Abstract. **Objective:** To evaluate the anti-thrombotic action of glucosamine, a naturally occurring amino monosaccharide, platelets were stimulated with ADP in the presence of glucosamine, and its effects on platelet functions were examined.

**Materials and Methods:** Human platelet-rich plasma was stimulated with 2.5 μM ADP in the presence of glucosamine (0.01 ~ 1 mM) or other aminosugars (N-acetyl-glucosamine, galactosamine or N-acetyl-galactosamine, 1 mM), and platelet aggregation was monitored. Furthermore, the effects of glucosamine on thromboxane A2 production, release of granule contents, intracellular calcium mobilization and phosphorylation of Syk (a 72 kD protein tyrosine kinase) were evaluated following ADP-stimulation. In addition, the binding of [3H] ADP to its receptors was examined.

**Results:** Glucosamine (>0.01 mM) dose-dependently suppressed platelet aggregation in response to ADP (p < 0.05), whereas N-acetyl-glucosamine, galactosamine or N-acetyl-galactosamine (1 mM) did not affect the ADP-induced platelet aggregation. Furthermore, glucosamine (>0.1 mM) inhibited the extracellular release of granule contents (ATP and platelet factor 4) and production of thromboxane A2 from ADP-stimulated platelets (p < 0.05). Moreover, glucosamine significantly repressed the intracellular calcium mobilization at >0.1 mM and phosphorylation of Syk at >0.01 mM upon ADP-stimulation (p < 0.05). In addition, glucosamine (>0.1 mM) inhibited the binding of ADP to its receptors (p < 0.05).

**Conclusion:** Glucosamine is able to suppress platelet aggregation, release of granule constituents, thromboxane A2 production, calcium mobilization and phosphorylation of Syk possibly via the inhibition of ADP-binding to the receptors. Glucosamine could be expected as a novel anti-platelet agent for thrombotic disorders due to its suppressive actions on platelets.

**Key words:** Glucosamine – Platelet – ADP – Aggregation – Thrombosis

Correspondence to: I. Nagaoka

Glucosamine, a naturally occurring amino monosaccharide, suppresses the ADP-mediated platelet activation in humans

J. Hua1, S. Suguro2, K. Iwabuchi1, Y. Tsutsumi-Ishii1, K. Sakamoto2 and I. Nagaoka1

1 Department of Host Defense and Biochemical Research, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan, Fax: +81 3 3813 3157, e-mail: nagaokai@med.juntendo.ac.jp
2 Koyo Chemical Co., Ltd., Iidabashi, Chiyoda-ku, Tokyo 102-0072, Japan

Received 18 June 2004; returned for revision 11 July 2004; accepted by M. Katori 9 August 2004

Introduction

Glucosamine, a naturally occurring amino monosaccharide, is present in the connective and cartilage tissues, and contributes to maintaining the strength, flexibility and elasticity of these tissues. Thus, glucosamine has been widely used to treat osteoarthritis in humans [1]. Several short- and long-term clinical trials in osteoarthritis have shown the significant symptom-modifying effect of glucosamine [2, 3]. According to the recent biochemical and pharmacological studies, administration of glucosamine normalizes cartilage metabolism, so as to stimulate the synthesis and inhibit the degradation of proteoglycans, and to restore the articular function [4, 5].

In addition to its chondroprotective action, glucosamine is expected to exert anti-inflammatory actions by inhibiting neutrophil functions such as superoxide generation, phagocytosis, granule enzyme release and chemotaxis [6]. Moreover, glucosamine is demonstrated to prolong the allogeneic cardiac allograft survival by suppressing the activation of T-lymphoblasts and dendritic cells [7]. More recently, glucosamine has been reported to suppress the growth of parasite (Plasmodium falciparum) by interfering with glycosylphosphatidylinositol biosynthesis [8].

Platelets have a critical role in normal hemostasis, whereas they also contribute to thrombotic disorders such as myocardial infarction and peripheral vascular diseases [9]. In recent years, a number of anti-platelet drugs such as aspirin, ADP receptor inhibitors (clopidogrel and ticlopidine) and glycoprotein IIb/IIIa receptor antagonists (e.g., abciximab) have been clinically used to treat or prevent thrombotic disorders [9–11]. However, most of these agents have a complication of bleeding upon treatment of thrombotic disorders [9–12]. In addition, aspirin causes gastrointestinal damage [13], and ticlopidine and abciximab exhibit adverse effects such as neutropenia and thrombocytopenia, respectively [10, 11].

In preliminary experiments, we found that platelet aggregation was suppressed after oral administration of glucosamine (1.5 g/day) to humans (see the Results). Based on
these observations, we have hypothesized that glucosamine may possess an anti-thrombotic activity to suppress platelet functions. Furthermore, long-term clinical trials with oral administration of glucosamine for 3 years to humans have indicated no apparent side effects for treatment of osteoarthritis [3]. Thus, glucosamine could be expected as a novel and safe anti-platelet agent for thrombotic disorders.

In this study, we evaluated the effects of glucosamine on the platelet functions in vitro using human peripheral blood platelets. The results indicated that glucosamine is able to suppress platelet aggregation, release of granule contents, thromboxane (TX) A2 production, calcium mobilization and phosphorylation of Syk possibly via the inhibition of ADP-binding to the receptors.

Materials and methods

Materials

ADP was purchased from Oriental Yeast Co. (Tokyo, Japan). Apyrase (grade I), bovine serum albumin (BSA), indomethacin, ethylendiaminetetraacetate (EDTA), ethylenglycoltetraacetic acid (EGTA), piceatannol (a specific inhibitor for protein tyrosine kinase Syk), D-galactosamine and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). A thromboxane B2 enzyme immunoassay (EIA) kit was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Glucosamine hydrochloride was obtained from Koyo Chemical Co. (Tokyo, Japan). N-Acetyl-D-glucosamine, N-acetyl-D-galactosamine and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were purchased from Wako Pure Chemicals (Osaka, Japan). Fura-2 acetoxy-methyl ester (fura-2-AM) was obtained from Dojindo Laboratories (Kumamoto, Japan).

Platelet aggregation

This study was approved by the Institutional Human Subject's Review Board (Juntendo University, School of Medicine). Informed consent was obtained from healthy and drug-free volunteers, and blood was obtained from cubital vein. To prepare platelet-rich plasma (PRP), blood was anticoagulated with 0.38% sodium citrate and centrifuged at 120 g for 10 min at room temperature. PRP contained approximately 10^9 platelets/ml. To further isolate wash-platelets, PRP was centrifuged at 1500 g for 10 min at 4°C. The pelleted platelets were washed twice in Tyrode's solution at 37°C for 15 min at 1000 rpm.

In some experiments, aliquots (0.2 ml) of washed-platelets (1:25 dilution in PBS) were preincubated with 1 mM glucosamine for 5 min in Tyrode's solution. Platelets were then washed in Tyrode's solution and stimulated with 10 μM ADP in the presence of 500 μg/ml fibrinogen (Sigma) at 37°C for 15 min at 1000 rpm.

Measurement of ATP

Release of ATP was determined by using an ATP bioluminescent assay, as described elsewhere [14]. In brief, 10 μl aliquots of supernatants from ADP-activated platelets (1:50 dilution in PBS) or standard ATP were added to 0.1 ml of reaction buffer (50 mM Tris-acetate, 2 mM EDTA, 60 mM diithiothreitol, 0.072% BSA, 10 mM Mg-acetate, pH 7.7) containing 1 μg/ml luciferase (Roche Molecular Biochemicals, Mannheim, Germany) and 0.1 mM D-luciferin (Wako). Luminescence assays were performed with a Lumat LB9501 luminometer (Berthold Japan, Tokyo, Japan), and light output was given as the integral relative light units over the 10 s measuring period. ATP concentration was expressed as a percentage of total ATP released from platelets by sonication (Tomy Ultrasonic Disrupter UD-201, Tominaga Works, Tokyo, Japan).

Measurement of PF4 and TXB2

PF4 was quantified by using a double antibody sandwich enzyme linked immunosorbent assay (ELISA), as described elsewhere [15]. In brief, 25 μl of anti-PF4 antibody (1 μg/ml in 0.1 M NaHCO3; PeproTech EC, London, UK) was added to ELISA plate, and incubated at 4°C overnight. After blocking with BlockAce (Dainippon Pharmaceutical, Osaka, Japan) for 1 h, 25 μl aliquots of supernatants from ADP-activated platelets (1:25 dilution in PBS) or PF4 fragment 58–70 (Sigma) as a standard were added, and incubated for 2 h at room temperature. After washing, the plates were incubated with biotinylated anti-PF4 antibody (1:100 dilution) for 1 h, and further incubated with streptavidin-horseradish peroxidase (HRP) (1:2000 dilution; Eymed Laboratories, Inc., San Francisco, CA) for 30 min. Then, 3,3',5,5'-tetramethyl-benzidine substrate (Sigma) was added, and after 15 ~ 30 min the enzyme reaction was stopped by putting in 25 μl/well of 1 M H2SO4. The optical density was photometrically determined at 450 nm, and the concentration of PF4 was expressed as a percentage of total PF4 released from platelets by sonication. Anti-PF4 antibody was biotinylated using N-hydroxysuccinimide-biotin, according to the manufacturer's protocol (Pierce, Rockford, IL).

TXB2, a stable metabolite of TXA2, was measured with a TXB2 enzyme immunoassay kit by using supernatants from ADP-activated platelets (1:25 dilution in PBS), according to the manufacturer's instructions.

Measurement of intracellular Ca2+ ([Ca2+]i) mobilization in platelets

Measurements of [Ca2+]i, in fura-2-loaded platelets were performed essentially as described previously [16]. PRP was incubated at 37°C in the presence of 3 μM fura-2-AM for 30 min in the presence of 1 mM EGTA and 40 μg/ml apyrase, which inhibit platelet activation during incubation by chelating Ca2+ and degrading ADP released from the addition of 0.8 ml phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) containing 10 mM EDTA and 10 μM indomethacin. The PRP aliquots were then centrifuged at 3000 g for 5 min at 4°C, and the supernatants were used for measuring ATP, platelet factor 4 (PF4) and thromboxane B2 (TXB2).

In the preliminary experiments, we confirmed that pH values of plasma and Tyrode's solution were not changed by the addition of glucosamine (0.01 ~ 10 mM) or other aminosugars (N-acetyl-glucosamine, galactosamine or N-acetyl-galactosamine, 1 mM).

In some experiments, to determine whether the effect of glucosamine on platelet aggregation is reversible or not, platelets (10^9/ml) were preincubated with 1 mM glucosamine for 5 min in Tyrode's solution. Platelets were then washed in Tyrode's solution and stimulated with 10 μM ADP in the presence of 500 μg/ml fibrinogen (Sigma) at 37°C for 15 min at 1000 rpm.