Cardiovasc Toxicol (2007) 7:192–201
DOI 10.1007/s12012-007-0027-z

Potentially Reduced Exposure Cigarettes Accelerate Atherosclerosis: Evidence for the Role of Nicotine

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Published online: 25 July 2007

Abstract The tobacco industry markets potentially reduced exposure products (PREPs) as less harmful or addictive alternatives to conventional cigarettes. This study compared the effects of mainstream smoke from Quest, Eclipse, and 2R4F reference cigarettes on the development of atherosclerosis in apolipoprotein E-deficient (apoE –/–) mice. Mice were exposed to smoke from four cigarette types for 12 weeks beginning at age of 12 weeks, and in a separate study for 8 weeks, beginning at age of 8 weeks. In both studies, mice exposed to smoke from high-nicotine, high-tar Quest 1, and 2R4F cigarettes developed greater areas of lipid-rich aortic lesions than did non-smoking controls. Exposure to smoke from the lower-nicotine products, Eclipse, and Quest 3, was associated with smaller lesion areas, but animals exposed to smoke from all of the tested types of cigarette had larger lesions than did control animals not exposed to smoke. Urinary levels of isoprostane F2 alpha VI, increased proportionally to cigarette nicotine yield, whereas induction of pulmonary cytochrome P4501A1 was proportional to tar yield. Lesion area was associated with both nicotine and tar yields, although in multiple regression analysis only nicotine was a significant predictor of lesion area. Smoke exposure did not alter systolic blood pressure (SBP), heart rate (HR), blood cholesterol, or leukocyte count. Taken together, these observations suggest that smoking may accelerate atherosclerosis by increasing oxidative stress mediated at least in part via the actions of nicotine.

Keywords Isoprostane · Oxidative stress · cytochrome P450 · Sympathetic nervous system · Complete blood count

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Introduction

The tobacco industry promotes potentially reduced exposure products (PREPs) with claims that they are less harmful or less addictive than standard cigarettes because they deliver lower doses of toxic, carcinogenic, and/or addictive agents. Among the currently marketed PREPs are the cigarettes Eclipse and Quest. Eclipse heats the air inhaled through the cigarette to volatilize its contents without burning the tobacco resulting in lower levels of nicotine and tar delivery than from conventional cigarettes. A recent study showed lower tumorigenicity of smoke condensate from Eclipse compared to a conventional reference cigarette (1R4F) [1]. Quests 1–3 are a group of conventionally burned cigarettes in which genetically modified low-nicotine tobacco is blended with normal tobacco to provide three cigarette types containing different nicotine levels ranging between 0.6 mg and 0.05 mg/cigarette. Quest cigarettes are marketed as

providing smokers the opportunity to gradually reduce their level of nicotine intake. The health risks associated with the use of these products have not been adequately investigated.

The cardiovascular risks associated with cigarette smoking include atherosclerosis, thrombosis, and vascular dysfunction [2]. Cigarette smoke may promote atherosclerosis by inducing inflammation, vasomotor dysfunction and proatherogenic changes in lipid profile [2, 3]. In humans, smoking causes a proatherogenic lipid profile in which serum triglycerides, cholesterol, and LDL-cholesterol are elevated, while HDL-cholesterol decreases [2, 4, 5]. Smoking may also affect the levels of circulating monocytes, and induce expression of adhesion molecules that accelerate the initiation and progression of atherosclerosis [6].

Despite the well-known association of tobacco smoke exposure with cardiovascular disease, few studies have sought to identify the components of tobacco smoke that promote atherosclerosis, in part because of the lack of suitable animal models [7–12]. Several recent studies have examined the effects of smoke and smoke components on atherosclerosis in apolipoprotein E-deficient (apoE –/–) mice, which develop lesions similar to those of humans over a relatively short time frame (8–16 weeks of age) [13]. Exposure to cigarette sidestream smoke has been shown to stimulate atherosclerosis in apoE –/– mice [14] and oral administration of either nicotine or benzo[a]pyrene, two principal smoke components, can stimulate the growth of atherosclerotic lesions in apoE –/– mice [12, 15]. However, neither the effects of mainstream smoke nor the relative contributions of inhaled nicotine and tar to the atherogenicity of tobacco smoke have been investigated.

The objectives of the present study were to determine if exposure to mainstream tobacco smoke affects aortic atherosclerosis in apoE–/– mice and if lesion areas differ after exposure to smoke from conventional cigarettes compared to smoke from the PREPs, Quest, and Eclipse.

Materials and Methods

All animal experimental procedures were approved by the Weill Medical College Institutional Animal Care and Use Committee. Male apoE –/– mice obtained from Jackson Laboratories were maintained on a normal laboratory diet containing 4.5% fat (Labdiet 5053). In Study 1, groups of 10 mice were exposed to smoke from each cigarette type for 12 weeks beginning at age of 12 weeks. In Study 2, groups of 20 mice were exposed to smoke from each cigarette type for 8 weeks beginning at age of 8 weeks.

Smoke Exposure

Nicotine and tar yields of the cigarettes used in these studies are shown in Table 1. Nicotine and tar yields are as stated by the manufacturer based on their implementation of Federal Trade Commission (FTC) protocols. 2R4F research cigarettes were obtained from the Kentucky Tobacco Research Institute, and were stored at 4°C. Eclipse, Quests 1 and 3 cigarettes were obtained freshly from retail sources every 1–2 weeks and stored at room temperature. All cigarettes were equilibrated at ambient temperature and humidity for at least 18 h before use.

Smoke exposure was carried out using a 24-port smoke delivery manifold (CH Technologies, Westwood NJ). Mice were placed in Plexiglas holders fitted with an aluminum end cap through which they directly breathed the tobacco smoke from the manifold. Mice were smoke-exposed for 1 h each day from Monday through Friday of each week, except for holidays. Animals in Study 1, the age of 12–24 weeks exposure group, were smoke-exposed for exactly 1 h/session, during which 10–12 cigarettes were burned. Animals in Study 2, the age of 8–16 weeks exposure group, were exposed to smoke from exactly 12 cigarettes per session, which took approximately 1 h. Smoke delivery was controlled by a computer-driven pump/valve mechanism that smoked cigarettes serially one at a time, delivering the smoke directly to the manifold chamber. Cigarettes were smoked according to a modified FTC protocol at the rate of 2 puffs/min, 2 s/puff, and 35 mL/puff. Airflow through the smoke manifold was 4 L/min, which diluted the smoke stream and filled the manifold with fresh air between puffs. Total smoke particulate (TSP) was quantified by aspirating smoke at a fixed rate through a glass micro fiber filter attached to one of the manifold ports, and the weight of smoke product collected on the filter divided by the total aspirated volume was calculated as the TSP in mg/m^3. Control animals were placed in the smoking chambers for 1 h/day on a similar schedule, but were not exposed to cigarette smoke during that time.

Physiological Measurements

Animals were weighed weekly. Systolic blood pressure (SBP) and heart rate (HR) were measured by non-invasive tail-cuff plethysmography using a Visitech 2000 automated device as described previously [16]. SBP was measured in the mornings, before animals were smoke exposed. Blood was collected by retrorbital puncture after light anesthesia induced with isoflurane. For Study 1, hematocrit was measured by centrifugation of whole blood collected in heparinized hematocrit tubes. For Study 2, blood was diluted 4-fold in 10 mmol/L EDTA/5% BSA and a complete blood count (CBC) carried out using a Bayer ADVIA 120 analyzer. At the midpoint and last week of each study, urine was collected from animals housed in metabolic cages. Urine was collected after the mice had been smoke

Table 1 Cigarette nicotine and tar. FTC yields and total smoke particulate exposure

Length of exposure (weeks)	FTC nicotine yield (mg)	FTC tar yield (mg)	TSP Study 1 (mg/m^3) 8	TSP Study 2 (mg/m^3) 12
Quest 1	0.6	10	395 ± 16	475 ± 2
Quest 3	0.05	10	383 ± 15	446 ± 2
2R4F	1.0	10	334 ± 12	459 ± 2
Eclipse	0.2	5	187 ± 12	272 ± 1
Control	NA	NA	NA	NA

NA, not applicable

exposed for four consecutive days. Urinary total nicotine and cotinine were analyzed as described previously [17]. Total and HDL cholesterol were measured using kits from WAKO diagnostics following the manufacturer's instructions. Isoprostane F2 alpha VI, a sensitive and specific marker of lipid peroxidation, was measured in urine as described previously [18].

Tissue Collection and Measurements

After the final smoke exposure, animals were deeply anesthetized with isoflurane and exsanguinated. The heart and vasculature were perfused for 10 min with 4% paraformaldehyde in 0.15 M NaCl, delivered at 100 mmHg pressure via a needle placed in the left ventricle, removed *en bloc*, and then immersed in the same fixative for 24 h before further processing. The aorta was divided into three sections: aortic arch (aortic sinus to the brachiocephalic artery), suprarenal aorta (brachiocephalic artery to the left renal artery), and infrarenal aorta (left renal artery to the iliac bifurcation). A circular cross section approximately 2 mm long was taken from the distal end of each of these fixed segments and paraffin-embedded for histology. The remaining aortic segments were opened longitudinally and stained *en face* with Oil Red O to visualize lipid deposits corresponding to lesions. Atherosclerotic lesion area was quantified by digital microscopy and expressed as the percentage of the lumenal area covered by lipid-rich lesions. All measurements were carried out by an observer blinded to the treatment group of the animals. Measurements were repeated by a second independent observer also blinded to treatment. Lungs were stored frozen in liquid nitrogen. Lung cytochrome P450 1A1 (CYP1A1) content was determined by western blotting as described previously [19].

Statistical Analysis

Data were analyzed using Dunnet's test for multiple comparison to single control. For multiple comparisons between groups Student's *t*-test was used, applying the Bonferroni adjustment as appropriate. $p < 0.05$ was considered significant. Statistical analysis was carried out using Statview software Version 5.0.1 (SAS Institute, Cary, NC).

Results

Smoke Exposure

In Study 1 (12–24 weeks exposure), cigarettes burned according to the modified FTC protocol over 1 h produced a TSP of 300–400 mg/m^3 except for Eclipse, which produced a TSP of ~190 mg/m^3, proportional to the lower tar content of this product (Table 1). There were no significant differences in TSP among Quests 1 and 3 and 2R4F, over the 12 weeks of the study as evaluated on a daily basis. In Study 2 (8–16 weeks exposure), TSP for each cigarette type was 20–40% higher than in Study 1, although the variability in TSP for each type of cigarette was much lower because a fixed number of cigarettes was burned in each session.

Total nicotine and cotinine measured in the urines of Study 1 mice were proportional to the FTC nicotine yield of each cigarette, although the slope of the relationship tended to be lower at high FTC nicotine yield (Fig. 1).

Atherosclerotic Lesion Area

Atherosclerotic lesion area was measured *en face* in Oil Red O stained whole mounts of aortic arch, suprarenal aorta, and infrarenal aorta (Fig. 2). Comparisons were made between each smoke exposed group and control, and each smoke exposed group and 2R4F (Fig. 2). In Study 1, lesion areas were approximately double the area of lesions in controls in the aortic arch, infrarenal aorta, and all aortic segments combined (total) of animals exposed to high-nicotine 2R4F and Quest 1 smoke. Among animals exposed to smoke, only in the Eclipse group was the measurement of total lesion area significantly lower than in the 2R4F group.

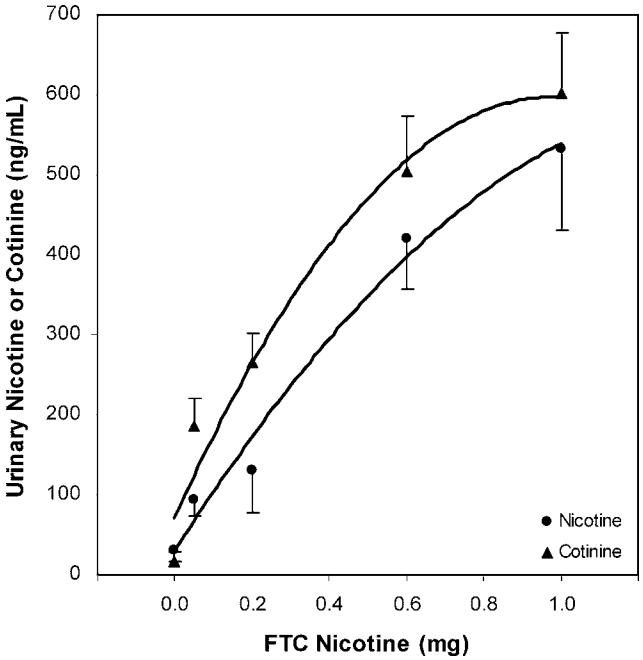

Fig. 1 Relationship of urinary nicotine and cotinine excretion after 12 weeks smoke exposure in Study 1 mice to FTC nicotine yield stated by the manufacturer. Two or three mice were placed in each metabolic cage overnight. Each point represents the mean and SEM of four separate group collections

In Study 2 animals, lesion areas were generally smaller than in Study 1 animals, presumably reflecting both younger age and the shorter period of smoke exposure. Lesion areas in mice varied with type of cigarette: animals exposed to high-nicotine 2R4F and Quest 1 smoke had lesions 0.5 to 3-fold larger than in control mice. Although lesion areas in mice exposed to the lower nicotine Quest 3 and Eclipse smoke were smaller than those in mice exposed to 2R4F or Quest 1 smoke, they were still significantly larger than controls. Moreover, suprarenal and total aortic lesion areas in Quest 3 and Eclipse exposed mice were significantly smaller than in 2R4F exposed mice. Similarly, Quest 1 exposed mice had significantly smaller lesion areas in the total aorta than did 2R4F exposed mice. These conclusions were supported by measurements made by two independent observers.

Relationship between Lesion Area, Nicotine, and Tar

Plots of total lesion areas against the FTC nicotine and tar yields of the cigarettes tested revealed dose–response relationships between both nicotine and tar with total lesion area for both studies (Fig. 3). Multiple regression analyses indicated that only nicotine was a significant predictor of lesion area in the 12–24 weeks study ($r^2 = 0.289$, $p < 0.002$), whereas in the 8–16 weeks study, both nicotine ($p < 0.001$) and tar ($p < 0.02$) were significant predictors

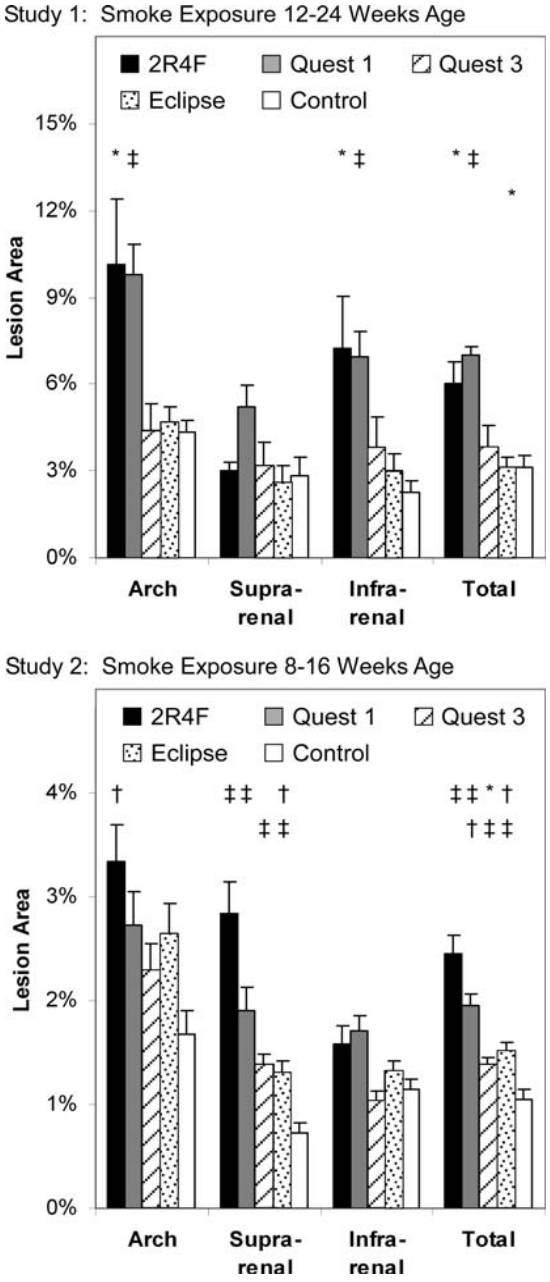

Fig. 2 Lesion areas measured *en face* in aortic segments (mean and SEM). Study 1 utilized 10 animals per group; Study 2 had 20 animals per group. A cube root transformation was applied to the data to normalize the variance between groups (raw data are shown in Fig. 3). Lesion areas were compared between each smoke exposure group and control (p values, upper row of symbols), and between each smoke exposure group and 2R4F (p values, lower row of symbols). p values are adjusted for seven comparisons. *$p < 0.05$, †$p < 0.005$, ‡$p < 0.001$

of lesion area that together accounted for almost half of the variance ($r^2 = 0.47$). A large part of the effect of tar observed in the 8–16 weeks study may reflect the smaller lesion area in the control group. When the smoke exposed groups were compared to one another, nicotine content alone remained a significant predictor of lesion area. Both

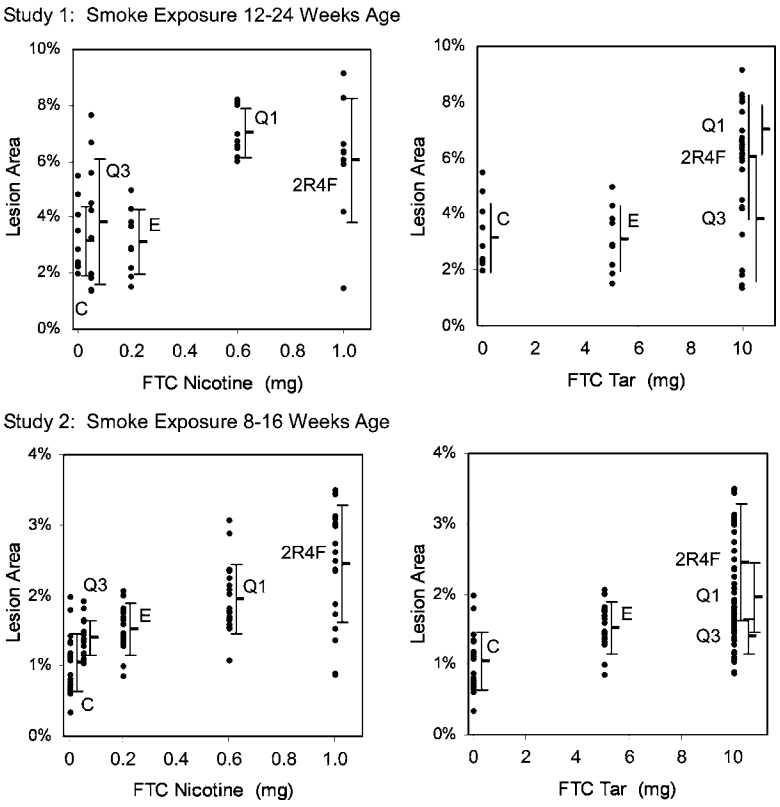

Fig. 3 Relation between total lesion area and FTC nicotine and tar yield among treatment groups (C, control; E, Eclipse; Q1, Quest 1; Q3, Quest 3; 2R, 2R4F). Raw data points are shown above nicotine or tar yield for each product. Means±SD are shown immediately to the right. Multiple regression analyses were carried out using data subjected to transformation in order to normalize the variances. See text for details

the co-linearity between the independent variables, nicotine, and tar ($r = 0.60$) and the small number of different tar levels in the cigarettes tested limit our ability to determine their relative contributions whether total aorta or subregions were evaluated.

Relationship between Nicotine and Oxidative Stress

Isoprostane F2 alpha VI, a sensitive and specific marker of lipid peroxidation and oxidative stress in vivo, was measured in urine collected overnight commencing immediately after smoke exposure for the 8–16 weeks study. Isoprostane F2 alpha VI levels were elevated in each of the smoke-exposed groups except for Quest 3, which has the lowest nicotine yield of all products tested (Fig. 4A). There was also a clear dose–response relationship between nicotine yield and urinary levels of isoprostane F2 alpha VI that tended to flatten with higher nicotine exposures (Fig. 4B).

Relationship between Tar and CYP1A1 Induction

CYP1A1 levels were measured by Western blot in lung tissue from animals in the 12–24 weeks study. CYP1A1 levels in the lungs increased in all smoke-exposed animals relative to controls (Fig. 5). Compared to levels in mice

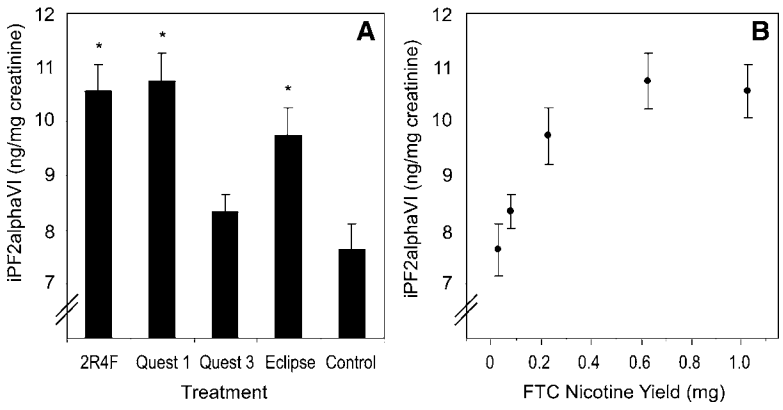

Fig. 4 Isoprostane F2 alpha VI levels (mean and SEM) measured in urine samples normalized to urine creatinine content plotted by treatment (**A**) and against the corresponding FTC nicotine yield (**B**). *$p < 0.05$, Dunnet's test for comparisons to control

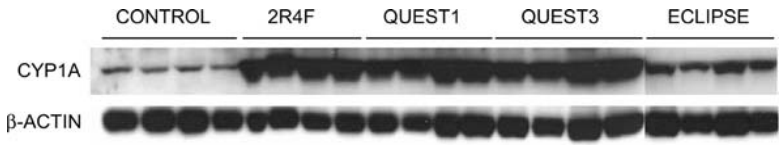

Fig. 5 CYP1A1 levels in lungs of selected mice from Study 2. Lungs were obtained from four mice in each group. Tissue lysate proteins (100 μg) were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and sequentially probed with antibodies specific for CYP1A1 and β-actin

exposed to 2R4F or Quest, CYP1A1 levels were less elevated in mice exposed to Eclipse cigarette smoke.

Physiological Measurements

All animals gained weight during the course of the study (Table 2), without differences between control and smoke-exposed groups except for the Eclipse group in Study 1. Mice grow most rapidly up to 8 weeks age after which growth slows, so that the greater weight gain of animals in Study 2 was most likely due to their younger age.

In Study 1, hematocrit was significantly higher at the end of the study in all smoke-exposed animals compared to control mice, except for the Quest 1 group, in which the hematocrit increase did not reach significance (Table 2). In the shorter 8–16 weeks study, there was a trend to increased hematocrit in all smoke-exposed mice compared to controls. The differences in hematocrit between the two studies are most likely due to the different analytic methods used. Elevated hematocrit is consistent with exposure to CO in cigarette smoke. A CBC on all mice from Study 2 showed no significant differences in leukocyte counts with smoke exposure, although mean platelet volume was slightly (1–3%), but significantly decreased in all smoke-exposed mice compared to controls (data not shown).

SBP and HR were measured before treatment, at midpoint and at the end of both studies. Generally it was difficult to detect a tail pulse immediately after smoke exposure, suggesting peripheral vasoconstriction. Therefore, SBP was routinely measured before smoke exposure, usually >20 h after the previous smoke exposure. SBP increased with time in all groups in both studies (Table 2). However, there were no significant differences in SBP among treatment groups at each time point compared to the control group. HR tended to decrease with age in all groups. However, there were no significant differences in HR among treatment groups at each time point compared to the control group with the exception of a small (11%) reduction in HR observed in the 2R4F group at 12 weeks compared to controls.

Total cholesterol increased with age (Table 2). Although total cholesterol at the end of both studies had increased by 4–16% in most smoke exposed groups compared to control, none of these differences were statistically significant. Conversely, HDL levels tended to fall with age, but not significantly with the exception of the Eclipse group in which HDL levels were slightly, although significantly increased compared to controls after 6 weeks smoke exposure in the 12–24 weeks study but not at the end.

Histology and Morphometry

Oil Red O staining of thoracic aortas showed that lipid-rich lesions occurred primarily at the branch points of side arteries, but also occurred in regions of the arterial wall in which there were no branch arteries (Fig. 6A). Treatment groups with greater lesion areas had larger lesions both at branch points and on uninterrupted sections of wall. Similar patterns were observed in other aortic segments.

Histological examination revealed early plaque development consisting mainly of intimal infiltration by macrophages and foam cells (Fig. 6B). Significantly increased numbers of breaks in the elastic laminae were observed at each level of the aortas of mice from the 2R4F, Quests 1 and 3 groups compared to controls (Figs. 6B and 7). There were also small increases in the number of elastic laminae present in the aortic arch of mice in the 2R4F, Quest 1, and Eclipse groups (Fig. 7). Morphometry revealed that lumenal and medial cross-sectional areas were similar between controls and each treatment group (data not shown).

Discussion

In two separate studies, we exposed atherosclerosis-prone apoE –/– mice to a variety of cigarettes for either 12 weeks beginning at 12 weeks age (Study 1), or for 8 weeks beginning at 8 weeks age (Study 2). The findings from both studies indicate that nicotine exposure is important in stimulating arterial lesion development, although tar content may also play a role. In both studies, we observed larger lipid rich lesion areas in aortas of mice exposed to the high-nicotine 2R4F and Quest 1 cigarettes (1 and 0.6 mg nicotine yield per cigarette, respectively). In the shorter Study 2, we also observed that lesion areas in the mice exposed to smoke from lower nicotine Eclipse and

Table 2 Physiological parameters for mice exposed to cigarette smoke between 12–24 weeks age (Study 1) and 8–16 weeks age (Study 2)

	Weight gain (g)	Hct (%)	Total cholesterol (mg/dL)			HDL cholesterol (mg/dL)			SBP (mmHg)			HR (bpm)		
Length of Exposure (weeks)	12	12	0	6	12	0	6	12	0	6	12	0	6	12
Study 1: 12–24 weeks age														
Quest 1	0.6 ± 0.3	43.4 ± 0.8	413 ± 23	555 ± 38	515 ± 26	28 ± 1	25 ± 1	20 ± 2	106 ± 1	107 ± 2	106 ± 3	625 ± 10	622 ± 13	617 ± 13
Quest 3	0.8 ± 0.3	45.6 ± 1.5*	377 ± 25	564 ± 43	546 ± 39	28 ± 2	27 ± 1	23 ± 2	103 ± 2	105 ± 2	105 ± 2	640 ± 12	617 ± 13	631 ± 10
2R4F	1.3 ± 0.3	46.3 ± 0.6*	446 ± 23	577 ± 28	542 ± 34	25 ± 1	23 ± 1	19 ± 1	106 ± 1	105 ± 1	110 ± 1	629 ± 9	624 ± 5	582 ± 18*
Eclipse	0.2 ± 0.3*	45.6 ± 1.2*	445 ± 32	532 ± 51	515 ± 17	27 ± 1	30* ± 2	19 ± 2	102 ± 1	102 ± 1	108 ± 3	629 ± 12	629 ± 10	621 ± 17
Control	1.3 ± 0.3	42.4 ± 0.6	421 ± 23	451 ± 31	470 ± 34	26 ± 1	24 ± 1	22 ± 1	102 ± 2	104 ± 2	110 ± 3	625 ± 11	624 ± 9	648 ± 9
Length of exposure (weeks)	8	8	4	8		4	8		0	4	8	0	4	8
Study 2: 8–16 Weeks Age														
Quest 1	2.2 ± 0.2	57.4 ± 1.0	543 ± 22	622 ± 15		20 ± 1	16 ± 1		102 ± 1	102 ± 2	107 ± 2	672 ± 9	600 ± 8	612 ± 10
Quest 3	3.5 ± 0.8	56.3 ± 0.9	521 ± 20	605 ± 20		21 ± 1	17 ± 1		98 ± 2	100 ± 1	104 ± 2	672 ± 8	590 ± 10	626 ± 13
2R4F	2.5 ± 0.1	57.0 ± 1.0	523 ± 19	621 ± 23		19 ± 1	16 ± 1		98 ± 1	103 ± 2	107 ± 2	660 ± 8	602 ± 8	633 ± 11
Eclipse	2.9 ± 0.1	56.3 ± 2.3	514 ± 20	579 ± 19		20 ± 1	18 ± 1		101 ± 1	106 ± 2	106 ± 1	675 ± 10	636 ± 11	619 ± 11
Control	2.6 ± 0.2	54.0 ± 0.5	516 ± 16	583 ± 22		19 ± 1	17 ± 1		102 ± 1	100 ± 2	103 ± 1	668 ± 8	631 ± 11	632 ± 8

*$p < 0.05$ compared to control (Dunnet's test)

Quest 3 cigarettes (0.2 and 0.05 mg nicotine yield per cigarette, respectively) were significantly smaller than in mice exposed to 2R4F and Quest 1 cigarettes, yet were significantly larger than lesion areas in controls. Although Quests 1 and 3 and 2R4F cigarettes all have the same tar yield (10 mg/cigarette) and produced similar TSPs under identical smoking conditions, mice exposed to smoke from the high-nicotine 2R4F and Quest 1 cigarettes developed larger aortic lesion areas than did mice exposed to smoke from Quest 3, which has the lowest nicotine content of all the products tested. Lesion areas in mice exposed to smoke from Eclipse cigarettes, which yield an intermediate amount of nicotine per cigarette (0.2 mg) and 50% less tar than Quest and 2R4F, were smaller than lesion areas in mice exposed to cigarettes with high tar and high nicotine, but were nonetheless significantly larger than in control mice.

Relationships between Smoke Components and Lesion Area

Regression analyses revealed a strong dose dependency between nicotine yield and lesion area in both studies, suggesting that nicotine plays an important role in stimulating lesion development. Our data suggested that lesion area also may be related to tar yield, but the limited range of tar yields in the products tested, and the close correlation between tar and nicotine yields limited our ability to establish an association between tar content and lesion size.

Our ability to detect significant differences in lesion areas between the lower nicotine Eclipse and Quest 3 products versus controls in the 8–16 weeks study may relate to the younger age of animals in that study, so that lesion areas in the smoke treated groups were compared to smaller lesions in the control group, giving a larger relative difference. In contrast, in the 12–24 weeks study lesion areas in the smoke treated groups were compared to relatively more evolved lesion areas in the control group so that the increase in lesion size in smoke treated groups appeared relatively less marked. The greater number of animals in the 8–16 weeks study also increased the power to detect differences. It should be noted, however, that under our study conditions in which animals were fed a normal low fat (4.5%) laboratory diet, lesion areas were relatively small both in smoke exposed animals and in controls, and there was no significant hypertrophy of the vascular walls during the observation period.

Possible Involvement of Oxidative Mechanisms

Increased oxidative stress has been widely implicated in promoting atherogenesis, and levels of isoprostanes, a measure of oxidative stress, are increased both in human

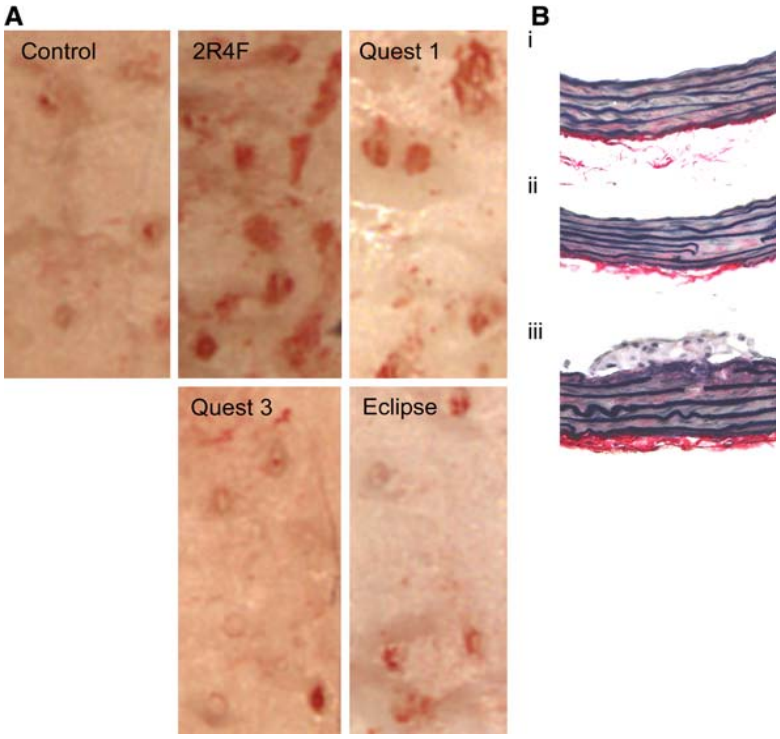

Fig. 6 (**A**) Oil Red O staining *en face* of thoracic aortas representative of each treatment group. (**B**) Aortic cross sections showing (i) normal aorta; (ii) breaks in elastic laminae under normal intima; and (iii) a typical early intimal lesion containing macrophages and breaks in the underlying media

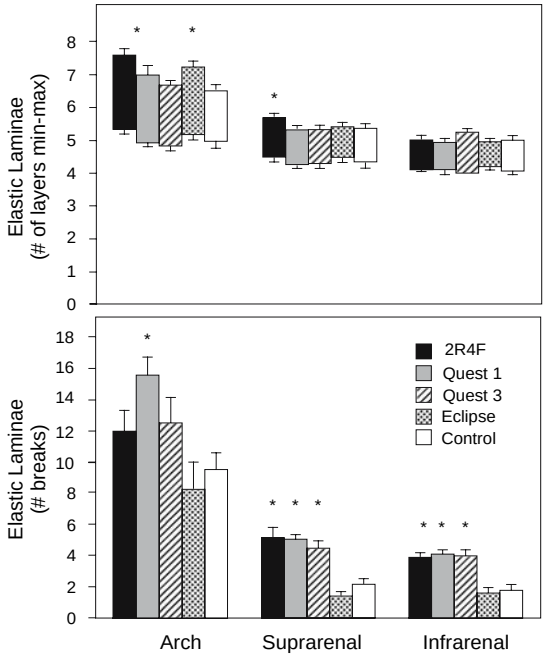

Fig. 7 Morphometric analysis of aortic cross sections. *$p < 0.05$, Dunnet's test for comparisons to control

and animal models of atherosclerosis [20]. Urinary excretion of isoprostane F2 alpha VI was significantly increased in mice exposed to smoke from 2R4F, Quest 1, and Eclipse cigarettes, but not in those exposed to Quest 3, suggesting that nicotine content is key in the production or augmentation of oxidative stress.

Cigarette smoke contains thousands of compounds that may foster development of atherosclerosis and cardiovascular disease [7–12]. The atherogenic potential of the few compounds that have been studied appears to be independent of their carcinogenicity, suggesting different effector mechanisms [9]. Recent studies suggesting that oxidative stress may play a role in atherosclerosis have focused on the involvement of plasma membrane-associated NAD(P)H oxidases [21], although other sources of reactive oxygen species have also been implicated [22]. The ability of various cigarette tar components to stimulate atherogenesis may be related to reactive oxygen species generated during their metabolism by cytochrome P450 enzymes [23]. Depletion of the cellular antioxidant, glutathione, by cigarette smoke components may further exacerbate these effects [24, 25]. In the present study, we found that mice exposed to smoke from high tar cigarettes exhibited higher levels of CYP1A1 than mice exposed to Eclipse smoke, in which enzyme levels were intermediate between the high tar groups and controls. This observation suggests that microsomal enzyme induction in response to cigarette smoke exposure may represent a source of oxidative stress stimulating atherogenesis. However, there was no direct relationship between cigarette tar yield and urinary excretion of isoprostane F2 alpha VI, a marker of oxidative stress (see below), suggesting the involvement of additional or alternative mechanisms.

Oxidative mechanisms also represent a target for interactions with other processes mediated by nicotine. Nicotine blocks nitric oxide production [26], and studies in humans

clearly demonstrate that nicotine can inhibit the protective functions of endothelium normally mediated by nitric oxide [27, 28]. Thus, while vascular nitric oxide generally counteracts proatherogenic effects of oxidative stress, if that oxidative stress is induced by cigarette smoking the nicotine absorbed from the smoke may suppress this vasculoprotective mechanism. The finding that isoprostane F2 alpha VI was increased in mice exposed to smoke from all cigarettes tested except Quest 3, which delivers minimal nicotine, is consistent with this hypothesis.

Other Physiological Parameters Involved in Atherogenesis

Many cardiovascular effects of cigarette smoke have been attributed to activation of the sympathetic nervous system by nicotine [29]. While we did not detect overall increases in SBP or HR among the different groups of mice exposed to smoking, which might indicate a sustained increase in sympathetic activity, our measurements were made >20 h after smoke exposure, by which time any short-term hemodynamic effects of smoke exposure due to acute sympathetic stimulation could have resolved. The observation that tail pulse was blunted immediately after smoke exposure suggests that there may be peripheral vasoconstriction, which could result in an increase in central aortic blood pressure. Histological studies of the aortas showed significant increases in the number of sharp breaks in the elastic laminae of smoke-exposed mice characterized by retraction of the broken elastic laminae. This type of break is associated with hemodynamic stress, in contrast with the more diffuse breaks associated with proteolytic degradation of the elastic laminae by macrophage proteases [30]. However, increases in breaks in elastic laminae were associated with exposure to smoke from cigarettes with high tar yield, rather than high nicotine yield, and therefore the evidence does not support the involvement of sympathetic activation by nicotine leading to sustained increased hemodynamic stress. Nicotine alone has also been shown to increase plaque vascularization in chronic models of mouse atherosclerosis in which lesions were more advanced than in the present study which focused on early vascular changes [15].

In human smokers, serum triglycerides, cholesterol, and LDL-cholesterol are elevated while HDL-cholesterol decreases [2]. In the present study, total cholesterol increased with age, as expected in this genetically hypercholesterolemic mouse strain, while HDL cholesterol decreased. However, despite a trend to increased LDL, there were no significant differences between smoking groups and controls, suggesting that the limited smoke exposure used in our model does not accelerate hypercholesterolemia. Similarly, we observed no differences in blood leukocyte counts that might contribute to increased atherogenesis.

In this study, total nicotine and cotinine values in urine were generally in proportion to the FTC nicotine yield of the cigarettes, with the dose–response relationship tending to flatten out with higher nicotine yield. These observations are consistent with the relatively flat dose response relationship between cigarette consumption and urinary nicotine and cotinine content reported in human studies [31]. Notably, the cotinine excretion in mice exposed to 2R4F cigarettes was only modestly lower than that of human smokers [32], suggesting that the nicotine exposures in this study were representative of the typical exposure in human smokers.

Study Limitations

A limitation of this animal model is that it does not reproduce the typical human temporal smoking pattern. While the mice were smoke exposed for only 1 h/day, humans tend to smoke through the course of the day to maintain plasma nicotine levels. Additionally, humans using low nicotine cigarettes tend to alter their smoking behaviors to maintain constant nicotine levels [33]. However, even with this limited duration of smoke exposure, effects of smoking on promoting lesion formation were clearly discernable in our model. Additional studies are required to determine whether a more typical 'human' pattern of smoke exposure would further accelerate atherosclerosis in this mouse model.

The regression modeling analysis performed in this study was also limited by the small range of tar exposures (0, 5, and 10 mg). To accurately ascertain the relative contributions of nicotine and tar to atherosclerosis would require the independent manipulation of tar yield in smaller increments within the range of available tobacco products.

Conclusions

Although nicotine is the addictive component of cigarette smoke, its contribution to the cardiovascular risk associated with cigarette smoking remains controversial. Our present findings suggest that nicotine is an important contributor to atherogenesis in this in vivo smoking model. This finding suggests that lower nicotine cigarettes might be "safer". However, smokers adjust their tobacco consumption to maintain constant nicotine levels even when they select low nicotine cigarettes [33]. Therefore, the cardiovascular risks of smoking are very difficult to diminish or eliminate in habitual smokers.

Acknowledgements We thank Dr. Stephen S. Hecht for carrying out measurements of urinary nicotine and cotinine, and Dr. Sharon Murphy for helpful comments on the manuscript. This work was supported in part by NIH grants HL 64660 to Daniel F Catanzaro and HL 55627 to Babette B. Weksler.

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