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Surfactin C inhibits platelet aggregation

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Abstract

This study was designed to investigate the effect of surfactin C, which is derived from *Bacillus subtilis*, on platelet aggregation and homotypic leucocyte aggregation. Surfactin C strongly and dose-dependently inhibited platelet aggregation, which was stimulated both by thrombin (0.1 U mL^{-1}), a potent agonist that activates the G protein-coupled protease receptor, and by collagen (5 μg mL^{-1}), a potent ligand that activates $\alpha_{IIb}\beta_3$ with IC50 values (concentration inhibiting platelet aggregation by 50%) of 10.9 and 17.0 μM, respectively. Moreover, surfactin C significantly suppressed the intracellular Ca^{2+} mobilization in thrombin-activated platelets. Surfactin C, however, did not affect various integrin-mediated U937 cell aggregation, implying that the anti-platelet activity of surfactin C was not due to its detergent effect but by its action on the downstream signalling pathway. Therefore, the results suggest that surfactin C may have a beneficial therapeutic effect on aberrant platelet aggregation-mediated cardiovascular diseases.

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Introduction

Platelets play an important role in haemostasis and thrombosis through aggregation (Shattil et al 1998; Stouffer & Smyth 2003). Aberrant intravascular thrombosis is the main cause of a wide variety of cardiovascular diseases (Grenache et al 2003; Huo & Ley 2004). Upon the activation of a platelet receptor on a plasma membrane, the bioactive substances (e.g. calcium, growth factor and aggregation-related materials) in granules are released in an energy-dependent process that requires ionized calcium (Savage et al 2001; Leclerc 2002). This process is referred to as inside-out signalling (Shattil et al 1998). On the other hand, intracellular signals or ligands, such as thrombin and collagen, lead to a conformational change of the integrins with a switch from a low- to a high-affinity state. The ligand-occupied integrin signals downstream to stabilize platelet aggregation through a reorganization of the actin cytoskeletal network, which is called outside-in signalling (Shattil et al 1998). Both inside-out and outside-in signalling of the platelets is the main factor of platelet aggregation, and can be a cause of serious cardiovascular diseases, including atherosclerosis, stroke and diabetes (Huo & Ley 2004). Of the many molecules, the adhesion molecules (i.e. $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$) of platelets are particularly critical in both inside-out and outside-in signalling, thus leading to a stable thrombus formation. Indeed, these adhesion molecules are abundantly expressed at the platelet surface and have been shown to be defective in platelet–platelet aggregation and thrombus formation if they are knocked-out (Vinogradova et al 2002).

Surfactin isomers are heptapeptides arranged in a lactone ring and are bound to a beta-hydroxy fatty acid, which are produced by various strains of *Bacillus subtilis* (Arima et al 1968). These biosurfactants have been reported to display various biological actions, including anti-fungal and anti-bacterial activity (Bernheimer & Avigad 1970), as well as anti-viral and anti-tumour activity (Kameda et al 1974). In addition, several reports on whether they inhibited fibrin clot formation (Arima et al 1968), platelet cytosolic phospholipase A_2 (PLA_2) activity (Kim et al 1998) and inflammatory mediators, such as nitric oxide (NO) and cytokines (Hwang et al 2005), have indicated that surfactins may be regarded as valuable drugs against platelet-mediated cardiovascular diseases.

Therefore, to confirm this possibility, the anti-platelet properties of surfactin C, a major active surfactin isoform isolated from *Bacillus subtilis*, were investigated using a platelet aggregation assay.

Materials and Methods

Materials

Thrombin was obtained from the Sigma Co. (St Louis, MO, USA). Collagen was procured from the Chronolog Co. (Havertown, PA, USA). A fura 2-acetoxymethylester (Fura 2-AM) was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were of reagent grade. The following antibodies were used in this study for aggregation: CD29 (MEM 101A, IgG1, ascites, were kindly provided by V. Horejsi); CD43 (161-46, ascites, IgG1, R. Villela); CD98 (ANH-18, purified IgG1, K. Skubiz); and CD147 (MEM M6/1, IgG1, ascites, V. Horejsi) (Cho et al 2004).

Purification and chemical characterization of surfactin C from bacterial strains

The isolation of *Bacillus subtilis* BC1212 has been previously described (Hwang et al 2005). Briefly, *Bacillus subtilis* ATCC 21332 was used as a control strain to compare surfactin isoform compositions. Both strains were cultivated in a shaking incubator (150 rev min^{-1}; 37°C) in a liquid medium. The culture broth was collected by centrifugation at 5700 rev min^{-1} for 40 min, and then bacterial cells were re-suspended in distilled water. The suspension was centrifuged again at 5700 rev min^{-1}, and then the supernatant was combined with the culture broth. The combined mixture was then extracted with ethyl acetate–methanol (4:1, v/v). The organic phase was evaporated and the crude lipopeptide mixture was purified with silica gel 60 (70-230 mesh; Merck, Germany) using n-hexane–acetone (6:4, v/v) as an eluent. Purified surfactins were separated into isoforms and identified by liquid chromatograph/mass spectrometry (LC/MS). Briefly, the MS analysis of surfactin isoforms revealed that it was a mixture of *quasi*-molecular ions at $m/z = 1008$, 1022, 1036, 1050 ($[M+H]^+$), and $m/z = 1030$, 1044, 1058, 1072 ($[M+Na]^+$). By reversed-phase HPLC, the surfactins were found to be a mixture of at least 5 compounds on the basis of their molecular weight (surfactin A, $8.9 \pm 2.7\%$; surfactin B_1, $23.9 \pm 0.1\%$; surfactin B_2, $10.6 \pm 2.6\%$; surfactin C, 56.6 ± 5.3; and surfactin D, $0.1 \pm 0.1\%$; based on the relative amount).

Platelet preparation

The preparation of the platelets was by a method described previously (Rhee et al 1993; Park et al 1995) with minor modifications. Rat blood (8 mL) was obtained via venipuncture using a 23 g needle inserted into the abdominal aorta and transferred to a 15-mL test tube containing 1 mL of a citrate phosphate dextrose solution (CPD; 90 mM $Na_3C_6H_5O_7 \cdot 2H_2O$, 14 mM $C_6H_8O_7 \cdot H_2O$, 128.7 mM $NaH_2PO_4 \cdot H_2O$, 2.55 g/100 mL dextrose). Blood was centrifuged at 1000 rev min^{-1} for 7 min to achieve platelet-rich plasma (PRP). To remove residual erythrocytes, the PRP samples were again centrifuged at 500 rev min^{-1} for 7 min. To isolate the platelets and to remove the CPD solution, the PRP was centrifuged twice at 2500 rev min^{-1} for 10 min and the supernatant was allocated to platelet-poor plasma (PPP), which was used as a reference solution for aggregation assay. The platelets in the precipitate were adjusted to the proper number (10^8 mL^{-1} for aggregation assay with a Tyrode buffer (composition in mM: 137 NaCl, 12 $NaHCO_3$, 5.5 glucose, 2 KCl, 1 $MgCl_2$ and 0.3 $NaHPO_4$, pH 7.4). All steps for platelet preparation were conducted at room temperature, and all experimental procedures and protocols used in this investigation were reviewed and approved by the Ethics Committee of our University.

Platelet aggregation assay

Platelet aggregation was performed as previously described (Rhee et al 1993; Park et al 1995). Aggregation was monitored by measuring light transmission via an aggregometer (Chronolog Co., Havertown, PA, USA). The washed platelets were pre-incubated at 37°C for 5 min with either surfactin C or vehicles. The reaction mixture was further incubated for 8 min, with stirring, at 1200 rev min^{-1}. The concentration of the vehicle was kept at less than 0.5% so as to exclude the artificial effect.

Measurement of intracellular Ca^{2+}

PRP was incubated with 5 μM of Fura 2-AM at 37°C for 60 min. Since Fura 2-AM is light-sensitive, the tube containing PRP was covered with aluminium foil during loading. The Fura 2-loaded washed platelets were also prepared using the procedure described above. The Fura 2-loaded washed platelets (10^8 mL^{-1}) were pre-incubated for 3 min at 37°C with various concentrations of surfactin C in the presence of 2 mM $CaCl_2$. The mixture was stimulated with 0.1 U mL^{-1} thrombin for 8 min for the evaluation of intracellular calcium concentration ($[Ca^{2+}]_i$). Fura 2 fluorescence was measured with a spectrofluorometer (SFM 25; Bio-Tek Instruments, Italy) with an excitation wavelength altering between 340 nm and 380 nm every 0.5 s; the emission wavelength was at 510 nm. The $[Ca^{2+}]_i$ values were calculated using the method of Schaeffer (Schaeffer & Blaustein 1989).

Statistical analysis

Data were analysed by a one-way analysis of variance followed by post-hoc Dunnett's test to determine the statistical significance of the differences. All data are presented as means ± s.e.m. $P \leq 0.05$ was considered to be statistically significant.

Results and Discussion

We have previously reported that surfactin isoforms (A, B_1, B_2, C and D) were produced in greater abundance in *Bacillus subtilis* BC1212 than in the standard strain of *Bacillus subtilis*

(Hwang et al 2005). Among the surfactin isomers, surfactin C enhances fibrin degradation in-vivo due to the pharmacological relaxation of the plasminogen conformation (Kikuchi & Hasumi 2002) and surfactin C suppresses pro-inflammatory activity, such as the inhibition of lipopolysaccharide-induced nitric oxide (NO) production and the gene expression of IL-1β and iNOS (Hwang et al 2005). Therefore, we examined the potential role of surfactin C as a potential therapeutic agent by using a platelet aggregation assay.

Thrombin, the most potent platelet agonist ex-vivo, elicits its effect via G protein-coupled protease-activated receptors, thus leading to the activation of inside-out signalling (Savage et al 2001). Thrombin initiates outside-in signalling through $\alpha_{IIb}\beta_3$ amplification, which is necessary for full platelet aggregation, granule secretion and for the formation of stable thrombus (Stouffer & Smyth 2003). Under thrombin treatment, we also obtained an appropriate amount of platelet aggregation up to $62.1 \pm 1.3\%$, which was effectively inhibited by ginsenoside Rg_3 (IC50 (concentration inhibiting platelet aggregation by 50%) of $25.2 \pm 4.3\ \mu$M). This suggests that the assay was in correlation with previous reports (Lee et al 1997). In this assay, surfactin C strongly inhibited thrombin-induced platelet aggregation with an IC50 value of $10.9 \pm 2.5\ \mu$M (Figure 1A). Furthermore, surfactin C clearly blocked the thrombin-induced intracellular Ca^{2+} mobilization (Figure 1B), suggesting that it may also affect early signalling events in thrombin-induced platelet aggregation. To confirm the anti-platelet activity of surfactin C, collagen, another strong agonist, was employed. Two collagen receptors were identified on the platelet surface: integrin $\alpha_2\beta_1$ (also known as GP Ia/IIa) (Jung & Moroi 2000) and glycoprotein VI (Savage et al 2001; Leclerc 2002). In particular, integrin $\alpha_2\beta_1$, which is abundantly expressed on the platelet surface, is required for platelet interaction with a subendothelial extracellular matrix (e.g. collagen) and for platelet–platelet interaction leading to aggregate and thrombus formation (Jung & Moroi 2000; Grenache et al 2003). As with thrombin, collagen-induced outside-in signalling plays an important role in the overall process of platelet aggregation. It is significant that surfactin C potently inhibited collagen-induced platelet aggregation with an IC50 value of $17.0 \pm 2.8\ \mu$M (Figure 2). Ginsenoside Rg_3, as a positive control, also displayed an inhibitory effect with an IC50 value of $85.8 \pm 6.3\ \mu$M. Therefore, our results suggest that surfactin C may be a potent anti-platelet aggregation agent.

In cardiovascular diseases, clustering, which is composed of platelets and leucocytes, is also considered as a pathological mechanism (Huo & Ley 2004). Therefore, we also tested whether surfactin C is able to modulate leucocyte aggregation by using U937 homotypic aggregation. This model was established using specific antibodies to adhesion molecules (including β_1-integrins (CD29) and other β_1- or β_2-integrin-activating molecules (CD43, CD98 and CD147)) and phorbol 12-myristate 13-acetate (PMA, a potent activator of CD18). The numbers of the non-aggregated and total cells were counted in a haemocytometer and the percentage of the cells in aggregates was determined (Cho et al 2004). Thus far, it is known that aggregation events are mainly mediated by outside-in signalling via direct activation of adhesion molecules (Cho et al 2004; others related to integrin signalling). Surfactin C, however, did not block U937 homotypic aggregation, which was induced by specific antibodies (e.g. CD29, CD43, CD98, CD147) and PMA (data not shown). This suggests that surfactin C may not be able to block β_1- or β_2-integrin-mediated leucocyte aggregation.

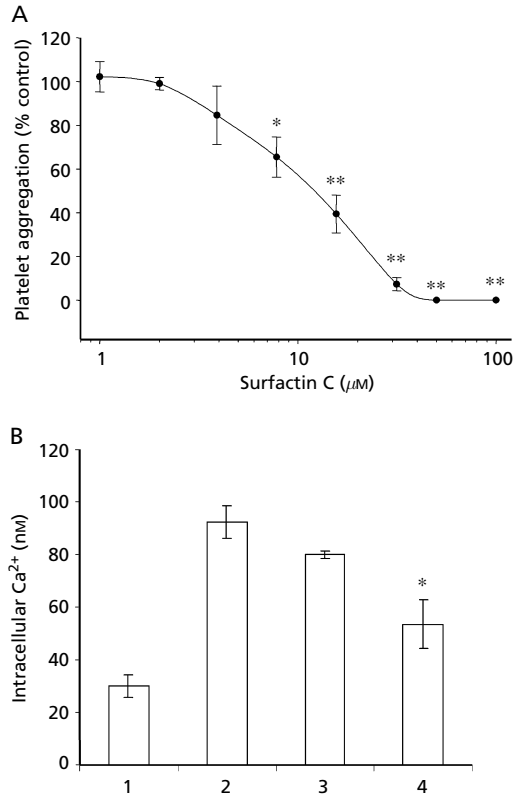

Figure 1 Surfactin C inhibits thrombin ($0.1\ U\ mL^{-1}$)-induced rat platelet aggregation. A. Dose–response curves of surfactin C on thrombin-induced platelet aggregation. Platelets were pre-incubated with indicated concentrations of surfactin C for 2 min and then thrombin was added to trigger aggregation for a further incubation of 8 min. The data are presented as means $\pm$ s.e.m., n = 5. B. The inhibitory effect of surfactin C on thrombin-induced intracellular Ca^{2+}. The platelets with Fura-2 AM were incubated and were employed to calculate intracellular Ca^{2+} mobilization as described in Materials and Methods. Data are presented as means $\pm$ s.e.m., n = 4. 1, Basal; 2, thrombin $0.1\ U\ mL^{-1}$; 3, thrombin $0.1\ U\ mL^{-1}$ with $12.5\ \mu$M surfactin C; 4, thrombin $0.1\ U\ mL^{-1}$ with $50\ \mu$M surfactin C. $^*P < 0.05$, $^{**}P < 0.01$ vs thrombin.

The mechanism of inhibition by which surfactin C effectively suppressed both thrombin- and collagen-induced platelet aggregation is not fully understood yet. The fact that surfactin C blocked thrombin- or collagen-induced platelet aggregation, but not β_1- or β_2-mediated U937 homotypic aggregation, suggests that this compound may only alter inside-out signalling rather than outside-in signalling in platelet activation. Recently, it has been reported that surfactin C is a strong inhibitor of cytosolic PLA_2 ($cPLA_2$), in that it has anti-inflammatory (Kim et al 1998) and anti-eosinophil adhesion effects (Zhu et al 1999) under the assumption that this enzyme is the potential target. It is known that $cPLA_2$ is fully activated in the platelet aggregation process (Purdon et al 1987; Kim et al 1998; Puri 1998; Savage et al 2001), and that

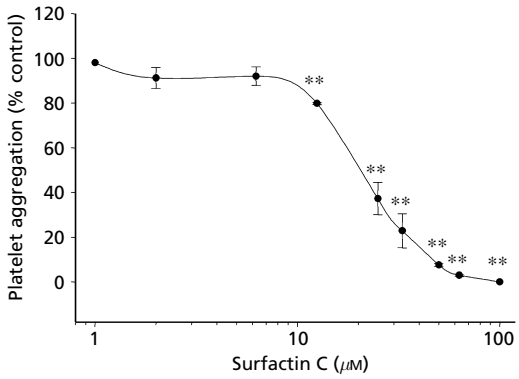

Figure 2 Surfactin C inhibits collagen ($5\,\mu g\,mL^{-1}$)-induced rat platelet aggregation. Dose–response curves of surfactin C on collagen-induced platelet aggregation. Platelets were pre-incubated with indicated concentration levels of surfactin C for 2 min and then collagen was added to trigger aggregation for a further incubation of 8 min. Data are presented as means ± s.e.m., n = 5. $^{**}P < 0.01$, vs control.

thrombin and collagen act as the most potent activators of $cPLA_2$ (Puri 1998). We found that surfactin C, however, strongly diminished the intracellular level of Ca^{2+} (Figure 1B), which is a critical upstream step for $cPLA_2$ activation (Puri 1998; Savage et al 2001), suggesting that $cPLA_2$ is not the drug's only target. According to our results (Figure 1B), it appears that the inhibition of the intracellular Ca^{2+} release provides it with a limited platform for the activation of $cPLA_2$.

Conclusions

We found that surfactin C selectively blocked thrombin- and collagen-induced platelet aggregation, but not U937 homotypic aggregation, which was mediated by function-activating antibodies to adhesion molecules such as CD18, CD29, and other CD18- or CD29-activating molecules, by interrupting Ca^{2+} mobilization. Therefore, in considering the following points – the enhancement of fibrinolysis in-vivo (Kikuchi & Hasumi 2002), the inhibition of thrombin- and collagen-induced platelet aggregation and anti-inflammatory activity in murine RAW264.7 cells in previous research (Hwang et al 2005) – surfactin C may have potential as a therapeutic drug in treating cardiovascular diseases.

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