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Guanosine exerts antiplatelet and antithrombotic properties through an adenosine-related cAMP-PKA signaling

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ABSTRACT

Background: Guanosine is a natural product and an endogenous nucleoside that has shown to increase during myocardial ischemia. Platelets are critically involved in ischemic coronary events. It remains unknown, however, whether guanosine may affect platelet activation and function. We sought to investigate the potential antiplatelet and antithrombotic properties of guanosine and decipher the mechanisms behind.

Methods: We firstly assessed the effects of guanosine on platelet activation/aggregation upon stimulation with several platelet agonists including adenosine diphosphate (ADP), collagen, arachidonic acid (AA), and TRAP-6. Guanosine antithrombotic potential was also evaluated both in vitro (Badimon perfusion chamber) and in vivo (murine model). In addition we assessed any potential effect on bleeding. At a mechanistic level we determined the release of thromboxane B2, intraplatelet cAMP levels, the binding affinity on platelet membrane, and the activation/phosphorylation of protein kinase A (PKA), phospholipase C (PLC) and PKC.

Results: Guanosine markedly inhibited platelet activation/aggregation-challenged by ADP and, although to a lesser extent, also reduced platelet aggregation challenged by collagen, AA and TRAP-6. Guanosine significantly reduced thrombus formation both in vitro and in vivo without significantly affects bleeding. Guanosine antiplatelet effects were associated with the activation of the cAMP/PKA signaling pathway, and a reduction in thromboxane B2 levels and PLC and PKC phosphorylation. The platelet aggregation and binding affinity assays revealed that guanosine effects on platelets were mediated by adenosine.

Conclusion: Guanosine effectively reduces ADP-induced platelet aggregation and limits thrombotic risk. These antithrombotic properties are associated with the activation of the cAMP/PKA signaling pathway.

1. Introduction

Cardiovascular disease (CVD) is the leading clinical and public health problem worldwide [1]. The incidence of thrombosis is the most common underlying pathology of the three major CVD disorders including ischemic heart disease (acute coronary syndrome), stroke and venous thromboembolism (VTE) [2]. Platelet activation plays a key role in the progression of thrombosis [3].

As such, exposure of thrombogenic surfaces upon atherosclerotic plaque rupture favors platelet interaction with the vessel wall and the consequent activation of the coagulation cascade leading to platelet activation, aggregation and the formation of a superimposed thrombus [4].

Data from epidemiological studies consistently indicate that the consumption of healthy diets rich in functional foods (e.g. Mediterranean diet), exerts cardioprotective effects in both primary and secondary prevention of CVD [5,6]. Particularly, several active food components have demonstrated to exert antiplatelet activities leading to a lesser pro-atherothrombotic profile [7].

Guanosine is a natural product and an endogenous nucleoside. Guanosine, like its adenine-based counterpart adenosine, is a primitive

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molecule which produces a wide spectrum of biological activities [8]. As such, guanosine acts as an intercellular signaling molecule with anti-inflammatory and neuroprotective effects, protects several cell types against staurosporine- and beta-amyloid-induced apoptosis, and has been reported to increase intracellular cAMP levels in astrocytes [9–14]. Under basal conditions, guanosine concentration is around 0.47 μmol/L, however, during specific pathological conditions such as hypoxia and/or hypoglycemia, guanosine concentration is largely increased [15]. In this context, guanosine interstitial levels has been shown to increase up to ~10 μmol/L during myocardial ischemia [16] and after 2 h of focal stroke, and remain high for 7 days [16–18]. Yet, whether guanosine exerts any effect on platelet activity or function remains to be determined. In the present study we aimed to determine whether guanosine modulates platelet function and thrombotic risk (both in vitro and in vivo) and examined the potential mechanisms behind.

2. Materials and methods

2.1. Reagents

Adenosine 5′-diphosphate (ADP), thrombin receptor activator peptide 6 (TRAP-6), collagen, arachidonic acid (AA), acetylsalicylic acid, guanosine, ZM241385, MSX-2, rose bengal, prostaglandin E_1 (PGE1), papaverine hydrochloride and triton X-100 were obtained from Sigma-Aldrich (St. Louis, Missouri/MO, U.S.A.). Antibodies (anti-CD62P-PE, anti-CD61-FITC, anti-glycoprotein (GP) IIb/IIIa-FITC PAC-1 and anti-CD61-PE) were obtained from BD Pharmingen (BD Biosciences, San Diego, CA, USA). [^{3}H] guanosine was obtained in Hartmann Analytic GmbH, and anti-phospho-protein kinase A (PKA)α/β/γ cat (Thr 198), anti-phospho (Tyr753)-phospholipase (PLC)-γ2, anti-phospho (S660)-PKC-β2 and an anti−γ-tubulin antibodies were from Santa Cruz (Biotechnology, CA, USA). Anti-PKA was obtained from Sigma-Aldrich (St. Louis, Missouri/MO, U.S.A.). The final concentration of dimethyl sulfoxide (DMSO) used as diluent was 0.2% (v/v) which does not affect platelet function and all assays incorporated appropriate vehicle controls [19].

2.2. Platelets isolation

Platelets were obtained as previously described [20,21]. Briefly, blood was drawn by phlebotomy (needle 21G × 1½″) in 3.2% citrate (9:1 v/v) from healthy, aspirin-free, human volunteers with written informed consent. Then, blood samples were centrifuged (DCS-16 Centrifugal Presvac RV) at 240*g* for 10 min to obtain platelet-rich plasma (PRP). Two-thirds of PRP was removed and centrifuged at 650*g* for 10 min. The platelet pellet was washed with HEPES-Tyrode's buffer containing PGE1 (120 nmol/L) and finally resuspended in HEPES-Tyrode's buffer at a final concentration of 200×10^9 platelets/L (Bayer Advia 60 Hematology System, Tarrytown, NY, USA) [20].

2.3. Flow cytometric analyses

Flow cytometric studies were conducted to assess P-selectin expression and GPIIb/IIIa activation as previously reported [20]. The PRP was pre-incubated with guanosine (10 to 500 μmol/L) for 5 min at 37 °C and stimulated with ADP 4 μmol/L for 6 min. The PRP sample was mixed with saturated concentrations of anti-CD62P-PE and anti-CD61-FITC and incubated for 30 min in the dark to determine platelet P-selectin expression. For the analysis of platelet GPIIb/IIIa activation, the PRP sample was incubated with saturated concentrations of anti-GPIIb/IIIa antibody PAC-1 and anti-CD61-PE for 30 min in the dark. After quench-dilution (≈6-fold), the samples were analyzed in an Accuri C6 flow cytometer (BD, Biosciences, USA), and platelet populations were gated on cell size using forward scatter (FSC) vs. side scatter (SSC). CD61 positivity was assessed to discard the electronic noise. The mean fluorescence intensity (over 5.000 events per sample) was calculated by using BD Accuri C6 Software (BD Biosciences, USA).

2.4. Platelet aggregation

Platelet aggregation was monitored by light transmission aggregometry using a lumi-aggregometer (Chrono-Log, Haverton, PA, USA) [20]. Washed platelets (200×10^9 platelets/L) were pre-incubated with guanosine (10 to 500 μmol/L) for 5 min at 37 °C while stirring (150*g*). Then, platelet aggregation was triggered by different agonists including ADP (4 μmol/L), collagen (1 μg/mL), AA (1 mmol/L) or TRAP-6 (30 μmol/L) in the presence of fibrinogen (275 μg/mL). Platelet aggregation was recorded for 6 min. We also evaluated the platelet aggregation response to ADP upon previous pre-incubation with ZM241385 (1 or 10 μmol/L) or MSX-2 1 μmol/L. ZM241385 and MSX-2 are adenosine A2A receptor antagonists.

2.5. Measurement of thromboxane B2 release and intraplatelet cAMP levels

The supernatants of aggregated platelets were incubated with papaverine hydrochloride 0.5 mmol/L (phosphodiesterase inhibitor) and centrifuged (6.000*g*, 10 min, 4 °C) for further analysis of thromboxane B2 release with the according ELISA kit. In addition, the platelet suspension was lysed (lysis buffer: 50 mmol/L Tris-HCl, 50 mmol/L NaCl, 1 mmol/L MgCl2, 1 mmol/L EDTA, 0.1% Triton® X-100 at pH 7.4) for intraplatelet cAMP determination using the corresponding ELISA kits. cAMP levels were normalized to total protein amount measured with the Bradford assay [22].

2.6. Immunoblotting study

Stimulated platelets were lysed and 20 μg of proteins were resolved in SDS-PAGE and transferred to nitrocellulose filters. The membranes were incubated with anti-phospho-PKAα/β/γ cat (Thr198), anti-phospho (Tyr753)-PLC-γ2, anti-phospho (S660)-PKC-β2, anti−γ-tubulin or anti-PKA antibodies overnight at 4 °C. Specific reactive proteins were detected by an enhanced chemiluminescence method employing an antibody linked to horseradish peroxidase. Analysis of individual protein was quantified by densitometry using the ImageJ software [20].

2.7. Guanosine binding to platelet membranes

The platelet membrane fraction was isolated from PRP obtained from healthy human volunteers, as previously described [23]. A total of 1 mg of protein/mL was used for binding site analysis using [^{3}H] guanosine. The values of binding were determined based on [^{3}H] guanosine alone or in combination with ZM241385 (1 or 10 μmol/L).

2.8. In vitro model of thrombosis: the Badimon perfusion chamber

A perfusion system (the Badimon chamber) was used to investigate the potential inhibitory effect of guanosine on platelet deposition and thrombus formation under controlled blood flow conditions as previously described [24]. To this end, porcine aortic tunica media specimens (thrombogenic substrate), obtained fresh from local slaughterhouse, were mounted in the Badimon perfusion chamber. After overnight fasting, 50 mL of blood was drawn from healthy pigs and platelets were labeled with indium oxine-111 (^{111}In) (Amersham Biosciences). Blood was pre-incubated for 30 min at 37 °C with guanosine (0.5 and 1 mmol/L) and then perfused through the chamber for 5 min at 2 different shear rates of 212/s and 1690/s, mimicking venous and moderately stenotic arterial flow conditions, respectively. Then, saline 0.9% was perfused for 1 min under the same rheological conditions. The porcine specimens were immediately fixed in 4% paraformaldehyde and counted in a gamma counter for quantitation of deposited platelets. The number of platelets deposited on each specimen was calculated from the indium activity on the perfusion area and normalized by blood ^{111}In activity, platelet counts in blood, and area of exposed surface.

2.9. Study of thrombosis formation in an in vivo mice model

Arterial thrombosis formation induced by endothelial cell damage was performed as described [25]. Briefly, C57BL/6 mice (14–18 weeks old) were anesthetized (tribromoethanol 270 mg/kg and xylazine 13 mg/kg) and the mesenteric artery was exposed by performing a central incision in the abdomen. Endothelial damage was induced by injection of rose bengal 50 mg/kg through the tail vein followed by illumination of the exposed mesenteric area with a 1.5-mW green light laser (532 nm). Thrombosis formation was monitored for 30 min and stable occlusion was defined as a blood flow of 0 mL/min for 3 min. Vehicle (control group, $n = 6$) or guanosine (50, 100 and 200 mg/kg, $n = 6$) were administered intraperitoneally 30 min before green light exposure. Thrombosis formation was recorded with an intravital microscopy (AXIO Examiner Z.1, Zeiss, Oberkochen, Germany) and then analyzed with ImageJ software (version 1.26 t, NIH, USA). [26]. In control experiments, neither injection of rose bengal without green laser or without rose bengal with green laser resulted in any alterations in blood flow (data not shown).

The protocol was authorized by the ethics committee of the Universidad de Talca in accordance with the Declaration of Helsinki (approved by the 18th World Medical Assembly in Helsinki, Finland, 1964).

2.10. Bleeding time assay

Guanosine (50, 100 and 200 mg/kg) was administered intraperitoneally to anesthetized C57BL/6 mice ($n = 6$ animals per dose) as reported above. After 30 min an incision was made on the ventral surface of the mice tail (about 2 mm from the tip) and immersed for 10 min in 1 mL saline warmed up to 37 °C. Bleeding time was recorded until blood cessation and reported in seconds [27]. The administration of acetylsalicylic acid 200 mg/kg ($n = 6$) was used for control purposes.

2.11. Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed using Prism 6.0 software (GraphPad Inc., San Diego CA, USA). The half maximal inhibitory concentration (IC_{50}) of guanosine on agonist-induced platelet activation was calculated from the dose-response curves. Differences between groups were analyzed using non-parametric Friedman test. p values <0.05 were considered significant.

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