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FRONT ROWS OF SCIENCE

Quantitative analysis of 8-isoprostane and hydrogen peroxide in exhaled breath condensate

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Quantitative analysis of 8-isoprostane and hydrogen peroxide in exhaled breath condensate. P.G.A. Van Hoydonck, W.A. Wuyts, B.M. Vanaudenaerde, E.G. Schouten, L.J. Dupont, E.H.M. Temme. ©ERS Journals Ltd 2004.

ABSTRACT: Exhaled breath condensate (EBC) provides a noninvasive means of sampling the lower respiratory tract. Collection of EBC might be useful in the assessment of airway oxidative stress in smokers. The aim of this study was to determine 8-isoprostane and hydrogen peroxide levels in EBC, and, in addition, to investigate the reproducibility of these measurements.

EBC samples were collected from 12 healthy male smokers at three time points within 1 week. 8-isoprostane and H_2O_2 were measured in nonconcentrated EBC using immunochemical and colorimetric assays, respectively.

8-isoprostane and H_2O_2 were detected in only 36 and 47% of all EBC samples, respectively. It was not possible to calculate the within-subject variation in a reliable manner since only three of the 12 smokers exhibited detectable 8-isoprostane concentrations on all three occasions (mean 4.6 pg·mL^{-1}; range 3.9–7.7 pg·mL^{-1}), whereas H_2O_2 could not be detected on all three occasions in any of the smokers. Spiking experiments revealed a recovery of 83.5–109.5% for 8-isoprostane and 69.9–129.0% for H_2O_2 in fresh EBC samples.

It was concluded that levels of 8-isoprostane and hydrogen peroxide cannot be reproducibly assessed in exhaled breath condensate from healthy smokers because of their low concentration and/or the lack of sensitivity of the available assays.

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The airways of smokers are exposed to high levels of oxidative stress, caused by oxidants and free radicals inhaled with cigarette smoke and reactive oxygen species produced by activated inflammatory cells [1, 2]. The oxidative burden of the smoker's airways can be investigated using traditional methods of sampling alveolar fluids or bronchial secretions, such as sputum induction and bronchoscopy with bronchoalveolar lavage. Sampling of these lung secretions is, however, invasive, and this may influence oxidative status markers. Recently, collection of exhaled breath condensate (EBC) has been suggested as a simple and easily repeatable procedure for monitoring airway inflammation and oxidative stress [3]. The method entails cooling exhaled air, which results in condensation. This EBC contains aerosolised airway epithelial lining fluid particles and volatile compounds. There is increasing evidence that exhaled markers may reflect biochemical changes in airway lining fluid [3]. The collection of EBC might, therefore, be useful in identifying smokers at a greater risk of developing smoking-related pulmonary diseases and investigating the potential effects of extra antioxidants. A number of studies have focused on markers of oxidative processes in the EBC of smokers, including hydrogen peroxide [4–6], 8-isoprostane [7], nitrogen oxides [8–10] and nitrosothiols [11]. However, it should be emphasised that the analysis and interpretation of EBC results are not straightforward. Several methodological issues regarding sampling and the analytical techniques used for EBC need to be taken into account, such as issues related to the collection procedure and the analysis of oxidative markers in EBC.

The aim of the present study was to determine whether 8-isoprostane and H_2O_2 are detectable in nonconcentrated EBC from healthy male smokers, when measured using immunochemical and colorimetric assays, respectively. In addition, the reproducibility of these measurements was investigated.

Subjects and methods

Subjects

The study population consisted of 12 male smokers (age range 24–61 yrs; mean±SD 44±14 yrs). All of the smokers were healthy as assessed by a medical questionnaire. Before the start of the study, the volunteers completed a short questionnaire about their smoking habits and measurement of weight and height was performed. Body mass index was 23.5±2.9 kg·m^{-2} and cumulative cigarette consumption 29.8±30.9 pack-yrs. The Ethics Committee of University Hospital Gasthuisberg, Leuven, Belgium, approved the study protocol, and all subjects gave informed consent before participating.

For editorial comments see page 183.

Collection of exhaled breath condensate

For each subject, EBC samples were collected at three time points within 1 week (day 0, day 3 or 4, and day 7) using a commercially available condenser (EcoScreen®; Erich Jaeger GmbH, Hoechberg, Germany). Subjects breathed through a mouthpiece and a two-way nonrebreathing valve, in which inspiratory and expiratory air were separated, and saliva trapped. They were asked to breathe at a normal frequency, wearing a noseclip, for a period of 15 min to obtain $\sim \geqslant 1$ mL condensate. The subjects refrained from smoking for 2 h before EBC collection and were asked not to change their smoking habits in the week during which the collection took place. All EBC samples were taken at the same location and, for each subject, at the same time of day. The EBC samples were immediately divided into aliquots, stored at -80°C and thawed at room temperature immediately before analysis.

Laboratory analysis

EBC 8-isoprostane concentrations were quantified using a specific enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA) based on competition with an 8-isoprostane/acetylcholinesterase conjugate for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. The observed absorbance, determined spectrophotometrically, was inversely related to the amount of free 8-isoprostane in EBC.

EBC H_2O_2 was measured colorimetrically by means of horseradish peroxidase-catalysed oxidation of tetramethylbenzidine according to the method previously described by GALLATI and PRACHT [12]. Briefly, 100 μL 3,3′,5,5′-tetramethylbenzidine (dissolved in 0.42 M citrate buffer (pH 3.8)) and 10 μL horseradish peroxidase (Sigma Chemicals, St Louis, MO, USA; 52.5 U·mL^{-1}) were reacted with 100 μL EBC for 10 min at room temperature. Subsequently, the mixture was acidified to pH 1 with 10 μL 36 M sulphuric acid. The reaction product was measured spectrophotometrically at 450 nm. A separate standard curve for H_2O_2 was constructed for each assay.

All EBC samples were analysed in duplicate and in one run to circumvent interassay variation. In addition, EBC aliquots were pooled in one sample. Intra-assay variation was calculated using a pooled sample containing detectable concentrations, which was analysed in triplicate in each run, and was 7.5% for 8-isoprostane and 3.3% for H_2O_2.

Assays of 8-isoprostane and H_2O_2 were performed on undiluted and nonconcentrated EBC samples. The lower limit of detection (LOD), defined as the lowest concentration of the standard curve, was 3.9 pg·mL^{-1} for 8-isoprostane and 0.31 μM for H_2O_2. Concentrations below the LOD were designated not detectable.

Results

8-isoprostane and H_2O_2 concentrations were below the LOD in many samples (table 1). 8-isoprostane was detectable in EBC samples from only three smokers on all three occasions within 1 week (mean 5.3, 4.0 and 4.5 pg·mL^{-1}). These three smokers were not different from the others with respect to age, body mass index and cumulative cigarette consumption. Eight of the 12 smokers exhibited 8-isoprostane concentrations above the LOD of 3.9 pg·mL^{-1} for at least one measurement (36% of all EBC samples contained detectable 8-isoprostane). The concentration of 8-isoprostane in these samples ranged 3.9–10.6 pg·mL^{-1}.

Table 1. – Concentration of 8-isoprostane and hydrogen peroxide (H_2O_2) in exhaled breath condensate

Subject No.	8-isoprostane pg·mL^{-1}			Hydrogen peroxide μM		
	Time 1	Time 2	Time 3	Time 1	Time 2	Time 3
1	ND	ND	ND	0.41	ND	0.31
2	ND	ND	ND	0.31	ND	ND
3	ND	ND	10.6	ND	0.31	ND
4	ND	ND	6.6	0.31	0.32	ND
5	ND	ND	6.2	0.31	ND	0.49
6	ND	4.8	ND	ND	ND	0.42
7	7.7	4.4	3.9	0.31	ND	ND
8	3.9	4.0	4.0	ND	ND	0.31
9	5.1	4.5	3.9	ND	0.34	ND
10	ND	ND	ND	ND	ND	ND
11	4.0	ND	8.3	ND	0.31	ND
12	ND	ND	ND	ND	0.32	0.31

ND: nondetectable (<3.9 pg·mL^{-1} for 8-isoprostane and <0.31 μM for H_2O_2).

None of the smokers showed detectable concentrations of H_2O_2 for all three measurements within 1 week, but 11 of the 12 subjects exhibited H_2O_2 concentrations above the LOD of 0.31 μM for at least one measurement (47% of all EBC samples contained detectable H_2O_2). The concentration of H_2O_2 in these EBC samples ranged 0.31–0.49 μM.

The reproducibility of the measurements was calculated: the intraclass correlation coefficient was 0.33 for 8-isoprostane and -0.34 for H_2O_2. However, these values were calculated from only a few detectable concentrations.

Separate spiking experiments were performed by adding 3.1–50 pg·mL^{-1} 8-isoprostane or 0.5–10 μM H_2O_2 to fresh and thawed (after 5 h at -80°C) EBC collected from a healthy subject. These experiments, which were performed in duplicate, showed a recovery of exogenous 8-isoprostane of 83.2–109.5% for fresh samples and 114.5–145.6% for thawed samples. Whereas, recovery of exogenously added H_2O_2 ranged 69.9–129.0% for fresh samples and even reached >200% in thawed samples.

Discussion

The present report shows that levels of 8-isoprostane and H_2O_2 cannot be reproducibly assessed in EBC from healthy smokers because of their low concentration and/or the lack of sensitivity of the available assay methods.

It is believed that cigarette smoking causes an increase in levels of free radicals and reactive oxygen species, and that oxidative status may be monitored by analysis of EBC markers [3, 5, 13]. 8-isoprostane, a prostaglandin $F_{2\alpha}$ isomer that is formed by free radical lipid peroxidation of arachidonic acid, may be a quantitative marker of *in vivo* oxidative stress in the airways of smokers [14]. MONTUSCHI *et al.* [7] previously investigated the effect of smoking on 8-isoprostane levels using a similar condensing device (the commercially available EcoScreen®) and immunochemical assay (with the same LOD) to those used in the present study. They observed higher mean concentrations of 8-isoprostane in 12 healthy smokers (24.0±2.6 pg·mL^{-1}), as well as in 10 healthy nonsmokers (10.8±0.8 pg·mL^{-1}), than observed in the present study (5.4±2.0 pg·mL^{-1} for all subjects combined). The difference in concentration in the smokers might be due to differences in baseline characteristics. The present smokers were younger and had a lower cumulative cigarette consumption than the smokers of the study of MONTUSCHI *et al.* [7] (44 *versus* 60 yrs; 29 *versus* 34 pack-yrs).

It might be argued that the broad range of age (24–61 yrs) and cumulative cigarette consumption (3–57 pack-yrs) of the present smokers implicates variation in susceptibility to oxidative stress and might subsequently affect the level of 8-isoprostane measured in EBC. However, whether age and cumulative cigarette consumption indeed increase EBC 8-isoprostane concentrations has not been addressed in previous studies.

In the present study, smokers refrained from smoking for a period of only 2 h prior to producing EBC, whereas, in other studies on 8-isoprostane in EBC, smokers abstained from smoking for $\geqslant$12 h. However, MONTUSCHI *et al.* [7] have shown that a short period of abstinence from smoking enhances exhaled 8-isoprostane concentrations. This cannot, therefore, explain the low 8-isoprostane concentrations detected in the present experiments.

The concentrations of 8-isoprostane found in the present healthy smokers resembled more closely those found in healthy nonsmoking subjects (4.5 ± 0.5 pg·mL^{-1}; n=15; eight males; mean age 42 yrs) in the study of CARPAGNANO *et al.* [15], also analysed using the same EIA kit. The investigators reported that 8-isoprostane was detectable in the EBC of all subjects. However, figure 1 of their report shows that four of their 15 healthy subjects exhibited 8-isoprostane concentrations below the LOD (3.9 pg·mL^{-1}). These results and the present findings indicate that more sensitive assays than the sole commercially available EIA kit are required to detect 8-isoprostane concentrations in EBC from healthy (smoking) subjects in a more accurate manner. In addition, data obtained by immunoassay need to be compared with data obtained by more sensitive methods such as mass spectrometry. Moreover, the specificity of the available EIA kit should be further investigated.

Isoprostanes are poorly volatile substances, and, consequently, it may be expected that their concentrations in EBC will be very low in healthy smokers. The condensate samples were not enriched by extraction or lyophilisation, because the possibility that the method used for enrichment of the samples might influence 8-isoprostane measurement could not be excluded. In addition, previous studies did not enrich EBC samples for measurement of 8-isoprostane concentrations [7, 15, 16]. However, in a recent abstract of VAN DER MEER *et al.* [17], all EBC samples were concentrated three-fold using vacuum centrifugation, and a mean concentration of 5.2 pg·mL^{-1} was observed in healthy subjects.

EBC consists mainly of water and volatile and water-soluble organic compounds, such as H_2O_2. These substances are able to reach the exhaled breath and can easily be detected in EBC. The detectable concentration of H_2O_2 in the present male smokers was within the concentration range of all previous observations in healthy smokers (0.10–0.75 μM) [5, 6, 18]. This wide range of exhaled H_2O_2 concentration may be explained, in part, by the variety of condensing devices of different design that were used for the collection of EBC samples or by differences in the analytical methods used for measurement of H_2O_2. For example, GUATURA *et al.* [18] explained their higher levels of H_2O_2 (0.75 μM) in healthy subjects by the use of a face mask and nasal breathing. The most frequently used devices for EBC collection are Teflon-lined tubes in buckets of ice, a specially designed double-walled glass vessel that is placed in ice or liquid nitrogen, or double-jacketed cooling tubes. The material of the cold tubes (glass or Teflon), as well as the length of the tube, may be important for the capture of the compounds under examination. The present study employed a new commercially available device (Ecoscreen®), which is composed of a lamellar condenser (aluminium, length ~10 cm) and an electric refrigeration system. Before recommending its use, the performance of this device should be tested further and compared with other condensers. Furthermore, a standardised method for analysing EBC H_2O_2 is still lacking. The choice of reaction substrate (tetramethylbenzidine, *p*-hydroxyphenylacetic acid or homovanillic acid), as well as use of fresh *versus* thawed EBC samples, might explain another part of the variation in H_2O_2 concentration. In a study by VAN BEURDEN *et al.* [4], a different assay was used, with a reported LOD of 0.02 μM, which was lower than that reported in the present study (0.31 μM). This assay was based on the H_2O_2-dependent oxidation of *p*-hydroxyphenylacetic acid to a highly fluorescent dimer. The *p*-hydroxyphenylacetic acid reagent was added within 30 min after collection, the samples frozen at -70°C and the highly fluorescent dimer measured after 2 weeks. However, another study assessing EBC H_2O_2 reported an LOD of 0.1 μM using the same analytical method as VAN BEURDEN *et al.* [4] used [19]. Conversely, HORVATH *et al.* [20] and GANAS *et al.* [8] reported a LOD of H_2O_2 of ~0.1 μM using the same analytical method as in the present study. Moreover, two previous studies using an assay based on H_2O_2-dependent oxidation of homovanillic acid reported an LOD of 0.1 μM [5, 21]. The different measurement techniques and reported LODs of H_2O_2 in the available studies may hamper adequate comparison between these study results and indicate the need for standardisation of analysis of EBC samples.

Although the present study contradicts published studies regarding the levels of 8-isoprostane and H_2O_2 in EBC, the present authors are confident that every effort was made to avoid error during all phases of the study. After 15 min of breathing through the condenser, the samples were transported in <5 min to a -80°C freezer and stored there until analysis. All analyses were performed within 2 months after collection.

In the present study, several analytical problems that might interfere with the assessment of 8-isoprostane and H_2O_2 in EBC were addressed. All of the guidelines of the manufacturer of the EIA kit were followed, including processing a positive control (maximum binding absorbance), which was in the expected range. The assay plates for 8-isoprostane and H_2O_2 were also read at different times after addition of the reagent and no differences were observed. In a separate experiment, different concentrations of the reaction substrate (tetramethylbenzidine) were added to the H_2O_2 assay. This influenced neither the results nor the LOD. Finally, recovery experiments (using fresh and thawed (after 5 h at -80°C) samples) were also performed to assess whether components of EBC might mask the detection of 8-isoprostane and H_2O_2. Recoveries of 83.2–109.5% (fresh samples) and 114.5–145.6% (thawed samples) were obtained for 8-isoprostane. Whereas, recovery of exogenously added H_2O_2 ranged 69.9–129.0% for fresh samples and even reached >200% in thawed samples.

Owing to the lack of sensitivity of the methodology used in the present study, reproducibility could not be assessed in a reliable manner. Recently, two studies, by VAN RENSEN *et al.* [22] and VAN DER MEER *et al.* [17], reported that the levels of 8-isoprostane or H_2O_2 measured in EBC on two consecutive days were not reproducible in patients with asthma or healthy volunteers. They used the intraclass correlation coefficient to assess the reproducibility of their measurements, which resulted in values of -0.35 (asthma) and 0.08 (healthy) for 8-isoprostane and -0.14 (asthma) and -0.08 (healthy) for H_2O_2. The poor reproducibility of the present study confirms the results of VAN RENSEN *et al.* [22] and VAN DER MEER *et al.* [17].

In conclusion, levels of 8-isoprostane and hydrogen peroxide cannot be reproducibly assessed in exhaled breath condensate from healthy smokers because of their low concentrations in most exhaled breath condensate samples and/or the lack of sensitivity of the assays used. In order to

use these exhaled breath condensate markers for research purposes or as possible diagnostic test for the identification of smokers at higher risk of developing smoking-related pulmonary diseases, more sensitive assays for the detection of low concentrations are essential. In addition, the performance of different exhaled breath condensate collection devices needs to be compared.

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