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The effect of the trolox equivalent antioxidant capacity (TEAC) in plasma on the formation of 4-aminobiphenylhaemoglobin adducts in smokers

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Smokers are exposed to a number of carcinogenic compounds including aromatic amines such as 4-aminobiphenyl. Antioxidants are thought to be involved in the defence against the damaging effect of such carcinogens. Recently it has been shown that plasma antioxidant status in smokers is diminished compared with non-smokers. In this study we investigated in 40 smokers whether the trolox equivalent antioxidant capacity (TEAC) in plasma could be quantitatively related to exposure to cigarette smoke. The biomarkers 4-aminobiphenylhaemoglobin (4-ABP–Hb) adduct and cotinine were determined as indices of cigarette smoke exposure. A correlation between 4-ABP–Hb adduct levels and plasma cotinine levels was found for the whole population studied, who smoked 4–70 cigarettes per day ($n=40$, $r^2=0.12$, $p=0.03$). A significant inverse relationship was found between TEAC and 4-ABP–Hb levels ($n=40$, $r^2=0.17$, $p=0.008$). Multiple regression analysis showed a strong relationship between 4-ABP–Hb levels and plasma TEAC and cotinine levels ($n=40$, $r^2=0.29$, $p=0.002$). These findings provide strong evidence that the 4-ABP–Hb adduct represents a valuable biomarker of (internal) exposure to tobacco smoke, and also that the formation of this marker is dependent on the plasma antioxidant status. The multiple regression analysis results show that the measure of effect (4-ABP–Hb adduct formation) is largely determined by dose (cotinine) and protection (TEAC).

Keywords: antioxidant, 4-aminobiphenylhaemoglobin adduct, smokers, TEAC.

Abbreviations: 4-ABP, 4-aminobiphenyl; 4′-F-ABP, 4′-fluoro-4-aminobiphenyl; Hb, haemoglobin; GC-MS, gas chromatography-mass spectrometry; TEAC, trolox equivalent antioxidant capacity.

Introduction

Various studies on the relationship between 4-aminobiphenylhaemoglobin (4-ABP–Hb) adduct levels and smoking behaviour have demonstrated enhanced 4-ABP–Hb levels for smokers versus non-smokers (Bryant *et al.* 1987, Falter *et al.* 1994) and significant correlations between 4-ABP–Hb adduct levels and the number of cigarettes smoked (Vineis *et al.* 1996, Dallinga *et al.* 1998).

Serum total radical-trapping antioxidant potential (TRAP) has been reported to be significantly lower in smokers compared with non-smokers (Sharpe *et al.* 1996, Erhola *et al.* 1997, Nyyssönen *et al.* 1997), showing a correlation and

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assuming an influence of smoking behaviour on the antioxidant status. Recently it has been shown that nuclear factor-κB, an oxidative stress-sensitive transcription factor, is higher in the peripheral blood mononuclear cells of smokers compared with non-smokers (Van den Berg *et al.* 2001a). Antioxidants are expected to protect against oxidative stress and the adduct formation of smoking-related carcinogens with macromolecules.

In this study we describe a population of 40 smoking volunteers who donated blood samples for the investigation of 4-ABP–Hb adduct levels. These adduct levels were related to the self-reported number of cigarettes smoked daily, to plasma cotinine levels and to the plasma antioxidant status measured using a trolox equivalent antioxidant capacity (TEAC) assay (Van den Berg *et al.* 1999), in order to establish a possible relationship between these parameters.

Materials and methods

Study population

The study population consisted of 26 female and 14 male cigarette-smoking volunteers, with a mean age of 43 ± 9 years. Questionnaires were used to obtain information on smoking, medication, possible occupational exposure, etc. No confounding due to occupational exposure or medication was anticipated and thus all questioned persons were included in the study. The cigarette consumption of the volunteers was 27 ± 12 cigarettes per day (range 4–70 per day, median 25 per day). All subjects had smoked for at least 9 years at the time of the sampling. No significant differences in the number of cigarettes smoked, in cotinine levels or in adduct formation due to gender or age of the subjects could be detected. The study was approved by the Medical Ethical Commission of the Netherlands Cancer Institute and informed consent was obtained from all volunteers.

Isolation of haemoglobin

Peripheral blood was sampled by venipuncture into ethylene diamine tetra-acetic acid tubes and erythrocytes were isolated according to the method described by Bryant *et al.* (1987), with minor modifications. After centrifugation the packed red blood cells were washed twice with 0.9% saline and frozen at −80°C overnight. After thawing, the lysation of the erythrocytes was completed by adding three volumes of ice-cold deionized water. After 20 min at 0°C the solution was buffered with phosphate at pH 6.6. The cell membranes were removed by centrifugation at 4°C. The remaining haemoglobin solution was dialysed against deionized water for 48 h at 4°C to remove ions and molecules with a molecular weight below 12 000 Da. The haemoglobin concentration of the dialysate was determined using Drabkin's method (Sigma kit 525a) and the dialysate was stored at −20°C until further use.

Isolation and derivatization of 4-aminobiphenyl

The dialysate (20 ml) was spiked with 1 ng of 4-fluoro-u-aminobiphenyl (4′-F-ABP) and incubated for 30 min at room temperature. 4-ABP was released from the haemoglobin by the addition of 0.2 ml 10 N NaOH and incubation for 2.5 h at room temperature. The dialysate was extracted twice with 20 ml hexane; the resulting foamy layer between the aqueous and hexane layers was broken down by the addition of 50 µl of ethanol, followed by centrifugation. The combined hexane layers were dried over anhydrous $Na_2SO_4/MgSO_4$, collected in deactivated glass vessels (Hecht *et al.* 1994) and evaporated to a volume of about 2 ml. 4-ABP and 4′-F-ABP were derivatized by the addition of 5 µl of triethylamine in hexane (1:5) and 3 µl of pentafluoropropionic anhydride, followed by a waiting time of 2 h at room temperature (Skipper and Stillwell 1994). The hexane was then evaporated and the residue redissolved in 20 µl of dichloromethane.

Determination of 4-ABP

Aliquots of 0.5 µl of the 4-ABP/4′-F-ABP solution in dichloromethane were introduced splitlessly into the gas chromatograph-mass spectrometer and ions at m/z 295 and m/z 313, from 4-ABP and 4′-F-ABP, respectively, were measured using negative ion chemical ionization mass spectrometry under selected ion monitoring conditions (Bryant *et al.* 1987). The GC-MS instrument consisted of a HP 5890 Series II capillary gas chromatograph (Hewlett Packard, Portland, Oregon, USA) coupled to a Jeol SX 102A double focusing mass spectrometer of reversed geometry (JEOL Ltd, Tokyo, Japan). The gas chromatograph was operated at an injector temperature of 260°C, using a 25 m, ID 0.32 mm, film

thickness 0.25 µm fused silica SGE BPX35 column (SGE Inc., Austin, Texas, USA). The column temperature was 60°C for 2 min, then rising at 30°C min^{-1} to 320°C. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The mass spectrometer was operated at an ion source temperature of 230°C, an ionizing energy of 200 eV and an accelerating potential of 10 000 V; methane was used as the reaction gas. The 4-ABP concentration in haemoglobin was calculated using the known haemoglobin and internal standard concentrations of the dialysate. The mean inter-assay variation and standard error for the 4-ABP determination starting from duplicate blood samples was $22.0 \pm 3.5\%$ ($n = 5$); duplicate measurements starting from the same dialysate showed a variation of $10.4 \pm 5.2\%$ ($n = 4$). This variation could be explained almost entirely by duplicate injections from the same extract into the GC-MS system, which had a variation of $9.2 \pm 0.8\%$ ($n = 82$). The detection limit was < 1 pg 4-ABP g^{-1} Hb.

Cotinine measurements

Cotinine, a principal metabolite of nicotine, was analysed in plasma by means of an ^{125}I-radioimmunoassay (Knight *et al.* 1989).

TEAC

The TEAC assay, as described by Miller *et al.* (1993), has been modified by Van den Berg *et al.* (1999). The assay is based on scavenging 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical anions ($ABTS^{\bullet -}$), the main modification of the assay being the pre-generation of the ABTS radical anions. For our TEAC measurements an $ABTS^{\bullet -}$ solution was prepared by mixing 2.5 mM 2,2′-azobis-(2-amidinopropane). HCl with 20 mM $ABTS^{2-}$ stock solution in 100 mM phosphate buffer pH 7.4, containing 150 mM NaCl. The solution was heated for 12 min at 60°C, protected from light and stored at room temperature. For measuring antioxidant capacity 40 µl of the sample was mixed with 1960 µl of the radical solution. The plasma was deproteinated by the addition of an equal volume of a 10% trichloroacetic acid solution in water. Absorbance was monitored at 734 nm for 6 min. The decrease in absorbance after 6 min was used for calculating the TEAC.

Statistics

Linear regression analysis was used to determine correlations between smoking parameters and between smoking parameters and TEAC. Because of the normal distribution of the study population, there was no need for a log-transformation of the data; calculations using such data led to essentially the same results as the non-transformed data. In addition, a multiple regression analysis was performed where appropriate. The Mann–Whitney U test was applied to investigate differences in TEAC in relation to the gender of the test population.

Results

The numbers of cigarettes smoked by the 40 volunteers were recorded using a questionnaire. This parameter, and the biological (effect) parameters, such as cotinine, 4-ABP–Hb and TEAC levels, were compared with each other using simple regression analysis.

Cotinine levels are often used as a measure of nicotine exposure (Jarvis *et al.* 1984, Muranaka *et al.* 1988, Pérez-Stable *et al.* 1992); in our population the cotinine levels were not significantly correlated with the self-reported number of cigarettes smoked. When those smoking more then 26 cigarettes per day were excluded from the calculations, a significant correlation was found between the number of cigarettes smoked and the plasma cotinine level ($n = 28$, $r^2 = 0.16$, $p = 0.03$). The ratio of plasma cotinine concentration to the number of cigarettes smoked decreased with an increasing number of cigarettes smoked, meaning that the cotinine levels tend to diminish *relatively* with increasing self-reported cigarette consumption.

The 4-ABP–Hb adduct levels of all smokers showed no significant correlation with the number of cigarettes smoked; however, restricting the analysis to those smoking 26 cigarettes per day or less, a significant correlation between 4-ABP–Hb adduct levels and the number of cigarettes was found ($n = 28$, $r^2 = 0.14$, $p = 0.05$).

4-ABP–Hb adducts appeared to correlate with cotinine levels in all subjects ($n = 40$, $r^2 = 0.12$, $p = 0.03$) (figure 1a).

The TEAC in plasma was determined in order to investigate a possible relationship between this parameter and adduct formation. The TEAC appeared to be inversely related to 4-ABP–Hb adduct levels ($n = 40$, $r^2 = 0.17$, $p = 0.008$) (figure 1b). However, no significant correlation between TEAC and plasma cotinine levels was found. Furthermore, a significant difference was found for the TEAC of men compared with women (Mann–Whitney U test, $p = 0.02$), the

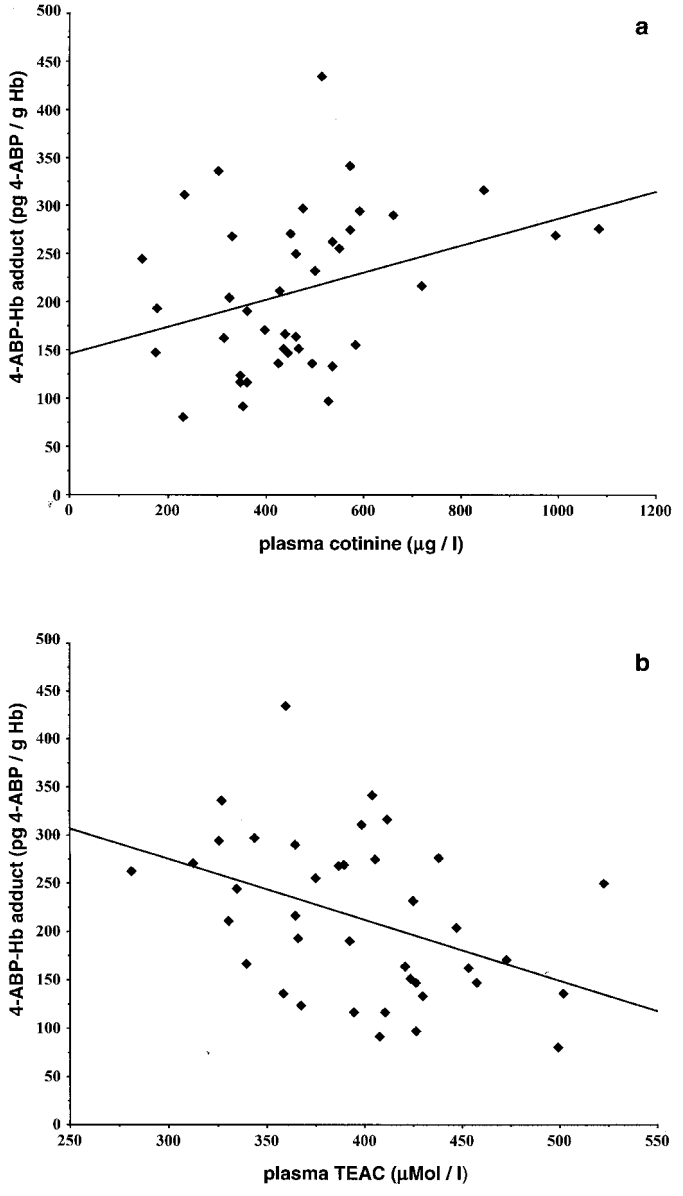

Figure 1. Linear regression analysis plot of 4-ABP–Hb adduct levels and (a) plasma cotinine concentrations ($n = 40$, $r^2 = 0.12$, $p = 0.03$); and (b) plasma TEAC ($n = 40$, $r^2 = 0.17$, $p = 0.008$).

concentration (mean ± SEM) being 424 ± 17 μmol l^{-1} for men and 384 ± 8 μmol l^{-1} for women. These results resemble the findings of Sharpe *et al.* (1996) for TRAP values. In our study no relationship was found between TEAC and age.

Multiple regression analysis of 4-ABP–Hb adduct levels with plasma cotinine and plasma TEAC resulted in a strong relationship, such that the 4-ABP adduct level (pg g^{-1} Hb) = 0.14 × plasma cotinine concentration (μg l^{-1}) −0.61 × plasma TEAC (μM) + 391 ($n = 40$, $r^2 = 0.29$, $p = 0.002$).

Discussion

Recent publications have demonstrated a difference in plasma antioxidant status (by TRAP assay) between smokers and non-smokers, smokers having lower antioxidant levels than non-smokers (Sharpe *et al.* 1996, Erhola *et al.* 1997, Nyyssönen *et al.* 1997). In this study we investigated whether there is a quantitative relationship between smoking dose and TEAC. The numbers of cigarettes smoked and plasma cotinine levels were available as markers of exposure. The relatively declining plasma cotinine levels with the increasing *self-reported* number of cigarettes smoked makes the latter parameter less suited to be used as a dose parameter. Apparently the intake of nicotine per cigarette becomes less when a person smokes more cigarettes per day; this effect might be due to an overrating of the number of cigarettes smoked, or reduced inhaling by people who report smoking large numbers of cigarettes per day. This effect has been reported before (Djordjevic *et al.* 1995, Hee *et al.* 1995). Therefore the plasma cotinine level was used as the biomarker of choice for exposure to cigarette smoke.

The idea that cigarette smoke components might be responsible for the lowering of the antioxidant capacity was not supported, since no correlation between dose (cotinine levels) and plasma TEAC was found. Several explanations for this lack of correlation can be found, such as inter-individual differences in enzymatic metabolism of tobacco-derived compounds, including nicotine, varying modulations of the TEAC due to differences in the antioxidant levels in the diet (Morabia *et al.* 1999, Ma *et al.* 2000, Van den Berg *et al.* 2001b), or the likelihood that components of cigarette smoke *other than nicotine* are responsible for lowering the antioxidant status.

One effect of the chemical exposure associated with smoking cigarettes is the formation of macromolecular adducts. The level of 4-ABP–Hb adducts was related to the number of cigarettes smoked when the data was restricted to a maximum number of cigarettes smoked per day. The adduct levels seemed to plateau at increasing self-reported doses. This phenomenon has previously been explained as a saturation effect (Van Schooten *et al.* 1997, Dallinga *et al.* 1998), but, since the same effect was found for plasma cotinine levels, it is probably due to a lower effective intake at higher cigarette consumption caused by decreased inhaling at a higher smoking rate (Djordjevic *et al.* 1995, Hee *et al.* 1995).

Since the plasma cotinine levels can be regarded as a measure of internal dose, we compared adduct formation with this parameter. Simple regression analysis of 4-ABP–Hb adduct concentration with plasma cotinine concentration resulted in a significant relationship ($n = 40$, $r^2 = 0.12$, $p = 0.03$). Apparently, the formation of this type of adduct is related to the plasma cotinine concentration and thus to the actual internal dose. The good correlation between the short-term (2–3 days) exposure marker cotinine and the long-term (90 days) biomarker of exposure 4-

ABP can be explained by the fairly constant smoking behaviour of the volunteers over many years.

The observed inverse correlation between 4-ABP–Hb adduct levels and the plasma TEAC ($n = 40$, $r^2 = 0.17$, $p = 0.008$) might be explained in two ways. Since 4-ABP–Hb adduct levels could be regarded as a marker of exposure to tobacco smoke, and antioxidant concentrations are known to be different (lower) for smokers compared with non-smokers (Sharpe *et al.* 1996, Erhola *et al.* 1997, Nyyssönen *et al.* 1997), the relationship could be regarded as a measure for the quantitative effect of tobacco smoke exposure on the TEAC. However, it would be difficult to explain the existence of such a quantitative relationship between TEAC and 4-ABP–Hb adduct levels but not between TEAC and plasma cotinine levels. Another explanation of the relationship opposes the first suggestion. For various reasons, including gender, smoking behaviour, possibly genetic variability and differences in diet (Morabia *et al.* 1999, Ma *et al.* 2000, Van den Berg *et al.* 2001b), there is an inter-individual variance in the plasma TEAC. This variance leads to differences in the protection against the various smoking-related carcinogens, including 4-ABP. A relatively low TEAC would lead to ineffective trapping of the reactive metabolites of these carcinogens and thus to a higher rate of adduct formation. This second idea is supported by the observations described in this paper: significant correlations were found between plasma TEAC and 4-ABP–Hb adducts and between plasma cotinine and 4-ABP–Hb adducts, but not between plasma cotinine and TEAC. It therefore seems reasonable to conclude that the plasma antioxidant status may be influenced by smoking behaviour, as was demonstrated by the different levels in smokers and non-smokers (Sharpe *et al.* 1996, Erhola *et al.* 1997, Nyyssönen *et al.* 1997), but is not quantitatively determined by smoking dose, which could be the case if the inter-individual variation is larger than the effect of smoking. The proposed cause-effect relationship is further supported by the results of multiple regression analysis with 4-ABP–Hb adduct levels as the dependent variable and cotinine and TEAC as the independent variables ($n = 40$, $r^2 = 0.29$, $p = 0.002$). The negative correlation between adduct levels and antioxidant status is particularly strong. The elevated levels of 4-ABP–Hb adducts caused by smoking cigarettes appear to be reduced when plasma antioxidant status (plasma TEAC) is high. This observation might present an indication that the adverse effects of smoking could be partly reduced by a sufficient antioxidant intake. This might also apply to non-smokers exposed to aromatic amines.

While (plasma) cotinine levels only reflect recent exposure to tobacco smoke, 4-ABP–Hb adducts could be expected to represent a steady-state situation, reflecting long-term continuous exposure. A distinct difference between these levels in smokers compared with non-smokers and quantitative relationships between smoking doses and adduct levels has been reported before, and this study confirmed these relationships. Moreover, we reported and explained another part of the variation in adduct levels due to the protective action of plasma antioxidant capacity on adduct formation. Apparently, one biological effect of smoking, the formation of 4-ABP–Hb adducts, can be described quantitatively as a function of smoking dose (cotinine levels) and protection by antioxidants (TEAC).

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