Nitrite circumvents platelet resistance to nitric oxide in patients with heart failure preserved ejection fraction and chronic atrial fibrillation

Alessandra Borgognone1, Eduard Shantsila1,2, Sophie M. Worrall1, Eakkapote Prompunt1, Thomas Loka1, Brodie L. Loudon3, Myriam Chimen1, G. Ed Rainger1, Janet M. Lord4, Ashley Turner2, Peter Nightingale5, Martin Feelisch6, Paulus Kirchhof1,2, Gregory Y. H. Lip1,2, Steve P. Watson1, Michael P. Frenneaux3†, and Melanie Madhani1*,†

1Institute of Cardiovascular Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK; 2Sandwell and West Birmingham NHS Trust, City Hospital, Birmingham B18 7QH, UK; 3Norwich Medical School, University of East Anglia, Norwich NR4 7UQ, UK; 4Institute of Inflammation and Ageing, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK; 5Wellcome Trust Clinical Research Facility, Queen Elizabeth Hospital, Edgbaston, Birmingham B15 2TT, UK; and 6Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton SO16 6YD, UK

Received 5 December 2016; revised 21 November 2017; editorial decision 29 March 2018; accepted 9 April 2018

Time for primary review: 43 days

Aims
Heart failure (HF) is a pro-thrombotic state. Both platelet and vascular responses to nitric oxide (NO) donors are impaired in HF patients with reduced ejection fraction (HFrEF) compared with healthy volunteers (HVs) due to scavenging of NO, and possibly also reduced activity of the principal NO sensor, soluble guanylate cyclase (sGC), limiting the therapeutic potential of NO donors as anti-aggregatory agents. Previous studies have shown that nitrite inhibits platelet activation presumptively after its reduction to NO, but the mechanism(s) involved remain poorly characterized. Our aim was to compare the effects of nitrite on platelet function in HV vs. HF patients with preserved ejection fraction (HFpEF) and chronic atrial fibrillation (HFpEF–AF), vs. patients with chronic AF without HF, and to assess whether these effects occur independent of the interaction with other formed elements of blood.

Methods and results
Platelet responses to nitrite and the NO donor sodium nitroprusside (SNP) were compared in age-matched HV controls (n = 12), HFpEF–AF patients (n = 29), and chronic AF patients (n = 8). Anti-aggregatory effects of nitrite in the presence of NO scavengers/sGC inhibitor were determined and vasodilator-stimulated phosphoprotein (VASP) phosphorylation was assessed using western blotting. In HV and chronic AF, both nitrite and SNP inhibited platelet aggregation in a concentration-dependent manner. Inhibition of platelet aggregation by the NO donor SNP was impaired in HFpEF–AF patients compared with healthy and chronic AF individuals, but there was no impairment of the anti-aggregatory effects of nitrite. Nitrite circumvented platelet NO resistance independently of other blood cells by directly activating sGC and phosphorylating VASP.

Conclusion
We here show for the first time that HFpEF–AF (but not chronic AF without HF) is associated with marked impairment of platelet NO responses due to sGC dysfunction and nitrite circumvents the ‘platelet NO resistance’ phenomenon in human HFpEF, at least partly, by acting as a direct sGC activator independent of NO.

Keywords
Nitrite • Platelets • Nitric oxide • Heart failure with preserved ejection fraction • Atrial fibrillation

© The Author(s) 2018. Published by Oxford University Press on behalf of the European Society of Cardiology.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
1. Introduction

Heart failure (HF) with preserved ejection fraction (HFrEF) accounts for ~50% of HF cases.1 HFrEF is associated with morbidity and mortality close to that of HF with reduced ejection fraction (HFrEF) and there are no effective therapies.2,3 Both HFrEF and HFrEF are associated with impaired endothelial function and a number of studies in patients with HFrEF have demonstrated that tissue responsiveness to direct nitric oxide (NO) donors in blood vessels and platelets are diminished.4,5

Atrial fibrillation (AF) commonly accompanies HFrEF (up to 40%) and its presence is associated with substantial embolic risk. Although warfarin or non-vitamin K antagonist oral anticoagulants are commonly prescribed in this setting the haemorrhagic risk in these patients (mainly elderly, often with comorbidities) is high. Trials have shown that aspirin is relatively ineffective in reducing embolic risk in patients with chronic AF6 and additional therapies that might reduce embolic risk, particularly in those at high risk of bleeding complications from warfarin would be potentially valuable.

Recent onset AF is itself associated with platelet hyperaggregability, in part related to impaired NO signalling.7 It is well-established that NO is an important mediator in the regulation of vascular tone and inhibitor of platelet aggregation.8 These effects are predominantly mediated by the activation of soluble guanylate cyclase (sGC) and cyclic guanosine-3′,5′-monophosphate (cGMP), which subsequently activates protein kinase G and leads to phosphorylation of vasodilator-stimulated phosphoprotein (VASP).9

Circulating blood platelets exhibit abnormalities in patients with acute coronary syndrome (ACS), stable angina and ischaemic HFrEF, and potent antplatelet therapy plays a pivotal role in management of patients with ACS.10 The phenomenon of ‘platelet NO resistance’ has been well described in these patient cohorts,11 but the mechanism of this diminished anti-aggregatory effect of NO in platelets remains poorly defined. However, it has been postulated that NO resistance is likely to be associated with NO scavenging by superoxide believed to be derived predominantly from neutrophils.12 Other proposed mechanisms include, oxidation or loss of the haem moiety and/or oxidation of specific cysteine thiols of the sGC, thus resulting in an impairment of NO-induced sGC activity.13,14 NO resistance has also been described as an independent predictor of increased mortality and morbidity in patients with high risk ACS.15 An alternative route to activate the NO–sGC–cGMP pathway would therefore be beneficial.

We have previously demonstrated that short-term intravenous sodium nitrite improves cardiac and pulmonary haemodynamics in patients with HFrEF,16 and very recent studies have shown that nebulized nitrite improves rest and exercise haemodynamics in HFrEF and that this effect was not impaired when compared with that seen in HV and chronic AF. and that this effect was not impaired when compared with that seen in HV and chronic AF.

2. Methods

2.1 Subjects

We studied 12 HVs (8 males and 4 women), 29 patients diagnosed with HFrEF-AF (21 males and 8 women), 8 age-matched patients with chronic AF without HF or known coronary artery disease (4 males and 4 women). The HVs and patients were randomly assigned to the pharmacological experiments, with 8–11 participants per treatment group. Sixteen young HVs (9 males and 7 females) were also recruited for western blotting experiments. HVs were non-smokers free of any cardiovascular risk factors, not on any regular medications, and in particular had not taken anti-platelet drugs in the 10 days prior to the study. All patients met the established criteria for the diagnosis of HFrEF-AF.20,21 This included participants who have permanent AF and ejection fraction >55% (established by echocardiography during screening).22,23 Chronic AF participants included persistent AF with no evidence of HF or known coronary artery disease. All participants gave written consent before participation in the study. The investigation conforms to the principles outlined in the Declaration of Helsinki. The studies were approved by the University of Birmingham Ethics Review Committee (ERN_10-0625 and ERN_12-1184R2) and West Midlands Coventry and Warwickshire research ethics committee (14/WM/1211 and 12/WM/0344).

2.2 Blood sampling and platelet preparation

Venous blood was drawn in 9NC coagulation sodium citrate 3.2% vacutainer tube (Greiner Bio-One, Austria). A preparation of washed platelets was obtained as previously described in.24,25 Whole blood was centrifuged at 200 g for 20 min and platelet rich plasma (PRP) was collected up to 0.5 cm from the interface with the red blood cell (RBC) pellet in order to minimize RBCs contamination. Platelets were isolated from PRP by centrifugation at 1000 g for 10 min following addition of PGI2 (0.1 μg/ml to inhibit platelet activation; Sigma Aldrich). The resulting platelet pellet was resuspended in Tyrode’s buffer (134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 2 mM HEPES, 5 mM glucose, 1 mM MgCl2, pH 7.3) and centrifuged at 1000 g in the presence of 0.1 μg/ml PGI2. The supernatant was discarded and the platelet pellet was resuspended in Tyrode’s buffer. The washed platelet suspensions were allowed to rest for 1 h prior to experimentation to allow the effects of PGI2 to decay. The level of contamination of our washed platelet preparation with plasma constituents was determined using the Bio-Rad assay for protein determination.

2.3 Assessment of RBC and leukocyte contamination in washed platelet preparations

Flow cytometry was used to determine RBC and leukocyte contamination in washed platelet preparations. Platelets (2 × 10^9/ml in Tyrode’s containing 10% heat deactivated human serum) and RBC (diluted 1:500 in Tyrode’s buffer) were stained with the RBC surface marker PE-conjugated anti-CD235a (eBioscience) or isotype IgG control (eBioscience) for 20 min in the dark. Cells were washed and acquired with a C6 Accuri flow cytometer. RBC was gated using anti-CD235a (eBioscience) and applied to the washed platelet preparation to assess...
Nitrite improves platelet function in HFpEF-AF

RBC contamination. To assess leukocyte contamination in preparations of washed platelets, platelets and leukocytes were labelled with anti-CD45 antibody-allophycocyanin (APC; Beckman Coulter) or isotype-APC control (Beckman Coulter) for 30 min at 4°C. In total 0.3 ml of blood was fixed using 2% formaldehyde for 10 min and after centrifugation at 500 g for 5 min, blood was resuspended in 3 ml ACK (Ammonium-Chloride-Potassium) lysis buffer for 10 min to remove RBCs. Cells were labelled and washed in PBS at 500 g for 5 min and resuspended in 300 µl PBS for analysis on a C6 Accuri flow cytometer. Leukocyte populations isolated from whole blood were separated based on forward scatter and CD45 expression. Gates based on this distribution were used to assess leukocytes present in preparations of washed platelets.

2.4 Oxyhaemoglobin preparation
Human haemoglobin (Sigma Aldrich) was dissolved in water (20 mg/ml) and reduced by a 10-fold molar excess of sodium dithionite (Na2S2O4; Sigma Aldrich). Excess reductant was removed by gel filtration over Sephadex G-25 (PD10 desalting column; GE Healthcare) according to the manufacturer’s instructions. Oxyhaemoglobin (OxyHb) was eluted with 3.5 ml of water, and only the middle run was collected. The concentration of OxyHb was determined spectrophotometrically, as described in.26 Aliquots of the OxyHb stock solution were kept at -80°C, thawed on the day of experimentation and discarded after use.

2.5 Platelet aggregation
Washed platelets were suspended at 2 x 10^9/ml for light transmission aggregation using a lumi-dual aggregometer (model 460VS; Chronolog, Labmedics) under continuous stirring at 1200 rpm, as previously described in.24 Sodium nitrite, sodium nitrate, sodium nitroprusside (SNP), 1H-[1, 2, 4]Oxadiazolo[4, 3-a]quinoxalin-1-one (ODQ), 2-Phenyl-4, 4, 5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) (all purchased from Sigma Aldrich), OxyHb, BAY 41-2272, or vehicles were incubated for the stated time and concentrations as indicated in the figure legends before platelet activation with 3 μg/ml collagen (Nycomed).

2.6 Western blotting
Washed platelets were suspended at 5 x 10^9/ml for western blot experiments. Platelets were incubated as indicated in the figure legends. Incubations were stopped by adding 5 x reducing sample buffer at the indicated time point. Samples were boiled for 5 min and spun prior to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transferred onto a polyvinylidene fluoride (PVDF; GE Healthcare) membrane. PVDF membranes were incubated with antibodies against p-Serine 239 VASP (Cell signalling Technology) and α-tubulin (Sigma Aldrich) overnight at 4°C. Membranes were then incubated with appropriate secondary antibodies [anti-rabbit (GE healthcare) and anti-mouse (Dako)] as detailed in figure legend text. Band densitometry was performed as previously described in.27

2.7 Statistical analysis
Results are presented either as representative experiment of n experiments performed or as average ± SEM. Differences between groups were analysed using the Fisher’s exact test, unpaired t test, one- or two-way ANOVA by Dunnett’s or Sidak’s multiple comparisons test as appropriate. Within group differences was assessed by Wilcoxon matched-pairs signed rank test. P < 0.05 was considered significant. All analyses were performed using Prism version 7.0 (GraphPad Inc., Lo Jolla, CA).

3. Results

3.1 HVs and HF patients’ characteristics
Table 1 summarizes the subject characteristics including drug therapies. Patients and healthy controls were well matched in terms of age (P = 0.40; one-way ANOVA) and gender (P = 0.53; Fisher’s exact test). Young HVs (whose blood was used for platelet isolation and western blotting) were well matched in terms of gender to HFpEF-AF group (P = 0.33; Fisher’s exact test).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HVs (n = 12)</th>
<th>HFpEF with chronic AF (n = 29)</th>
<th>Chronic AF (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>8/4</td>
<td>21/8</td>
<td>4/4</td>
</tr>
<tr>
<td>Age, year (mean ± SD)</td>
<td>71.3 ± 5.9</td>
<td>74.3 ± 6.2</td>
<td>73.9 ± 7.9</td>
</tr>
<tr>
<td>NYHA-Class</td>
<td>–</td>
<td>23/11/2</td>
<td>–</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>–</td>
<td>2 2</td>
<td>–</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
<td>–</td>
<td>10 2</td>
<td>–</td>
</tr>
<tr>
<td>Angiotensin II receptor blocker</td>
<td>–</td>
<td>2 2</td>
<td>–</td>
</tr>
<tr>
<td>Diuretics</td>
<td>–</td>
<td>2 4</td>
<td>–</td>
</tr>
<tr>
<td>β-adrenoceptor antagonists</td>
<td>–</td>
<td>8 6</td>
<td>–</td>
</tr>
<tr>
<td>α-adrenoceptor antagonist</td>
<td>–</td>
<td>2 –</td>
<td>–</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>–</td>
<td>9 1</td>
<td>–</td>
</tr>
<tr>
<td>Statins</td>
<td>–</td>
<td>10 2</td>
<td>–</td>
</tr>
<tr>
<td>Cardiac glycoside</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Digoxin</td>
<td>–</td>
<td>4 2</td>
<td>–</td>
</tr>
<tr>
<td>Anti-arrhythmic</td>
<td>–</td>
<td>– 1</td>
<td>–</td>
</tr>
<tr>
<td>Anti-diabetic</td>
<td>–</td>
<td>2 2</td>
<td>–</td>
</tr>
<tr>
<td>Anti-coagulant</td>
<td>–</td>
<td>26 8</td>
<td>–</td>
</tr>
</tbody>
</table>

Values are mean ± SD.
NYHA, New York Heart Association classification; HFpEF, Heart Failure with preserved ejection fraction; ACE-inhibitors, angiotensin-converting enzyme inhibitors.
plasma proteins concentration was confirmed to be negligible in the second washed platelets (-2 ± 0.01%) when compared with PRP (100 ± 0.55%) and first wash (20 ± 0.01%; Figure 1E).

### 3.3 Nitrite circumvents ‘platelet NO resistance’ in patients with HFP EF and chronic AF

Concentration response to collagen (1, 3, and 10 µg/ml) was conducted to determine the concentration used for platelet aggregation experiments (data not shown). A collagen dose (3 µg/ml) that achieved a 50% aggregatory response in platelets was selected for this study. The effects of nitrite on platelet aggregation in HV, HFP EF-AF, and chronic AF patients alone were studied using 3 µg/ml collagen (Figure 2A). Incubation with nitrite in HVs induced significant concentration-dependent inhibition of platelet aggregation in response to collagen (P < 0.0001 at 100 µM and 1 mM; n = 10; Figure 2A), indicating that nitrite is able to affect platelet function in the absence of other cell types or extracellular proteins. A similar trend was also observed with the NO donor, SNP, with significant concentration-dependent attenuation of
Nitrite improves platelet function in HFpEF-AF

To determine whether increased concentrations (1 and 10 mM) of nitrite significantly attenuated platelet aggregation with 1 mM nitrite (mainly dependent on cGMP elevation) was assessed from young HVs and patients with HFpEF-AF. Nitrite and SNP induced concentration-dependent attenuation of platelet aggregation in response to collagen (Figure 2A; P < 0.0001 nitrite (1 mM); and Figure 2B; P < 0.01 (10 mM) and P < 0.0001 (100 mM) SNP, respectively). Representative traces for HV, HFpEF-AF and cAF alone are shown in Figure 3.

To ascertain whether the platelet responses observed with nitrite and SNP in HFpEF-AF patients were attributable to the HF, the chronic AF or both we then measured platelet aggregation responses in a sub-group of patients with chronic AF alone. Both nitrite and SNP induced concentration-dependent attenuation of platelet aggregation in response to collagen (Figure 2A; P < 0.0001 nitrite (1 mM); and Figure 2B; P < 0.01 (10 mM) and P < 0.0001 (100 mM) SNP, respectively). Representative traces for HV, HFpEF-AF and cAF alone are shown in Figure 3.

The differences in nitrite and SNP response to collagen at the highest doses used to their respective control HFpEF-AF. As shown in Figure 2C, 10 μM SNP (13.15 ± 3.5% inhibition) demonstrated impaired responses compared with 1 mM nitrite (29.12 ± 5.4% inhibition; P < 0.05).

To ascertain whether the platelet responses observed with nitrite and SNP in HFpEF-AF patients were attributable to the HF, the chronic AF or both we then measured platelet aggregation responses in a sub-group of patients with chronic AF alone. Both nitrite and SNP induced concentration-dependent attenuation of platelet aggregation in response to collagen (Figure 2A; P < 0.0001 nitrite (1 mM); and Figure 2B; P < 0.01 (10 mM) and P < 0.0001 (100 mM) SNP, respectively). Representative traces for HV, HFpEF-AF and cAF alone are shown in Figure 3.

We next investigated whether nitrite activated sGC, the normal physiology vs disease physiology of nitrite mechanism in washed platelets. VASP serine-239 phosphorylation was determined using the sGC-dependent effect of nitrite on HV platelets exhibiting synergistic activity with the NO-independent sGC activator, Bay 41-2272 (P < 0.001 30 nM Bay 41-2272 plus 10 and 100 μM nitrite, respectively; n = 11, see Supplementary material online, Figure S3B). A similar trend was also observed with SNP as a positive control (30 nM Bay 41-2272 plus 10 nM SNP (P < 0.01) and 100 nM SNP (P < 0.001), respectively; see Supplementary material online, Figure S3C).

3.4 Nitrite phosphorylates serine-239 VASP

To confirm that the effects of nitrite on platelet aggregation occur via the activation of the sGC-cGMP pathway, we next studied the status of phosphorylation of the cyclic nucleotide downstream substrate VASP. In order to understand the normal physiology vs disease physiology of nitrite mechanism in washed platelets, VASP serine-239 phosphorylation (mainly dependent on cGMP elevation) was assessed from young HVs vs. HFpEF-AF (Figure 5A and B). VASP serine-239 phosphorylation was increased with nitrite in a concentration-dependent manner as compared with the NO donor SNP. All concentrations of nitrite reached a peak after 5–10 min of incubation and then decreased to low levels at 45 min. VASP serine-239 phosphorylation with 100 μM and 1 μM nitrite were similar to that triggered by treatment with 10 and 100 nM SNP, respectively (n = 5).

To determine whether the underlying mechanism of nitrite was NO dependent or independent, we measured serine-239 phosphorylation of VASP following the use of PTIO and OxyHb. We found that in the presence of NO scavengers, VASP serine-239 was phosphorylated by nitrite in both young HVs (Figure 6A and B; n = 9) and HFpEF-AF patients (Figure 6C and D; n = 10). We next investigated whether nitrite activated sGC

Figure 2 Platelet aggregation is inhibited by NaN3O2. 2 × 109/ml washed platelets from HVs, HFpEF-AF and chronic AF alone were incubated with increasing concentrations of NaN3O2 or SNP for 5 min, activated with 3 μg/ml collagen (A and B) and studied by light transmission aggregometry. Statistical differences were determined by repeated measures two-way ANOVA with Dunnett’s test for multiple comparisons with the control dose values (**P < 0.01, ****P < 0.0001; n = 8–10). (C) Platelet responses to nitrite and SNP in the HFpEF-AF group. The difference between nitrite (1 mM) and SNP (10 μM) responses to collagen with their respective control HFpEF were analysed. *P < 0.05 unpaired t test with Welch’s correction (n = 8–10).
to phosphorylate serine-239 VASP. VASP phosphorylation on Serine-239 triggered by nitrite was completely blocked by ODQ in both HVs and patients with HfPEF-AF, thus corroborating our findings from platelet aggregation experiments (Figure 4A–F).

3.5 Nitrate does not affect platelet aggregation and VASP phosphorylation

The effects of oral inorganic nitrate are usually considered to be dependent on enterosalivary circulation of nitrate with reduction of salivary nitrate to nitrite by oral bacteria, but a direct effect of nitrate cannot be excluded. We therefore assessed the effect of a high concentration of sodium nitrate (1 mM) added in vitro on collagen-induced aggregation in young HVs (Figure 7A). In the absence of a functional nitrate reductase, the addition of nitrate to platelets in vitro had no effect on aggregation (P > 0.05; n = 5) or phosphorylation of VASP serine-239 (Figure 7B).

4. Discussion

In this study, we show several important new findings: first, platelet NO resistance exist in patients with HfPEF with associated chronic AF compared with age-matched HVs. Second, while NO resistance has been observed in recent onset AF we did not observe it in age-matched patients with chronic AF in the absence of HF. Taken together, these data suggest that the platelet NO resistance is attributable predominantly to the HfPEF rather than the associated chronic AF. Third, this NO resistance was observed in washed platelets indicating that platelet NO resistance is largely intrinsic to the platelet. Fourth, nitrite inhibits platelet aggregation in HfPEF-AF patients by circumventing the platelet NO resistance phenomenon by directly activating sGC and phosphorylating VASP serine 239 independent of NO and the interaction with other formed elements of blood and their constituents such as mitochondria (neutrophils) or haemoglobin (RBCs).
Approximately half of all cases of HF have HfPEF and the syndrome is frequently associated with AF. The pathophysiology of HfPEF is associated with abnormalities in left ventricular diastolic reserve, impaired systolic reserve, peripheral and pulmonary vasodilatation, endothelial dysfunction, and right ventricular dysfunction related to the presence of pulmonary hypertension (PH).

Recent studies have postulated that impairment of the NO-cGMP pathway may also play a contributing factor in the hemodynamic abnormalities observed in HfPEF. However, to our knowledge the role of the NO-cGMP pathway on platelet function in patients with HfPEF with chronic AF has not been previously evaluated. It is well-documented that impaired platelet responsiveness to NO donors (organic nitrates and SNP) occurs in a number of cardiovascular disease states, such as ischaemic heart disease, HFrEF, and in new onset of AF. Herein, we sought to determine whether NO donors also have a similar impact on the function of platelets isolated from HfPEF patients with chronic AF. We demonstrate for the first time that ‘platelet NO resistance’ exists in HfPEF associated with chronic AF. We used SNP, a well-established NO donor, to evaluate the impact of activation of the sGC-cGMP pathway on platelet aggregation. Platelet responsiveness to the NO donor SNP from HfPEF-AF were diminished following platelet activation with collagen when compared with platelets from HVs.

It is now appreciated that under certain conditions nitrite can serve as an alternative source of NO in the vasculature and other tissues. Recent studies have shown that the nitrate-nitrite-NO pathway inhibits platelet activation. It has also been suggested that platelets possess the ability to generate transient NO signals from nitrite in the absence of other blood cell types through an unidentified mechanism.

Although the role of endothelial-derived NO on platelet function is well characterized, the mechanism(s) by which nitrite inhibit platelet aggregation remain poorly defined. Platelets are exposed not only to endothelial NO but also to circulating nitrite, and this could represent a source of NO and an alternative mechanism of control of platelet activation. Basal circulating nitrite levels in human plasma range are typically in...
the submicromolar range\textsuperscript{38,39} and this can rise to levels >8 $\mu$M upon therapeutic nitrite application.\textsuperscript{17,18} However, local concentrations in the microcirculation of mucosal membranes are likely considerably higher. In the oral cavity, nitrite concentrations reach micromolar concentrations even under normal physiological conditions, and salivary nitrite concentrations may reach 1–2 mM after dietary nitrate.\textsuperscript{40,41} If nitrite were to be used as a therapeutic agent to treat HFpEF-AF, concentrations of this mediator would exceed those physiological levels by far, with possible effects on platelet function. A similar scenario is likely to occur at the alveolar-arterial interface of the pulmonary circulation following therapeutic application of nebulized nitrite.

This study was motivated by the paucity of information on the signaling cascade leading to platelet inhibition by nitrite. Although it is generally assumed that nitrite requires prior reduction to NO to become bioactive, this process is remarkably inefficient under normoxic conditions, and not all of its actions appear to be accompanied by the generation of free NO.\textsuperscript{19,42} To determine whether nitrite inhibits platelet aggregation independently of haemoglobin, we used a well-established washed platelet aggregation technique.\textsuperscript{43} Washed platelets are routinely used to assess pharmacological agents on platelet function and mechanism in the absence of plasma proteins, enzymes, and blood cells. Our results demonstrate that platelets from HV are inhibited by nitrite through the activation of sGC (as assessed via the sGC inhibitor ODQ; Figure 4). We next sought to determine whether these effects were NO dependent. Both NO scavengers (PTIO and OxyHb) effectively reverted the effects of an inhibitory concentration of SNP on platelet aggregation (Figure 4). In contrast neither PTIO nor OxyHb were able to revert the inhibition caused by a high concentration of nitrite on platelet aggregation from HV. VASP phosphorylation on Ser239 triggered by nitrite was completely blocked by ODQ (Figure 6), but inhibition was only partial with PTIO and OxyHb (Figure 6). Collectively, these data indicate that the antiaggregatory effects of nitrite are only partly mediated by NO. This is consistent with additional findings showing that the effects of nitrite are potentiated by the NO-independent sGC activator Bay

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{NaNO$_2$ triggers phosphorylation of VASP. $5 \times 10^6$/ml washed platelets from healthy young volunteers were incubated with increasing concentrations of NaNO$_2$ or SNP and lysed by addition of SDS containing $5 \times$ sample buffer at the indicated time point. Aliquots of the lysates were used for SDS-PAGE (10%). Blots were probed with anti p-VASP Serine 239 (1:1000) or $\alpha$-tubulin (1:15000) and the appropriate secondary antibodies (anti-rabbit or anti-mouse 1:10000). Representative blots (A) and densitometrical analysis of all the experiments performed (B, n = 4–5) are shown.}
\end{figure}
Nitrite improves platelet function in HFpEF-AF

Recent studies have demonstrated that acute nitrite infusion or nebulized nitrite improves rest and exercise haemodynamics and exercise capacity in HFpEF. Thus, this remains a major limitation to the current investigation and further studies are warranted to clarify the role of nitrite on platelet function in this particular patient cohort.

Figure 6 NaNO2 upregulates VASP serine-239 phosphorylation NO-independently. S × 10⁹/ml washed platelets from HVs (A and B; n = 8–9) and HFpEF-AF patients (C and D; n = 10) were incubated with ODQ, PTIO, or OxyHb before addition of NaNO2 for 10 min and lysed by addition of S× sample buffer. Aliquots of the lysates were used for SDS-PAGE (10%) and western blot as in Figure 5. Representative blots (A and C) and densitometrical analysis of all the experiments performed (B and D) are shown. Statistical differences were determined by one-way ANOVA and Dunnet’s post-test. Differences within groups (control NO scavengers/sGC inhibitors vs. nitrite) were evaluated by Wilcoxon matched-pairs signed rank test (*P < 0.05; *P < 0.01; **P < 0.001).

41-2272 (see Supplementary material online, Figure S3). It is conceivable, therefore, that part of the effects of nitrite are mediated by a modulation of one or more cysteine thiols of sGC, whether this is via S-nitrosation or S-thiolation (e.g. sulphhydration or formation of a mixed disulphide with another low-molecular weight thiol such as cysteine or glutathione, and therefore oxidation of a regulatory thiol in sGC) or another mechanism (such as the loss of haeme, which renders sGC unresponsive to NO) was beyond the remit of the present study and warrants further investigation.

Previous studies have suggested that nitrite therapy may not be subject to the development of tolerance. Furthermore, we and others have very recently explored nitrite as a potential treatment of HF. We have shown that short-term intravenous sodium nitrite improves cardiac and pulmonary in patients with HFrEF, and very recent studies by Borlaug et al. have demonstrated that acute nitrite infusion or nebulized nitrite improves rest and exercise haemodynamics and exercise capacity in HFpEF. To assess whether nitrite inhibits platelet aggregation in this group of patients, we next assessed the effects of nitrite on platelet function. In the presence of ‘NO resistance’, nitrite inhibits platelet aggregation. To assess the potential mechanism by which nitrite mediates these promising effects, platelet responses to nitrite was assessed in the presence of sGC inhibitor or NO scavengers. We show that the platelet responsiveness to nitrite was significantly attenuated in the presence of ODQ (Figure 4), but both NO scavengers were unable to revert the inhibition caused by a high concentration of nitrite on platelet aggregation and VASP serine 239 phosphorylation (Figure 6). Herein, we show for the first time that nitrite circumvents the platelet NO resistance in patients with HFpEF-AF. A similar result was also observed with our western blot data. However, haeme-containing components (cytochrome C) of the mitochondrial respiratory chain of platelets may convert nitrite to NO inside of the platelet, thus avoiding the scavenging by OxyHb or PTIO.

A number of studies have associated AF with inflammation, endothelial dysfunction and in particular in the new onset AF with impaired platelet NO response. Procter et al. (2015) have previously reported that acute onset AF is associated with platelet NO resistance, whilst responses to NO donor (SNP) were not impaired in chronic AF patients. To validate whether the platelet NO resistance observed in the HFpEF-AF with SNP was HFpEF dependent and not AF, we also assessed the effects of nitrite and SNP in a sub-group of patients with chronic AF alone. There was approximately two-fold variability between HFpEF-AF vs chronic AF sub-groups with SNP, therefore further supporting platelet NO resistance in patients with HFpEF-AF. In addition, nitrite also inhibited platelet aggregation in a dose-dependent manner in the chronic-AF sub-group.

ACE inhibitors have previously been shown to reduce platelet NO resistance. Although 10 of the 29 patients with HFpEF-AF were taking ACE inhibitors, NO resistance was observed whether or not the patients were taking ACE inhibitors. Two of the eight patients with chronic AF alone were taking ACE inhibitors, but in contrast platelet NO responsiveness was not observed in either of these two patients or the six who were not taking ACE inhibitors.

Previous studies have demonstrated that cGMP plays a key role in determining the functional and haemodynamic abnormalities developing in HFrEF. In this study, we show that cAF in the absence of HF respond to NO donors and there is a clear difference in response of ‘NO resistance’ in patients with HFpEF-AF. This suggests that phenotyping of patients according to their ‘NO-stimulatable’ component in platelet reactivity might allow to stratify them to different pharmacological treatment (e.g. personalised medicine).

Herein, we have compared the effects of nitrite on platelet function in patients with chronic AF with and without HFpEF to conclude about HFpEF. This study could have benefited in having an additional sub-group of HFpEF patients with sinus rhythm to further validate the study. However, as local practice, the majority of HFpEF in sinus rhythm were taking aspirin and therefore we were unable to recruit for this study. Thus, this remains a major limitation to the current investigation and further studies are warranted to clarify the role of nitrite on platelet function in this particular patient cohort.

A major limitation of the current findings is that the concentrations of nitrite used in this study are clearly pharmacological, therefore the observations do not imply a physiological role for normal plasma nitrite
concentrations in the modulation of platelet function. Nevertheless, they may have implications for therapy. Recent studies have suggested a potentially beneficial effect of the nitrate/nitrite pathway on haemodynamics and exercise capacity in HFpEF, this study raises the potential that such therapy may also reduce the burden of thromboembolic disease seen HFpEF patients with chronic AF.

Furthermore, we observed a decreased platelet aggregation response to collagen in the control chronic AF when compared with age-matched control groups from HVs and HFpEF-AF patients. To our knowledge the effects of collagen on washed platelet aggregation from chronic AF has not been investigated before, but we have previously assessed platelet aggregation (PRP) in patients with AF on no therapy to have 24% inhibition of aggregation in response to 2 mg/ml collagen.47 We have reported the complexity of the events of platelet activation in AF and therefore further studies are warranted to clarify the role of nitrite and NO donors in the disease states investigated herein.

In conclusion, we show for the first time that HFpEF is associated with marked impairment of platelet NO responses and that nitrite circumvents the ‘platelet NO resistance’ phenomenon in human HFpEF, at least in part by acting as a sGC activator. Our findings are consistent with the notion that thiol oxidation at the level of the NO-receptor, sGC may play a major role in the phenomenon of ‘NO resistance’; this would seem to warrant further investigation. Our findings may also have relevance to the condition of PH since coagulation has been implicated in the pathogenesis of this disease. Although the use of anticoagulation as part of the treatment in PH remains controversial, recent experimental studies suggest that nitrite may provide some benefit in this setting.48

Thus, our data implicate an alternative strategy to potentiate efficacy of direct sGC activation, and perhaps may reduce the risk of thrombotic complications in PH.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We would like to thank Professor Adrian Hobbs (Queen Mary University of London, UK) for providing the sGC activator, Bay 41-2272.

Conflict of interest: none declared.

Funding

This work was supported by the UK Medical Research Council (G1001536). J. ML is supported by the Birmingham NIHR Biomedical Research Centre in Inflammation.

References

Nitrite improves platelet function in HFpEF-AF

21. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, Falk V, Nitrite improves platelet function in HFpEF-AF

15. Willoughby SR, Stewart S, Holmes AS, Chirkov YY, Horowitz JD. Platelet nitric oxide


