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The *ex vivo* antiplatelet activation potential of fruit phenolic metabolite hippuric acid

Abishek Bommannan Santhakumar,^{*a,b} Roger Stanley^c and Indu Singh^a

Polyphenol-rich fruit and vegetable intake has been associated with reduction in platelet hyperactivity, a significant contributor to thrombus formation. This study was undertaken to investigate the possible role of hippuric acid, a predominant metabolite of plant cyclic polyols, phenolic acids and polyphenols, in reduction of platelet activation-related thrombogenesis. Fasting blood samples were collected from 13 healthy subjects to analyse the effect of varying concentrations of hippuric acid (100 μM , 200 μM , 500 μM , 1 mM and 2 mM) on activation-dependant platelet surface-marker expression. Procaspase activating compound-1 (PAC-1) and P-selectin/CD62P monoclonal antibodies were used to evaluate platelet activation-related conformational changes and α -granule release respectively using flow cytometry. Platelets were stimulated *ex vivo* via the $\text{P}_2\text{Y}_1/\text{P}_2\text{Y}_{12}$ – adenosine diphosphate (ADP) pathway of platelet activation. Hippuric acid at a concentration of 1 mM and 2 mM significantly reduced P-selectin/CD62P expression ($p = 0.03$ and $p < 0.001$ respectively) induced by ADP. Hippuric acid at 2 mM concentration also inhibited PAC-1 activation-dependant antibody expression ($p = 0.03$). High *ex vivo* concentrations of hippuric acid can therefore significantly reduce P-selectin and PAC-1 expression thus reducing platelet activation and clotting potential. However, although up to 11 mM of hippuric acid can be excreted in the urine per day following consumption of fruit, hippuric acid is actively excreted with a recorded C_{max} for hippuric acid in human plasma at 250–300 μM . This is lower than the blood concentration of 1–2 mM shown to be bio-active in this research. The contribution of hippuric acid to the protective effects of fruit and vegetable intake against vascular disorders by the pathways measured is therefore low but could be synergistic with lowered doses of antiplatelet drugs and help reduce risk of thrombosis in current antiplatelet drug sensitive populations.

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Introduction

According to a recent World Health Organisation report, 17.5 million people died from cardiovascular disease in 2012, representing 31% of all global deaths.¹ Amongst several risk factors including smoking, hypertension, genetic association and obesity, poor nutrition has also been one of the important contributors. Several studies have reported the benefits of a healthy diet of fruit and vegetables in reducing the risk of vascular disorders.^{2–5} Interest in plant derived antioxidants and

polyphenols such as anthocyanins and quercetin continues to grow as a result of their reported antithrombotic and cardiovascular benefits.^{6–13} Analysis of the pharmacokinetics and bioavailability of plant derived compounds after polyphenol/anthocyanin rich food intake has shown that the concentrations of anthocyanins and structurally related metabolites only reach micro-molar concentrations *in vivo* (less than 5 μM).¹⁴ However hippuric acid is a major metabolite post polyphenol consumption that is excreted in urine in up to millimolar concentrations *in vivo*.^{15,16} Consumption of prunes,¹⁶ black and green tea solids,¹⁷ cranberry juice,¹⁸ red wine, grape juice,¹⁹ and fruits/vegetables in general¹⁵ have been shown to increase urinary hippuric acid levels. According to a dietary intervention study by Toromanovic and colleagues,¹⁶ urinary excretion of hippuric acid reached levels of up to 11 mM per 24 hours after consumption of 300 g of edible fruits.¹⁶ The increased levels are believed to be due to plant derived simple phenolic compounds and the microbial degradation of anthocyanins, chlorogenic acids and quinic

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acid in the colon and their catabolism to benzoid type compounds.^{20–22} In spite of hippuric acid being a consistent biomarker/metabolite that can be excreted *via* the urine in grams per day, there are no well-documented cardiovascular benefits associated with it.

Several *in vitro* and *in vivo* trials have demonstrated the prospective bioactivity of natural dietary phenolics rich in antioxidants, such as anthocyanins^{6,23,24} and quercetin derivatives²⁵ in reducing thrombotic risk by alleviating platelet hyperactivity/aggregation. Hippuric acid, however, occurs in very high concentrations *in vivo* versus very low levels of most other phenolics.²⁶ Similar to the action of current antiplatelet drugs such as aspirin and clopidogrel, polyphenols have been demonstrated to block platelet activation pathways (cyclooxygenase-1 – COX-1 and P₂Y₁/P₂Y₁₂) consequently reducing the risk of thrombosis.^{9,27,28} We hypothesize that, active metabolites occurring *in vivo* at high concentration such as hippuric acid, could also be partially responsible for the claimed antiplatelet properties of plant derived phenolic acids and polyphenols.

This *ex vivo* trial aimed to evaluate the effect of hippuric acid, a potential active metabolite, in targeting the P₂Y₁/P₂Y₁₂ pathway of platelet activation. We have analysed the effect of various concentrations of the metabolite on activation-dependant platelet surface receptor P₂Y₁/P₂Y₁₂ induced by adenosine diphosphate (ADP), a physiological platelet activator, by evaluating the expression of platelet activation-related conformation change and degranulation.

Materials and methods

Subject recruitment and sample collection

The study protocol was approved by the Griffith University Human Research ethics committee (GU Protocol no. MSC/13/11/HREC) and was performed in compliance with the relevant laws and institutional guidelines. Thirteen healthy subjects were recruited from the general population by means of advertisements placed around Griffith University, Gold Coast campus. Subjects were healthy, non-smokers with no history of metabolic or cardiovascular diseases and were not on any anti-inflammatory, antiplatelet medications or health supplements at least 7 days prior to blood collection. Informed consent was obtained from all volunteers prior to commencement of the study. Fasting whole blood samples were collected from the median cubital vein by a trained phlebotomist at the School of Medical Science, Griffith University. Care was taken to ensure minimal sample handling or agitation to prevent platelet activation. No samples were obtained from traumatic phlebotomy procedures or contained obvious clots. Blood was drawn into tri-potassium EDTA (1.8 mg mL⁻¹) anticoagulant tubes (used for full blood examination – FBE) before collection into tri-sodium citrate (28.1 g mL⁻¹) tubes (used for platelet surface-marker expression) to avoid risk of collecting venepuncture activated platelets. Baseline FBE and biochemical profile was carried out for initial subject screening and recruitment using a Coulter® Ac.T™ 5diff CP haematology analyser (Beckman

Coulter, Inc., Lane Cove, NSW, Australia) and Cobas Integra 400® plus biochemistry analyser (Roche diagnostics, Basel, Switzerland) respectively.

Hippuric acid

Hippuric acid used in the analysis was purchased from Sigma-Aldrich, Castle Hill, NSW, Australia. Working solutions of 100 µM, 200 µM, 500 µM, 1 mM and 2 mM were prepared from a stock solution of 20 mM concentration diluted in 1× concentration of phosphate buffered saline (PBS). Aliquots of different concentrations of hippuric acid were stored at 4 °C for a maximum of 2 weeks.

Activation dependant platelet surface-marker expression

Venous blood collected into tri-sodium citrate tubes were used to evaluate platelet surface marker expression. This analysis was performed and interpreted using the BD LSRFortessa cell analyser (BD Biosciences, North Ryde, NSW, Australia) and BD FACSDiva software (version 6.1.3, BD Biosciences, North Ryde, NSW, Australia) respectively. Platelet activation dependant monoclonal antibodies (mAb) were used to evaluate the expression of positive activated platelets induced by ADP agonist. CD42b-peridinin chlorophyll protein (CD42b-PerCp-Cy5.5) conjugated mAb was used to identify the GPIIb-IX receptors, the most abundant receptor on the platelet population. Platelet activity was assessed by activation-dependant platelet surface marker expression using PAC-1-fluorescein isothiocyanate (PAC-1-FITC recognises activated conformational changes in the fibrinogen binding receptor GPIIb-IIIa) and P-selectin/CD62P (expressed in activated de-granulated platelets) conjugated mAb. The antibodies and their respective isotype controls were purchased from BD Biosciences (BD Biosciences, North Ryde, NSW, Australia).

Whole blood was incubated with respective concentrations of hippuric acid and control (PBS) for 15 min at room temperature (25 °C). After incubation, the samples were then diluted in filtered modified tyrode's buffer (pH 7.2) and incubated with CD42b, PAC-1, P-selectin mAb's for 15 min in the dark at room temperature. ADP (5 µM) was incubated with the blood-antibody mixture for 10 min in the dark at room temperature to stimulate the platelets *via* the P₂Y₁₂/P₂Y₁ platelet activation pathway. The suspension was then fixed with 1% paraformaldehyde solution (pH 7.2), incubated in the dark at room temperature for 15 min and analysed in the flowcytometer for antibody expression. The setup and optimal fluorescence compensation of the flowcytometer was validated using BD Cytometer Setup and Tracking beads and BD CompBead compensation particles (BD Biosciences, North Ryde, NSW, Australia). Ten thousand platelet events were acquired, gated on the basis of light scatter and CD42b mAb expression. Activation dependant-mAb (PAC-1 and P-selectin) expression by activated platelets was articulated as mean fluorescence intensity (MFI).

Statistical analysis

A two-way ANOVA following Tukey's post comparison test was performed using GraphPad Prism version 6.0 for Windows

(GraphPad Software, La Jolla, California, USA). A minimum sample size of 12 subjects in each group is required for 80% power to detect a 5% variation in the laboratory parameters measured, where a 3–5% standard deviation exists in the population, assuming an alpha error of 0.05. All the data is expressed as mean \pm standard deviation. Differences between the groups were considered to be significant when $p < 0.05$. Any significant statistical interactions were included in the analysis where applicable.

Results

The baseline full blood cell counts, inflammation marker and biochemical profile of the subjects under study were within normal reference ranges (Table 1) as established by the Royal College of Pathologists Australasia.²⁹

It was observed that whole blood *ex vivo* treatment with 1 mM and 2 mM concentrations of hippuric acid reduced ADP-induced P-selectin/CD62P expression by 7.5% ($p = 0.03$) and 14% ($p < 0.001$) respectively (Fig. 1). Lower concentrations of hippuric acid (100 μ M, 200 μ M or 500 μ M) did not have a statistically significant effect on P-selectin platelet surface marker expression but demonstrated a trend in inhibition of the activation-dependant monoclonal antibody expression (Fig. 1).

Hippuric acid at a concentration of 2 mM inhibited the initial platelet activation phase, by 11% ($p = 0.03$), involving its conformational change targeting the GPIIb-IIIa receptor, evident from PAC-1 activation-dependant monoclonal antibody expression (Fig. 2). Lower concentrations of hippuric acid did not affect PAC-1 expression.

Table 1 Baseline parameters of subjects under study

Parameters	Mean \pm SD	Reference ranges
<i>n</i> (M/F)	13	NA
Age (y)	30 \pm 3	NA
Body mass (kg)	73 \pm 8	NA
BMI (kg m ⁻²)	23.6 \pm 0.7	20–25
Haemoglobin (g L ⁻¹)	140 \pm 10	120–180
Haematocrit (%)	42 \pm 2	40–54
RBC ($\times 10^{12}$ L ⁻¹)	4.9 \pm 0.4	3.8–6.5
WBC ($\times 10^9$ L ⁻¹)	6.2 \pm 1.2	4.0–11.0
Platelet ($\times 10^9$ L ⁻¹)	217 \pm 13	150–400
MPV (fL)	9.0 \pm 0.9	7.5–11.5
Total cholesterol (mmol L ⁻¹)	4.40 \pm 0.81	3.10–6.50
HDL (mmol L ⁻¹)	1.52 \pm 0.32	>1.0
Triglycerides (mmol L ⁻¹)	0.85 \pm 0.35	0.20–1.90
LDL (mmol L ⁻¹)	2.49 \pm 0.72	<6.00
Glucose (mmol L ⁻¹)	4.87 \pm 0.62	3.0–6.0
Uric acid (μ mol L ⁻¹)	274.7 \pm 75.5	200–430
HS-CRP (mg L ⁻¹)	0.85 \pm 0.76	0.0–6.0

BMI, body mass index; RBC, red blood cell; WBC, white blood cell; MPV, mean platelet volume; HDL, high density lipoprotein; LDL, low density lipoprotein, HS-CRP, high sensitivity C-reactive protein, NA, not applicable.

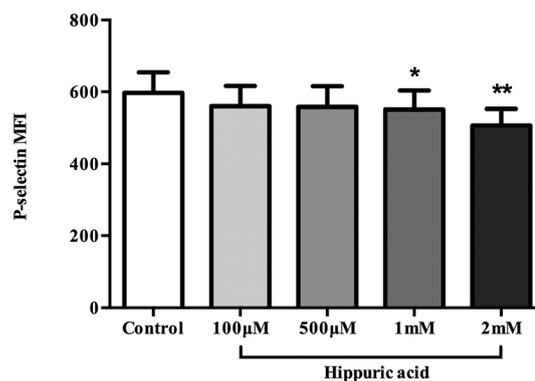


Fig. 1 The effect of varying concentrations of hippuric acid on P-selectin/CD62P surface marker expression. Hippuric acid at 1 mM and 2 mM concentration inhibits P-selectin activation dependant monoclonal antibody expression and consequently platelet degranulation/ α -granule release ($\downarrow 45.9 \pm 50.3$, $p = 0.03$ and $\downarrow 89.6 \pm 74.6$, $p < 0.001$ respectively). $N = 13$ and the data is represented as mean fluorescence intensity (MFI) versus hippuric acid concentrations. *signifies statistical significance $p < 0.05$. **signifies greater statistical significance $p < 0.001$. Error bars expressed as mean \pm SE.

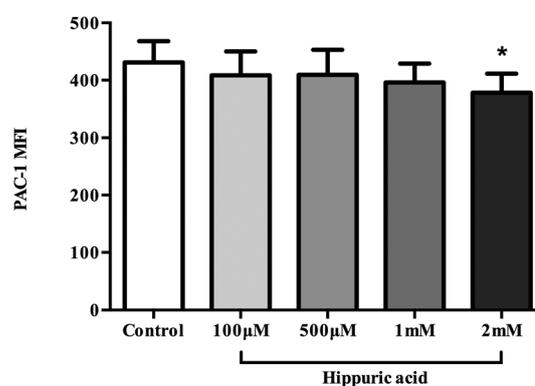


Fig. 2 The effect of different concentrations of hippuric acid on PAC-1 activation-dependant surface marker expression. Hippuric acid at 2 mM concentration reduces activation-dependant conformational changes in platelets ($\downarrow 53.0 \pm 81.2$, $p = 0.03$). $N = 13$ and the data is represented as mean fluorescence intensity (MFI) versus hippuric acid concentrations. *signifies statistical significance $p < 0.05$. Error bars expressed as mean \pm SE.

Discussion

In this trial we have demonstrated the potential of hippuric acid in alleviating platelet activation stimulated by an exogenous agonist, ADP, which is responsible for thrombus formation and platelet adhesion *in vivo*. This potent platelet stimulant was used to activate resting platelets *ex vivo* and target the P₂Y₁/P₂Y₁₂ receptor of platelet activation. Similar to the action of the antiplatelet drug clopidogrel, (i) hippuric acid at 1 mM and 2 mM concentrations reduces P-selectin/CD62P mAb expression indicating its ability to inhibit platelet degranulation consequently platelet activation (ii) 2 mM concentration of hippuric acid reduces PAC-1 mAb expression therefore sup-

pressing the initial phase of activation – GPIIb-IIIa platelet receptor-related conformational change induced by ADP platelet agonist. However we did not observe inhibition of platelet activation by lower concentrations of hippuric acid in relation to the expression of activation-dependant conformational change or degranulation markers.

In platelet related thrombogenesis, platelets adhere to damaged endothelium undergoing a conformational change followed by activation and degranulation.³⁰ This activation results in the binding of fibrinogen to platelet surface receptors consequently leading to thrombus formation.³⁰ Current antiplatelet drugs target different receptors on the surface of platelets or their activation pathways in order to reduce platelet hyper-activation or hyper-aggregability. For example, aspirin, the most commonly used antiplatelet drug and salicylate derived from fruit and vegetables in the diet,³¹ inhibit platelet activity by targeting the cyclooxygenase-1 (COX-1) enzyme hence blocking the production of thromboxane A₂ (TxA₂), a pro-thrombotic agent.³² Furthermore, clopidogrel binds irreversibly to the P₂Y₁/P₂Y₁₂-ADP platelet surface-receptor consequently reducing ADP-induced platelet activation.³³ In the current *ex vivo* trial it was observed that 1 mM and 2 mM concentrations of hippuric acid efficiently target the P₂Y₁/P₂Y₁₂ platelet surface-receptor induced by ADP. In an *in vitro* study performed by Ostertag and colleagues, 100 μM hippuric acid did not inhibit collagen induced P-selectin expression but reduced platelet aggregation.²⁶ Furthermore, Rechner *et al.* demonstrated that lower concentrations of hippuric acid (10 μM) did not exhibit an inhibitory effect on thrombin receptor activating peptide induced P-selectin expression in an *in vitro* trial evaluating the effect of anthocyanins and colonic metabolites of polyphenols on platelet function.²³ The action of hippuric acid on platelet activation inhibition could also potentially be due to the internalization and metabolism by enzymes in platelets capable of modifying molecules by the addition of a methyl, sulphate or a glucuronide group. These functional groups, conjugated to polyphenols or their metabolites within the platelets, might impact the regulation of activation.³⁴ Our results are in agreement with the above *in vitro* trials, where low concentrations of hippuric acid did not effectively block platelet activation dependant receptors. Studies had not previously been extended into the higher concentrations of 1–2 mM. Consumption of a 300 g portion of blueberries, cranberries and prunes resulted in excretion of 11 mM hippuric in 24 hours and other fruits such as raspberry, melon and blackberry resulting in excretions of 5–6 mM hippuric in 24 hours peaking at 10–15 hours.¹⁶ Children and adolescents eating fruit and vegetable averaged 2 mM hippuric acid per day in urine.¹⁵ Since this millimolar excretion of hippuric acid occurs *via* the portal circulation it is believed that high concentrations could also be detected in plasma post polyphenol rich fruit or vegetable consumption. Hippuric acid, following a bolus dose of benzoic acid, has a recorded maximum concentration (C_{max}) in human plasma of 250–300 μM with elimination of hippuric acid occurring at a first-order rate or in a non-saturable manner with the maximum urinary excretion

rates of hippuric acid independent of the dose size.³⁵ Serum hippuric acid concentrations were in the range 1.1–6.1 mM when 5 g (28 mM) hippuric acid was bolus dosed for anti-bacterial effects.³⁶ While the *in vivo* levels of hippuric acid in plasma that can be reached following fruit consumption have not been tested they are not likely to reach the recorded C_{max} of 250–300 μM but could be higher than the 100 μM hippuric acid shown by Ostertag and colleagues to reduce platelet aggregation *in vitro*.²⁶ Although several dietary intervention and *in vivo* studies have shown the mechanism of inhibition of platelet function and anti-clotting activity by polyphenols, the prospective of active metabolites such as hippuric acid being a possible reason for the observed anti-thrombotic effect has not been demonstrated. We have shown the potential of the metabolite in reducing platelet activation related conformational change and inhibition of α-granule release although at a level at least three times higher than what is currently known to occur *in vivo* as a result of fruit and vegetable consumption.

Microbial degradation of polyphenols especially anthocyanins and quinic acid or chlorogenic acid in the colon, followed by hepatic conjugation with glycine, is believed to be responsible for the observed increased levels of hippuric acid.^{20,21} It has also been suggested that majority of the hippuric acid seen in urine is due to synthesis from quinic acid present in antioxidant rich fruits.²¹ Ronald W. Pero recently proposed that quinic acid and its end catabolite, hippuric acid may themselves not be efficacious but may lead to the production of increased levels of nicotinamide and tryptophan as antioxidants through the shikimate pathway.²¹ The present study shows the anti-thrombotic effect of acute *in vitro* addition of varying concentrations of hippuric acid to whole blood directly. It is unclear whether a similar effect of active metabolites can be seen after prolonged consumption of large quantities of polyphenol rich food.

In addition to increased levels of hippuric acid post consumption of a polyphenol rich diet, it is also a metabolite of acetylsalicylic acid (aspirin). The other major metabolites of aspirin include salicylic acid and gentisuric acid which have very similar structural moieties as hippuric acid.³⁷ Acetylsalicylic acid is converted to salicylic acid and conjugates with benzoic acid in the liver to result in the synthesis of hippuric acid. This pharmacokinetics of acetylsalicylic acid might partly explain the reason behind the anti-thrombotic activity of its potential metabolite, hippuric acid. Aspirin alleviates platelet activation by blocking the COX-1 pathway resulting in reduced arachidonic acid production hence preventing thromboxane synthesis consequently thrombus formation. Further mechanistic studies evaluating the potential of hippuric acid in reducing arachidonic acid induced platelet activation by blocking the COX-1 pathway of platelet activation is warranted.

It has also been demonstrated that thiol derivatives of anti-platelet drugs such as clopidogrel and ticlopidine bind in a covalent manner to the P₂Y₁/P₂Y₁₂ ADP receptor and block the activation of platelets.³⁸ Hippuric acid at concentrations attained *in vivo* post polyphenols rich food consumption could

potentially help block the ADP receptor of activation thereby mimicking the action of antiplatelet drugs. We hence believe that consumption of polyphenols rich foods could be synergistic with lowered doses of antiplatelet drugs and help platelet activation inhibition and thus reduce risk of thrombosis in current antiplatelet drug sensitive populations.

Conflict of interest and funding disclosure

The authors of this manuscript have no conflicts of interest to declare. This work was not financially supported by any external funding or grants.

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