Serum levels of nicotinamide-adenine dinucleotide phosphate oxidase 4 are associated with non-valvular atrial fibrillation

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Abstract. Recent evidence indicates that nicotinamide-adenine dinucleotide phosphate oxidase (NOX)-derived reactive oxygen species have a pivotal role in the development of atrial fibrillation (AF). The present study aimed to investigate the potential association between serum levels of NOX4, as well as inflammatory biomarkers and AF. In total, 108 patients with AF (71 with paroxysmal AF and 37 with persistent/permanent AF) and 68 patients without AF, as the controls, were enrolled. The demographic, clinical, laboratory, electrocardiographic and echocardiographic characteristics were carefully recorded. Serum levels of myeloperoxidase (MPO), high-sensitivity C-reactive protein (hs-CRP) and NOX4 were assessed. Left atrial diameter (LAD), left ventricular end-diastolic diameter and P-wave dispersion were significantly increased in patients with paroxysmal AF and persistent/permanent AF compared with the controls, while NOX4 levels were significantly higher in patients with paroxysmal AF and persistent/permanent AF compared to the controls (155.57 ± 90 and 155.88 ± 64.79 vs. 126.72 ± 23.51 pg/ml, respectively, $P<0.05$). A significant correlation between serum NOX4 levels and hs-CRP, and between NOX4 levels and MPO was also evident ($r=0.170$; $r=0.227$, $P<0.05$, respectively). Multivariate analysis demonstrated that the baseline serum NOX4 level was independently associated with paroxysmal AF [odds ratio (OR)=1.014; 95% confidence interval (CI), 1.001-1.027; $P<0.05$] and with persistent/permanent AF (OR=1.022; 95% CI, 1.000-1.044; $P<0.05$). There appears to be an association between increased NOX4 levels and AF, suggesting NOX4 involvement in the pathophysiology of human AF. Further studies are required to elucidate its role in atrial remodeling and to examine its potential prognostic impact.

Introduction

Atrial fibrillation (AF) represents an evolving, global epidemic that is associated with increased morbidity and mortality (1,2). Recently, there has been an increasing interest for new biomarkers that may have an impact on the management and prognosis of AF (3). Notably, accumulating evidence suggests the involvement of inflammation and oxidative stress in the development and maintenance of AF (4-6). Increased levels of serum inflammatory biomarkers have been associated with AF development and perpetuation, AF burden, and with thromboembolic events (6). Additionally, excessive production of reactive oxygen species (ROS) appears to be implicated in atrial electrical and structural remodeling. The nicotin-amide-adenine dinucleotide phosphate oxidases (NOXs) represent a family of multi-subunit enzymatic complexes that have a major role in the production of ROS in the cardiovascular system. In particular, the isoforms NOX2 and NOX4 are the major NOX isoforms expressed in the heart while their activation is involved in the pathogenesis of AF (7). NOX2 was upregulated in atrial tissue of a goat rapid-pacing model or in the serum of patients with paroxysmal/persistent AF (8,9). With regard to NOX4, the study by Zhang *et al* (10) showed that H_2O_2 production in the left atrial (LA) appendage tissue was more than doubled in AF patients compared with controls, and had an association with higher *NOX4* mRNA expression. In this prospective pilot observational study, the aim was to investigate the potential association between serum NOX4 levels, as well as inflammatory biomarkers and AF.

Materials and methods

Patients. In the period between August 1, 2012 and June 30, 2013, 180 consecutive patients with AF who were admitted to the Department of Cardiology, Second Hospital of Tianjin Medical University (Tianjin, China) were screened for inclusion in the study. The final study population consisted

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of 108 consecutive AF patients. The patients were divided into 3 groups: Paroxysmal AF (n=71), persistent/permanent AF (n=37) and patients without AF as the controls, which were matched for gender, age and atherosclerotic risk factors (n=68). The control group patients were individuals who were admitted to the department during the same period for evaluation of atypical chest pain, initial evaluation of mild hypertension (no hypertensive urgencies or emergencies) or for regular clinical examination without history of arrhythmias.

AF subtypes. An electrocardiographic documentation of AF was obligatory. The subtypes of AF were defined according to European Society of Cardiology Guidelines (11). Exclusion criteria were as follows: i) Severe congestive heart failure (New York Heart Association functional class III or IV); ii) history of coronary artery bypass grafting; iii) history of myocardial infarction; iv) significant valvular heart disease; v) hypertrophic or dilated cardiomyopathy; vi) congenital heart disease; vii) serious liver or kidney dysfunction [serum creatinine (Cr) >3 mg/dl]; viii) history of acute coronary syndrome in the past 6 months; ix) stroke or transient ischemic attack within the previous 3 months; x) acute infection or inflammatory/neoplastic diseases; xi) thyroid dysfunction; and xii) current smokers or those prescribed antioxidants/anti-inflammatory drugs. The study was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University and written informed consent was obtained from all the patients.

Baseline characteristics. Baseline demographic and clinical characteristics were carefully recorded. White blood cell count, red blood cell distribution width, serum Cr, fasting blood-glucose, alanine aminotransferase and aspartate aminotransferase were measured after 12-h overnight fasting. As a measure of renal function, the baseline glomerular filtration rate was estimated (eGFR) using the abbreviated Modification of Diet in Renal Disease study equation: eGFR (ml/min/1.73 m^2 of body surface area) = 186 x (serum Cr in mg/dl)$^{-1.154}$ x (age in years)$^{-0.203}$ (x 0.742 in female subjects) (12). A transthoracic echocardiographic examination was performed in all the patients using the Vivid-7 system equipped with a 2.4 MHz transducer (GE Medical Systems, Milwaukee, WI, USA). LA diameter (LAD), interventricular septal thickness, left ventricular posterior wall thickness and left ventricular end-diastolic diameter were assessed. Left ventricular ejection fraction was determined from apical 4-chamber and 2-chamber views using Simpson's biplane formula. All the echocardiographic data were analyzed by the same investigator who was blinded to the clinical status of the participants. A 12-lead electrocardiogram at a signal size of 10 mm/mV and paper speeds of 50 mm/sec was recorded in all the patients with paroxysmal AF and controls, when the patients were in sinus rhythm. P-wave duration was measured from the beginning (junction between the isoelectric line and the beginning of P-wave deflection) and the end of the P-wave (junction between the end of P-wave deflection and isoelectric line). P-wave dispersion (PWD) was measured by calculating the difference between the duration of the longest and the shortest P-waves in lead II in msec by one independent investigator.

Assessment of biomarkers. After 12-h overnight fasting and supine rest for ≥10 min, blood samples were collected and centrifuged at 1,000 x g for 10 min to obtain the supernatant, which was stored at -80˚C until use for the determination of specific inflammatory and oxidative biomarkers. Serum high-sensitivity C-reactive protein (hs-CRP) was measured using a high-sensitivity immmunonephelometric assay. Serum myeloperoxidase (MPO) was detected by a colorimetry method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum levels of NOX4 were determined by an ELISA method (Cusabio Biotech Co., Ltd., Wuhan, China). The minimum detectable concentration of NOX4 was ranged linear from 9.38 to 600 pg/ml, and intra-assay and inter-assay variabilities were <8 and <10%, respectively.

Statistical analysis. Categorical variables are reported as numbers (percentages) while continuous variables are mean ± standard deviation or median (interquartile range) values. The normality of the distribution of each variable was tested by the Kolmogorov-Smirnov test. Differences between >2 study groups were analyzed using the one-way analysis of variance test or the non-parametric Kruskal-Wallis test as appropriate, while differences between 2 groups were assessed by the unpaired t-test or Student-Newman-Keuls-q test for continuous variables, while the χ^2 test was used to compare categorical variables. Bivariate analysis was performed with Pearson correlation analysis. Appropriate non-parametric tests (Mann-Whitney U test and Spearman rank correlation test) were used for all the other variables. Multivariable regression analyses were performed in order to investigate the association between NOX4 and different types of AF. P<0.05 was considered to indicate a statistically significant difference. All the tests were two-tailed and analyses were performed using SPSS 17.0 statistical package program for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Clinical characteristics. The clinical characteristics of the studied patients are presented in Table I. The prevalence of cardiovascular risk factors and the associated medications were comparable between the 3 groups. There were no significant differences in hs-CRP, MPO and eGFR between the 3 groups. However, serum NOX4 levels were significantly higher in the patients with paroxysmal AF and persistent/permanent AF compared to the controls. No significant difference in serum NOX4 levels was identified between the patients with paroxysmal AF and those with persistent/permanent AF. Furthermore, correlation analysis showed a significant correlation between serum NOX4 levels and hs-CRP, and between NOX4 and MPO levels (r=0.17; r=0.227, P<0.05, respectively).

Multivariate analysis. The multivariate analysis showed that independent predictors of paroxysmal AF were serum NOX4, LAD and PWD (Table II). By contrast, independent predictors of persistent/permanent AF were serum NOX4 and LAD (Table III).

Discussion

In the present study, an independent association was demonstrated between serum NOX4 levels and the presence of AF.

Table I. Baseline clinical and echocardiographic characteristics of the study population.

Characteristics	Controls (n=68)	Paroxysmal AF (n=71)	Persistent/permanent AF (n=37)	P-value
Clinical features				
Age, years	66.4±10.6	67.9±11.5	67.8±10.2	0.715
Gender, male, no. (%)	28 (41.2)	31 (43.7)	20 (54.1)	0.432
Hypertension, no. (%)	45 (66.2)	45 (63.4)	28 (75.7)	0.427
Coronary artery disease, no. (%)	34 (50.0)	34 (47.9)	22 (59.5)	0.238
Diabetes mellitus, no. (%)	9 (13.2)	11 (15.5)	10 (27.0)	0.180
History of stroke, no. (%)	1 (1.5)	10 (14.1)	5 (13.5)	0.020
Hypercholesterolemia, no. (%)	23 (33.8)	27 (38.0)	12 (32.4)	0.807
Smoking habit, no. (%)	4 (5.9)	10 (14.1)	5 (13.5)	0.248
Laboratory examinations				
WBC count, $10^9/l$[a]	6.70±1.76	7.62±1.23	7.07±1.86	0.527
RDW, % CV	12.59±0.59	12.68±0.73	12.72±0.60	0.537
Cr, μmol/l	66.12±17.86	70.33±33.40	71.53±17.76	0.486
eGFR, ml/min/1.73 m^2	96.51±23.85	95.84±31.19	91.63±22.95	0.654
AST, U/l	16.52±17.17	16.91±9.02	15.69±3.93	0.886
ALT, U/l	18.20±15.91	19.18±13.84	17.18±9.31	0.772
FBG, mmol/l	5.89±1.63	6.08±1.74	6.09±1.93	0.780
NOX4, pg/ml	126.72±23.51	155.57±90.00	155.88±64.79	0.038
hs-CRP, mg/l	0.78 (4.88)	1.20 (7.68)	1.36 (7.62)	0.551
MPO, U/l	0.149±0.029	0.160±0.040	0.158±0.036	0.177
PWD, msec	47.16±13.31	58.58±17.41		<0.0001
Echocardiogram parameters				
LAD, mm	33.89±4.86	37.38±6.30	44.21±6.92	<0.0001
LVEDD, mm	47.19±5.51	46.90±5.20	50.65±6.77	0.006
LVPWT, mm	9.06±3.26	9.32±2.28	9.29±1.48	0.388
IVST, mm	9.09±1.05	9.59±2.36	9.24±1.82	0.832
LVEF, %	60.93±5.58	59.67±6.48	54.27±10.20	<0.0001
Previous medication, no. (%)				
Aspirin	54 (79.4)	55 (77.5)	31 (83.8)	0.742
Statins	55 (80.9)	59 (83.1)	29 (78.4)	0.833
ACEIs/ARBs	21 (30.9)	33 (46.5)	16 (43.2)	0.152
β-blocker	20 (29.4)	23 (32.4)	13 (35.1)	0.827
CCB	28 (41.2)	31 (43.7)	20 (54.1)	0.432

[a]Data are expressed as mean ± standard deviation or median (range). AF, atrial fibrillation; WBC, white blood cell; RDW, red blood cell distribution width; CV, coefficient of variation; Cr, serum creatinine; eGFR, estimated glomerular filtration rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FBG, fasting blood-glucose; NOX4, nicotinamide-adenine dinucleotide phosphate oxidase 4; hs-CRP, high-sensitivity C-reactive protein; MPO, myeloperoxidase; LAD, left atrial diameter; IVST, interventricular septal thickness; LVPWT, left ventricular posterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; PWD, P-wave dispersion; ACEIs, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blocking agents; CCBs, calcium channel blockers.

To the best of our knowledge, this is the first human study implicating this novel oxidative stress marker in AF. Of note, no significant difference in NOX4 levels was evident between paroxysmal and persistent/permanent forms. However, no firm conclusions can be made regarding its association with the AF burden.

A large body of evidence indicates that apart from the triggers, AF development and perpetuation depends on the electrophysiological and structural remodeling of the atria (13). The pathophysiology of atrial remodeling is extremely complex and the molecular pathways indicated in the initiation and perpetuation of AF show a high diversity and variability across different underlying substrates (14,15). During the past few years the role of pathophysiological pathways that involve inflammatory and oxidative processes is under investigation (4,16-18). NOXs have been proposed to be critical enzymatic sources of ROS and appear to have an important role in the pathogenesis of hypertension, atherosclerosis and heart failure (19-21). Thus far, a total of 5 NOX isoforms (NOX1-5) and 2 related dual oxidase enzymes have been discovered (22). The major NOX isoforms expressed in the cardiovascular system are NOX2 and NOX4, found in

Table II. Multivariate logistic regression analysis on predictors of paroxysmal atrial fibrillation.

Characteristics	β	SE	Wald	P-value	OR (95% CI)
LAD	0.106	0.043	6.003	0.014	1.111 (1.021-1.209)
LVEDD	-0.096	0.054	3.171	0.075	0.908 (0.817-1.010)
LVEF	-0.042	0.040	1.101	0.294	0.959 (0.886-1.037)
eGFR	0.005	0.008	0.334	0.563	1.005 (0.989-1.020)
PWD	0.046	0.017	7.561	0.006	1.047 (1.013-1.082)
NOX4	0.014	0.007	4.676	0.031	1.014 (1.001-1.027)
hs-CRP	-0.046	0.056	0.670	0.413	0.955 (0.856-1.066)

LAD, left atrial diameter; LVEDD, left ventricular end diastolic diameter; LVEF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate; PWD, P-wave dispersion; NOX4, nicotinamide-adenine dinucleotide phosphate oxidase 4; hs-CRP, high-sensitivity C-reactive protein; OR, odds ratio; CI, confidence interval; β, regression coefficient; SE, standard error.

Table III. Multivariate logistic regression analysis on predictors of persisitent/permanent atrial fibrillation.

Characteristics	β	SE	Wald	P-value	OR (95% CI)
LAD	0.323	0.079	16.512	<0.0001	1.381 (1.182-1.613)
LVEDD	0.053	0.071	0.551	0.458	1.054 (0.917-1.212)
LVEF	-0.072	0.056	1.662	0.197	0.930 (0.833-1.038)
eGFR	-0.006	0.016	0.134	0.714	0.994 (0.962-1.027)
NOX4	0.022	0.011	3.967	0.046	1.022 (1.000-1.044)
hs-CRP	0.034	0.049	0.499	0.480	1.035 (0.941-1.139)

LAD, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate; NOX4, nicotinamide-adenine dinucleotide phosphate oxidase 4; hs-CRP, high-sensitivity C-reactive protein; OR, odds ratio; CI, confidence interval; β, regression coefficient; SE, standard error.

endothelial cells, vascular smooth muscle cells, cardiomyocytes and fibroblasts. In addition, recent findings indicate that NOX4 predominantly generates H_2O_2 in contrast to NOX2, which generates O_2 (19-21). In particular, NOX4 has been demonstrated to participate in cell differentiation (23), fibroblast proliferation (24) and myofibroblast activation (25), while it induces apoptosis, cardiac hypertrophy and fibrosis (20,26).

Previous evidence implies that NOX-derived ROS have a critical role in the development and maintenance of AF7. The study by Dudley *et al* (27) reported that decreased nitric oxide (NO) and increased superoxide production in the LA appendage was associated with increased NOX activity in an experimental model of atrial tachy-pacing. Notably, the NOX inhibitor apocynin reduced the superoxide production by 91%. Additionally, Kim *et al* (28) examined the sources of superoxide production from the right atrial appendage of patients undergoing cardiac surgery and reported that NOX2 was possibly the main source of atrial superoxide production in human atrial myocytes during sinus rhythm and AF. Of note, Cangemi *et al* (9) demonstrated that serum levels of soluble NOX2-derived peptide were upregulated in patients with paroxysmal/persistent AF compared to those with permanent AF, as well as compared to the controls. Zhang *et al* (10) indicated that angiotensin II significantly upregulates NOX4 expression and H_2O_2 production in atrial HL-1 cells. Therefore,

NOX4-induced H_2O_2 production may be involved in the pathogenesis of AF.

In this context, the present study supports the role of NOX4 activation in AF, suggesting an independent association. However, the cause-effect association is not clear. Whether NOX4 inhibition modulates atrial remodeling and prevents AF promotion and perpetuation is a noteworthy area for future investigation. NOX4 was also demonstrated to correlate with MPO and hs-CRP. Thus, the possibility that the upregulation of NOX4 reflects the inflammatory status, which often complicates the clinical course of AF, cannot be excluded. In addition, serum NOX4 levels between paroxysmal and persistent/permanent AF had no significant difference. A plausible explanation could be the relatively small size of the patient groups. The increase in oxidative stress is also dependent on different sources of ROS according to the type of AF. Other sources of ROS, such as uncoupled endothelial NO synthase and mitochondrial oxidases, have been indicated in the setting of permanent AF (8).

Previous studies have shown that increased levels of inflammatory biomarkers, including hs-CRP and MPO, are associated with an increased risk of AF (6,29). They can also predict AF recurrence following successful electrical cardioversion (5) or catheter ablation (29). However, this association was not observed in the present study and may be due to the relatively small sample size and to differences in the study population.

Certain potential limitations should be acknowledged. First, the study population was small. Second, there are no data on the exact AF burden (number of episodes and duration). Additionally, data on the prognostic significance of NOX4 in terms of future AF burden and other future untoward events is unavailable. The intracellular levels of NOX4 in order to investigate the association with plasma levels were not assessed. Finally, the effect of agents with pleiotropic properties on this oxidative stress marker was not studied.

In conclusion, there appears to be an association between increased NOX4 levels and AF, suggesting NOX4 involvement in the pathophysiology of human AF. Further studies are required to elucidate its role in atrial remodeling and to examine its potential prognostic impact.

Acknowledgements

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