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Role of inorganic nitrate and nitrite in driving nitric oxide/cGMP-mediated inhibition of platelet aggregation *in vitro* and *in vivo*

Running head: Nitrite/nitrite and platelet aggregation

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Summary. *Background:* Nitric oxide (NO) is a critical negative regulator of platelets implicated in the pathology of thrombotic diseases. Platelets generate NO but the presence and functional significance of NO synthase (NOS) in platelets is unclear. Inorganic nitrate/nitrite is increasingly recognised as a source of bioactive NO although its role in modulating platelets during health and vascular dysfunction is incompletely~~poorly~~ understood. *Methods:* We investigated the functional significance and upstream sources of NO-cGMP signalling events in platelets using established methods for assessing *in vitro* and *in vivo* platelet aggregation and assessed bioconversion of inorganic nitrate to nitrite during deficiency of endothelial NOS (eNOS). *Results:* The phosphodiesterase 5 (PDE5) inhibitor sildenafil inhibited human platelet aggregation *in vitro*. This inhibitory effect was abolished by a guanylyl cyclase inhibitor and NO scavengers but unaffected by NOS inhibition. Inorganic nitrite drove cGMP-mediated inhibition of human platelet aggregation *in vitro* and nitrate inhibited platelet function in eNOS$^{-/-}$ mice *in vivo* in a model of thromboembolic radiolabelled platelet aggregation associated with~~following~~ enhanced ~~bioconversion to~~plasma nitrite concentration compared to wild-type mice. *Conclusions:* Platelets generate transient, endogenous cGMP signals downstream of NO that are primarily independent of NOS and may be enhanced by inhibition of PDE5. Furthermore, nitrite can generate transient NO-cGMP signals in platelets. The absence of eNOS leads to ~~an~~ enhanced plasma nitrite levels following nitrate administration ~~capacity to bioconvert nitrate to nitrite~~ *in vivo* which negatively impacts platelet function. Our data suggest that inorganic nitrate exerts an antiplatelet effect during eNOS deficiency and, potentially, that dietary nitrate may reduce platelet hyperactivity during endothelial dysfunction.

Keywords: nitric oxide, nitrites, pharmacology, platelets, thrombosis,

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Introduction

Platelet activation is governed by a variety of positive and negative stimuli that act to precisely regulate the process of haemostasis. Positive stimulators of platelets include subendothelial collagen, thrombin generated *via* the coagulation cascade as well as ADP and thromboxane A_2 which are released from platelets themselves. The major negative regulators of platelets are prostacyclin (PGI_2) and nitric oxide (NO), generated by the vascular endothelium. An imbalance between positive and negative platelet stimuli contributes to the pathogenesis of thrombotic disorders such as myocardial infarction. NO is conventionally described as being generated by NO synthase (NOS) enzymes which catalyse the conversion of L-arginine to L-citrulline resulting in NO release [1]. Many of the effects of NO are mediated through activation of soluble guanylyl cyclase (sGC), subsequent cGMP production and protein kinase activation leading to further signalling events including phosphorylation of the vasodilator-stimulated phosphoprotein (VASP). The actions of cGMP are terminated by phosphodiesterase 5 (PDE5) which hydrolyses active cGMP to inactive GTP. PDE5 is expressed at high levels in platelets so that the effects of cGMP are transient due to its rapid hydrolysis. PDE5 inhibitors, such as sildenafil citrate are used therapeutically in conditions associated with regional blood flow deficiency such as erectile dysfunction and pulmonary arterial hypertension. Sildenafil was reportedly associated with adverse cardiovascular events [2], however risk was more recently shown to arise from the cardiovascular risk profile of patients with erectile dysfunction [3]. Focus can therefore shift towards exploration of PDE5 inhibition in cardioprotection [3]. Sildenafil has also been shown to enhance NO-mediated inhibition of human aggregation *in vitro* [4] and to enhance collagen-induced aggregation *ex vivo* [5]. The ability of sildenafil to directly modulate platelet activation in the absence of exogenous or endothelial NO by investigating its effect upon isolated platelets *in vitro* is poorly understood although it had no effect on ADP-induced aggregation in platelet-rich plasma [6].

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Endogenous NO derived from endothelial NOS (eNOS) in the vascular endothelium acts as a critical negative regulator of platelet function *in vivo* [7, 8] and deficiency of eNOS is associated with endothelial dysfunction and the pathology of a range of cardiovascular diseases [9, 10]. The intrinsic expression of eNOS in platelets remains contentious however [11, 12] and a number of studies have reported a lack of eNOS protein or mRNA in platelets as well as a lack of a functional role of platelet-derived NO [13, 14]. There is, however, considerable evidence that platelets generate NO [15, 16] so that the source of endogenous NO in platelets is unclear. More recently, NO has been shown to be derived not only *via* NOS but *via* reduction of inorganic nitrite [17]. In humans, dietary nitrate is concentrated and secreted by the salivary glands and reduced to nitrite by anaerobic bacteria on the tongue [18, 19]. Nitrite is absorbed into the circulation and is chemically reduced to NO by a variety of mechanisms including enzymatic processes that provide a mechanism for the localised delivery of NO to cells independent of NO synthases [17, 20]. Oral administration of nitrate lowered blood pressure [21] and modestly reduced platelet aggregation *ex vivo* in healthy volunteers [22] demonstrating the potential value of inorganic nitrate as a modulator of cardiovascular function. The ability of nitrate/nitrite to impact platelets in the context of endothelial dysfunction *in vivo* is unknown.

We hypothesised that platelets generate transient NO-cGMP signals from upstream nitrate/nitrite. These signals may be amplified by PDE5 inhibition to reveal endogenous inhibitory signalling processes. Secondly, we hypothesised that during endothelial dysfunction, exogenously administered inorganic nitrate acts as an alternative source of bioactive NO to counteract impaired eNOS activity by inhibiting platelet activation following bioconversion to nitrite.

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Materials and methods

Materials

Materials used were as follows: ^{111}indium oxine (GE Healthcare, Amersham, UK); collagen (Takeda Pharmaceuticals International, Linz, Austria); anti-vasodilator-stimulated phosphoprotein (VASP) and anti-VASP-P(Ser239) (Cell Signalling, Hertforshire, UK); iloprost (Cayman Chemicals, Washington, USA); Anti-rabbit horseradish peroxidase-conjugated antibody (Dako, Cambridgeshire, UK); anti-GAPDH (Santa Cruz, California, USA). Sildenafil citrate was kindly donated by Pfizer (Peapack, NJ, USA). All other materials were purchased from Sigma-Aldrich (Poole, UK).

Light transmission aggregometry

Blood was taken into ACD (1:9) from aspirin-free volunteers aged 23 to 55 years and with an even sex distribution. Informed consent from all blood donors was obtained and procedures were approved by the National Research Ethics Service. Platelet-rich plasma (PRP) was prepared by centrifugation at 100 *g* for 20 mins. Washed platelets (WP) were prepared by the addition of ACD (1:80) and PGE_1 (175 nM) to PRP and centrifuged at 1400 *g* for 10 mins. The pellet was resuspended in tyrodes-HEPES buffer and the final centrifugation step was repeated. WP were resuspended to a platelet count of $250x10^3$ μL^{-1} in tyrodes-HEPES buffer. Platelet preparations were incubated for 5 mins with test compounds prior to stimulation with agonists and aggregation was measured at 37°C under stirring conditions in an optical aggregometer (Chrono-log Corporation, Havertown, USA).

Western blotting

Human WP were incubated with test compounds (as detailed for light transmission aggregometry) before centrifugation and pellet resuspension in RIPA lysis buffer. Western blotting was performed as

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detailed [23]. The antibody concentrations used were: rabbit anti-GAPDH (1:500), VASP (1:1000) and phosphor-VASP(Ser239) (1:1000) and incubations were at 4°C overnight.

Nitrate/nitrite colorimetric assay

Human washed platelets (500µL) were incubated in the presence or absence of test compounds for 5 mins before they were snap frozen and stored at -80°C. Nitrate/nitrite concentration was determined in supernatants using a nitrate/nitrite colorimetric assay kit (Cayman Chemicals , Ann Arbor, MI, USA).

Animals

Male C57BL/6 mice (20–30 g) were purchased from Harlan (Bicester, UK). eNOS knock-out mice (eNOS$^{-/-}$, Strain: 0026847) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house. Protocols involving the use of animals were licensed by the UK Home Office and approved by the Ethical Review Panel at Imperial College London. Procedures involving animals were conducted and are reported in accordance with ARRIVE guidelines [24].

Ozone~~Gas phase~~ chemiluminescence

Wild-type or eNOS$^{-/-}$ mice were pre-treated with saline or sodium nitrate 1 h before they were anaesthetised and plasma and salivary glands extracted [25]. 1 h has previously been shown to provide adequate time for increases in plasma nitrate/nitrite following i.p. or oral administration of inorganic nitrate [18, 26, 27]. All samples were snap frozen and stored at -80°C until further analysis. Mouse salivary glands were homogenised with phosphate-buffered saline, using a Mixer Mill MM 400 homogeniser, vibrational frequency 30 Hz (1800 min $^{-1}$) for 3 mins. Salivary gland homogenates

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or plasma were deproteinised by incubation with sodium hydroxide (0.5 M) and zinc sulphate (10% w/v) for 15 mins at room temperature. Samples were centrifuged and the supernatant was extracted and analysed for nitrate/nitrite concentration using a Sievers nitric oxide analyser (280, Analytix). Samples were refluxed in vanadium III chloride (0.1 M) and hydrochloric acid (1 M) at 95°C (nitrate analysis) or in sodium iodide (0.3 M) and glacial acetic acid at 35°C (nitrite analysis). Nitrate/nitrite concentrations were detected using ozone chemiluminescence as previously reported [28].

In vivo platelet aggregation

Platelets were isolated from wild-type or $eNOS^{-/-}$ donor mice and radiolabelled with 1.8 MBq indium oxine (In^{111}) as previously described [29]. Radiolabelled platelets of the same genetic background were administered to anaesthetised (urethane 25% w/v, 10 μl g^{-1}) recipient wild-type or $eNOS^{-/-}$ mice *via* the femoral vein and platelet aggregation responses were measured as increases in platelet-associated counts in the pulmonary vascular bed following intravenous injection of collagen (50 μg kg^{-1}). In a typical experiment, 5 donor mice were bled and the resulting platelet pool evenly distributed into 4 recipient mice. The experimental protocol for sodium nitrate involved the pre-treatment of the recipient mice with saline (0.9% w/v) or sodium nitrate (1 mmol kg^{-1}, i.p) 1 h before collagen and sildenafil involved the administration of vehicle (DMSO <0.05%) or sildenafil (50 μg kg^{-1}, i.v) 5 mins before collagen.

Data analysis and statistics

All data were expressed as mean ± standard error of the mean (SEM). *In vivo* platelet aggregation data was expressed as the percentage increase in maximal radioactive counts from the baseline recording. *In vitro* platelet aggregation data were arbitrary 'area under the curve' values generated

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by the Aggrolink software (version 5.2.1, Chrono-log, Havertown, USA). All statistical tests were performed on raw data. Where statistical comparisons were made, Student's t-test, one-way analysis of variance (ANOVA) or a two-way ANOVA followed by a Bonferroni post-hoc multiple comparison test were used to compare mean values. P-value < 0.05 was considered to denote statistical significance.

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Results

Sildenafil inhibits platelet aggregation in vitro *and* in vivo

We investigated the presence of a functionally relevant, intrinsic NO-cGMP signalling cascade in isolated human platelets by enhancing transient cGMP signals *via* inhibition of PDE5. The selective PDE5 antagonist sildenafil (10-1000 nM) caused a significant and concentration-dependent inhibition of collagen- (Fig 1A-B) and thrombin-induced washed platelet aggregation *in vitro* (Fig 1C) but had no effect on ADP-induced aggregation (Fig 1D). *In vivo*, collagen-induced platelet aggregation was significantly reduced following pre-treatment of mice with 50 μg kg^{-1} sildenafil (Fig 1 E-F).

Sildenafil selectively amplifies endogenous NO-mediated signalling independent of NOS

Sildenafil (10 nM) significantly enhanced NO-mediated inhibition of platelet aggregation (Fig 2A) but in contrast had no effect upon the inhibitory effect exerted by the prostacyclin mimetic iloprost (Fig 2B). Sildenafil also induced a concentration-dependent increase in VASP-239P phosphorylation (Fig 1C-D) when applied to isolated platelets. Sildenafil-mediated inhibition of platelet aggregation was abolished in the presence of the sGC antagonist ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; Fig 2E) and the NO scavengers haemoglobin and hydroxocobalamin (Fig 2F). In contrast, pre-treatment of platelets with the NOS inhibitor L-NAME had no effect on sildenafil-mediated inhibition of aggregation compared to both vehicle and D-NAME control (2G). Similarly, sildenafil-mediated VASP-P239 phosphorylation was abolished by ODQ but not significantly affected by L-NAME (Fig 2H-I).

Nitrite reduction drives cGMP-mediated inhibition of platelet aggregation

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Having shown that isolated platelets generate inhibitory NO-cGMP signals that arise predominantly from sources other than NOS, we explored the ability of nitrate and nitrite to drive NO-cGMP-mediated inhibition of platelet aggregation. We firstly demonstrated the presence of both nitrate and nitrite in platelet extracts and undetectable levels in experimental buffers using a colorimetric assay (Fig 3A). Washing of platelet suspensions with the mild reducing agent ascorbic acid lowered their nitrate and nitrite content presumably due to chemical reduction to NO (Fig 3B). Nitrate/nitrite depletion and any accompanying NO release were associated with inhibition of platelet aggregation (Fig 3C). The inhibitory effect of nitrate/nitrite reduction by ascorbic acid was abolished by ODQ and haemoglobin (Fig 3D).

Sodium nitrite (0.01 – 100 μM) exerted a concentration-dependent inhibition of aggregation in the presence of sildenafil (Fig 3E) whereas equivalent concentrations of sodium nitrate had no effect (Fig 3F.) The inhibitory effect of nitrite was abolished by ODQ and did not occur in the absence of sildenafil (Fig 3E). Similarly, nitrite increased VASP-P239 phosphorylation in the presence of sildenafil, an effect that was prevented by ODQ, whereas nitrate had no effect (Fig 3G-H).

Inorganic nitrate inhibits platelet aggregation following enhanced bioconversion to nitrite during endothelial dysfunction in vivo

Sodium nitrate (1 mmol kg^{-1}) administration to mice led to increased concentrations of nitrate in salivary glands (Fig 4A) and increased plasma nitrate concentration (Fig 4B) in wild-type and $eNOS^{-/-}$ mice as measured by ozone~~gas-phase~~ chemiluminescence. There was also an accompanying increase in plasma nitrite that was not significant in wild-type mice but was significantly increased in $eNOS^{-/-}$ mice compared to saline-treated controls (Fig 4C). Nitrate administration had no significant effect on subsequent collagen-induced platelet aggregation *in vivo* in wild-type mice but significantly reduced platelet aggregation in $eNOS^{-/-}$ mice (Fig 5A-B).

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Discussion

Sildenafil has already been shown to amplify the inhibitory effect of exogenously applied NO upon human platelet aggregation [4] and to reduce ADP-induced glycoprotein IIb/IIIa activation [30] and aggregation [5] *ex vivo* indicating the ability of sildenafil to enhance the platelet inhibitory activity of exogenous and endothelial NO respectively. The ability of sildenafil to enhance intrinsic endogenous NO signals in platelets is less well studied although a lack of effect upon ADP-induced aggregation has been reported [6]. We found a similar lack of effect when platelets were stimulated with ADP in the absence of exogenous NO but, in contrast, found an inhibition of collagen- and thrombin-induced aggregation in isolated platelet suspensions. This finding indicates, not only the ability of sildenafil to directly modulate platelet activation in the absence of exogenous or endothelial NO, but demonstrates the presence of transient, endogenous signals upstream of PDE5 in platelets. The ability of sildenafil to modulate activation downstream of collagen and thrombin suggests modulation of pathways that were not triggered by the relatively weaker agonist ADP. In addition, experiments with ADP in work published previously [6] and in the current study were conducted in the presence of plasma proteins whereas experiments with collagen and thrombin were conducted in preparations lacking plasma proteins. The lack of effect of sildenafil in ADP experiments may therefore be partially pharmacokinetic due to interaction of relatively lipophilic sildenafil [31] with plasma proteins.

We confirmed that, in isolated platelets, sildenafil acts downstream of the NO-cGMP signalling cascade by showing that its inhibitory effects were completely abolished by the sGC antagonist ODQ and two distinct NO scavengers. We further confirmed that sildenafil mediates inhibitory signalling events in platelets by demonstrating phosphorylation of VASP at Ser^{239}. Given the reported cross-talk between cyclic nucleotides and PDEs [32], particularly at the level of protein kinases [33, 34], we contrasted the selectivity of sildenafil upstream of these signalling events and showed an ability of sildenafil to enhance the inhibitory effect of NO (Fig 2A) but not prostacyclin (Fig 2B). Sildenafil

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therefore selectively amplifies the inhibitory effect of NO with no measurable functional effect on prostacyclin-mediated inhibition despite downstream crosstalk between these pathways.

A number of groups, including ours, have previously suggested a lack of functional relevance or expression of NOS in human platelets [13, 14]. This led us to consider the source of NO acting upstream of sildenafil to mediate inhibition of platelet aggregation. Studies with a NOS inhibitor (Fig 2G) suggested~~revealed~~ that the effect of sildenafil occurred independently of NOS suggesting alternative sources of bioactive NO. The validity of this conclusion depends upon effective blockade of NOS at the concentration of L-NAME employed. L-NAME inhibits NOS with an IC_{50} of 0.81 μM [35] and off-target effects in platelets emerge at around 500 μM to 1 mM [14]. Our working concentration of 100 μM can therefore be reasonably assumed to result in effective and selective inhibition of NOS activity in platelets as previously reported [36]. Nonetheless, although we have data suggestive of ~~conclusively demonstrated~~ NOS-independent inhibitory activity upstream of PDE5, we cannot exclude entirely the possibility that NO derived from NOS, if expressed in platelets, may have relevance under certain circumstance, albeit insignificant in the present study. We proceeded by exploring the ability of inorganic nitrate and nitrite (administered as sodium nitrate and sodium nitrite) to modulate platelet activation and VASP phosphorylation.

In mammals, inorganic nitrate can be bioconverted to nitrite and subsequently reduced to NO by a variety of mechanisms [20, 37, 38]. We firstly confirmed that platelets contain nitrate/nitrite that could potentially be reduced to NO to explain the presence of endogenous, NOS-independent NO signals in platelets. Nitrate and nitrite levels in platelets were then successfully depleted by incubation of platelets with the mild and relatively non-toxic reducing agent ascorbic acid in buffer prepared with nitrate free water. We hypothesised that reduced endogenous nitrite would generate a NO/cGMP signal that could be amplified by sildenafil to inhibit agonist induced aggregation and found evidence to support this when ascorbic acid induced ODQ-sensitive inhibition of aggregation. In fact, the inhibitory effect of ascorbic acid was entirely abolished by ODQ or NO scavenging

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suggesting that, under the prevailing experimental conditions, ascorbic acid exerted functional effects that were entirely mediated *via* sGC and NO. Reducing agents exert a range of effects including reduction of reactive oxygen species and peroxynitrite and demonstrating that reducing agents can drive inhibitory signalling, whilst indicating that, in principle, platelet function can be impacted by reduced nitrite/nitrate, does not demonstrate an endogenous ability of platelets to reduce nitrate or nitrite to NO. We therefore investigated the functional impact of nitrate/nitrite in the absence of exogenous reducing agents to directly link nitrate/nitrite with platelet activity. Nitrite has previously been reported to directly inhibit platelet aggregation although the concentrations required were higher than those found in plasma following nitrate administration to humans [21, 39]. In contrast, lower, more relevant concentrations of nitrite were shown to have no effect on isolated platelets [22] reflecting either an inability of platelets to reduce nitrite or the generation of a transient signal without functional impact. The application of a fixed concentration of sildenafil in the current study revealed the ability of nitrite but not nitrate to generate ODQ-sensitive inhibitory signals in platelets in the presence of sildenafil suggesting an endogenous capacity to reduce nitrite. In line with earlier studies [22], nitrite had no effect on platelet aggregation in the absence of sildenafil suggesting the generation of transient signals which are, under normal circumstances, rapidly hydrolysed by PDE5. Although some evidence of sGC-independent effects of NO [40-42] has been reported, the effects of nitrite reported here are entirely-sGC dependent. This is in line with more recently reported data demonstrating absolute dependence of NO-mediated signalling upon sGC in platelets [43]. The mechanism by which platelets reduce nitrite remains unclear. In other cell types such as vascular endothelial cells, nitrite is reduced enzymatically by xanthine oxidases [17, 20] and aldehyde dehydrogenase [44], however these enzymes are not part of the platelet proteome [45]. In addition, nitrite reduction is also suggested to occur in erythrocytes [22, 46, 47], implying an enhanced capacity to reduce nitrite in whole blood and *in vivo* compared with isolated platelets or plasma. Therefore, alternative mechanisms in platelets such as mitochondrial activity or the presence of as yet unidentified mediators with reducing capacity may explain the efficacy of nitrite in

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isolated platelets reported here. Given the absence of many of the proposed nitrate reductase systems from platelets, we tentatively speculate that the most likely mechanism of nitrite reduction in platelets to be a mitochondrial nitrite reductase such a cytochrome C [48]. Demonstrating conclusively the role of mitochondrial components with critical roles in mitochondrial respiration and cellular metabolism in nitrite reduction and inhibition of platelet activation is likely to be challenging.

The presence of NOS in platelets has been contentious for some time now [11, 12] and there is increasing evidence of a lack of importance of NOS--derived NO in regulating platelet function [13, 14] (the primary source of NO affecting platelets physiologically being the vascular endothelium [7]). Nonetheless, platelets are widely reported to generate NO [15, 16]. Our data provide one potential explanation of these apparently contradictory observations in addition to those previously suggested such as NO production *via* s-nitrosothiols [49] and protein disulphide isomerases [50].

We also explored the *in vivo* relevance of our data obtained with isolated platelets. Sildenafil has previously been reported to improve coronary artery patency in a model of cyclic coronary occlusion [51]. The effect was suggested to be potentially platelet-mediated but may also have been due to coronary vasodilation. The model used in the current study was selected because it has previously been shown to measure platelet aggregation independent of any effect on vascular tone [7]. We can therefore conclude that as well as exerting a direct inhibitory effect upon platelets *in vitro*, sildenafil inhibits agonist induced platelet aggregation *in vivo via* a direct effect upon the platelet rather than *via* a secondary vascular effect. Since *in vivo* preparations contain a fully functional vascular endothelium, it is reasonable to conclude that the effect of sildenafil upon platelets *in vivo* is at least in part mediated *via* enhancement of NO derived from the vascular endothelium as well as *via* any direct platelet-mediated effect of nitrite.

Nitrate administration in humans has previously been shown to induce a fall in blood pressure and to inhibit *ex vivo* platelet aggregation [21, 22]. In previously reported mouse studies, a lowering of plasma nitrite concentration was associated with enhanced platelet aggregation *ex vivo* [52]. 0.2

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mmol kg^{-1} d^{-1} of nitrate has been estimated to be produced endogenously by NOS [26, 53]. One nitrate-rich vegetable portion contains more nitrate that that produced by all forms of NOS daily [54]. Therefore the dose of 1 mmol kg^{-1} nitrate used in the current study reflects a realistic dose that could be achieved through dietary choices or supplementation. In the present study, the increase in plasma nitrite concentration following administration of nitrate to wild-type mice, although not significant, was similar to that reported previously in humans following consumption of high nitrate beetroot juice (approximately 0.2 μM) [21]. This increase in plasma nitrite did not lead to a change in platelet aggregation *in situ* in our study suggesting that NO was not a limiting factor in the context of a healthy vasculature. In $eNOS^{-/-}$ mice however, nitrate administration led to an approximately 5-fold greater increase in plasma nitrite (approximately 1 μM) indicating that bioconversion of nitrate to nitrite was certainly evident and indeed greater than that observed in wild-type mice ~~a greater capacity for the bioconversion of nitrate to nitrite in the absence of endogenous eNOS activity~~. These data suggest that $eNOS^{-/-}$ mice may compensate for the absence of NO from conventional enzymatic sources by increasing NO generation from nitrate. Our data showing nitrate reduction in $eNOS^{-/-}$ mice also indicate that although eNOS has been shown to mediate nitrite reduction in a previous study[55], this was not a primary mechanism of systemic reduction in our study. Our data raise the question of whether a similar switch in the physiological source of NO from NOS to nitrite occurs in humans with vascular disease. This issue has not been addressed in the current study and additional studies in humans and, in particular, patients with cardiovascular conditions associated with deficient eNOS activity are required to translate our mechanistic linking of eNOS with enhanced nitrite reduction to human pathology. If this translation were established, then nitrate/nitrite derived from the diet may become critical as a source of bioactive NO during endothelial dysfunction. We tested the functional relevance of the differential changes in plasma nitrite in wild-type and $eNOS^{-/-}$ mice by measuring platelet aggregation *in vivo*. Interestingly, the higher bioconversion of nitrate occurring in $eNOS^{-/-}$ mice was associated with a significant reduction in platelet aggregation, an effect not seen in wild-type mice. Our data therefore suggest that nitrate exerted a specific effect upon platelet

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function during conditions of vascular dysfunction, namely eNOS deficiency, whilst during vascular health, associated with wild-type mice, normal platelet function was retained. This data is potentially of great interest since it suggests targeted efficacy during conditions of endothelial dysfunction. In summary, we have shown that platelets generate transient, endogenous cGMP signals that may be pharmacologically enhanced by inhibition of PDE5 activity. These signals are generated downstream of NO but are primarily independent of NOS activity. Furthermore, nitrite is able to generate transient NO-cGMP signals in platelets which can be enhanced by sildenafil. The absence of eNOS leads to an enhanced capacity to bioconvert nitrate to nitrite which in turn negatively impacts platelet function. Our study adds to the increasing body of evidence suggesting that dietary nitrate may account, at least partly, for the beneficial effects of healthy diets, particularly those rich in green vegetables with high nitrate content. Furthermore, inorganic nitrate may potentially exert an antiplatelet effect specifically during endothelial dysfunction whilst allowing retention of normal platelet function in conditions of vascular health. Our study, when combined with growing literature concerning the impact of dietary nitrate on cardiovascular health, suggests that the potential use of dietary nitrate supplementation in the primary prevention of platelet-driven cardiovascular events should be further explored.

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Authorship details

G.L Apostoli: design, conduct and analysis of experiments, drafting of manuscript; A. Solomon: concept and design of experiments; M.J Smallwood: design, conduct and analysis of experiments; P.G Winyard: concept, design of experiments and drafting of manuscript; M. Emerson: concept, design of experiments, drafting of manuscript, final approval of manuscript.

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Figure Legends

Figure 1: *Sildenafil inhibits platelet aggregation* in vitro *and* in vivo*.* **A** - **D**: Isolated human platelets were pre-incubated with vehicle or sildenafil citrate (10 nM - 1 μM) for 5 mins before stimulation with (A-B) collagen (5 μg mL^{-1}), (C) thrombin (0.1 U mL^{-1}) and (D) adenosine diphosphate (ADP, 0.3 – 30 μM). Platelet aggregation was measured as light transmission. (A) Example traces are representative of 8 independent experiments. (B-C) Data are expressed as mean±S.E.M. One-way ANOVA with Bonferroni post-hoc test, *P<0.05, **P<0.01, ***P<0.001 compared to vehicle (D) Vehicle EC_{50} = 0.585 μM, sildenafil EC_{50} = 0.447 μM. **E** - **F**: Sildenafil (50 μg kg^{-1}) or vehicle was administered to mice 5 mins before collagen (50 μg kg^{-1}). Platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. (E) Mean trace of collagen response is expressed as percentage increase from baseline, error bars omitted for clarity. (F) Maximum percentage increase from baseline is expressed as mean ± S.E.M., unpaired Students t-test, *P<0.05 compared to vehicle, n=5-8.

Figure 2: *Sildenafil selectively amplifies endogenous NO-mediated signalling independent of nitric oxide synthase (NOS).* **A-B:** Sildenafil (10 nM) significantly enhanced the inhibition of collagen-induced (5 μg mL^{-1}) washed human platelet aggregation mediated by (A) SNP (0.01-100 μM), but not (B) iloprost (0.1-1000 pM). **C-D**: sildenafil (10 – 1000 nM) and SNP (positive control) induced phosphorylation of VASP at serine 239 (VASP-239P). Data are shown as (C) Western blot representative of 4 independent experiments, (D) percentage of VASP-239P compared to total VASP. Data are expressed as mean±S.E.M. **E-G:** The inhibitory effect of sildenafil (10 nM – 1 μM) on collagen-induced aggregation was significantly inhibited by (E) ODQ (10 μM) and (F) haemoglobin (Hb, 5 μM) or hydroxocobalamin (HXB, 100 μM), whereas (G) L-NAME (100 μM) or D-NAME (100 μM) had no effect. **H** - **I**: sildenafil (100 nM) induced VASP-239P phosphorylation was abolished by ODQ whereas L-NAME had no effect, data are presented as (H) Western blot representative of 4

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independent experiments and (I) percentage of VASP-239P compared to total VASP. Data are expressed as mean±S.E.M., One-way ANOVA with Bonferroni post-hoc test, *P<0.05, **P<0.01, ***P<0.001 compared to vehicle treated, ns = not significant (P>0.05) compared to sildenafil treated, n=4-6.

Figure 3: *Nitrite drives cGMP-mediated inhibition of platelet aggregation.* **A-B**: Nitrate and nitrite concentration was measured in human platelets (A) untreated or (B) pre-incubated with vehicle or ascorbic acid (10, 1000 µM). Data are expressed as mean±S.E.M, one-way ANOVA with Bonferroni post-hoc test. **C**: Collagen (5 µg mL^{-1}) induced aggregation was inhibited in nitrate/nitrite depleted platelets treated with ascorbic acid, one-way ANOVA with Bonferroni post-hoc test. **D:** Ascorbic acid-induced (2.5mM) inhibition of aggregation was reversed in the presence of ODQ (10 µM) and haemoglobin (Hb; 5µM), one-way ANOVA with Bonferroni post-hoc test. **E-F**: Sodium nitrite ($NaNO_2$, 0.01-100 µM) caused concentration-dependent inhibition of platelet aggregation in the presence of sildenafil (10 nM) which was reversed by ODQ (10 µM), an effect not seen with (F) sodium nitrate ($NaNO_3$, 0.01-100 µM). Data are expressed as mean±S.E.M, two-way ANOVA with Bonferroni post-hoc test. **G-H**: Pre-incubation of platelets with NO_2 (100 µM) and sildenafil (10 nM) resulted in significant phosphorylation of VASP at serine 239 (VASP-239P) that was reversed in the presence of ODQ (10 µM). This effect was not seen with NO_3 (100 µM). Sodium nitroprusside (SNP, 1 µM) was used as a positive control. Data are presented as (G) Western blot representative of 4 independent experiments and (H) percentage of VASP-239P compared to total VASP. Data are expressed as mean±S.E.M., one-way ANOVA with Bonferroni post-hoc test. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle treated, n=4-7.

Figure 4*: Bioconversion of nitrate to nitrite is enhanced in the absence of eNOS.* **A-B:** Following treatment of mice with saline or sodium nitrate (1 mmol kg^{-1} i.p.) for 1 h, nitrate concentrations were

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significantly increased in (A) salivary glands and (B) plasma in both wild-type (W.T) and $eNOS^{-/-}$ mice. **C:** Plasma nitrite concentration was significantly increased in $eNOS^{-/-}$ but not W.T. mice following nitrate treatment. Data are expressed as mean±S.E.M., unpaired Students t-test, *P<0.05, **P<0.01, ***P<0.001, ns= non-significant (P>0.05), n=5-7.

Figure 5: *Inorganic nitrate inhibits platelet aggregation during endothelial dysfunction in vivo.* Wild-type (W.T) and $eNOS^{-/-}$ mice were treated with saline or sodium nitrate (1mmol kg^{-1} i.p.) 1 h prior to collagen (50 µg kg^{-1}) and radiolabelled platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. **A:** Mean trace of collagen response (percentage increase from the baseline radioactive counts) *versus* time. Data are expressed as mean (error bars omitted for clarity). **B:** Maximum percentage increase from baseline radioactive counts. Data are expressed as mean ± S.E.M., unpaired Students t-test, *P<0.05, ns = non-significant (P>0.05), n=4-6.

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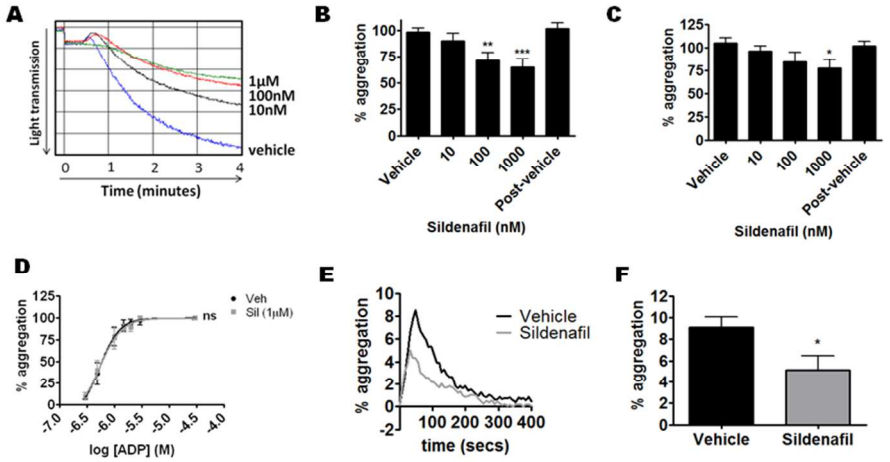

Figure 1: *Sildenafil inhibits platelet aggregation* in vitro *and* in vivo*.* **A - D**: Isolated human platelets were pre-incubated with vehicle or sildenafil citrate (10 nM - 1 µM) for 5 mins before stimulation with (A-B) collagen (5 µg mL^{-1}), (C) thrombin (0.1 U mL^{-1}) and (D) adenosine diphosphate (ADP, 0.3 – 30 µM). Platelet aggregation was measured as light transmission. (A) Example traces are representative of 8 independent experiments. (B-C) Data are expressed as mean±S.E.M. One-way ANOVA with Bonferroni post-hoc test, *P<0.05, **P<0.01, ***P<0.001 compared to vehicle (D) Vehicle EC_{50} = 0.585 µM, sildenafil EC_{50} = 0.447 µM. **E - F**: Sildenafil (50 µg kg^{-1}) or vehicle was administered to mice 5 mins before collagen (50 µg kg^{-1}). Platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. (E) Mean trace of collagen response is expressed as percentage increase from baseline, error bars omitted for clarity. (F) Maximum percentage increase from baseline is expressed as mean ± S.E.M., unpaired Students t-test, *P<0.05 compared to vehicle, n=5-8.
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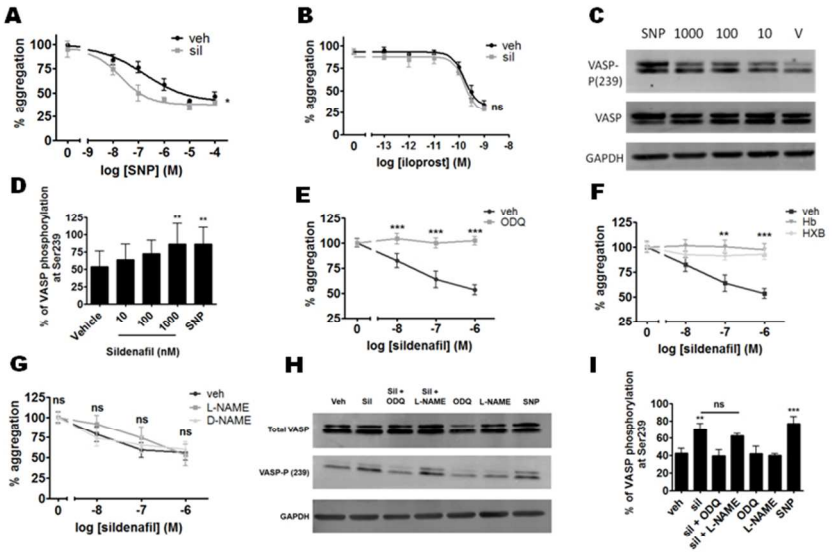

Figure 2: *Sildenafil selectively amplifies endogenous NO-mediated signalling independent of nitric oxide synthase (NOS).* **A-B**: Sildenafil (10 nM) significantly enhanced the inhibition of collagen-induced (5 µg mL^{-1}) washed human platelet aggregation mediated by (A) SNP (0.01-100 µM), but not (B) iloprost (0.1-1000 pM). **C-D**: sildenafil (10 – 1000 nM) and SNP (positive control) induced phosphorylation of VASP at serine 239 (VASP-239P). Data are shown as (C) Western blot representative of 4 independent experiments, (D) percentage of VASP-239P compared to total VASP. Data are expressed as mean±S.E.M. **E-G**: The inhibitory effect of sildenafil (10 nM – 1 µM) on collagen-induced aggregation was significantly inhibited by (E) ODQ (10 µM) and (F) haemoglobin (Hb, 5 µM) or hydroxocobalamin (HXB, 100 µM), whereas (G) L-NAME (100 µM) or D-NAME (100 µM) had no effect. **H - I**: sildenafil (100 nM) induced VASP-239P phosphorylation was abolished by ODQ whereas L-NAME had no effect, data are presented as (H) Western blot representative of 4 independent experiments and (I) percentage of VASP-239P compared to total VASP. Data are expressed as mean±S.E.M., One-way ANOVA with Bonferroni post-hoc test, *P<0.05, **P<0.01, ***P<0.001 compared to vehicle treated, ns = not significant (P>0.05) compared to sildenafil treated, n=4-6.
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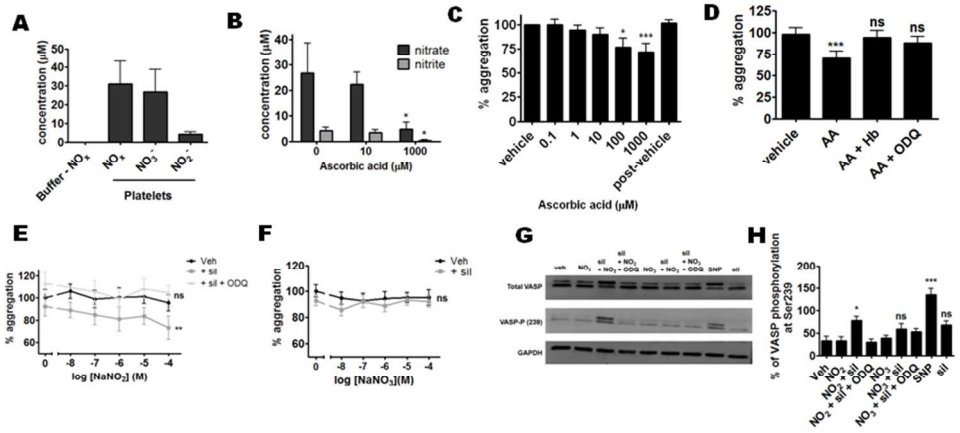

Figure 3: *Nitrite drives cGMP-mediated inhibition of platelet aggregation.* **A-B**: Nitrate and nitrite concentration was measured in human platelets (A) untreated or (B) pre-incubated with vehicle or ascorbic acid (10, 1000 μM). Data are expressed as mean±S.E.M, one-way ANOVA with Bonferroni post-hoc test. **C**: Collagen (5 μg mL^{-1}) induced aggregation was inhibited in nitrate/nitrite depleted platelets treated with ascorbic acid, one-way ANOVA with Bonferroni post-hoc test. **D**: Ascorbic acid-induced (2.5mM) inhibition of aggregation was reversed in the presence of ODQ (10 μM) and haemoglobin (Hb; 5 μM), one-way ANOVA with Bonferroni post-hoc test. **E-F**: Sodium nitrite ($NaNO_2$, 0.01-100 μM) caused concentration-dependent inhibition of platelet aggregation in the presence of sildenafil (10 nM) which was reversed by ODQ (10 μM), an effect not seen with (F) sodium nitrate ($NaNO_3$, 0.01-100 μM). Data are expressed as mean±S.E.M, two-way ANOVA with Bonferroni post-hoc test. **G-H**: Pre-incubation of platelets with NO_2 (100 μM) and sildenafil (10 nM) resulted in significant phosphorylation of VASP at serine 239 (VASP-239P) that was reversed in the presence of ODQ (10 μM). This effect was not seen with NO_3 (100 μM). Sodium nitroprusside (SNP, 1 μM) was used as a positive control. Data are presented as (G) Western blot representative of 4 independent experiments and (H) percentage of VASP-239P compared to total VASP. Data are expressed as mean±S.E.M., one-way ANOVA with Bonferroni post-hoc test. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle treated, n=4-7.
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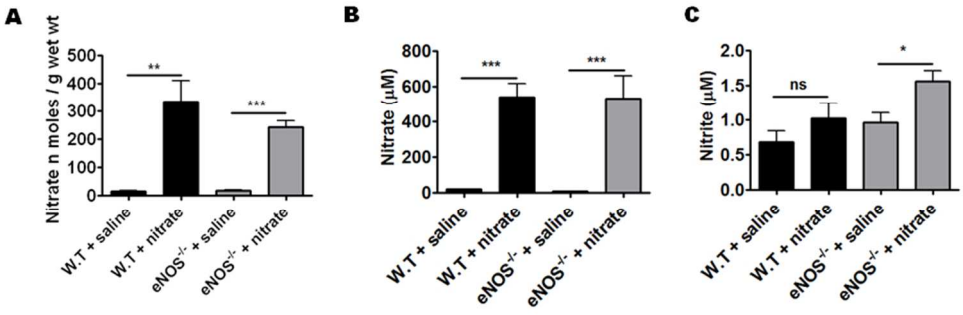

Figure 4: *Bioconversion of nitrate to nitrite is enhanced in the absence of eNOS.* **A-B**: Following treatment of mice with saline or sodium nitrate (1 mmol kg^{-1} i.p.) for 1 h, nitrate concentrations were significantly increased in (A) salivary glands and (B) plasma in both wild-type (W.T) and $eNOS^{-/-}$ mice. **C**: Plasma nitrite concentration was significantly increased in $eNOS^{-/-}$ but not W.T. mice following nitrate treatment. Data are expressed as mean±S.E.M., unpaired Students t-test, *P<0.05, **P<0.01, ***P<0.001, ns= non-significant (P>0.05), n=5-7.
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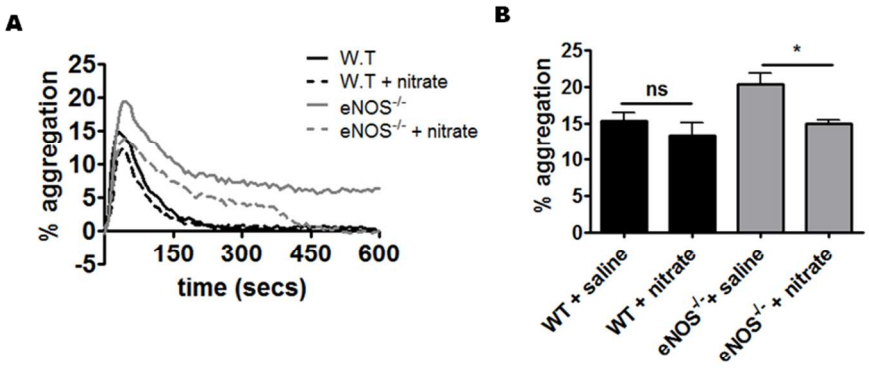

Figure 5: *Inorganic nitrate inhibits platelet aggregation during endothelial dysfunction* in vivo. Wild-type (W.T) and eNOS$^{-/-}$ mice were treated with saline or sodium nitrate (1mmol kg^{-1} i.p.) 1 h prior to collagen (50 µg kg^{-1}) and radiolabelled platelet aggregation was measured as changes in radioactive counts in the pulmonary vasculature. **A**: Mean trace of collagen response (percentage increase from the baseline radioactive counts) versus time. Data are expressed as mean (error bars omitted for clarity). **B**: Maximum percentage increase from baseline radioactive counts. Data are expressed as mean ± S.E.M., unpaired Students t-test, *P<0.05, ns = non-significant (P>0.05), n=4-6.
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