Early increase of NOX2-derived oxidative stress in children: relationship with age

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BACKGROUND: Studies conducted in the prepubertal period showed that biomarkers of oxidative stress decreased with increasing age in normocholesterolemic children (NC), and, conversely, they are persistently high in hypercholesterolemic children (HC). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the most important cellular source of reactive oxygen species. No data have been reported concerning the behavior of age-related oxidative stress generated by NOX2, the catalytic subunit of NADPH oxidase, in children.

METHODS: We performed a cross-sectional study comparing oxidized low-density lipoprotein (ox-LDL), as a marker of oxidative stress, and NOX2 activity, as assessed by blood levels of soluble NOX2-derived peptide (sNOX2-dp), in a population of 250 children, including 125 NC and 125 HC.

RESULTS: HC had higher sNOX2-dp (25.8 ± 16.1 pg/ml) and ox-LDL (23.6 ± 14.4 U/l) levels as compared with NC (15.4 ± 10.1 and 11.6 ± 6.4 pg/ml, respectively; $P < 0.001$). Multiple linear regression analysis showed that ox-LDL and total cholesterol were the only independent predictive variables associated with sNOX2-dp. In NC, ox-LDL and sNOX2-dp significantly decreased from the first to the second quintile of age. In HC, ox-LDL and sNOX2-dp levels did not show significant differences among quintiles of age.

CONCLUSION: This study suggests that NOX2 contributes to generating ox-LDL during the early phase of life in NC and to the persistent high oxidative stress in the prepubertal period of HC.

Atherosclerosis represents the leading cause of death in Western countries (1). Although this pathologic process is a typical adult disease, it is already detectable in children with classic cardiovascular risk factors. Autopsy studies performed in children or youths with established risk factors demonstrated an association with the presence and extent of atherosclerotic lesions in the aorta and coronary arteries (2–4). Among the classic risk factors for atherosclerosis, hypercholesterolemia seems to be a major determinant of early atherosclerosis (2–4). Therefore, autopsy studies documented an association between cholesterol and the extent and severity of atherosclerosis in infants, children, and adolescents (2–4).

Early increase of cholesterol is also relevant in the progression of atherosclerosis in adults (5).

Oxidative stress plays a crucial role in the initiation and progression of atherosclerosis (6). In particular, an imbalance between superoxide and nitric oxide production could be responsible for endothelial dysfunction, the *primum movens* of the atherosclerotic process (7). Nicotinamide adenine dinucleotide phosphate (NADPH) activation has been recognized as the major reactive oxygen species (ROS) source (8). We have provided evidence of a major role for NADPH oxidase in modulating artery tone and generating oxidative stress in hypercholesterolemic children (HC) (9).

Studies conducted in healthy children showed that biomarkers of oxidative stress decreased with increasing age from 1 to 11 y (10). In particular, during the prepubertal period, there is an age-related decrease of oxidative stress markers such as 8-hydroxy-2'-deoxyguanosine levels (10), oxidized low-density lipoprotein (ox-LDL) (10), urinary F₂-isoprostanes (11), and plasma levels of myeloperoxidase (10). Of note, such "physiologic" decline of oxidative stress is not detectable in HC (10), who are, therefore, exposed to the potential damage of oxidative stress even before the pubertal period.

No data have been reported concerning the age-related behavior of oxidative stress generated by NOX2, the catalytic subunit of NADPH oxidase, in healthy children or HC. The aim of this study was to analyze the role of NOX2 in normocholesterolemic children (NC) and HC during the prepubertal period.

RESULTS

Clinical characteristics of the population are reported in [Table 1](#). Apart from serum cholesterol, no significant difference was found between the groups for BMI, systolic and diastolic blood pressure, fasting blood glucose, age, or gender ([Table 1](#)).

Oxidative Stress Indexes According to Hypercholesterolemia

HC had higher ox-LDL and soluble NOX2-derived peptide (sNOX2-dp) levels as compared with NC ([Table 1](#)). No significant difference for oxidative stress indexes was found between males and females in HC and NC ([Table 2](#)). Bivariate

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analysis in the overall population showed that sNOX2-dp was significantly associated with ox-LDL ($r = 0.523$; $P < 0.001$) (Figure 1a), total cholesterol (TC) ($r = 0.333$; $P < 0.001$) (Figure 1b), LDL cholesterol ($r = 0.298$; $P < 0.001$) (Figure 1c), and BMI ($r = 0.185$; $P = 0.003$) (Figure 1d).

sNOX2-dp levels were significantly associated with ox-LDL in both the HC ($r = 0.485$; $P < 0.001$) and NC ($r = 0.291$; $P = 0.001$) population (Figure 1a).

To establish the independent predictors of sNOX2-dp in the overall population, a multiple linear regression analysis, including the variables linearly associated with the dependent

variable, was performed; ox-LDL (SE: 0.069; standardized coefficient β : 0.460; $P < 0.001$) and TC (SE: 0.019; standardized coefficient β : 0.169; $P = 0.005$; $R^2 = 33\%$) were the only independent predictive variables associated with sNOX2-dp levels (Table 3).

Age-Related Oxidative Stress Indexes

To investigate the behavior of sNOX2-dp in children according to age distribution, we summarized the age of the overall population in five quintiles (Quintile (Q)1: <6 y, $n = 25$; Q2: $6-7$ y, $n = 25$; Q3: $8-9$ y, $n = 25$; Q4: $10-11$ y, $n = 25$; Q5: $12-13$ y, $n = 25$); clinical characteristics of each quintile in HC and NC are reported in Table 4.

In NC, TC, high-density lipoprotein cholesterol, and triglycerides did not show any significant differences among quintiles of age; by contrast, sNOX2-dp levels ($P = 0.02$) significantly decreased from the first to the second quintile of age (Figure 2). In HC, the lipid pattern parameters ox-LDL and sNOX2-dp did not show any significant differences among quintiles of age (Figure 2). ox-LDL and sNOX2-dp were significantly higher in HC as compared with NC ($P < 0.001$) from the second to the fifth quintile of age (Figure 2 and Table 4).

DISCUSSION

The main result of the current study is that NOX2 activity is persistently elevated during the prepubertal period in HC subjects, indicating that hypercholesterolemia elicits an early oxidative burden via overactivation of NOX2.

NADPH oxidase constitutes the main source of superoxide in phagocytic and vascular cells (8). The NADPH oxidase-derived ROS are involved in several physiological functions (aerobic metabolism, immunologic responses, cellular signaling, regulation of gene expression, and cell differentiation (8,12–14)) and pathologic (e.g., atherosclerosis, tumorigenesis, and neurodegenerative diseases (8,12–14)) processes. In vascular cells, the NADPH oxidases are implicated in the atherosclerotic process (13). Upregulation of NADPH expression seems to be involved also in the anatomic and functional changes of the arterial wall occurring in HC (15,16). As compared with

Table 1. Clinical characteristics of healthy subjects and hypercholesterolemic children (HC)

	NC	HC	<i>P</i>
	<i>n</i> = 125	<i>n</i> = 125	
Age (y)	8.3 ± 3.2	8.2 ± 2.9	0.671
Gender	62 Males, 63 females	61 Males, 64 females	0.899
Systolic blood pressure (mm Hg)	106 ± 9	107 ± 9	0.505
Diastolic blood pressure (mm Hg)	68 ± 8	69 ± 8	0.764
Total cholesterol (mg/dl)	154.1 ± 18.5	217.7 ± 42.0	<0.001
LDL cholesterol (mg/dl)	91.1 ± 23.3	148.9 ± 42.8	<0.001
HDL cholesterol (mg/dl)	51.8 ± 13.7	53.7 ± 23.3	0.387
Triglycerides (mg/dl)	63.5 ± 32.5	77.3 ± 51.4	0.012
Fasting blood glucose (mg/dl)	77 ± 10	78 ± 10	0.660
BMI	19.3 ± 4.7	18.6 ± 4.1	0.203
ox-LDL (U/l)	11.6 ± 6.4	23.6 ± 14.4	<0.001
sNOX2-dp (pg/ml)	15.4 ± 10.1	25.8 ± 16.1	<0.001

Bold values represent $P < 0.05$.

HDL, high-density lipoprotein; LDL, low-density lipoprotein; NC, normocholesterolemic children; ox-LDL, oxidized LDL; sNOX2-dp, soluble NOX2-derived peptide.

Table 2. Clinical characteristics of healthy subjects and hypercholesterolemic children (HC)

	NC			HC		
	62 Males	<i>P</i>	63 Females	61 Males	<i>P</i>	64 Females
Systolic blood pressure (mm Hg)	107 ± 9	0.398	106 ± 9	107 ± 9	0.286	108 ± 7
Diastolic blood pressure (mm Hg)	67 ± 9	0.855	66 ± 8	69 ± 8	0.638	69 ± 9
Total cholesterol (mg/dl)	152.9 ± 20.4	0.473	155.3 ± 16.6	216.1 ± 38.7	0.674	219.3 ± 45.2
LDL cholesterol (mg/dl)	89.2 ± 21.6	0.347	93.1 ± 25.0	147.5 ± 41.7	0.728	150.2 ± 44.2
HDL cholesterol (mg/dl)	50.9 ± 13.7	0.525	52.5 ± 13.7	52.1 ± 14.4	0.397	54.4 ± 15.7
Triglycerides (mg/dl)	64.3 ± 35.5	0.786	62.7 ± 29.5	82.0 ± 55.1	0.319	72.8 ± 47.4
Fasting blood glucose (mg/dl)	79 ± 10	0.167	77 ± 10	80 ± 8	0.703	79 ± 9
BMI	19.4 ± 4.2	0.895	19.3 ± 5.3	19.1 ± 4.1	0.109	18.0 ± 3.5
ox-LDL (U/l)	13.5 ± 8.3	0.408	12.3 ± 8.2	22.3 ± 13.1	0.692	24.0 ± 15.7
sNOX2-dp (pg/ml)	16.1 ± 10.9	0.454	14.7 ± 9.4	25.1 ± 15.5	0.461	26.4 ± 17.1

HDL, high-density lipoprotein; LDL, low-density lipoprotein; NC, normocholesterolemic children; ox-LDL, oxidized LDL; sNOX2-dp, soluble NOX2-derived peptide.

Figure 1. Linear regression analysis between (a) sNOX2-dp and ox-LDL, (b) total cholesterol, (c) LDL cholesterol, and (d) BMI. Black circles: NC group; white circles: HC group. HC, hypercholesterolemic children; NC, normocholesterolemic children; ox-LDL, oxidized low-density lipoprotein; sNOX2-dp, soluble NOX2-derived peptide.

Table 3. Multiple linear regression analysis

Model	95% Confidence interval	Unstandardized coefficients	Standardized coefficients	P
		SE	β	
ox-LDL	0.368–0.624	0.069	0.460	0.000
Total cholesterol	0.018–0.090	0.019	0.169	0.005

Dependent variable: sNOX2-dp.

ox-LDL, oxidized low-density lipoprotein.

NC, we previously observed an overexpression of platelet gp91 (phox), reduced flow-mediated dilation, and enhanced intima media thickness in HC and, conversely, higher flow-mediated dilation and lower intima media thickness and platelet gp91 (phox) in children with hereditary deficiency of NOX2 (15).

Although this finding is consistent with our previous study demonstrating NOX2 upregulation in children with atherosclerotic risk factors (17), it underscores the fact that the

burden of oxidative stress-related atherosclerotic risk appears in the early phase of life and is dependent on NOX2 regulation.

Therefore, data on the behavior of oxidative stress in childhood consistently showed an enhanced oxidative stress in the first years of life, which has been suggested to reflect a response to higher infection rate (18) or the more rapid basal metabolic rate occurring at this age (17); subsequently, oxidative stress tends to gradually decline until the age of 11 y (19). It is likely that more than one pathway generating ROS is implicated in enhancing oxidative stress during the first years of life. Consistent with this hypothesis is the previous demonstration of myeloperoxidase upregulation and the results of the current study showing NOX2 upregulation.

We also examined if during the prepubertal period, children with hypercholesterolemia show a different behavior of NOX2 and its impact with oxidative stress. This issue is of potential interest because HC have early manifestations of atherosclerotic disease that may be related to prolonged exposure to oxidative stress. In this study, we found that, as compared with

Table 4. Clinical characteristics of healthy subjects and hypercholesterolemic children (HC) according to quintile

	First quintile		Second quintile		Third quintile		Fourth quintile		Fifth quintile	
	NC	HC	NC	HC	NC	HC	NC	HC	NC	HC
	25	25	25	25	25	25	25	25	25	25
Age (y)	3.9±1.2	3.9±1.2	7.0±0.7	6.9±0.6	8.7±0.6	8.4±0.5	10.9±0.5	10.2±0.4	12.5±0.5	12.1±0.3
Gender (females/males)	11/14	13/12	14/11	16/9	14/11	10/15	13/12	16/9	11/14	9/16
Systolic blood pressure (mm Hg)	105±8	105±8	106±7	106±9	107±8	107±7	109±11	110±9	111±11	112±9
Diastolic blood pressure (mm Hg)	68±8	68±9	67±9	68±9	69±8	70±8	70±9	72±7	69±8	71±7
Total cholesterol (mg/dl)	153±18*	224±46*	157±17*	209±33*	160±16*	213±46*	150±22*	216±35*	151±18*	227±48*
LDL cholesterol (mg/dl)	92±17*	163±50*	94±25*	140±30*	93±22*	142±47*	88±21*	141±36*	89±30*	157±45*
HDL cholesterol (mg/dl)	50±14	49±9	57±13	55±14	54±16	56±16	48±10*	57±17*	50±12	54±16
Triglycerides (mg/dl)	57±15*	82±38*	51±16*	73±61*	68±38*	72±37*	76±45	80±68	64±32*	78±46*
Fasting blood glucose (mg/dl)	69±8	72±6	74±8	76±6	74±9	85±8	81±8	85±8	84±8	83±8
BMI	16.2±2.4	16.2±1.4	17.1±3.4	17.6±3.6	18.8±3.4	18.7±3.8	20.8±5.7	19.7±4.5	20.9±4.1	20.9±5.1
ox-LDL (U/l)	14.6±7.3*	21.4±11.6*	10.5±4.7*	22.6±13.6*	10.0±6.5*	22.1±13.9*	12.0±6.0*	26.2±18.4*	10.9±6.7*	25.9±14.1*
sNOX2-dp (pg/ml)	20.3±13.6	21.7±10.3	11.6±6.8*	24.1±14.6*	14.3±11.1*	23.5±19.5*	15.0±8.6*	26.9±16.7*	15.7±7.6*	32.6±16.9*

* $P < 0.05$, NC vs. HC.

HDL, high-density lipoprotein; LDL, low-density lipoprotein; NC, normocholesterolemic children; ox-LDL, oxidized LDL; sNOX2-dp, soluble NOX2-derived peptide.

those of NC, sNOX2-dp levels were higher in HC from the second to the fifth quintile, suggesting a precocious role for this ROS-generating pathway in enhancing oxidative stress. Furthermore, the significant correlation between sNOX2-dp and ox-LDL suggests a role for this ROS-generating pathway in promoting LDL oxidation. ox-LDL elicits an array of pro-atherogenic and proinflammatory responses (20) that could contribute to vascular complications in HC.

Together, these results show that hypercholesterolemia in children elicits functional changes that ultimately lead to an early oxidative stress. Such changes are not dependent only on the activation of NOX2 because activation of myeloperoxidase is also involved (10). The mechanism accounting for the upregulation of these two enzymatic pathways is still unknown and deserves further investigation.

This study has some limitations that should be acknowledged. One of the limitations is the small sample size of our population; larger studies need to evaluate the effect of NADPH oxidase in children. Moreover, we cannot exclude that other

NADPH oxidase isoforms, such as NOX1 (8) and NOX4 (8), or other ROS-generating pathways, such as the xanthine oxidase pathway (21), could be potentially implicated in the oxidative stress formation observed in NC and HC.

In conclusion, this study suggests that in NC, NOX2 contributes to generating ox-LDL during the early phase of life and is implicated in the persistent high oxidative stress observed in the prepubertal period of HC.

METHODS

A total of 250 consecutive subjects, matched for age and gender, including 125 NC and 125 HC, were enrolled through a screening program performed in Italian schools for metabolic disease prevention in childhood. In this population, we measured serum ox-LDL, as a marker of oxidative stress, and NOX2 activity as assessed by blood levels of sNOX2-dp. BMI was determined according gender- and age-specific growth charts (22). All the children with hypercholesterolemia had a polygenic hypercholesterolemia, classified as the presence of TC >90th age- and gender-specific percentile and without clear family transmission (15). Exclusion criteria for all subjects included the existence of hypothyroidism, renal disease, malignancy,

Figure 2. sNOX2-dp and ox-LDL levels according to quintile. (a) sNOX2-dp and (b) ox-LDL values in HC and NC according quintiles. Black circles: NC group; white triangles: HC group. * $P < 0.05$. HC, hypercholesterolemic children; NC, normocholesterolemic children; ox-LDL, oxidized low-density lipoprotein; sNOX2-dp, soluble NOX2-derived peptide.

treatment with immunosuppressive drugs, connective tissue disease, and acute illness.

All subjects were enrolled from the same geographic area and followed a typical Mediterranean diet. None of the patients had clinical evidence of cardiovascular disease (as shown by clinical history, physical examination, or electrocardiogram), diabetes mellitus, or hypertension. Patients with hypercholesterolemia had not taken any lipid-lowering agents.

Blood Sampling

Between 8.00 and 9.00 AM, subjects underwent routine biochemical evaluations including fasting TC and glucose levels. After overnight fasting (12 h) and supine rest for at least 10 min, blood samples were collected in vacutainers (Vacutainer Systems, Belliver Industrial Estate, Plymouth England, UK) and centrifuged at 5,000g for 10 min to obtain supernatant, which was stored at -80°C until use.

Serum TC, high-density lipoprotein cholesterol, and triglycerides were measured by an enzymatic colorimetric method. LDL-C levels were calculated according to the Friedwald formula.

Informed written consent was obtained from all subjects. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of Sapienza University.

sNOX2-dp. NOX2-dp, a marker of NADPH oxidase activation, was detected in serum by an enzyme-linked immunosorbent assay method as previously described by Pignatelli *et al.* (23). The peptide was recognized by the specific monoclonal antibody against the amino acid sequence (224–268) of the extra membrane portion of NOX2. Values were expressed as pg/ml; intraassay and interassay coefficients of variation were 5.2 and 6%, respectively.

ox-LDL. Serum levels of ox-LDL were measured by commercially available immunoassays (Tema Ricerca, Bologna, Italy). Intraassay and interassay coefficients of variation were 4.0 and 8.3% for ox-LDL.

Statistical Analyses

Comparisons between groups were carried out by Student's *t*-test. Data were presented as mean $\pm$ SD unless otherwise indicated.

Comparisons to detect differences among age groups were carried out by ANOVA for multiple measures, with Bonferroni correction for *post hoc* analysis. Proportions and categorical variables were tested by the χ^2 test. The correlation analysis was carried out by Pearson's test. To determine the independent predictors of sNOX2-dp, multiple linear regression analysis was performed using a forward-selection method. Statistical significance was defined at $P < 0.05$. Statistical analysis was performed with SPSS 18.0 software for Windows (SPSS, Chicago, IL).

Sample Size Determination

We computed the minimum sample size with respect to a two-tailed one-sample Student's *t*-test, considering (i) a difference for sNOX2-dp to be detected between the healthy subjects and HC as $|\delta| \geq 10$ pg/ml, (ii) homogeneous SDs between groups as 16, and (iii) type I error probability as $\alpha = 0.05$ and power $1 - \beta = 0.90$. This resulted in a minimum sample size of $n = 54$ for group.

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