, JHMI. OKF6/TERT6 cell line was originally generated by Dr. J.G. Rhinewald (Division of Dermatology, Department of Medicine and Harvard Skin Disease Research Center, Brigham and Women’s Hospital, Boston, MA, USA) and provided to the Head and Neck Cancer Division at the JHMI through a Material Transfer Agreement [25]. Cell lines were authenticated by a short tandem repeat profiling analysis at the JHMI through a Material Transfer Agreement [25]. Cell lines were authenticated by a short tandem repeat profiling analysis at the JHMI through a Material Transfer Agreement [25]. Cell lines were authenticated by a short tandem repeat profiling analysis at the JHMI through a Material Transfer Agreement [25].

Reverse transcription-PCR (RT-PCR)

The First Strand cDNA Synthesis kit (Invitrogen) was used for reverse transcription (RT). RT-PCR was performed with reconstituted Taq polymerase (Invitrogen) as follows: 24–30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 30 s as described elsewhere [17]. As a control, we amplified GAPDH mRNA with the following primers, sense primer, (121) 5'-CTACATGGTT TACATGT-3' and antisense primer, 5'-TGCCCTAACACGACACT-3' (920) giving rise to the 800 bp PCR product. For the NOS2 amplification, we used the following primers: sense primer, (1020) 5'-gga ctggagcagttgta-3' (1040) and antisense primer, (1500) 5'-ttgcc agggcagagggag-3' (1520) yielding the 480 bp PCR product. Quantitative PCR (qPCR) was performed using the same primers as previously described [38].

Chromatin immunoprecipitation (ChIP) assay

Cells (2 × 10⁶) were exposed to control medium, MSE and SSE followed by ChIP using a ChIP assay kit (Upstate Cell Signaling Solutions as previously described [26]). Chromatin was immunoprecipitated with 1 μg of primary antibodies against PEA3 (clone 1A2G3, sc-130661, Santa Cruz Biotechnology), NF-κb p65 subunit (ab7970, Abcam), \( \Delta Np63 \) (anti-p40, PC373, residues 5–17 epitope, EMD/Calbiochem), C/EBP (sc#3087, Cell Signaling Technology) and NF-YA (ab6558, Abcam). Immunoprecipitates were used as templates for PCR amplification of the NOS-2 promoter with the following primers: sense primer, (–600) 5'-AGTAAGAAGGCAATGTCAGA-3' (–581) and antisense primer, (–70) 5'-CTTATGAAAGAAAACAACCTC-3' (–51) yielding the PCR product of 449 bp, or antisense primer, (–320) 5'-CCATGTTGCTGCCCATAA-3' (–301) yielding the PCR product of 299 bp. Resulting PCR fragments were amplified as follows: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 30 s using a Platinum Taq DNA polymerase (Invitrogen) for 37 cycles [18]. As negative controls, we used the amplification of a non-specific region of the NOS2 promoter (that does not contain the cognate sequences for the tested transcription factors) with the following primers: sense primer, (1020) 5'-gaactgacagctaaattgta-3' (1040) and antisense primer, (1500) 5'-ttgagcagacagggag-3' (1520). ChIP binding was monitored by a quantitative PCR (qPCR) assay as described elsewhere [26].

Immunoblotting and immunoprecipitation

We used primary antibodies against \( \Delta Np63 \) (PC373, Calbiochem/EMD), IRF6 (clone H-140, sc-98829), NOS2 (sc-7271), and MAP LC3 \( \alpha / \beta \) (F-14, sc-16756) all from Santa Cruz Biotechnology; against C/EBP (sc#3087, Cell Signaling Technology); NF-κb p65 subunit (ab7970) and NF-YA (ab6558) both purchased from Abcam; against \( \beta \)-actin (Sigma) for immunoblotting and immunoprecipitation as previously described [26].

Mammalian 2-hybrid protein interaction assay

Efficiency of protein interactions was monitored with the Mammalian Matchmaker 2-hybrid Assay Kit (Clontech, #630305). The interactions were monitored by a non-radioactive SEAP assay (secreted alkaline phosphatase). PCR fragments for the \( \Delta Np63 \alpha \) domains were amplified with the following primers: for 1–138, sense primer, (1) 5'-ctgagacagctaaattgta-3' (1040) and antisense primer, (1500) 5'-ttgagcagacagggag-3' (1520). PCR fragments of the IRF6 domains were amplified with the following primers: for 1–86, sense primer, (1) 5'-ctgagacagctaaattgta-3' (1040) and antisense primer, (1500) 5'-ttgagcagacagggag-3' (1520).

E.A. Ratovitski / Archives of Biochemistry and Biophysics 506 (2011) 208–215
sense, (1003) 5′-ggaagaattgcccagatgga-3′ (1022) and antisense, (1292) 5′-ctctctttcttttttta-3′ (1311) using the pcMV-Sport6-IRF6 (ID#3449699, Invitrogen) plasmid as a template. Resulting PCR fragments were subcloned into the Xho I and Sal I sites of the pM plasmid. Pairs of bait and prey plasmids were introduced into HEK-293 cells along with the pGSEAP plasmid using the CLONectin transfection reagent. Transfection efficiency was normalized by a second reporter construct, pSEAP2-Control (Clontech, #631717). As a positive control, we used a PM-53/pVP16-T combination, and as a negative control, we used a PM-53/pVP16-CP combination according to the manufacturer’s recommendations. Each construct of interest was also tested for self-activation using additional combinations of the pVP16-ΔNp63α constructs with the pM-Gal4-BD vector or the pM-IRF6 constructs with the pVP16-AD vector. 48 h after transfection of cells, the SEAP activities were monitored with the Great EscApE™ SEAP Chemiluminescence Kit 2.0 (Clontech, #631736) using the chemiluminescent substrate CSPD (3-(4-methoxyxpiro [1,2-dioxetane-3, 2-tricyclo[3.3.1.1(3,7)] decan]-4-yl) phenyl phosphate, dioxetane relative) in triplicate using a Monolight TM2010 luminometer 60 min after substrate addition.

**Luciferase reporter assay**

The pGL3-NOS2 (1000 bp) promoter-luciferase reporter plasmid was purchased from SwitchGear Genomics. Cells (5 × 10⁴) were plated per well in a 24-well plate and 100 ng of the pGL3 luciferase reporter constructs plus 1 ng of the Renilla luciferase plasmid pRL-SV40 (Promega) were introduced into the cells using FuGENE 6 (Roche) as previously described [26]. Forty eight hours after transfection, luciferase assays were performed using the Dual luciferase reporter assay kit (Promega). For each experiment, wells were transfected in triplicate and each well was assayed in triplicate. Ten microlitre of cleared cell lysate was added to 50 μl of Firefly Luciferase substrate, and light units were measured in a luminometer. Renilla luciferase activity was measured in the same tube. The values of Firefly luciferase activity were normalized (divided) for the Renilla luciferase activity for each transfected well.

**NOS activity assay**

Cells were sonicated in 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 100 mM tetrahydrobiopterin, 2 mM dithiothreitol, 10% (v/v) glycerol, aprotinin (25 μg/ml), leupeptin (25 μg/ml), 100 μM PMSF, 10 μM FMN and 10 μM FAD. A reaction was performed in a mix of cell lysate containing 50 μg protein, 50 μl of [14C] arginine (50,000 cpm), 5 μl of 5 mM FAD, 5 μl of 100 μM tetrahydrobiopterin, 1 μl of 30 μM calmodulin, as previously described [27].

**Autophagy assay**

Cells were transiently transfected with scrambled siRNA, siRNA against p63, IRF6 or NOS2. Cells were exposed to control medium, 1% MSE for 24 h in the presence of lyzosomal protease inhibitors (10 mg/ml of both E64d and pepstatin A purchased from Sigma) as previously described [28–30]. Protein levels of LC3B-I and LC3B-II were tested with an antibody against MAP LC3B as previously described [28–30]. Protein levels of LC3B-I and (10 mg/ml of both E64d and pepstatin A purchased from Sigma) 1% MSE for 24 h in the presence of lyzosomal protease inhibitors. The LC3B-II/LC3B-I ratios were plotted as bars using the Microsoft Excel software with standard deviations (±SD) resulting from three independent experiments and three individual measurements of each experiment.

**Statistical analysis**

The data represent mean ± SD from three independent experiments and the statistical analysis was performed by Student’s t test at a significance level of p < 0.05 to < 0.001.

**Results**

ΔNp63α and NOS2 are up regulated in both HNSCC and OKF6/TERT2 cells upon smoking exposure

As previously reported by many investigators including us, HNSCC cells predominantly express ΔNp63α and no significant expression of TAp63 isoforms was found by RT-PCR or immunoblotting [19,20]. Therefore, current experimental efforts were entirely focused on the effect of cigarette smoking on the expression of ΔNp63α. HNSCC cells [18–20] were exposed to control medium, 0.5% MSE and 1% SSE for 24 h leading to increase in ΔNp63α protein levels upon MSE or SSE exposure (Fig. 1A, left columns). Furthermore, both MSE and SSE induced expression of NOS2 shown by immunoblotting and RT-PCR (Fig. 1B and C, left columns) suggesting that the NOS2 transcription could be modulated in HNSCC cells upon cigarette smoke exposure. Similar observations were made using the immortalized oral keratinocytes (OKF6/TERT2 cell line, Fig. 1 right columns). Moreover, qPCR analysis demonstrated a dramatic increase in the NOS2 mRNA levels after HNSCC cells and OKF6/TERT2 cells were exposed to SSE and MSE (Fig. 1D).

**Transcriptional regulation of NOS2 in HNSCC and OKF6/TERT2 cells upon cigarette smoke exposure**

To further examine the potential mechanism of NOS2 up regulation in HNSCC cells exposed to MSE or SSE, the potential transcription factors that are capable to regulate NOS2 expression were defined. Using the human NOS2 promoter sequence obtained from the UCSC web-engine (http://genome.ucsc.edu/) and subsequently analyzing this sequence by TFSEARCH software (http://mbs.cbrc.jp/research/db/TFSEARCH.html), the following putative transcription factors: NF-kB, STAT, IRF, p53, p63, ETS, C/EBPα, NF-Y, KLF6, and AABS (Fig. 2), were found in the NOS2 promoter sequence suggesting their potential role in regulation of NOS2 transcription [31–37].

To further test whether any of these transcription factors associate with the NOS2 promoter sequence the ChIP assay was performed. Using indicated antibodies, the binding of NF-kB (p65 subunit), IRF6, ΔNp63α, ETS, C/EBPα and NF-YA to the corresponding consensus elements in the NOS2 promoter was shown in HNSCC cells upon both MSE and SSE exposure (Fig. 3A, Input, upper panel, ChIP, middle panel). However, no binding was detected to the non-specific region of the NOS2 gene, which does not contain the cognate elements for the tested transcription factors (Fig. 3A, lower panel). Using qPCR, the binding of selected transcription factors to the NOS2 promoter was quantitatively monitored in HNSCC cells exposed to control medium, MSE and SSE (Fig. 3B). Interestingly, among all tested transcription factors, the highest up-regulation of binding to the NOS2 promoter was observed for IRF6 and ΔNp63α after both MSE and SSE treatments (Fig. 3B), while MSE significantly induced ETS and C/EBPα binding to the NOS2 promoter (Fig. 3B). Similarly, MSE and SSE dramatically increased the binding of both IRF6 and ΔNp63α to the specific region of the NOS2 promoter in immortalized oral keratinocytes (OKF6/TERT2) and this binding showed the greatest degree compared to other tested transcription factors (Fig. 4A).
To further examine whether various transcription factors bound to the NOS2 promoter are forming protein–protein complexes in HNSCC cells in response to cigarette smoke exposure, co-immunoprecipitation studies were performed. Using various antibodies, DNp63a was found to be associated with IRF6, DNp63, ETS, C/EBPβ and NF-YA in response of HNSCC cells (Fig. 4B) and OKF6/TERT2 cells (data not shown) to cigarette smoke extract (MSE and SSE) treatment. While a formation of protein complexes between DNp63a, ETS, IRF6 and C/EBPβ were dramatically induced in HNSCC cells upon tobacco exposure, the interaction of DNp63a with the NF-κB p65 subunit was slightly increased in HNSCC cells exposed to MSE (Fig. 4B, second panel). Cigarette smoking treatment was also shown to up regulate the complex formation between DNp63a, ETS, IRF6 and C/EBPβ in OKF6/TERT2 cells (data not shown).

Protein domains mediating DNp63α/IRF6 interaction

To further examine a nature of DNp63α/IRF6 protein–protein interactions, we generated expression constructs with specific domains of both DNp63α and IRF6 used for mammalian 2-hybrid assay. For the IRF6 protein, we designed the following bait constructs cloned into the pM vector: pM-IRF6 (1–138, DNA-binding domain), pM-IRF6 (139–334, maspin-associated domain), and pM-IRF6 (335–467, carboxyl terminus), while for DNp63α protein, we designed the following prey constructs cloned into the pVP16 vector: pVP16-DNp63α (1–86, DN/proline-rich domain), pVP16-DNp63α (87–294, DNA-binding domain), pVP16-DNp63α (295–446, oligomerization domain), and pVP16-DNp63α (447–586, sterile α-motif and carboxyl terminus) as shown in Fig. 5A. Then we introduced the resulting plasmids into HEK-293 cells and tested their ability to induce SEAP activity encoded by the pG5SEAP expression vector. First, we observed that the SEAP activity induced by a positive control pM-53/pVP16-T combination (132.5 ± 12.8 RLU), and failed to be induced by a negative control pM-53/pVP16-CP combination (22.4 ± 2.6 RLU). We further observed that the SEAP activity failed to be supported by self-activation of the both pVP16-DNp63a (28.9 ± 3.5 RLU) and pM-IRF6 (447–586) constructs. Finally, using the various bait/prey combinations, we found that all but [pM-IRF6 (336–467) + pVP16-DNp63α (447–586)] failed supporting protein association.
suggesting that the carboxyl termini of both IRF6 and DNp63a proteins involved in the protein–protein association (Fig. 5B).

DNp63a and IRF6 regulate NOS2 promoter activity and NOS2 activity

To further examine the effect of specific transcription factors on the reporter luciferase activity driven by the NOS2 promoter was tested in the absence or presence of siRNAs against DNp63a and IRF6. First, both DNp63a siRNA and IRF6 siRNA dramatically decreased the transactivation ability of DNp63a and IRF6 to induce the NOS2 promoter-driven reporter luciferase activity (Fig. 6A). Second, both DNp63a and IRF6 were implicated in the NOS2 expression and subsequently NOS2 enzymatic function, which was monitored by NO activity, since both DNp63a siRNA and IRF6 siRNA were shown to inhibit the NOS2 activity (Fig. 6B).

To assure the ability to induce NOS2, HNSCC cells and OKF6/TERT2 cells were exposed to 20 U/ml of interferon-gamma (IFN-γ) or 0.5% MSE for 16 h leading to a sufficient amount of NOS2 induced by both treatments (data not shown). Total NOS activity was tested under calcium-independent conditions thereby preventing the detection of the calcium-dependent NOS1 and NOS3 activities, while detecting NOS2 only [27,38].

Silencing of DNp63a, IRF6 or NOS2 inhibits smoke-induced autophagy

Finally, we examined whether DNp63a, IRF6 or NOS2 are involved in the cigarette smoke-induced autophagy recently reported to be implicated in COPD [17,28–30]. Microtubule-associated protein light chain 3 (LC3B), a mammalian homolog of yeast Atg8, has been used as a specific marker to monitor autophagy [28–30]. Upon induction of autophagy, the cytosolic form LC3B (LC3B-I) is conjugated to phosphatidylethanolamine (conversion into LC3B-II) and targeted to autophagic membranes. HNSCC cells and OKF6/TERT2 cells were transiently transfected with scrambled siRNA, siRNA against DNp63a, IRF6 or NOS2 for 24 h and then exposed to control media or 1% MSE for 24 h in the presence of 10 mg/ml of lysosomal protease inhibitors, E64d and pepstatin A, as recommended elsewhere [30]. Using the immunoblotting analysis of autophagosome marker, LC3B-I (16 kDa) and its conversion variant LC3B-II (14 kDa), we found that the MSE-induced autophagy-related changes of LC3B expression in HNSCC cells (Fig. 7A) and OKF6/TERT2 cells (data not shown). We further found that both siRNA against DNp63a and IRF6 modulated the LC3B-II protein levels in HNSCC cells (Fig. 7A) and OKF6/TERT2 cells (data not shown). We also observed that the siRNA-mediated NOS2

| Fig. 3. Cigarette smoke exposure induced binding of endogenous transcription factors to NOS2 promoter. HNSCC cells were exposed to control medium (C), 1% SSE (S) and 0.5% MSE (M) for 24 h. PCR was used to amplify the specific regions of the NOS2 promoter around the cognate sequences of p63 and other tested transcription factors. (A) Input of amplification assays used for ChIP (upper panel). Monitoring the binding of NF-YA, DNp63a, ETS and IRF6 to NOS2 promoter using ChIP assay with indicated antibodies (middle panel). To confirm specificity of the binding, we used ChIP assay with normal rabbit immunoglobulins (IgG, middle panel) and ChIP assay with the NOS2 non-specific region (lower panel). Experiments were performed twice. (B) qPCR quantification [expressed in relative units (RU)] of the binding of transcription factors to the specific region of the NOS2 promoter in HNSCC cells upon exposure to control media (C), SSE (S) and MSE (M). Values from control samples were designated as 1. Numerical values demonstrate the fold-change increase in NOS2 expression after smoking exposure compared to the control values. Experiments were performed three times (p > 0.05).

| Fig. 4. Protein complexes between various transcription factors induced by cigarette smoking exposure. (A) OKF6/TERT2 cells were exposed to control medium (C), 1% SSE (S) and 0.5% MSE (M) for 24 h. PCR was used to amplify the specific regions of the NOS2 promoter around the cognate sequences of p63 and other tested transcription factors. Quantification of the transcription factor binding to the NOS2 promoter specific region (expressed in relative units, RU) monitored by qPCR. Values from control samples were designated as 1. Numerical values demonstrate the fold-change increase in NOS2 expression after smoking exposure compared to the control values. Experiments were performed three times. (B) Immunoprecipitation (IP) was performed with Ab-1 antibody exclusively recognizing DNp63a protein. Protein complexes were detected with indicated antibodies. Separate panels represent independent gel experiments. Experiments were performed two times.
knockdown inhibited the autophagic process as seen by a decrease of the LC3B-II protein levels in HNSCC cells (Fig. 7A) and OKF6/TERT2 cells (data not shown) and LC3B-II/LC3B-I ratio in both cell lines (Fig. 7B).

Discussion

NO is a ubiquitous free radical that plays a key role in a broad spectrum of signaling pathways in physiologic and pathophysiologic processes [9,13,39–42]. As a critical signaling molecule and a component of inflammatory response, NO is involved in tumorigenesis [13,39–42]. Quantity of NO production and the cellular microenvironment influences the role of NO in tumor development [39–42]. NO is produced by three types of NO synthase, neuronal NOS1, inducible NOS2 and endothelial NOS3 [39–42]. NOS2 is implicated in immune and inflammation in vivo in both HNSCC and OKF/TERT2 cell lines. Additionally, ΔNp63α formed protein–protein complexes with ETS, NF-Ya, C/EBPb and IRF6 to the NOS2 promoter sequences and up-regulated the NOS2 mRNA and protein levels. The NOS2 promoter was shown to induce the binding of several transcription factors including NF-kB, STAT, IRF, p53, ETS, C/EBPb and NF-Y, KLF6, AABS potentially implicated in the NOS2 regulation.

Multiple molecular mechanisms are underlying control of NOS2 expression and activity [39–42]. Since NOS2 is capable of vast amount production of NO potentially leading to severe cellular damage and multiple pathologies, the tight mechanisms of NOS2 regulation were evolved and put in place protecting cells [39–42]. Modulation of NOS2 expression, on both the transcriptional and post-transcriptional level, is the major regulation mechanism for NOS2 [42]. Numerous transcription factors [NF-kb, IRF1, Stat1a, EGRF/Stat3, HIF1α, C/EBPb, KLF6, Ap1 (Jun/Fos), p53, Tcf/b-catenin, ETS (ESE1), and PARP1] and protein combinations thereof are implicated in regulation of NOS2 expression in mice and humans [43–46]. At the post-transcriptional level, NOS2 expression is regulated by the following RNA-binding proteins: AUF1, HuR, KSRP, PTB, TIAR and TTP [47–51].

In this study, cigarette smoke exposure of HNSCC cells or OKF6/TERT2 cells led to the ΔNp63α protein induction and subsequently to up-regulation of the NOS2 mRNA and protein levels. The NOS2 promoter was shown to contain the consensus sequences for many putative transcription factors [NF-kB, STAT, IRF, p53, ETS, C/EBPb and NF-Y, KLF6, AABS] potentially implicated in the NOS2 regulation. Some factors are well known regulators of NOS2 expression, and some are novel, including p63 responsive element (RE), which differed from the canonical p53RE sequence [37]. Tobacco exposure was shown to induce the binding of several transcription factors including ΔNp63α and IRF6 to the NOS2 promoter sequences in vivo in both HNSCC and OKF/TERT2 cell lines. Additionally, ΔNp63α formed protein–protein complexes with ETS, NF-YA, IRF6 and C/EBPb in HNSCC cells after tobacco exposure. The
carboxyl termini of both ΔNp63α and IRF6 proteins were further shown to mediate their mutual protein–protein interaction in transfected HNSCC cells. Interestingly, siRNA against ΔNp63α and IRF6 decreased the induction of the NO2 promoter-driven reporter luciferase activity and these siRNAs were shown to inhibit the NO2 activity in HNSCC cells and OKF6/TERT2 cells. Finally, cigarette smoke exposure was demonstrated to induce the autophagic changes in HNSCC cells and silencing of ΔNp63, IRF6 or NO2 reversed these changes. HNSCC cells and OKF6/TERT2 cells were transiently transfected with scrambled siRNA, siRNA against p63, IRF6 or NO2. Cells were exposed to control medium, 1% MCE for 24 h in the presence of lysosomal protease inhibitors (10 μg/ml of both E64d and pepstatin A, Sigma). Experiments were performed three times. (A) Protein levels of LC3B-I and LC3B-II were tested with an antibody against MAP LC3C/β. (B) Immunoblots were scanned using PhosphorImager (Molecular Dynamics) and quantified by ImageQuant software version 3.3 (Molecular Dynamics). Values of LC3B-II were expressed as percentage of LC3B-I values defined as 100%. The LC3B-I ratios were plotted as bars using the Microsoft Excel software with standard deviations (≤SD, p < 0.05) resulting from three independent experiments and three individual measurements of each experiment. HNSCC cells, black bars; OKF6/TERT-2 cells, grey bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Cigarette smoke induced the autophagic changes in HNSCC cells and silencing of ΔNp63, IRF6 or NO2 reversed these changes. HNSCC cells and OKF6/TERT2 cells were transiently transfected with scrambled siRNA, siRNA against p63, IRF6 or NO2. Cells were exposed to control medium, 1% MCE for 24 h in the presence of lysosomal protease inhibitors (10 μg/ml of both E64d and pepstatin A, Sigma). Experiments were performed three times. (A) Protein levels of LC3B-I and LC3B-II were tested with an antibody against MAP LC3C/β. (B) Immunoblots were scanned using PhosphorImager (Molecular Dynamics) and quantified by ImageQuant software version 3.3 (Molecular Dynamics). Values of LC3B-II were expressed as percentage of LC3B-I values defined as 100%. The LC3B-I ratios were plotted as bars using the Microsoft Excel software with standard deviations (≤SD, p < 0.05) resulting from three independent experiments and three individual measurements of each experiment. HNSCC cells, black bars; OKF6/TERT-2 cells, grey bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In summary, our data establishes a new link between ΔNp63α/IRF6 interplay and NO2 regulation underlying the autophagy-related response of cancer cells to tobacco exposure, therefore providing novel potential targets for pharmacological and biological therapeutic interventions with pathological conditions where DNA damage occurs and cells undergo the autophagic process.

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