Passive Smoking Exacerbates Nicotinamide-Adenine Dinucleotide Phosphate Oxidase Isoform 2–Induced Oxidative Stress and Arterial Dysfunction in Children with Persistent Allergic Rhinitis

Lorenzo Loffredo, MD1,2,*, Anna Maria Zicari, MD2,*, Francesca Occasi, MD2, Ludovica Perri, MD1, Roberto Carnevale, PhD1,3, Simona Battaglia, MD1, Francesco Angelico, MD1, Maria Del Ben, MD1, Francesco Martino, MD2, Cristina Nocella, PhD4, Alessio Farcomeni, MD2, Giovanna De Castro, MD2, Marzia Duse, MD2, and Francesco Violi, MD1

Objective To characterize nicotinamide-adenine dinucleotide phosphate oxidase isoform 2 (NOX2), oxidative stress, and endothelial function in children with and without allergic rhinitis and to ascertain the effect of passive smoke exposure on these factors, because there is an established association between allergic rhinitis and increased cardiovascular risk in adults.

Methods We recruited 130 children—65 with persistent allergic rhinitis and 65 healthy controls. A cross-sectional study was performed to compare endothelial function by flow-mediated dilation, blood levels of isoprostanes, serum activity of soluble NOX2-dp (sNOX2-dp), and nitric oxide bioavailability, in these 2 groups of children. Serum cotinine levels were assessed to measure exposure to passive smoking.

Results Compared with healthy controls, children with persistent allergic rhinitis had significantly higher sNOX2-dp and isoprostanes levels, lower flow-mediated dilation, and reduced nitric oxide bioavailability. Multivariable linear regression analysis showed that flow-mediated dilation, isoprostanes, and cotinine were independently associated with sNOX2-dp levels. Of note, sNOX2-dp serum levels were significantly higher in children with allergic rhinitis exposed to smoke, as compared with unexposed children with allergic rhinitis.

Conclusion NOX2 is activated in children with persistent allergic rhinitis and passive smoke exposure exacerbates this effect. We further demonstrate an association between higher sNOX2-dp and oxidative stress and endothelial dysfunction. (J Pediatr 2018; . . . . . . .).

Allergic rhinitis is a disease that affects up to 40% of the worldwide population, particularly children.1,2 Moreover, studies have demonstrated that participants suffering from allergic rhinitis have a greater risk of developing coronary and/or peripheral vascular disease, as well as all-cause death,3,4 presumably owing in part to systemic manifestations of allergic rhinitis–related inflammation. However, the mechanism by which allergic rhinitis predisposes or contributes to higher coronary and/or peripheral vascular disease incidence remains unclear.

Endothelial dysfunction is a hallmark of systemic atherosclerosis and an effective marker for assessing coronary and/or peripheral vascular disease risk.1 Flow-mediated dilation (FMD) is a widely used, direct measure of endothelial function in humans.5,6 Compared with controls, adults with chronic rhinitis have reduced FMD values and higher intima-media thickness (IMT).7 No studies have directly assessed these surrogate markers of atherosclerosis in children with allergic rhinitis. Equally relevant, why endothelial function is altered in these participants is unknown.

Oxidative stress, a well-known factor in atherosclerosis pathogenesis, is characterized by an imbalance between reactive oxygen species (ROS) production and scavenging and may predispose to reduced nitric oxide (NO) bioavailability owing to direct quenching of NO by ROS.5,9 These effects account for impaired endothelial dysfunction.8,10-12 Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase is a primary source of cellular superoxide anion production in humans,11-14 and studies performed in animals and humans suggest that NADPH oxidase activity modulates arterial tone.15 Accordingly, we have shown that lack of or deficient NADPH oxidase isoform 2...
(NOX2) activity is associated with arterial dilation in humans.11,12 Continuous exposure to allergens stimulates nasal eosinophils to produce ROS.13 NADPH oxidase activity represents a source of excessive ROS production and inflammation in patients with allergic rhinitis. Previous studies have shown that NADPH oxidase isoforms NOX1, NOX4, DOX1, and DOX2 are overexpressed in the airway epithelium and mucosa.14,15 NADPH oxidase is known to be concentrated in the subpollen particles, so that its contribution to rhinitis-associated oxidative stress could be considered direct.16,17

Second-hand tobacco smoke represents an important factor associated with persistent allergic rhinitis.20 Tobacco smoking activates NOX2, provoking endothelial dysfunction.21,22 Children exposed to passive smoke have a higher risk of cardiovascular events in adulthood.23

We sought to address whether NOX2-derived oxidative stress is involved in arterial dysfunction in children with persistent allergic rhinitis and whether NOX2 activation is associated with exposure to passive smoke in view of the symptom exacerbation (such as worsening nasal congestion, conjunctivitis, sneezing, and cough) seen with exposure to passive smoke in children with allergic rhinitis. To address these important unknown issues, we performed a cross-sectional study to assess NOX2 activity, oxidative stress, and endothelial function in children with persistent allergic rhinitis and healthy controls, and the association with exposure to passive smoke.

Methods

Consecutive children (n = 65) affected by severe persistent allergic rhinitis to dust mites and 65 healthy controls, matched for age and sex, were recruited between February 2013 and June 2017 at the Allergy and Rhinitis service at the Pediatric Clinic of “Sapienza” University of Rome. In the allergic rhinitis cohort only patients with a positive skin prick test were recruited.

Exclusion criteria were presence of acute or chronic cardiorespiratory diseases, neuromuscular diseases, chronic inflammatory diseases, liver disease, serious kidney disorders (eg, serum creatinine of >2.8 mg/dL), smoking, or vitamin consumption.

Persistent allergic rhinitis was defined according to the Allergic Rhinitis and its Impact on Asthma criteria (nasal symptoms for >4 days a week and for >4 consecutive weeks). The severity of rhinitis was defined by the Allergic Rhinitis and its Impact on Asthma items (presence of sleep disturbances, impairment of daily activities/sport/leisure, impairment of school performance, and troublesome symptoms) to classify pediatric patients into 3 severity groups: mild (no items affected), moderate (1-3 items affected), or severe (all 4 items affected). Healthy controls had to have no items of Allergic Rhinitis and its Impact on Asthma criteria. At enrollment, patients with allergic rhinitis had to be have not received anti-allergic treatment for at least 4 weeks.

As described elsewhere in this article, all participants underwent assessment of FMD and had blood collected to analyze markers of oxidative stress, namely, serum isoprostanes and NADPH oxidase activity via blood level measurements of soluble NOX2-derived peptide (sNOX2-dp), and NO bioavailability via Griess reaction. We assessed plasma cotinine levels to identify children exposed to second-hand smoke.

In addition to the cotinine level, parents completed a questionnaire assessing parental smoking habits, including nicotine consumption and time and place of nicotine exposure to determine the level and the type of passive smoking exposure. Parents were categorized as nonsmokers if they reported no current cigarette, cigar, or pipe smoking, or any other type of smoking during the past 5 years. To confirm non-smoking status, children reported as not exposed to second-hand smoking had to have plasma cotinine levels of less than 15 ng/mL, consistent with other studies.24,25

All procedures performed in this study were in accordance to the ethical guidelines of the 1975 Declaration of Helsinki; the study was approved by the Ethical Committee of “Sapienza” University of Rome. Written parental informed consent was obtained. For all participants, we performed a complete physical examination and elicited a full medical history.

Blood Sampling

Blood sampling was collected between 8:00 and 9:00 a.m. for routine biochemical evaluations, including fasting total cholesterol and glucose, and for oxidative stress analysis. Blood samples were collected in Vacutainers (Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK) after an overnight fast (12 hours). Samples were centrifuged at 300g for 10 minutes, and the supernatant was collected and stored at −80°C until dosage. Cholesterol analysis was assessed by an enzymatic colorimetric method on a Dimension RXL apparatus (Dade Behring AG, Ziegelbrucke, Switzerland).

FMD

Ultrasound assessment of basal brachial diameter and endothelial dependent FMD of brachial artery were investigated according to current guidelines,4 and as previously described.26 The variability of different measurements was evaluated by using an intraclass correlation coefficient. The intraclass correlation coefficient for brachial diameter at rest and FMD was 0.98 and 0.89, respectively.

IMT Measurement

IMT measurement was assessed via longitudinal ultrasound scans of the carotid artery as previously reported.11 The coefficient of variation is 2.6%.11

Enzyme-Linked Immunosorbent Assay Detection of sNOX2-dp

NOX2-derived peptide (NOX2-dp), a marker of NADPH oxidase activation, was assessed in serum by an enzyme-linked immunosorbent assay (ELISA) method, as previously described.27 Briefly, the assay is based on coating laboratory standards and serum samples into ELISA 96-well plates overnight at 4°C, addition of anti–NOX2-dp-horseradish peroxidase monoclonal antibody against the amino acidic sequence (224-268) of the extra membrane portion of NOX2, and...
quantification of immobilized antibody by the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically at 450 nm after addition of 2 mol/L sulphuric acid. Values were expressed as picograms per milliliter. Intra-assay and interassay coefficients of variation were 5.2% and 6.0%, respectively.

Serum NO and 8-iso-Prostaglandin F2α

NO bioavailability was measured in serum by ELISA method (Arbor Assays, Ann Arbor, Michigan). For this analysis, 50 μL of standards or samples are coated into ELISA 96-well plates and, after incubation, samples are read at 560 nm. Value are expressed as micromoles per liter.

The 8-iso-prostaglandin F2α (8-iso-PGF2α) levels were measured in serum by using a colorimetric assay kit (DRG International, Inc, Springfield Township, New Jersey). For this analysis, 50 μL of standards or hydrolyzed samples are coated into ELISA 96-well plates for 2 hours at room temperature and, after incubation, samples are read at 405 nm. Value are expressed as micromoles per liter.

Plasma Cotinine

Plasma cotinine was assessed by a human cotinine ELISA kit (TemaRicerca, Castenaso, Bologna, Italy). The cotinine kit is a solid phase competitive ELISA. The samples and cotinine enzyme conjugate are added to the wells coated with anticotinine antibody. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of cotinine in the samples. After incubation, samples are read at 550 nm and value are expressed as nanograms per milliliter.

Statistical Analyses

Continuous variables are reported as mean ± SD unless otherwise indicated. Pairwise comparisons were performed by t test; nonparametric tests (Kolmogorov–Smirnov [Z]) were used in case of nonhomogeneous variances as verified by the Levene test. Simple linear regression analysis was performed by Pearson test. Multivariable linear regression analysis was performed using a forward selection. Statistical significance was set at P < .05.

A subgroup analysis of only children with allergic rhinitis who were or were not exposed to passive smoke was performed, as was a subgroup analysis only control children with and without passive smoke exposure. Nonparametric tests were used in this subgroup analysis to assess the difference of sNOX2-dp, NO, isoprostanes, and FMD levels between participants exposed or not to passive smoking.

All analyses were carried out with SPSS V.18.0 (SPSS Statistics v. 18.0, SPSS Inc, Chicago, Illinois).

Sample Size Determination

The sample size for this cross-sectional study was computed with respect to a 2-tailed Student t test for independent groups, considering 2.1% (Δ) with a standard deviation of 3.5% as the difference for FMD between children with persistent allergic rhinitis and controls, with 0.05 (α) as type I error probability and 0.90 as power 1 − β. FMD was defined as a change in post-stimulus diameter and measured as a percentage increase of the baseline diameter. The sample size was 59. The sample size was increased by 10% (from 59 to 65) to account for the possibility of missing measurements.

Results

The clinical characteristics of children with persistent allergic rhinitis and controls are reported in Table 1. All children with allergic rhinitis had a severe form of the disease with a dust mite allergy. There were no differences between the 2 groups for age, fasting blood glucose, systolic and diastolic blood pressure, body mass index, or exposure to tobacco smoking.

Compared with controls, FMD and NO bioavailability were significantly lower in children with persistent allergic rhinitis (Table 1 and Figure, A and B). Conversely, sNOX2-dp and serum 8-iso-PGF2α were significantly higher in children with allergic rhinitis, compared with controls (Table 1 and Figure, C and D). No seasonal difference was observed between the groups for sNOX2-dp, serum 8-iso-PGF2α, and FMD in children with allergic rhinitis.

A subgroup analysis in the allergic rhinitis group showed that serum sNOX2-dp was higher in children exposed to passive smoking (n = 32), as compared with children not exposed (n = 33) (28.8 ± 6.6 pg/mL vs 23.4 ± 7.3 pg/mL; P = .006). The passive smoking-exposed children had higher levels of serum 8-iso-PGF2α (178 ± 48 pmol/L vs 161 ± 43 pmol/L; P = .056) and lower serum content of NO although these were not statistically significant (52 ± 12 μmol/L vs 58 ± 14 μmol/L; P = .111), and reduced FMD levels (5.1 ± 2.6% vs 6.4 ± 2.7%; P = .210). Furthermore, in accordance with our previous research,28 in the control group, compared with those not exposed (n = 36), participants exposed to passive smoking (n = 29) had significantly higher sNOX2-dp (13.2 ± 7.6 pg/mL vs 25 ± 11 pg/mL; P = .001), serum 8-iso-PGF2α (138 ± 18 pmol/L vs 159 ± 21 pmol/L; P < .001) and lower FMD (6 ± 3% vs 9 ± 3%; P = .001); conversely, no significant difference was found for NO (62 ± 8 μmol/L vs 58 ± 8 μmol/L; P = .474).

Table 1. Clinical characteristics of participants with persistent rhinitis and controls

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Children with rhinitis (n = 65)</th>
<th>Controls (n = 65)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>9.5 ± 3.5</td>
<td>9.5 ± 3.4</td>
<td>.821</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>50/15</td>
<td>50/15</td>
<td>1.0</td>
</tr>
<tr>
<td>Blood glucose levels</td>
<td>78 ± 8</td>
<td>80 ± 7</td>
<td>.149</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>105 ± 11</td>
<td>103 ± 10</td>
<td>.299</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>62 ± 7</td>
<td>65 ± 7</td>
<td>.186</td>
</tr>
<tr>
<td>Body mass index</td>
<td>18 ± 4</td>
<td>18 ± 5</td>
<td>.403</td>
</tr>
<tr>
<td>Children exposed to passive smoking (%)</td>
<td>32 (49)</td>
<td>29 (43)</td>
<td>.598</td>
</tr>
<tr>
<td>Cigarettes per day in smoker parents</td>
<td>18 ± 8</td>
<td>16 ± 9</td>
<td>.097</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>5.8 ± 3.7</td>
<td>7.8 ± 3.6</td>
<td>.001</td>
</tr>
<tr>
<td>IMT (mm)</td>
<td>0.52 ± 0.07</td>
<td>0.46 ± 0.09</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Serum isoprostanes (pmol/L)</td>
<td>170 ± 46</td>
<td>147 ± 22</td>
<td>.001</td>
</tr>
<tr>
<td>sNOX2-dp (pg/mL)</td>
<td>26 ± 7</td>
<td>19 ± 11</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>NO bioavailability (μmol/L)</td>
<td>55 ± 13</td>
<td>60 ± 6</td>
<td>.013</td>
</tr>
</tbody>
</table>

Continuous variables are expressed as mean ± SD.
Simple linear regression analysis in the overall population showed that sNOX2-dp levels were significantly correlated with FMD (r = −0.409; P < .001), serum 8-iso-PGF2α (r = 0.580; P < .001), NO bioavailability (r = −0.497; P < .001), and serum cotinine levels (r = 0.417; P < .001). Furthermore, FMD was significantly and inversely correlated with serum 8-iso-PGF2α levels (r = −0.373; P < .001), NO bioavailability (r = 0.192; P = .028), serum cotinine (r = −0.293; P = .001), and IMT (r = −0.238; P = .008).

Multivariable linear regression analysis, including the variables linearly associated with the dependent variable, was performed in the overall population to define the independent predictors of sNOX2-dp in the overall population. FMD (SE, 0.228; standardized coefficient β, -0.161; P = .031), serum isoprostanes (SE, 0.19; standardized coefficient β, 0.457; P < .001), and serum cotinine (SE, 0.40; standardized coefficient β, 0.263; P < .001) were independently associated with sNOX2-dp levels (R² = 45%; Table II).

We report that serum NADPH-2 oxidase activation levels are elevated in children with persistent allergic rhinitis and may be implicated in the endothelial dysfunction/reduced NO bioavailability that characterizes these participants.

Previous studies have shown that other isoforms of NADPH oxidase, such as NOX1, NOX4, DOX1, and DOX2, are

**Table II.** Multivariable linear regression analysis of the variables independently associated with sNOX2-dp levels

<table>
<thead>
<tr>
<th>Variables included in the model</th>
<th>B</th>
<th>Standard Error</th>
<th>Beta</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoprostanes</td>
<td>0.123</td>
<td>0.020</td>
<td>0.459</td>
<td>.000</td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>0.148</td>
<td>0.041</td>
<td>0.256</td>
<td>.000</td>
</tr>
<tr>
<td>FMD</td>
<td>−0.501</td>
<td>0.228</td>
<td>−0.162</td>
<td>.030</td>
</tr>
</tbody>
</table>
overexpressed in the airway epithelium and mucosa. The present study reports that the NADPH oxidase isoform, NOX2, is highly activated in the serum of young patients with persistent allergic rhinitis, as compared with controls.

Previous studies have also explored and confirmed the presence of elevated oxidative stress in patients with allergic rhinitis. For instance, isoprostane and NO levels were tested in children with persistent rhinitis. Accordingly, our study shows that serum isoprostane levels are increased and NO bioavailability is decreased in patients with persistent allergic rhinitis, thus, confirming that an imbalance occurs between the extent of ROS generation and its effective scavenging.

Environmental sources such as allergens, air pollution, and cigarette smoke could represent a trigger to produce excessive ROS in patients with persistent allergic rhinitis. Chronic exposure to allergens stimulates inflammatory cells, such as eosinophils and neutrophils, in the airway mucosa to produce oxidative stress by NADPH oxidase. In aggregate, these support the notion that NADPH oxidase–generated oxidative stress is a key player in the pathophysiology of rhinitis. In addition, we demonstrate a relationship between NOX2 and passive smoke exposure, as shown by the close association between cotinine and serum sNOX2-dp levels, supporting a study that demonstrated a positive association of tobacco smoke exposure with rhinitis. Finally, we demonstrate the presence of endothelial dysfunction in children with persistent allergic rhinitis.

Previous studies in animal models support the effect of passive smoking on NADPH oxidase. Another experimental study in human umbilical vein endothelial cells exposed to smoke showed a reduction of eNOS activity and stimulation of NADPH oxidase activity. Previously, we found in humans that NOX2 activation is higher in active smokers and in children exposed to passive smoke, but no data were available on the effect of passive smoke on NADPH oxidase in allergic rhinitis.

NADPH oxidase also plays a pivotal role in modulating endothelial function. Children at risk of atherosclerosis, such as those with obesity, obstructive sleep apnea, and hypercholesterolemia, have endothelial dysfunction and increased NADPH oxidase–derived oxidative stress. In contrast, when NADPH oxidase is downregulated, as in children with hereditary deficiency of gp-91 phox, the endothelial function is improved and the oxidative burden is lower than in controls.

A novel aspect of our study is the presence of endothelial dysfunction in children with persistent allergic rhinitis, as indexed by the lower FMD values found in the latter compared with healthy controls. Elcioglu et al previously showed reduced FMD values in adults with chronic rhinitis. The low FMD observed in persistent allergic rhinitis, exacerbated by passive smoke exposure, could be due to high NOX2 activity with impairment of NO bioavailability and/or biosynthesis.

As previously shown in the Bruneck and ARMY studies, we found enhanced atherosclerosis, as shown by the higher IMT values, in participants with persistent allergic rhinitis. Future studies must validate the association between surrogate markers of atherosclerosis, such as IMT and FMD, and cardiovascular events in participants with persistent allergic rhinitis.

Our findings must be interpreted in light of several limitations. We did not evaluate other NADPH isoforms, such as NOX1 and NOX4, that could also contribute to increased oxidative burden and further deteriorate endothelial dysfunction. However, their contribution to airway/mucosa dysfunction has been already documented. Future studies should determine to what extent therapies directed to treat allergic rhinitis also have an impact on NOX2-generated oxidative stress and endothelial dysfunction. The therapeutic implications of our findings are beyond the scope of the present work. This is a small, single-center sample that needs to validated in a larger sample. Because of this limitation, we cannot rule out selection bias. The cross-sectional design precludes the ability to establish a causal relationship and only associations can be reported between NOX2–mediated oxidative stress and endothelial function in children with persistent allergic rhinitis.

In conclusion, our study shows that NOX2 activation is a key determinant of oxidative stress and endothelial dysfunction in children with persistent allergic rhinitis, and that passive smoke exacerbates these relationships.

Submitted for publication Feb 20, 2018; last revision received Jun 14, 2018; accepted Jun 18, 2018
Reprint requests: Lorenzo Loffredo, MD, Divisione I Clinica Medica, Viale del Policlinico 155, Rome, 00161, Italy. E-mail: lorenzo.loffredo@uniroma1.it

References