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Impaired platelet nitric oxide response in patients with new onset atrial fibrillation

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ABSTRACT

Background: Clinical factors associated with thromboembolic risk in AF patients are well characterized and include new onset AF. Biochemically, AF is associated with inflammatory activation and impairment of nitric oxide (NO) signalling, which may also predispose to thromboembolism: the bases for variability in these anomalies have not been identified. We therefore sought to identify correlates of impaired platelet NO signalling in patients hospitalized with atrial fibrillation (AF), and to evaluate the impact of acuity of AF.
Methods: 87 patients hospitalized with AF were evaluated. Platelet aggregation, and its inhibition by the NO donor sodium nitroprusside, was evaluated using whole blood impedance aggregometry. Correlates of impaired NO response were examined and repeated in a "validation" cohort of acute cardiac illnesses.
Results: Whilst clinical risk scores were not significantly correlated with integrity of NO signalling, new onset AF was associated with impaired NO response ($6 \pm 5\%$ inhibition versus $25 \pm 4\%$ inhibition for chronic AF, $p < 0.01$). New onset AF was a multivariate correlate ($p < 0.01$) of impaired NO signalling, along with platelet ADP response ($p < 0.001$), whereas the associated tachycardia was not. Platelet ADP response was predicted by elevation of plasma thrombospondin-1 concentrations ($p < 0.01$). Validation cohort evaluations confirmed that acute AF was associated with significant ($p < 0.05$) impairment of platelet NO response, and that neither acute heart failure nor acute coronary syndromes were associated with similar impairment.
Conclusion: Recent onset of AF is associated with marked impairment of platelet NO response. These findings may contribute to thromboembolic risk in such patients.

1. Introduction

Atrial fibrillation (AF) represents a major cause of morbidity and mortality in ageing populations worldwide [1]. Apart from the adverse effects of AF on hemodynamic status, its major impact is the potential for development of intra-atrial thrombosis and thromboembolism. Thus, AF represents a substantial risk for cerebrovascular accident (CVA) [2]. Determinants of thromboembolic risk in patients with AF have historically been identified on the basis of epidemiological studies, yielding indices such as the $CHADS_2$ score [3]. More recently, these have been refined to include the recognition of increased risk in ageing females as a component of the CHA_2DS_2VASc score [4]. Finally, a number of studies have recently documented incremental thromboembolic risk in the setting of acute AF [5–7]. The pathophysiological bases for these associations remain incompletely understood.

Once perceived as a product purely of atrial distension, AF is now recognized to have a largely inflammatory basis. A number of studies have associated risk of AF with markers of inflammation, in particular with leukocyte infiltration of atria together with release of the inflammatory enzyme myeloperoxidase (MPO) [8,9]. Additionally, AF has been associated with markers of endothelial dysfunction: diminution of nitric oxide (NO) availability might in theory contribute to inflammation [10–14].

Abbreviations: AF, atrial fibrillation; CVA, cerebrovascular accident; MPO, myeloperoxidase; NO, nitric oxide; LVEF, left ventricular ejection fraction; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; TSP-1, thrombospondin-1; Txnip, thioredoxin-interacting protein; ACE, angiotensin-converting enzyme.

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Intriguingly, both inflammation and reduced availability of NO might also be relevant to thromboembolic risk in AF. Many clinical factors known to modulate risk of CVA in AF, such as hypertension, cardiac failure, advanced age and diabetes mellitus, have also been shown to be associated with impaired NO signalling [15]. This intersection of inflammation, NO signalling and thromboembolic risk can be demonstrated in platelets, which have been shown to have important roles in inflammatory mediation, haemostasis and under pathological conditions, atherogenesis [16]. Impairment of NO signalling in platelets has previously been shown to have adverse implications for morbidity and mortality in acute coronary syndromes [17]. Whilst platelet hyperaggregability has been shown to be present in AF, this has never been related closely to clinical risk [18–20]. However, these studies failed to address the potential impact that impairment of the NO signalling pathway within platelets (which functions to limit platelet activation and aggregation) may have on thromboembolic risk.

In the current investigation, we sought to determine correlates of: (a) Impaired platelet NO response and (b) Platelet hyperaggregability in a cohort of patients hospitalized with AF. We hypothesized that risk factors for thromboembolic complications of AF, including CHA_2DS_2VASc score [21] and acuity of AF [5,22,23] would be correlated with impaired platelet NO response and/or hyperaggregability. Our data provide evidence that recent onset AF represents a state of particular impairment of NO signalling.

2. Methods

2.1. Patient selection

2.1.1. Index cohort

The investigation was conducted as a single centre mechanistic sub-study of the Standard vs. Atrial Fibrillation spEcific managemenT studY (SAFETY), an investigation of non-pharmacological management strategies in patients hospitalized with AF [24]. Patients were considered for inclusion if they were admitted to hospital due to AF. Exclusion criteria for SAFETY were age <45 years, primary diagnosis of valvular heart disease, scheduled catheter ablation of AF, pre-existing NYHA class III–IV heart failure with a documented LVEF <45%, alcohol-induced AF and terminal illness requiring palliative care. Patients receiving $P2Y_{12}$ receptor antagonists were also excluded from the current sub-study because of potential impact of such agents on capacity to measure platelet response to NO.

2.1.2. Validation cohort

The results from the index cohort indicated that platelet NO response was impaired in new onset AF (see Results). Prospective validation of these results was performed in the following additional groups of patients:

(1) New onset AF (n = 15)
(2) Acute coronary syndromes (unstable angina pectoris/acute non-Q-wave myocardial infarction, "ACS" [n = 31])
(3) Stable angina pectoris ("SAP", n = 20)
(4) Acute heart failure (n = 25).

The study was approved by the institutional Ethics of Human Research Committee. Written informed consent was obtained in all cases.

2.2. Clinical data

All patients underwent standardized clinical assessment and routine biochemical investigation. Additional cardiac investigations were resting ECG (which was used for measures of admission heart rate) and transthoracic echocardiography: left ventricular ejection fraction (LVEF) was calculated from biplane images using Simpson's method [25].

2.3. Blood sampling

Blood samples were obtained following admission for biochemical/physiological investigations as follows.

2.3.1. Platelet aggregometry

Platelet aggregometry was performed using whole blood impedance aggregometry as previously described [26]. Briefly, venous blood was collected from an antecubital vein into 10 ml tubes containing 1:10 volume of acid citrate anticoagulant (2 parts 0.1 M citric acid to 3 parts of 0.1 M trisodium citrate). Aggregation was induced with ADP (2.5 μM), and responses were recorded for electrical impedance (Ω) via a computer interface system (Aggrolink, Chrono-Log, Havertown, Pennsylvania, USA). The NO donor sodium nitroprusside (SNP, 10 μM) was used to measure platelet response to NO. Inhibition of aggregation by SNP was evaluated as percentage of maximal aggregation in the absence of SNP. In order to minimize inaccuracies in calculation of inhibitory effect of SNP, at least 4 Ω of ADP response was required.

2.3.2. Plasma asymmetric and symmetric dimethylarginine concentrations

Plasma asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) concentrations were evaluated in view of the role of ADMA as an endogenous inhibitor of NO generation [27]. Peripheral blood was collected into sodium heparin tubes and placed immediately on ice. Plasma was stored at −70 °C until analysis. Plasma ADMA and SDMA levels were determined by high performance liquid chromatography as reported previously [28].

2.3.3. Plasma thrombospondin-1 concentrations

Plasma levels of thrombospondin-1 (TSP-1), which has been shown to inhibit NO generation and signalling [29–31], were determined by enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems, USA). Peripheral blood was collected into sodium heparin tubes and placed immediately on ice. Platelet poor plasma was stored at −70 °C until analysis. Intra-assay CV was 2.8% and inter-assay CV was 5.0%.

2.3.4. Plasma myeloperoxidase concentrations

Plasma levels of MPO, which has been implicated in the inflammatory response associated with AF, were determined by ELISA (Mercodia, Sweden). Peripheral blood was collected into sodium heparin tubes and placed immediately on ice. Platelet poor plasma was stored at −70 °C until analysis. Intra-assay CV was 7.6% and inter-assay CV was 8.6%.

2.3.5. Platelet thioredoxin-interacting protein determination

Thioredoxin-interacting protein (Txnip) is a pro-inflammatory α-arrestin protein which appears to modulate NO signalling [32,33]. Platelet Txnip content was determined semi-quantitatively using immunohistochemistry as previously described [32]. Briefly, EDTA-anticoagulated blood was centrifuged to obtain platelet rich plasma, which was smeared onto untreated slides and fixed using 4% (w/v) paraformaldehyde in PBS, then stored at −70 °C until assayed. Slides were blocked using 20% (v/v) goat serum in PBS, followed by Txnip detection using rabbit polyclonal anti-human VDUP-1 (Invitrogen, USA), 1% (w/v) BSA in PBS and incubating overnight at 2–4 °C. Secondary detection was performed using FITC-conjugated swine anti-rabbit polyclonal IgG (Dako, Denmark), as well as primary detection of platelet CD41 using RPE-conjugated mouse monoclonal anti-human CD41 (Dako, Denmark) in PBS. Fluorescence was developed using 'fluorescent mounting medium' (Dako, Denmark) and images acquired at 400× magnification using an Axio Scope.A1 microscope with apotome and AxioVision 4.8 software (Carl Zeiss, Germany). Images were analysed for densitometric fluorescence using AxioVision LE software. The intra-assay CV was 8.5% and the inter-assay CV was 18.6%.

2.3.6. Statistical methods

Data were analysed by evaluating potential univariate followed by multivariate correlations with (a) platelet response to NO and (b) platelet response to ADP. Clinical factors evaluated were age, sex, LVEF, acuity of AF (with "new onset" AF defined on the basis of de novo detection), admission heart rate and CHA_2DS_2VASc score. Biochemical parameters were ADMA, SDMA, TSP-1 and MPO concentrations as well as platelet Txnip content. ANCOVA was used to evaluate the ADP:NO response relationship in different patient cohorts. Patient characteristics were compared by non-paired t-test, Mann–Whitney U test or χ^2 test as appropriate. Data for the validation cohorts of patients were analysed using Dunnett's test for multiple comparisons. All data for normally distributed parameters are expressed as mean ± standard error of the mean unless otherwise stated. Skewed data are expressed as median and interquartile range. Data were analysed using the IBM SPSS Statistics 19 and GraphPad Prism 5 software packages.

3. Results

3.1. Patient characteristics

The clinical profiles of the index study cohort are shown in Table 1A. Patient characteristics for the AF cohort were typical for an ageing population with multiple risk factors for thromboembolism. Only 58.6% of AF patients from the index cohort had started oral anticoagulant therapy at time of discharge: this can largely be attributed to low perceived thromboembolic risk (i.e. $CHADS_2$ score of 0–1), the presence of severe comorbidities, or high bleeding risk (e.g. previous cerebral haemorrhage) among the non-anticoagulated AF population. Aspirin therapy was present in 33.3% of the cohort, however no other anti-aggregatory agents were used. The majority of patients were subject to rate control therapies. As regards comparisons between patients with chronic and those with new onset AF, there were a number of differences: patients with new onset AF were younger, with better renal function, and had substantially more rapid heart rates. However, plasma NT-proBNP concentrations did not differ significantly according to acuity of AF (p = 0.107). The characteristics of new onset AF patients in index and