Free Radical Research, Volume 37 Number 12 (December 2003), pp. 1299–1305

Taylor & Francis
healthsciences

Benzo[a]pyrene Enhances Lipid Peroxidation Induced DNA Damage in Aorta of Apolipoprotein E Knockout Mice

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Accepted by Professor H. Sies

(Received 26 May 2003; In revised form 28 July 2003)

The genotoxic compound benzo[a]pyrene (B[a]P) enhances atherosclerotic plaque progression, possibly by inducing oxidative stress and subsequent lipid peroxidation (LPO). Since LPO plays a key role in atherosclerosis, stable LPO derived DNA modifications such as 1,N^6-ethenodeoxy-adenosine (εdA) and 3,N^4-ethenodeoxy-cytidine (εdC) may be useful biomarkers for *in vivo* oxidative stress. In this study, benzo[a]pyrene-diol-epoxide (BPDE)-DNA, εdA and εdC were determined by ^{32}P-postlabelling in apolipoprotein E knockout (ApoE-KO) mice treated with 5 mg/kg B[a]P by gavage. After 4 days, BPDE-DNA adduct levels were higher in aorta (10.8 ± 1.4 adducts/10^8 nucleotides) than in lung (3.3 ± 0.7, $P < 0.05$), which is a known target organ for B[a]P. Levels of εdA were higher in aorta of B[a]P-exposed animals than in unexposed controls (8.1 ± 4.4 *vs* 3.4 ± 2.1 adducts per 10^8 parent nucleotides, $P < 0.05$). On the other hand, εdC levels were not affected by B[a]P exposure. Serum low density lipoprotein (LDL) levels were lower in B[a]P-exposed mice than in controls (9.3 ± 3.7 and 13.3 ± 4.0 mmol/l, respectively), whereas high density lipoprotein (HDL) levels were higher (1.4 ± 1.6 and 0.4 ± 0.3 mmol/l, respectively). Consequently, a three-fold difference in the LDL/HDL ratio was observed ($P = 0.001$). εdA levels were positively related with plasma HDL concentrations ($R = 0.68$, $P = 0.02$), suggesting that the HDL mediated protection of the vessel wall against reactive lipid peroxides was reduced in B[a]P-exposed apoE-KO mice. Our observations show that direct as well as lipid peroxidation induced DNA damage is formed by B[a]P in aorta of apoE-KO mice, which may be involved in atherosclerotic plaque progression. This study further indicates that etheno-DNA adducts are useful biomarkers for *in vivo* oxidative stress in atherosclerosis.

Keywords: Atherosclerosis; Benzo(a)pyrene; Etheno-DNA adducts; Lipid peroxidation

Abbreviations: ApoE-KO, apolipoprotein-E knockout mice; B[a]P, benzo(a)pyrene; εdA, 1,N^6-ethenodeoxyadenosine; εdC, 3,N^4-ethenodeoxycytidine; LDL, low density lipoproteins; HDL, high density lipoproteins

INTRODUCTION

Cardiovascular diseases and cancer are both characterized by uncontrolled proliferation of cells and share common risk factors, such as cigarette smoking and dietary habits [1]. Autopsy studies demonstrated that atherosclerosis and cancer tend to occur in the same individuals [2], and these observations led to the hypothesis that the process of atherogenesis may develop like a benign tumor according to an initiation–promotion mechanism [2]. In this monoclonal theory of atherosclerosis, it is hypothesized that monoclonal expansion of cells in atherosclerotic plaques may result from acquired mutations due to DNA damage, caused directly by mutagenic agents (e.g. benzo[a]pyrene, B[a]P) or agents endogenously generated by oxidative stress/lipid peroxidation inflicted by inflammation. Earlier studies indeed suggested that acquired mutations initiate at least part of the atherosclerotic lesion [3,4]. Although the monoclonal theory of atherosclerosis is disputed, it does not completely contradict the response-to-injury theory, in which it is hypothesized that atherosclerosis is a vascular reaction towards inflammatory insults.

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000162133

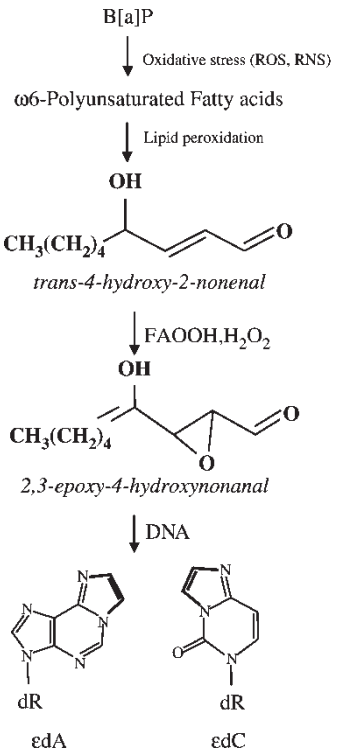

FIGURE 1 Scheme of formation of etheno-DNA adducts after B[a]P exposure. *Trans*-4-hydroxy-2-nonenal (HNE) is formed by the action of reactive oxygen and nitrogen species (ROS and RNS, resp.). HNE is converted to 2,3-epoxy-4-hydroxynonanal by fatty acid hydroperoxides, which subsequently reacts covalently with DNA to form etheno-bridged base modifications (indicated with bold lines in the chemical structures of 1,N^6-ethenodeoxyadenosine [εdA] and 3,N^4-ethenodeoxycytidine [εdC].

Oxidative stress, which plays a key role in the onset and progression of atherosclerosis and cancer, can cause DNA damage and many human cancers are thought to arise from mutations as a result of persistent DNA damage during cell replication. DNA bases damaged by reactive oxygen species and proteins modified by lipid peroxidation products have indeed been detected in human atherosclerotic lesions [5]. One of the lipid peroxidation products, *trans*-4-hydroxy-2-nonenal (HNE), is known to bind covalently to cellular DNA after its epoxidation to form exocyclic etheno-base adducts (Fig. 1) [6]. Among the etheno-bridged base modifications, 1,N^6-ethenodeoxyadenine (εdA) and 3,N^4-ethenodeoxycytosine (εdC) are the most studied ones and were found to be highly miscoding lesions [7]. Therefore, these types of DNA adducts are promising early stable biomarkers for DNA damage caused by oxidative stress/lipid peroxidation in the pathogenesis of atherosclerosis.

Major advances have been made in our understanding of the role of apolipoprotein E in the onset and development of atherosclerosis. Enhanced oxidative stress has been found in apolipoprotein E knockout mice (ApoE-KO mice), which develop severe spontaneous atherosclerosis [8]. Using antibodies directed against HNE-lysine conjugate, increased levels of low-density lipoprotein (LDL)-oxidation specific epitopes have been identified in aortic lesions of ApoE-KO mice. Moreover, high titers of autoantibodies against the malondialdehyde-lysine conjugate have been measured in serum of these animals [9]. Circulating lipoproteins in ApoE-deficient mice are more oxidized and more susceptible to oxidation than lipoproteins from wild-type animals [9]. Therefore, ApoE-KO mice offer an excellent animal model to investigate the role of oxidative stress induced DNA damage in vascular diseases.

It is known that diet and smoking are separately as well as synergistically acting risk factors in both carcinogenesis and atherogenesis. B[a]P is a well known genotoxic compound, present in cigarette smoke and pyrolysed food products, for instance grilled meat. Exposure to B[a]P may result in the formation of bulky DNA adducts, but is also linked to oxidative stress and subsequent lipid peroxidation [10], which might be associated with the B[a]P-related vascular toxicity. In the present study, we investigated whether exposure to this environmental carcinogen, which was found to influence plaque progression in ApoE-KO mice [11], affects lipid peroxidation *in vivo* and subsequent formation of etheno-DNA adducts in mouse aorta.

MATERIALS AND METHODS

Animals and Their Treatment

Twenty male 6–8 weeks-old apoE-knockout C57Bl/6 mice (purchased from IFFA CREDO S.A. a Charles River Company, Lyon, France) were used in our experiments. The animals were put either on a high fat/high cholesterol diet (HFC; 16% fat, predominantly cocoa-butter which contains 35% stearic acid, 35% oleic acid, 25% palmitic acid, 3% Linoleic acid and 2% others) or a normal mouse chow (LFC, 6.3% crude fat, SRM-A, Hope Farms, Woerden, The Netherlands) during 25 days (10 animals per group). Four days before the end of the experiment and after an overnight fasting period, all animals received either a single oral dose by gavage of 5 mg/kg bw B[a]P (Sigma, St. Louis, MO, USA) dissolved in tricaprylin (5 animals on HFC and 5 on LFC diet) or vehicle only. Animals were anaesthetized by i.p. injection of Nembutal® (0.5 ml sodium phenobarbital/kg bw). Approximately 0.5 ml blood was drawn from the inferior caval vein and used for measurement of lipid levels. The arterial tree was perfused *in situ* for 3 min with 0.9% NaCl via a catheter into the left ventricular apex. Subsequently, lungs and the arterial tree

(abdominal aorta, thoracic aorta and the aortic arch including the brachiocephalic trunk, left carotid artery and left subclavian artery) were dissected out and stored at −80°C until DNA isolation.

DNA Isolation

Lung tissue and the complete aorta were homogenized in lysis-buffer (DNA-isolation kit supplied by Qiagen, Hilden, Germany) using a polytron homogenizer (15,000 rpm for 30 s). Proteinase K and RNases were added, followed by a 2 h incubation at 50°C. Subsequently, DNA was isolated using Qiagen columns according to the manufacturer's protocol with the following modification of the supplied elution buffer; pH was set to 7.4 and the NaCl concentration was increased to 1.4 M. DNA was precipitated by the addition of 0.7 volume isopropanol, collected by centrifugation, washed twice with 70% ethanol and dried *in vacuo*. Before analysis, DNA was redissolved in water and quantified by spectrophotometry at 260 nm.

Analysis of εdA and εdC

εdA and εdC were analyzed in DNA by immunoaffinity/^{32}P-postlabelling [12]. In brief, ca. 25 μg of DNA was hydrolyzed to nucleotide 3′-monophosphates using micrococcal nuclease and spleen phosphodiesterase. Normal nucleotides were quantitated by high-performance liquid chromatography and adducts were enriched on immunoaffinity columns prepared from the monoclonal antibodies EM-A-1 (εdA) and EM-C-1 (εdC). The antibodies used in this study were provided by Rajewsky (Institute of Cell Biology, University of Essen, Essen, Germany). The adducts and the internal standard deoxyuridine 3′-monophosphate were labeled with [γ-^{32}P]ATP (>5000 Ci/mmol) and T4 polynucleotide kinase (Amersham Buchler, Braunschweig, Germany and Pharmacia Biotech, Freiburg, Germany, respectively). Adducts were resolved on polyethyleneimine-TLC plates using two-directional chromatography [D1 = 1 M acetic acid (pH 3.5), D2 = saturated ammonium sulfate (pH 3.5)]. After autoradiography, the adduct spots and the internal standard were marked, cut, and the radioactivity was measured in a liquid scintillation counter. The absolute adduct levels were quantitated using standards, and the relative adduct level per parent nucleotides was determined with the amount of deoxycytidine (dC) and deoxyadenosine (dA) obtained from high-performance liquid chromatography analysis of normal nucleotides obtained from the DNA digest. Typical chromatograms are shown in Fig. 2.

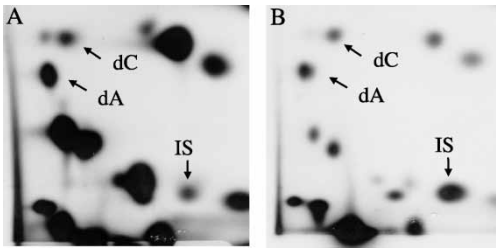

FIGURE 2 Adduct profiles after ^{32}P-postlabelling and thin layer chromatography of an aorta sample (panel A) and a standard mixture of 1,N^6-ethenodeoxyadenosine [εdA] and 3,N^4-ethenodeoxycytidine [εdC] (Panel B). IS = internal standard (dUp) used for quantitation purposes.

Analysis of BPDE-DNA Adducts

Nuclease P1 enriched ^{32}P-postlabelling analysis was performed [13] to assess benzo[a]pyrene-diol-epoxide (BPDE)-DNA adduct levels in aorta and lung. Briefly, DNA was enzymatically digested and unmodified nucleotides were dephosphorylated by nuclease P1. Labeling was carried out with excess [γ-^{32}P]-ATP and T4-polynucleotide kinase. 5′-Labeled adducts were resolved on polyethylenimine-cellulose TLC sheets (Merck, Darmstadt, Germany) using the solvents as described in Ref. [13]. In each experiment, 3 standards of [^{3}H]-BPDE modified DNA with known modification levels (1 per 10^7, 10^8 and 10^9 nucleotides) were run in parallel for quantification purposes. These standards were synthesized by incubations of calf-thymus DNA with [^{3}H]-BPDE (NCI, USA) and their modification levels were determined by ^{3}H-scintillation counting. Quantification was performed by using a phosphor-imager (Molecular Dynamics™, Sunnyvale, CA, USA).

Measurement of Blood Lipids

Standard enzymatic techniques were used for the assessment of plasma lipid levels, automated on the Cobas Fara centrifugal analyzer (Hoffmann-La Roche, LTD, Basel, Switzerland). Total plasma cholesterol and high density lipoproteins (HDL) were measured using kit no. 07-3663-5 and no. 543004 (Hoffmann-La Roche), total glycerol using kit no 337-40A/337-10B (Sigma) and free glycerol was measured using kit no. 0148270 (Hoffmann-La Roche). Precipath (standardized serum) was used as standard. Low density lipoprotein (LDL) levels were calculated in mmol/L using the formula: *LDL − cholesterol = total cholesterol − (triglycerides*/2.2) − *HDL cholesterol*.

Statistical Analysis

The results are presented as mean ± SD. Differences in etheno-DNA adduct levels and plasma lipid levels between B[a]P treated mice and vehicle

treated mice were compared by the non-parametric Mann–Whitney U test (no tied ranks present in the data). Relationships between various parameters were studied using simple regression analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Diet and B[a]P on Blood Lipids and Body Weight

Plasma levels of HDL, LDL, total cholesterol and triglycerides were found to be slightly increased in ApoE-KO mice that received a high fat diet as compared to those on a low fat diet (Table I). Treatment of these mice with a single oral dose of B[a]P (5 mg per kg bodyweight) and assessment of serum lipid levels 4 days after treatment, showed a significant reduction of LDL, and a concomitant increase of HDL-levels (Table I). Consequently, the overall LDL/HDL ratio was three-fold decreased from 47.1 ± 21.4 to 15.9 ± 13.9 ($P = 0.001$). Animals on a high fat diet gained more weight during the experiment as compared to the mice on a low fat diet and therefore weighed approximately 20% more than those on a low fat diet at the time of exposure to B[a]P (Table I).

Effect of Diet and B[a]P on Etheno-DNA Adduct Formation in Aorta

Low or high fat diets for a period of 25 days did not differently affect adduct levels (expressed per 10^8 parent nucleotides): εdA, 2.9 ± 1.6 and 3.9 ± 0.8 adducts, and εdC, 6.3 ± 3.9 and 5.9 ± 3.3 adducts, respectively in aorta of ApoE-KO mice. In control animals, mean εdA levels were approx. two-fold lower than εdC levels ($P = 0.07$), and both DNA adduct types were interrelated ($R = 0.62$, $P = 0.04$, $n = 10$), suggesting similar pathways in their formation.

εdA Levels were significantly higher in animals which were exposed to B[a]P as compared to unexposed controls (8.1 ± 4.4 and 3.4 ± 2.1, respectively, animals on low and high fat diet combined, $n = 10$ per group, $P < 0.05$). Similar effects were observed in animals on low or high fat diets separately (Table I). Levels of εdC were unaffected by acute oral exposure to B[a]P. As a result, there was no significant relationship between εdA and εdC levels in B[a]P-exposed animals ($R = 0.33$, $P = 0.34$).

Bulky Adduct Formation in Aorta and Lung

BPDE-DNA adduct formation in apoE-KO mice on a normal lab chow was three-fold higher in aorta (10.8 ± 1.4 BPDE-DNA adducts/10^8 nucleotides) than in lung (3.3 ± 0.7, $P < 0.05$). In animals kept on a high fat diet, DNA adduct levels were even >six-fold higher in aorta (13.1 ± 3.2) than in lung DNA (2.1 ± 0.5). A significant relationship was observed between BPDE-DNA adduct levels and levels of εdA ($R = 0.65$, $P = 0.002$), which was not found for εdC ($R = 0.07$, $P = 0.77$). BPDE-DNA adducts were not detectable in lung and aorta of unexposed controls.

Relationship between Etheno-DNA Adducts with Blood Lipid Levels

LDL/HDL ratios decreased significantly and εdA levels were concomitantly increased after treatment with B[a]P. As a result, an inverse relationship was observed between εdA and the LDL/HDL ratio in B[a]P exposed animals ($R = -0.60$, $P = 0.005$, Fig. 3A), but not in unexposed controls. Further analysis revealed that this was predominantly due to a strong positive relation between εdA and HDL

TABLE I Effect of B[a]P exposure and type of diet on serum levels of low density lipoproteins (LDL), high density lipoproteins (HDL), triglycerides and the formation of DNA adducts in mouse aorta

	Not exposed to B[a]P, (N=5)		Exposed to B[a]P, (N=5)	
	Low fat diet	High fat diet	Low fat diet	High fat diet
Body weight (g)				
Before experiment	22.7 ± 1.0	25.0 ± 0.9	23.9 ± 0.8	25.4 ± 1.0
After experiment	22.6 ± 1.5	27.4 ± 2.4	23.8 ± 1.0	28.1 ± 1.3
Weight gain	−0.1 ± 0.7	2.4 ± 2.1	−0.1 ± 0.8	2.7 ± 0.7
Lipoprotein levels (mmol/l)				
LDL	12.6 ± 2.6	14.2 ± 5.3	6.6 ± 2.4*	14.1 ± 3.1
HDL	0.2 ± 0.1	0.6 ± 0.4	0.6 ± 0.6**	2.5 ± 1.9**
Triglycerides	1.0 ± 0.2	1.2 ± 0.5	1.4 ± 0.4	0.8 ± 0.1
LDL/HDL	54.7 ± 12.2	39.5 ± 27.2	21.3 ± 16.1*	9.1 ± 8.0**
DNA adducts (adducts per 10^8 parent nucleotides)				
εdA	2.9 ± 1.6	3.9 ± 0.8	7.3 ± 4.4**	8.8 ± 4.7*
εdC	6.3 ± 3.9	5.9 ± 3.3	4.9 ± 2.3	6.5 ± 3.6
BPDE-DNA[†]	ND	ND	10.8 ± 3.3	13.1 ± 3.2

*: $P < 0.05$, **: $P < 0.07$ as compared to unexposed controls on same diet. ND: Not detectable. [†] Adduct level expressed as adducts per 10^8 total nucleotides.

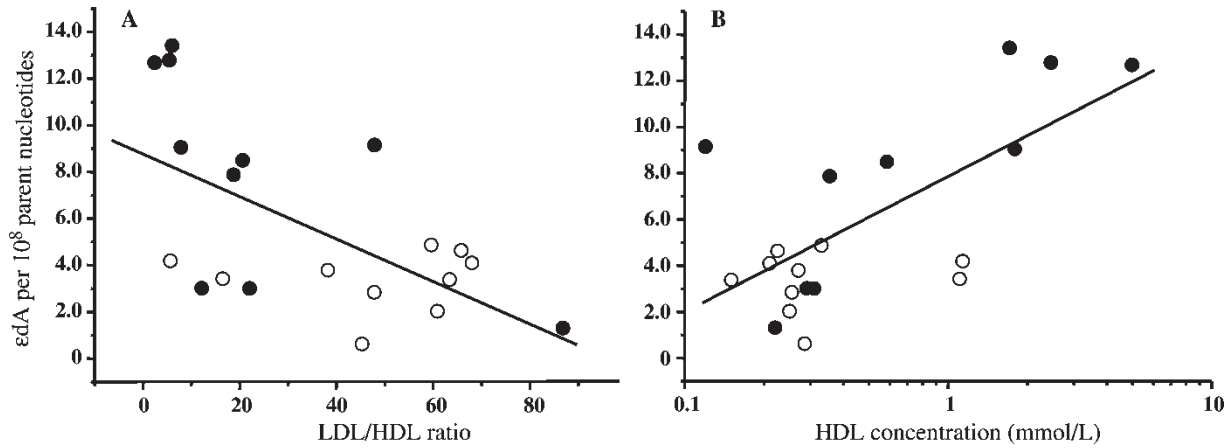

FIGURE 3 (A) Correlation between the LDL/HDL-ratio and levels of 1,N^6-ethenodeoxyadenosine (εdA) ($r = -0.60$, $P = 0.005$, $n = 20$). (B) Correlation between serum HDL-concentrations and levels of εdA ($R = 0.68$, $P = 0.02$, $n = 20$). Values of B[a]P-exposed animals are indicated as (•), and unexposed controls as (○).

concentrations ($R = 0.68$, $P = 0.02$, Fig. 3B). On the other hand, no such relationship was observed between εdA and LDL levels. Serum lipid concentrations were not related to levels of εdC in aorta of apo-KO mice (LDL, $R = 0.01$, $P = 0.98$; HDL, $R = 0.39$, $P = 0.09$).

DISCUSSION

The well-studied environmental carcinogen B[a]P was found to enhance atherosclerotic plaque progression in experimental animals [11], but the mechanism by which B[a]P modulated the atherogenic process is not known. Since lipid peroxidation seems to play a predominant role in the aetiology of atherosclerosis, we hypothesized that B[a]P accelerates plaque progression *via* induction of oxidative stress with subsequent lipid peroxidation in the vessel wall. Increased lipid peroxidation after exposure to B[a]P would result in an increased formation of etheno-DNA adducts in aorta, which are frequently applied as stable biomarkers for *in vivo* oxidative stress [7]. This was indeed observed in the present study; the lipid peroxidation related DNA lesion 1,N^6-ethenodeoxyadenosine (εdA) was found to be 2.5 fold increased in aorta of B[a]P exposed ApoE-KO mice as compared to unexposed controls. On the other hand, no increased levels of εdC were found in B[a]P-exposed mice, which is actually formed by similar pathways as compared with εdA. Differences in DNA repair efficiency may explain this discrepancy; εdA is repaired by 3-methyladenine DNA glycosylase, whereas εdC is repaired by a mismatch specific thymidine-DNA glycosylase [7]. DNA repair processes were found to be up regulated in aortas of hypercholesterolemic laboratory rodents [14], but no information is currently available on specific DNA repair processes and their dietary modulation (or up-regulation due to oxidative stress) in aorta of ApoE-KO mice.

Formation of BPDE-DNA adducts was found to be 3 (low fat diet) to 6 fold (high fat diet) higher in aorta as compared to lung, which is an important target organ for the genotoxicity of B[a]P. Next to the formation of BPDE-DNA adducts, B[a]P is metabolically converted into B[a]P-quinones, which can undergo redox-cycling to their corresponding B[a]P-diols, producing superoxide and hydroxyl radicals by the Haber-Weiss and Fenton reactions [10]. Superoxide and hydroxyl radicals can subsequently give rise to increased levels of oxidative stress and the LPO derived pro-mutagenic etheno-bridged base modifications. These data indicate that aorta is an additional biologically relevant target for B[a]P.

Although the formation of BPDE-DNA adduct levels in aorta as compared to lung was increased by a high fat diet, this type of diet did not alter etheno-DNA adduct levels. However, since we wanted to study the effect of B[a]P exposure on lipid peroxidation in the early phase of atherogenesis, the animals used in this study might have been too young and/or the feeding period too short to observe strong effects of diet on etheno-DNA adduct formation. Moreover, the high fat diet predominantly contained saturated fatty acids, which are much less susceptible for LPO than ω-6 polyunsaturated fatty acids (PUFA's).

After B[a]P exposure, an increase of HDL levels and a concomitant decrease of serum LDL concentrations was found, which was also observed in human volunteers who consumed charcoal-grilled meat containing B[a]P [15]. This effect of B[a]P on serum lipid levels was found to be due to the induction of hepatic cytochrome P450's [16], and was expected to be a beneficial change, because HDL may act as an antioxidant and thus may suppress

oxidative stress in the vessel wall. However, this was not in agreement with the positive relation between εdA and HDL levels as observed in the present study (Fig. 3B), which in fact suggests that HDL acted as a pro-oxidant in this experimental model of environmentally induced atherosclerosis. HDL in the basal state is anti-inflammatory and capable of destroying oxidized lipids that generate an inflammatory response [17]. However, HDL in ApoE-KO mice can be altered and become pro-inflammatory [18], which has also been observed during the acute phase responses after bacterial or viral infections [19] and exposure to cigarette smoke [20]. Changes in the functionality of HDL, rather than changes of plasma HDL levels determine the anti-atherogenicity of HDL. Acute exposure to B[a]P may induce a remodeling of HDL to its pro-inflammatory state. Furthermore, B[a]P is predominantly transported in rodents by HDL [21] and may thus be specifically taken up by HDL from the vessel wall during the reverse cholesterol transport. HDL was positively associated with εdA levels in animals exposed to B[a]P, which is consistent with the hypothesis that cigarette smoke constituents modify serum HDL such that there is an increased risk of coronary artery disease due to a diminished capacity to protect lipoproteins from oxidative stress [22]. More studies are necessary to elucidate the exact role of B[a]P or other tobacco smoke carcinogens in modulating the protective capacity of HDL against reactive lipid peroxides.

DNA damage remains a poorly examined field in atheroslerosis, but our current findings show that the analysis of oxidatively damaged DNA bases may provide relevant and stable biomarkers for *in vivo* oxidative stress in human atherosclerosis or animal models. The covalent interaction of reactive carcinogenic metabolites with DNA (i.e. DNA adducts) is thought to be a critical initial step in chemically induced carcinogenesis, and DNA adduct measurements are therefore frequently used as biomarkers for exposure to carcinogenic compounds. High levels of DNA adducts were also detected in human aorta [23], heart tissue of patients with atherosclerotic plaques [24] and in cardiovascular tissue of animal models [25], indicating that the vascular system may represent a relevant target organ. The etiological role of these DNA modifications in atherosclerosis, however, remains to be demonstrated. We conclude that B[a]P exposure results in increased lipid peroxidation in the vessel wall, which may explain the promoting effect of B[a]P on atherogenesis.

Acknowledgements

R. Godschalk was supported by a EU-Marie Curie Fellowship (QLK4-CT-2000-51241).

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