Atherosclerosis 211 (2010) 271–277

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Reduced LDL-cholesterol levels in patients with coronary artery disease are paralelled by improved endothelial function: An observational study in patients from 2003 and 2007

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ARTICLE INFO

Article history:
Received 19 November 2009
Received in revised form 11 January 2010
Accepted 11 January 2010
Available online 21 January 2010

Keywords:
Cholesterol
Endothelial function
Coronary disease
Statins
Oxidative stress

ABSTRACT

Objective: Recent guidelines recommend more aggressive lipid-lowering in secondary prevention protocols. We examined whether this resulted in improved endothelial function.
Methods: We studied saphenous vein specimens of patients undergoing surgical coronary revascularisation in 2007 and compared results with those of patients examined in 2003. Endothelium-dependent vasodilation was assessed by relaxation to calcium ionophore A23187, and vascular superoxide production by lucigenin enhanced chemiluminescence.
Results: Statin dose increased from 26 ± 16 mg/d in 2003 to 37 ± 17 mg/d in 2007 ($P < 0.001$), and total (4.0 ± 0.9 mmol/L *vs* 4.8 ± 1.0 mmol/L) and LDL-cholesterol levels (2.0 ± 0.7 mmol/L *vs* 3.0 ± 0.9 mmol/L) were lower in 2007 compared to 2003 ($P < 0.001$; $n = 90$ each). Endothelium-dependent vasodilation was greater in 2007 (44 ± 15%) compared to 2003 (28 ± 12%; $n = 36$ each; $P < 0.001$). Vascular superoxide derived from endothelial NO synthase (eNOS) was lower in 2007 than in 2003 (reduction by N^G-nitro-L-arginine-methyl ester, 0.29 ± 0.21 nmol/(mg min) *vs* 0.09 ± 0.20 nmol/(mg min); $P = 0.002$). In linear regression analysis, LDL-cholesterol levels have been shown to be the major determinant of endothelial function in the combined 2003 and 2007 cohort.
Conclusion: Intensive lipid-lowering is associated with improved endothelial function and reduced superoxide production from eNOS. Further improvement in vascular function could be achieved by targeting other sources of superoxide including xanthine oxidase.

1. Introduction

Increased LDL-cholesterol is an important risk factor for cardiovascular disease. We have recently shown that the levels of LDL-cholesterol determine endothelial function and oxidative stress in patients with severe coronary artery disease [1]. A plethora of clinical studies have demonstrated that statins improve endothelial function and reduce oxidative stress in patients at different stages of cardiovascular disease [2,3]. Statins and reduction of LDL-cholesterol levels have been found to improve outcome of patients with cardiovascular disease [4,5]. In light of these observations, guidelines for secondary prevention of cardiovascular disease have been updated. In particular, there is now a focus on lower optimum total and LDL-cholesterol targets in the US, Europe and UK compared to previous editions of the guidelines (supplementary Table 1). Patients known to be at cardiovascular risk may therefore receive more intensive lipid-lowering therapy to achieve current targets. A recent study, however, has found no additional benefit of aggressive lipid-lowering using simvastatin and ezetimibe compared to simvastatin alone on reduction of carotid intima-media thickness despite a significance between group difference in LDL-cholesterol [6]. The exact value of aggressive lipid-lowering and the choice of drugs to achieve this are therefore still under debate.

Endothelial function is an independent predictor of morbidity and mortality in patients with cardiovascular diseases [7–9]. Being a critical step in the cardiovascular continuum, endothelial function may serve as a surrogate marker to monitor efficacy of treatment. From our previous cross-sectional study [1] we have suggested that reductions in LDL-cholesterol levels will lead to improvement of endothelial function in patients with severe CAD. We aimed to

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0021-9150
doi:10.1016/j.atherosclerosis.2010.01.014

test this hypothesis by comparing lipid profiles, endothelial function and levels of vascular superoxide production in a group of patients who underwent coronary artery bypass graft surgery in 2003 with an age-matched group of patients undergoing surgery in 2007 in whom cholesterol levels were significantly lower in line with revised secondary prevention protocols.

2. Methods

2.1. Subjects

The present observational study derived from comparison of two studies which have been performed at our Centre. Data were collected from 105 patients with severe CAD undergoing coronary artery bypass surgery between November 2006 and February 2008 ("2007 group") and compared with data from a similar group of patients studied between February 2003 and February 2004 ("2003 group"; $n = 121$). Patients were operated in the same clinical centre by the same team of cardiothoracic surgeons. Blood samples were taken from patients between one and seven days prior to surgery after 3 h of fasting, and were stored at −70 °C for routine biochemistry including cholesterol levels. Saphenous vein segments surplus to requirement at the time of surgery were taken to the laboratory, cleaned of excess connective tissue and stored at 4 °C in a HEPES buffer for study of endothelial function and superoxide production the following day. Within the two patient groups data on endothelial function was available from 36 age-matched patients from 2003 and 2007 and from 33 age-matched patients for superoxide production. These subjects did not differ from the whole 2003 and 2007 groups in their clinical characteristics (supplementary Tables 2 and 3).

We also recruited 106 control subjects without evidence of cardiovascular disease. Seventy control subjects were patients who underwent surgery for the removal of varicose veins but were otherwise healthy ($n = 51$ in 2003 and $n = 19$ in 2007). Saphenous vein specimens from non-varicosed parts of vein were processed as above. We age-matched the 19 patients from 2007 with 19 patients from 2003 for analysis. Total (3.7 ± 0.7 mmol/L *vs* 4.8 ± 0.8 mmol/L) and LDL-cholesterol levels (1.8 ± 0.5 mmol/L *vs* 2.7 ± 1.0 mmol/L) were available in some of these patients and in 2003 ($n = 14$) and 2007 ($n = 9$), respectively. Patients were operated in the same hospital by the same team of vascular surgeons. Thirty-six control subjects were recruited in 2007 from local gyms or were employees of Glasgow University. They provided blood samples for assessment of whole blood superoxide release and underwent *in vivo* endothelial function testing.

The study complies with the Declaration of Helsinki and was approved by the local ethics committee. All participants gave informed written consent.

2.2. Endothelial function

These studies have been performed in 2003 and 2007 under the supervision of the same experienced researcher (C.A.H.) using the same equipment. Three millimetre rings of saphenous vein were studied in organ chambers as previously described [1]. In brief, vessels were constricted with phenylephrine (3 μmol/L) and relaxation to calcium ionophore A23187 (0.01–10 μmol/L) was studied. Maximum relaxation was calculated and expressed as a percentage of constriction to phenylephrine. In a subset of patients in 2007 ($n = 22$ patients with CAD, $n = 29$ control subjects) we also assessed endothelium-dependent vasodilation *in vivo*. We performed pulse wave analysis using a SphygmoCor Vx device (AtCor Medical Inc, Itasca, IL, USA) and examined the maximum change of radial augmentation index to inhaled salbutamol (400 μg) [10]. This was compared to maximum response to inhaled glycerol trinitrate (400 μg) to assess endothelium-independent vasodilation. Response to glycerol trinitrate was found to be similar between patients and control subjects ($-66 \pm 19\%$ *vs* $-70 \pm 20\%$, $P = 0.482$).

2.3. Superoxide production

Basal vascular superoxide production was measured in 3–4 mm rings by chemiluminescence as described previously [1] using lucigenin (5 μmol/L) by the same experienced researcher (C.A.H.) using the same equipment in 2003 and 2007. When sufficient tissue was available the effects of the following on superoxide production were also studied: N^G-nitro-L-arginine-methyl ester (L-NAME) 0.1 mmol/L (endothelial NO synthase inhibitor), allopurinol 0.1 mmol/L (xanthine oxidase inhibitor), diphenylene iodonium (DPI) 10 μmol/L (NAD(P)H oxidase inhibitor) and rotenone 3 μmol/L (mitochondrial superoxide inhibitor). Superoxide levels were calculated from a standard curve generated from xanthine and xanthine oxidase and standardised to mg of wet weight.

In the 2003 cohort measurements of vascular superoxide production were confirmed using dihydroethidine fluorescence as described previously [1]. In the 2007 cohort oxidative stress status was confirmed by analysing superoxide release from whole blood. In brief, venous blood was collected in lithium heparinate containing tubes, kept on ice and processed within half an hour. Superoxide levels were detected by electron paramagnetic resonance (e-scan R; Bruker BioSpin GmbH, Rheinstetten, Germany) with the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH; Noxygen, Elzach, Germany) to a final concentration of 500 μM [11]. Instrument settings were: centre field of 3375 G, modulation amplitude of 2.27 G, sweep time of 5.24 s, sweep width of 60 G and 10 scans. Superoxide levels were recorded once a minute for 10 min and the rate of superoxide anion production was calculated as counts per minute.

2.4. Statistical analysis

Statistical analyses were performed using SPSS (version 15; SPSS Inc., Chicago, IL, USA) and Minitab (version 12.1; Minitab Inc., State College, PA, USA) software. In text and tables, data are expressed as mean ± standard deviation or median [interquartile range] as appropriate. In figures, means and standard errors or box plots are given as indicated. Normal distribution of data was examined by the Kolmogorov-Smirnov test and by visual inspection of *Q*–*Q* plots. Paired and unpaired Student's *t*-tests have been performed for comparison of normally distributed data as appropriate. Wilcoxon test has been used for comparison of data that were not normally distributed. Fisher's exact test has been used for comparison of categorical data. Pearson's correlation coefficients have been calculated where indicated. Linear regression analysis was used to examine the independent effects risk factors on endothelial function in the whole study cohort. A *P*-value of less than 0.05 (two-sided) was considered significant.

3. Results

3.1. Clinical characteristics and cholesterol levels

Compared to the 2003 group, the 2007 group was older, had a slightly lower diastolic blood pressure and contained more male patients. Other clinical characteristics were not different between the two groups (Table 1). Control subjects undergoing varicose vein surgery were similar between 2003 ($n = 19$; age 48 ± 13 years) and 2007 ($n = 19$; age 48 ± 13 years).

Table 1
Clinical characteristics of patients with coronary artery disease.

	2003 $n=121$	2007 $n=105$	P-value
Age (years)	62 ± 9	65 ± 10	0.028
Male/female	77/44 (64%)	84/21 (80%)	0.003
Body mass index (kg/m^2)	28.7 ± 4.9	29.5 ± 5.0	0.243
Systolic blood pressure (mmHg)	140 ± 14	140 ± 24	0.999
Diastolic blood pressure (mmHg)	82 ± 11	78 ± 12	0.009
Diabetes (yes/no)	27/94 (22%)	28/77 (27%)	0.092
Active smoking (yes/no)	28/93 (23%)	18/87 (17%)	0.071
Aspirin (yes/no)	103/18 (85%)	88/17 (84%)	0.140
Beta blocker (yes/no)	75/46 (62%)	67/38 (64%)	0.106
ACEI or ARB (yes/no)	58/63 (48%)	55/50 (52%)	0.085
Statin (yes/no)	107/14 (88%)	99/5 (94%)	0.038
Simvastatin (%)	68	58	0.148
Atorvastatin (%)	19	30	0.073
Pravastatin (%)	11	4	0.069
Rosuvastatin (%)	0	2	n/a
Fluvastatin (%)	0	1	n/a

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

In patients with CAD, statin use increased from 88% in 2003 to 94% in 2007 ($P=0.038$). Average statin dose increased from 26 ± 16 mg/d in 2003 to 37 ± 17 mg/d in 2007 ($P<0.001$). This was associated with lower total (4.0 ± 0.9 mmol/L *vs* 4.8 ± 1.0 mmol/L; $P<0.001$; $n=91$ each) and LDL-cholesterol levels (2.0 ± 0.7 mmol/L *vs* 3.0 ± 0.9 mmol/L; $P<0.001$; $n=89$ each) in 2007 compared to 2003, in the absence of changes in HDL-cholesterol levels (1.1 [0.9;1.3] mmol/L *vs* 1.2 [1.0;1.4] mmol/L; $P=0.124$; $n=90$; Fig. 1).

The significant difference in cholesterol levels measured in the entire patient cohorts from 2003 and 2007 were also observed in the subgroups used for assessment of endothelial function (2003: 4.9 ± 0.9 mmol/L, 2007: 4.1 ± 1.0 mmol/L; $P<0.001$) and superoxide production (2003: 4.9 ± 1.2 mmol/L, 2007: 4.2 ± 1.1 mmol/L; $P=0.048$).

3.2. Endothelial function

Patients with CAD had impaired endothelial function compared to control subjects both *ex vivo* (maximum relaxation of saphenous veins to calcium ionophore A23187; 2003: 28 ± 13% *vs* 60 ± 11%, $P<0.001$; 2007: 44 ± 16% *vs* 65 ± 15%, $P<0.001$; Fig. 2a and b) and *in vivo* (maximum change in radial augmentation index to salbutamol; −3.1 [−7.0;6.1]% *vs* −6.8 [−10.5,−3.2]%, $P=0.021$; Fig. 2c).

In patients with CAD, endothelial function, measured as the maximum relaxation of saphenous veins to calcium ionophore A23187, was significantly greater in the 2007 compared to the 2003 group (44 ± 15% *vs* 28 ± 12%, $P<0.001$, $n=36$ each; Fig. 2d). The previously observed correlation between LDL-cholesterol and endothelial function in the 2003 group ($r=-0.275$, $P=0.012$; $n=83$) extended to the combined 2003 and 2007 cohorts ($r=-0.482$, $P<0.001$; Fig. 2e). We performed linear regression analysis with the vasodilatory response to A23187 as dependent variable and entered characteristics that were different between the 2003 and 2007 cohorts with P-values less than 0.10 (age, sex, diabetes status, smoking status, LDL-cholesterol levels, diastolic blood pressure, statin dose, and ACEI/ARB usage) together with a variable indicating the year of study (2003 or 2007; to adjust for factors not represented by the above variables) into the model (Table 2). This demonstrated that out of the above variables only LDL-cholesterol contributed significantly to endothelial function explaining 15.6% of its variability. The relationship between LDL-cholesterol and endothelium-dependent vasorelaxation held true if the analysis was restricted to male patients (supplementary Figure 1).

Endothelium-dependent vasorelaxation remained unchanged in saphenous vein from control subjects (maximum response to calcium ionophore A23187 in 2003: 60 ± 11%, in 2007: 65 ± 15%; $P=0.252$; $n=14$).

3.3. Vascular superoxide generation

Patients with CAD had greater vascular superoxide production compared to control subjects (lucigenin enhanced chemiluminescence; 2003: 0.78 [0.39;0.92] nmol/(mg min) *vs* 0.46 [0.28;0.67] nmol/(mg min), $P=0.003$; 2007: 0.65 [0.39;0.92] nmol/(mg min) *vs* 0.38 [0.25;0.64] nmol/(mg min), $P=0.011$). These results were confirmed by hydroethidine fluorescence in the 2003 cohort (Fig. 3a) and by assessment of whole blood superoxide release in the 2007 cohort (patients with CAD: 37.8 [32.1;75.3] kAU; control subjects: 23.8 [21.3;30.4] kAU; $P<0.001$; Fig. 3b).

Levels of total vascular superoxide were not significantly different between the 2003 and 2007 groups of patients with CAD (0.77 [0.53;1.08] nmol/(mg min) *vs* 0.50 [0.37;0.85] nmol/(mg min); $P=0.053$; $n=33$ each) (Fig. 3c). When the sources of superoxide were investigated, significant differences between the groups were found (Fig. 3d). A decrease in the amount of superoxide derived from uncoupled eNOS was observed from 2003 ($n=27$) to 2007 ($n=21$) (decrease in vascular superoxide production by L-NAME: −0.29 ± 0.21 nmol/(mg min) *vs* −0.09 ± 0.20 nmol/(mg min); $P=0.002$). Change of superoxide production by DPI (which will inhibit production from eNOS as well as NAD(P)H oxidase and mitochondrial sources) was

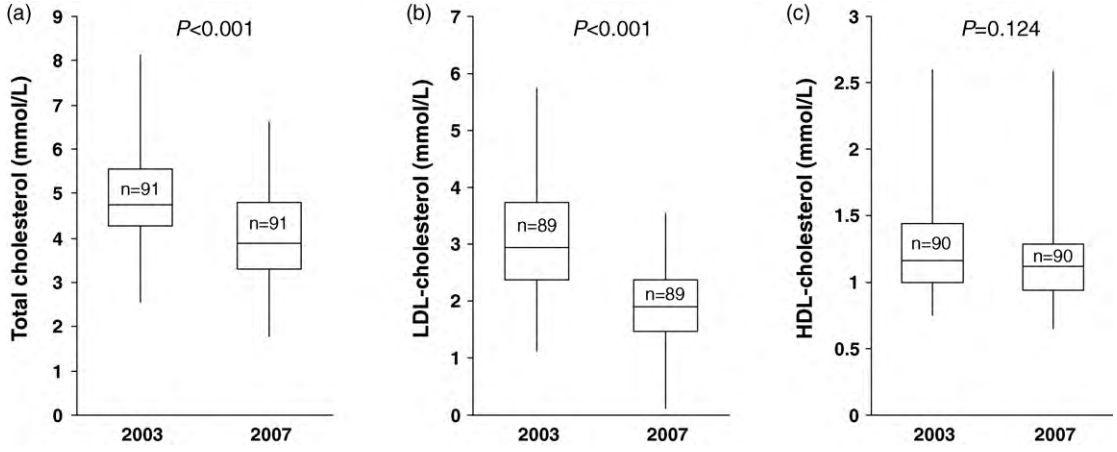

Fig. 1. Lipids: total (a), LDL (b) and HDL-cholesterol levels (c) in patients with coronary artery disease in 2003 and 2007. Lines, boxes and whiskers represent median, interquartile range and extremes, respectively. Numbers of experiments/samples are given in the boxes.

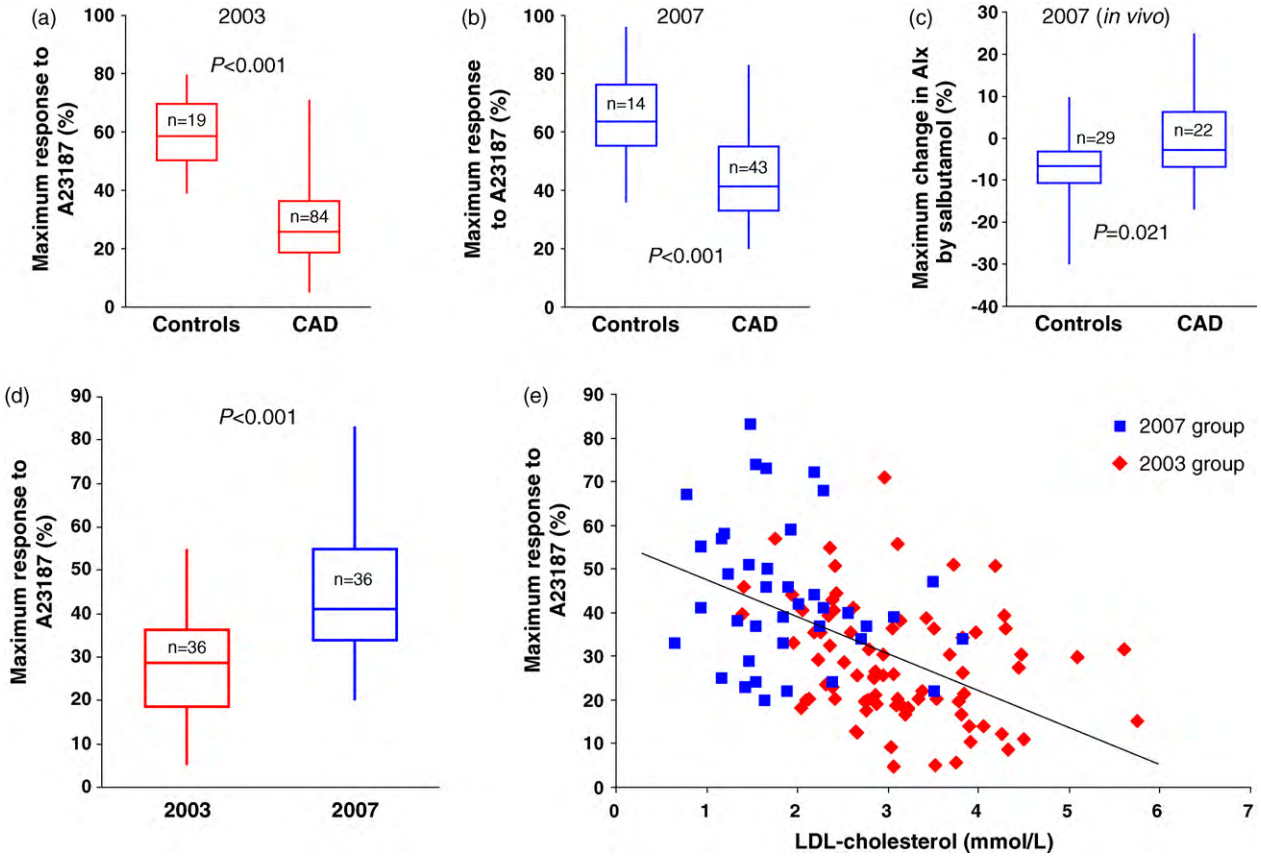

Fig. 2. Endothelial function: (a and b) endothelial function as assessed by maximum vasodilation of saphenous vein rings to calcium ionophore A23187 (10 μmol/L) in patients with coronary artery disease (CAD) and control subjects in 2003 (a) and 2007 (b). (c) Endothelial function as assessed by maximum change in radial augmentation index (AIx) to salbutamol (400 μg) in patients with CAD and control subjects. (d) Endothelial function as assessed by maximum vasodilation of saphenous vein rings to calcium ionophore A23187 (10 μmol/L) in age-matched patients with CAD from 2003 and 2007. (e) Scatterplot of maximum vasodilation of saphenous vein rings to calcium ionophore A23187 (10 μmol/L) *vs* LDL-cholesterol levels in 2003 (red diamonds) and 2007 (blue squares). There is significant correlation ($r = -0.482$; $P < 0.001$) between LDL-cholesterol and endothelial function in the combined 2003 and 2007 cohorts (black regression line). Lines, boxes and whiskers represent median, interquartile range and extremes, respectively. Numbers of experiments/samples are given in the boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

numerically different between 2003 (-0.43 ± 0.29 nmol/(mg min), $n = 26$) and 2007 (-0.26 ± 0.27 nmol/(mg min), $n = 13$) but did not reach statistical significance ($P = 0.054$). No difference in superoxide derived from xanthine oxidase was seen between the 2003 ($n = 27$) and 2007 ($n = 31$) cohorts (decrease by allopurinol, -0.12 ± 0.18 nmol/(mg min) *vs* -0.08 ± 0.22 nmol/(mg min); $P = 0.341$). Mitochondrial superoxide, examined for the first time in the 2007 study, was also identified as a significant source of superoxide in patients with CAD. Incubation with rotenone reduced superoxide from 0.51 [0.39;1.00] to 0.46 [0.32;0.76] nmol/(mg min) ($P < 0.001$; $n = 47$). In control subjects the amount of superoxide generated from mitochondria was found to be negligible (0.50 ± 0.34 and 0.49 ± 0.32 nmol/(mg min) in the absence and presence of rotenone, respectively; $P = 0.618$; $n = 10$; Fig. 3e).

Vascular superoxide production remained unchanged in control subjects between 2003 and 2007 (0.47 ± 0.26 nmol/(mg min) *vs* 0.43 ± 0.32 nmol/(mg min), $P = 0.697$; $n = 14$).

Table 2
Determinants of endothelial function in the combined 2003 and 2007 cohort.

	Full model ($R^2 = 0.275$)		Stepwise model ($R^2 = 0.156$)	
	β	P-value	β	P-value
Age	0.062	0.558	–	–
Sex (0 = female; 1 = male)	0.005	0.965	–	–
Diabetes (0 = no, 1 = yes)	−0.061	0.562	–	–
Active smoking (0 = no, 1 = yes)	0.128	0.245	–	–
Diastolic blood pressure	−0.163	0.140	–	–
LDL-cholesterol	−0.0286	0.022	−0.395	<0.001
ACEI/ARB (0 = no, 1 = yes)	−0.025	0.856	–	–
Statin dose	−0.058	0.597	–	–
Year of study	0.343	0.027	–	–

In the full model all variables were forced into the model. The stepwise model was developed using probabilities of F to enter and remove variables of ≤0.050 and ≥0.100, respectively. β indicates the partial correlation coefficients. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

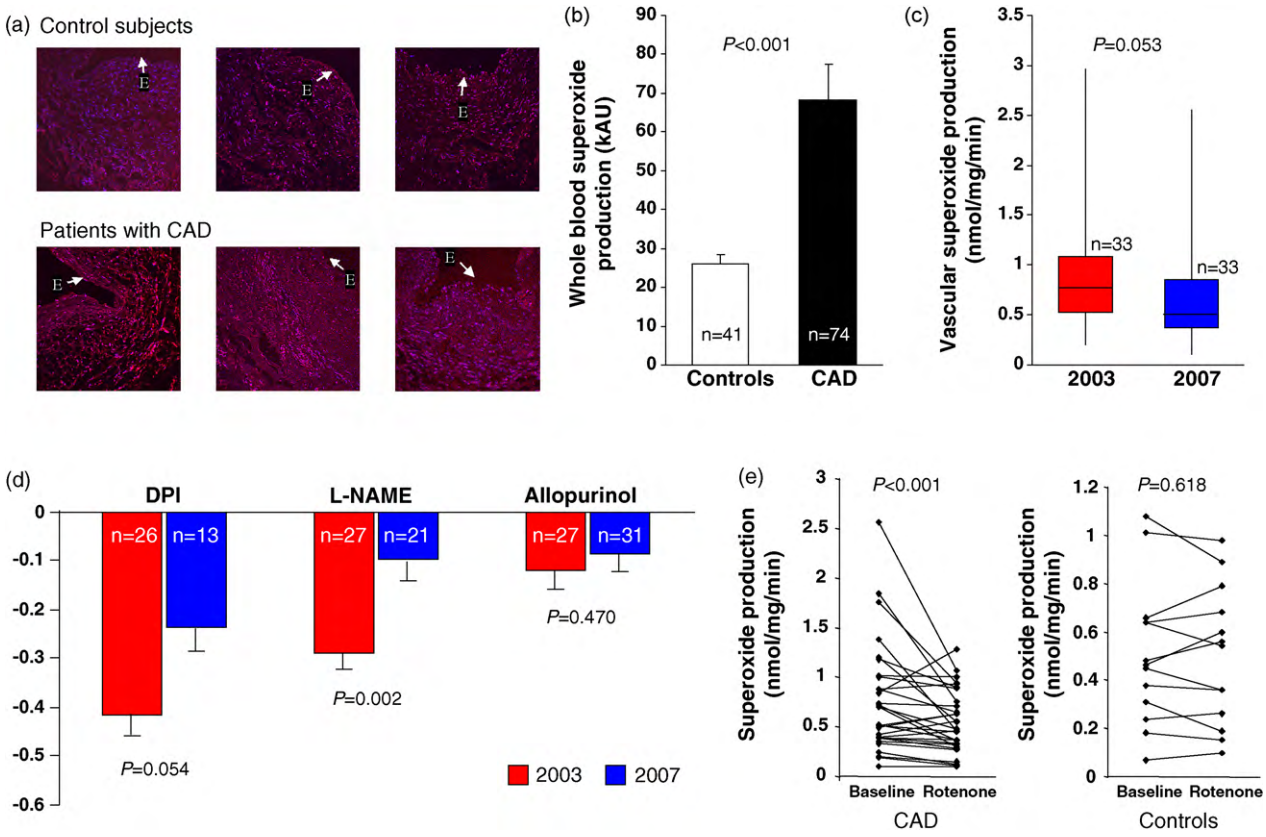

Fig. 3. Superoxide production: (a) increased vascular superoxide production in patients with coronary artery disease (CAD; top row) compared to control subjects (bottom row) in the 2003 cohort. Representative examples of hydroethidine fluorescence (red) in saphenous vein specimens are given. Background staining with 4′-6-diamidino-2-phenylindole (DAPI; blue). "E" indicates endothelium. (b) Whole blood superoxide production in patients with CAD (solid bar) compared to control subjects (open bar) in the 2007 cohort. (c) Superoxide production from saphenous vein assessed by lucigenin-enhanced chemiluminescence in age-matched patients with CAD from 2003 and 2007. Lines, boxes and whiskers represent median, interquartile range and extremes, respectively. Numbers of experiments/samples are given in the boxes. (d) Sources of vascular superoxide production in patients with CAD from 2003 (red bars) and 2007 (blue bars). Bars (means and standard errors) indicate the decrease in superoxide production following incubation with diphenylene iodonium (DPI; 10 μmol/L; NAD(P)H oxidase inhibitor), N^G-nitro-L-arginine-methyl ester (L-NAME; 0.1 mmol/L; endothelial NO synthase inhibitor) and allopurinol (0.1 mmol/L; xanthine oxidase inhibitor). (e) Vascular superoxide production assessed by lucigenin-enhanced chemiluminescence in the absence and presence of rotenone (3 μmol/L) in patients with coronary artery disease (CAD, $n = 47$) and control subjects ($n = 14$) of the 2007 group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

The central finding from this study is that LDL-cholesterol levels have been reduced between 2003 and 2007 in patients undergoing coronary artery bypass surgery and that this reduction in LDL-cholesterol levels is accompanied by an improvement in endothelial function. LDL-cholesterol has been shown to be a major determinant of endothelium-dependent vasorelaxation in patients with CAD in this and in our previous study [1]. Although a large proportion of the improvement of endothelial function from 2003 to 2007 remains unexplained, the potential contribution of lower LDL-cholesterol levels require some discussion.

Improvement of endothelial function by lipid-lowering therapy has been shown previously in clinical studies [2,3]. In particular, more powerful statins and higher doses of statins improve flow mediated brachial artery vasodilation [12]. However, our present work is to the best of our knowledge the first study demonstrating a marked effect of more aggressive treatment with statins on endothelial function in routine clinical care. Our data are particularly important against the background of recently published findings from a controlled clinical trial suggesting that aggressive lipid-lowering does not necessarily lead to better vascular outcome compared to standard therapy [6]. In our study we found that improvement of endothelial function was paralleled by lower cholesterol levels. This may have been a consequence of higher statin dose but also of recently more common prescription of more powerful statins such as atorvastatin although we cannot prove the latter due to small numbers and the observational nature of our study. At least for the use of statins, higher doses, more intensive LDL-cholesterol lowering, and improved vascular outcome appear to go hand in hand. However, LDL-cholesterol only explains about 16% of the variability of endothelial function in patients with CAD which leaves room for other factors that have not been addressed in the present study or for whose detection our study was not adequately powered.

A number of mechanisms may contribute to the adverse effects of LDL-cholesterol on endothelial function. Oxidative modification of LDL-cholesterol not only results in formation of oxidised LDL-cholesterol but also of free radicals which may interact directly with nitric oxide reducing its bioavailability. Oxidised LDL-cholesterol causes a depletion of caveolae cholesterol, a redistribution of eNOS away from caveolae and a diminished capacity to activate the enzyme [13,14]. Both LDL and oxidised LDL-cholesterols may cause decreased eNOS transcription, although a compensatory up-regulation of eNOS has been reported in hypercholesterolemic rabbits [15]. LDL and oxidised LDL-cholesterols also increase superoxide production; activation of xanthine oxidase, NAD(P)H oxidoreductase and eNOS uncoupling all having been reported to be involved in this cholesterol-dependent production of superoxide [16–18].

The attenuation of LDL-cholesterol levels in the 2007 patient group was associated with prescription of higher doses of statins. It

has been reported that the beneficial effects of statins on endothelial function extend beyond cholesterol lowering. Statins inhibit mevalonate synthesis leading not only to reduced cholesterol synthesis but also decreased isoprenylation of small GTPases and inhibition of these signalling molecules. An example of the latter would be the inhibition of RhoA and consequent inhibition of arginase activity [19] with arginase having recently been proposed as a novel target for treatment of atherosclerosis [20]. In addition statins can increase eNOS expression and induce phosphorylation and activation of eNOS via the phosphotidylinositol-3-kinase Akt pathway [21]. A reduction in superoxide production via inhibition of vascular NAD(P)H oxidase expression and inhibition of TGF-β-Smad 2/3 signalling has also been reported [22–24] as has recoupling of eNOS [25]. Cholesterol-independent effects of statins on endothelial function have been shown previously in clinical studies [2,3]. In addition to direct and indirect effects of statins a number of other factors may influence endothelial function. Diastolic blood pressure was lower in the more recent patient group and numerically, a higher proportion of patients in this group were taking angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, which could contribute both to better blood pressure control and decreased production of superoxide from NAD(P)H oxidase and thus lead to an improvement in endothelial function [26]. However, in regression analysis these factors were not independently associated with endothelial function.

In addition to excess superoxide generation from NAD(P)H oxidase and eNOS there is increasing evidence that over-production of reactive oxygen species by the mitochondria could contribute to development of atherosclerotic disease [27]. In this study we were able to detect a significant contribution from the mitochondria to vascular superoxide in patients with severe CAD but not in control subjects. Moreover, mitochondrial SOD deficiency has been associated with hypertension in mice [28]. Increasing mitochondrial anti-oxidant systems preserve endothelial function and prevent development of atherosclerosis in apoprotein E-deficient mice [29]. Parts of the effects of DPI on vascular superoxide production in our study may indeed reflect the substance's unspecific effects on mitochondrial superoxide generating systems. Increased production of superoxide from xanthine oxidase has been demonstrated previously in humans with coronary artery disease [1,30] whilst treatment with allopurinol has been reported to normalise endothelial function in diabetic subjects [31].

We should emphasise again that our analysis suggests that reduced LDL-cholesterol levels contribute to improvements of endothelial function between 2003 and 2007. However, the presented regression analysis is based on a combined analysis of the 2003 and 2007 cohorts where the differences in cholesterol levels were most striking. It clearly does not exclude the presence of other and possibly more important determinants of endothelial function but provide an estimate of the contribution of LDL-cholesterol lowering to improved endothelial function.

We acknowledge that our data do not derive from a controlled clinical trial. Combined analysis of data collected in 2003 and 2007 was complicated by differences in data collected in these individual projects. Nevertheless, this observational study demonstrates that effects predicted from controlled trials hold true in clinical practice. Another limitation of our study is that control subjects were younger than patients. Control subjects were mainly used in order to demonstrate consistency of our measures between 2003 and 2007; in this context age does not play a role. Results concerning mitochondrial superoxide production should, however, be interpreted against the background of a 17-year age difference. We would also like to point out that the 2007 control group is characterised by lack of cholesterol data in the majority of subjects. Whilst this does not affect the primary message of this study which focussed on patients with CAD, it is reassuring that in the 2003 cohort total and LDL-cholesterol levels were lower in the control group (*i.e.* in the group with better endothelium-dependent vasorelaxation) [1].

In summary, we have found a significant improvement of endothelial function in patients with severe CAD that is paralleled by treatment of lipids to lower targets. Being an independent predictor of morbidity and mortality, this improvement of endothelial function may serve as surrogate evidence for improved survival of patients with CAD as a result of new secondary prevention guidelines. Although a significant reduction in superoxide production from eNOS was observed the total superoxide production was not significantly lower in the 2007 compared to the 2003 group and was almost twice that observed in the control groups. Thus additional therapeutic strategies to lower vascular superoxide production should be considered. Our data suggest that mitochondrial and xanthine oxidase pathways involved in generation of reactive oxygen species may become novel drug targets for cardiovascular prevention.

Conflict of interest

The authors report no conflict of interest.

Funding

This work was supported by the British Heart Foundation Chair Grant to AFD and Programme Grant [BHF RG/07/005/23633] to AFD and CD and the European Union's Sixth Framework Programme InGenious HyperCare [LSHM-CT-2006-037093] to AFD and CD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2010.01.014.

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