Biochemical and Biophysical Research Communications xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

DMSO inhibits human platelet activation through cyclooxygenase-1 inhibition. A novel agent for drug eluting stents?

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ARTICLE INFO

Article history:
Received 14 December 2009
Available online xxxx

Keywords:
Platelet activation
Thrombosis
Drug eluting stents

ABSTRACT

Background: DMSO is routinely infused together with hematopoietic cells in patients undergoing myeloablative therapy and was recently found to inhibit smooth muscle cells proliferation and arterial thrombus formation in the mouse by preventing tissue factor (TF), a key activator of the coagulation cascade. This study was designed to investigate whether DMSO prevents platelet activation and thus, whether it may represent an interesting agent to be used on drug eluting stents. *Methods and results:* Human venous blood from healthy volunteers was collected in citrated tubes and platelet activation was studied by cone and platelet analyzer (CPA) and rapid-platelet-function-assay (RPFA). CPA analysis showed that DMSO-treated platelets exhibit a lower adherence in response to shear stress ($-15.54 \pm 0.9427\%$, $n = 5$, $P < 0.0001$ versus control). Additionally, aggregometry studies revealed that DMSO-treated, arachidonate-stimulated platelets had an increased lag phase ($18.0\% \pm 4.031$, $n = 9$, $P = 0.0004$ versus control) as well as a decreased maximal aggregation ($-6.388 \pm 2.212\%$, $n = 6$, $P = 0.0162$ versus control). Inhibitory action of DMSO could be rescued by exogenous thromboxane A2 and was mediated, at least in part, by COX-1 inhibition. *Conclusions:* Clinically relevant concentrations of DMSO impair platelet activation by a thromboxane A2-dependent, COX-1-mediated effect. This finding may be crucial for the previously reported anti-thrombotic property displayed by DMSO. Our findings support a role for DMSO as a novel drug to prevent not only proliferation, but also thrombotic complications of drug eluting stents.

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Introduction

Arterial thrombosis is the crucial event in a large number of cardiovascular conditions such as acute coronary syndromes (ACS) [6,19]. Even though coronary bare metal stents (BMS) and drug eluting stent (DES) implantation greatly improved prognosis of ACS patients, acute and sub-acute stent thrombosis still remain a serious concern [4,5,14,15,17,20,31,34]. In line with this, we recently showed that drugs released from DES enhance tissue factor (TF), a key trigger for thrombosis and thus could play a paradoxical role in eliciting stent thrombosis [29,32]. In this context, DMSO could represent an interesting alternative drug which not only inhibits human vascular smooth muscle cell (HVSMC) proliferation, but also prevents TF expression and thrombus formation *in vivo* [6].

Initiation of a luminal thrombosis involves both activation of the coagulation cascade by TF and factor VII [18,22,23,33], and platelet activation [13,26]. Under normal circumstances, platelets do not aggregate spontaneously; however, in the event of an injury, platelets adhere to the disrupted surface and release different biologically active substances which induce aggregation [13]. Abnormal platelet activation is implicated in a wide variety of cardiovascular disorders [1,3,16,20,28,36] and is also thought to be important in atherogenesis [3]. Anti-platelet therapy is an increasingly important aspect of secondary prevention in patients suffering from cardiovascular disease and especially in those who underwent DES implantation [20,35]. Discovery of novel agents capable of simultaneously preventing restenosis, thrombosis and platelet activation would offer an unprecedented opportunity to develop improved DES.

In light of its previously reported properties including inhibition of HVSMC proliferation and TF-mediated thrombosis [6,8], we investigated whether DMSO inhibits platelet activation and thus exerts its strong anti-thrombotic properties via a combined inhibitory effect.

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doi:10.1016/j.bbrc.2009.12.102

Please cite this article in press as: L. Asmis et al., DMSO inhibits human platelet activation through cyclooxygenase-1 inhibition. A novel agent for drug eluting stents?, Biochem. Biophys. Res. Commun. (2009), doi:10.1016/j.bbrc.2009.12.102

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Materials and methods

Study protocol. Citrated venous blood samples were drawn from healthy adult volunteers who had not taken medications known to affect platelet function for at least one week before blood sampling. Thirty milliliters of venous blood were drawn into a plastic syringe containing trisodium citrate (0.129 M). For cone and platelet analyzer experiments, 200 μl of whole blood were used; for aggregometry experiments, 225 μl of platelet-rich plasma (PRP) at a concentration of 250,000 platelets/ml were used. In DMSO experiments, samples were pretreated with DMSO 0.5% (6.3 mM) for 30 min.

Cone and platelet analyzer. To study platelet activation under shear stress conditions, CPA test was performed as previously described [10]. Briefly, 200 μl of citrated blood were placed in polystyrene wells and circulated at a high shear rate (1875 s^{-1}) for 2 min with a rotating Teflon cone. Wells were then washed with PBS, stained with May–Grünwald dye, and analyzed with an inverted-light microscope connected to an image analysis system. Results are expressed as percentage of the total well surface covered by platelets.

Aggregometry and rapid-platelet-function-assay. The rapid-platelet-function-assay (RPFA) is a semiquantitative platelet function test which measures turbidometric platelet aggregation as an increase of light transmittance due to platelet agglutination [25]. The test was performed according to the manufacturer's instructions. The RPFA reports results in "platelet aggregation units," a function of the rate at which platelets aggregate.

Briefly, turbidometric platelet aggregation was performed in platelet-rich plasma (250×10^3 cells/mm^3) and aggregation was assessed as maximal aggregation at 6 min after adding the agonist. Platelet aggregation was measured following stimulation with: Q1 arachidonate (2 mM), ADP (10 −5–1.25 μM), collagen (10^{-5}–1.25 μg/ml), ristocetin (1.25–0.62 mg/ml), U46619 (3 μM) or epinephrine (10^{-5}–1.25 μM) in the presence or absence of DMSO 0.5% (6.3 mM) preincubation 30 min before stimulation with the agonist.

Cyclooxygenase activity assay. Cyclooxygenase (COX) activity in response to vehicle (PBS) or DMSO 0.5% (6.3 mM) treatment was determined in PRP as peroxydise activity (Cayman chemical kit No. 760151, MI, USA). Generation of *N,N,N′*,-tetramethyl-*p*-phenylenediamine (TMPD) was measured colorimetrically at 590 nm. The assay was performed following the supplier's indications. Briefly, 1.5 ml of PRP (250,000 platelets/ml) was lysed in EDTA 1 mM and tris–HCl 0.1 M at pH 7.8 for 10 min by mechanical grinding. Samples were then centrifuged for 10 min at 14,000 rpm at 4 °C and resuspended in 150 μl of the same buffer. COX-1 specific inhibitor SC-560 and COX-2 specific inhibitor DuP-697 were used to identify which COX isoform is inhibited by DMSO.

Statistical analysis. Data are presented as means + SD. For the comparison of two groups, unpaired Student's *t*-test and Mann–Whitney test were applied for normally and non-normally distributed variables, respectively. ANOVA with Bonferroni's correction was used for comparison of greater than or equal to three groups. A *P*-value <0.05 was considered significant.

Results

DMSO inhibits platelet adherence

Exposure to shear stress (1875 s^{-1}) for 2 min triggered platelet activation and high rate platelet adherence (Fig. 1, upper panel). In control vehicle treated platelets, the area covered by the adhering platelets equalled 19.00 ± 0.6919% ($n = 5$) of the total dish surface. In samples pretreated with DMSO 0.5% (6.3 Mm) for 30 min, the area covered by adhering platelets drastically decreased to 3.465 ± 0.2656%, $n = 5$, $P < 0.0001$ (Fig. 1).

CPA is a validated technique also for the assessment of shear stress-induced platelet aggregation [27]. Following shear stress average platelet aggregate size in control conditions equalled 38.28 ± 7.440 ($n = 5$) and decreased to 8.926 ± 0.4291 ($n = 5$, $P = 0.0254$) in DMSO treated samples (data not shown).

DMSO delays and decreases maximal platelet aggregation

Aggregometry studies were performed on human PRP in response to several agonists. Time to aggregation (lag phase) was significantly increased (18.0% ± 4.031, $n = 9$, $P = 0.0004$) by DMSO pretreatment in arachidonate- (2 mM) stimulated platelets (Fig. 2A), but not in ADP- (10^{-5}, 1.25 μM), collagen- (10^{-5}, 1.25 μg/ml), ristocentin- (1.25, 0.62 mg/ml), U46619- (3 μM) or epinephrine- (10^{-5}, 1.25 μM) stimulated platelets (Table 1, upper panel). Additionally, DMSO pretreatment significantly decreased maximal aggregation in response to arachidonate by 6.388% ± 2.212 ($n = 6$, $P = 0.0162$, Fig. 2B). In contrast, aggregation in response to other agonists was not affected by DMSO (Table 1, lower panel).

To investigate whether the inhibitory action of DMSO could be rescued by exogenous thromboxane A2, platelets were incubated with the thromboxane A2 analogue U46619. Platelet stimulation with U46619 (3 μM) alone triggered comparable maximal aggregation to arachidonate (2 mM) ($n = 6$, P = NS, Fig. 2B). Co-incubation of U46619 with DMSO could restore arachidonate-stimulated maximal platelet aggregation ($n = 6$, $P = 0.0162$, Fig. 2B).

DMSO inhibits platelet aggregation via COX-1

To find out whether the effect of DMSO on platelet aggregation was caused by an inhibited COX activity, COX-dependent peroxydise activity was determined. Incubation of PRP with DMSO 0.5% (6.3 Mm) for 30 min significantly decreased total COX activity by 36.23% ± 15.31 ($n = 5$, $P = 0.0455$) compared to control (Fig. 3). Similarly, incubation of PRP with the COX-1 specific inhibitor SC-560 caused a comparable inhibition of total COX activity by 33.65% ± 10.16 ($n = 5$, $P = 0.0107$) compared to control. Co-incubation of DMSO and SC-560 caused no further decrease in COX activity compared to control and DMSO alone thus indicating that the residual COX activity is represented by COX-2 and that DMSO acts via inhibition of COX-1 (Fig. 3).

Discussion

This study demonstrates that 0.5% (6.3 mM) DMSO strongly inhibits platelet shear stress-induced adherence of human platelets. Additionally, DMSO prolongs lag phase and decreases maximal aggregation of human platelets in response to arachidonate but not to ADP, collagen, epinephrine and ristocetin. This finding underscores the specificity of the effect observed with DMSO. The inhibitory effect of DMSO could be prevented by exogenous thromboxane A2 and is mediated, at least in part, by inhibition of COX-1.

In patients undergoing myeloablative therapy, DMSO is infused intravenously together with hematopoietic progenitor cells with plasma concentrations readings of 1.6% (20.0 mmol/L) and only rarely causes adverse effects [9]; thus, the concentrations used in this study are well within clinically relevant levels. Furthermore, in previous in vitro studies we have shown that DMSO concentrations up to 1% are not toxic for human endothelial cells, smooth muscle cells and peripheral blood monocytes [6].

DMSO was previously suggested to inhibit platelet activation; however, this was postulated only indirectly and without any

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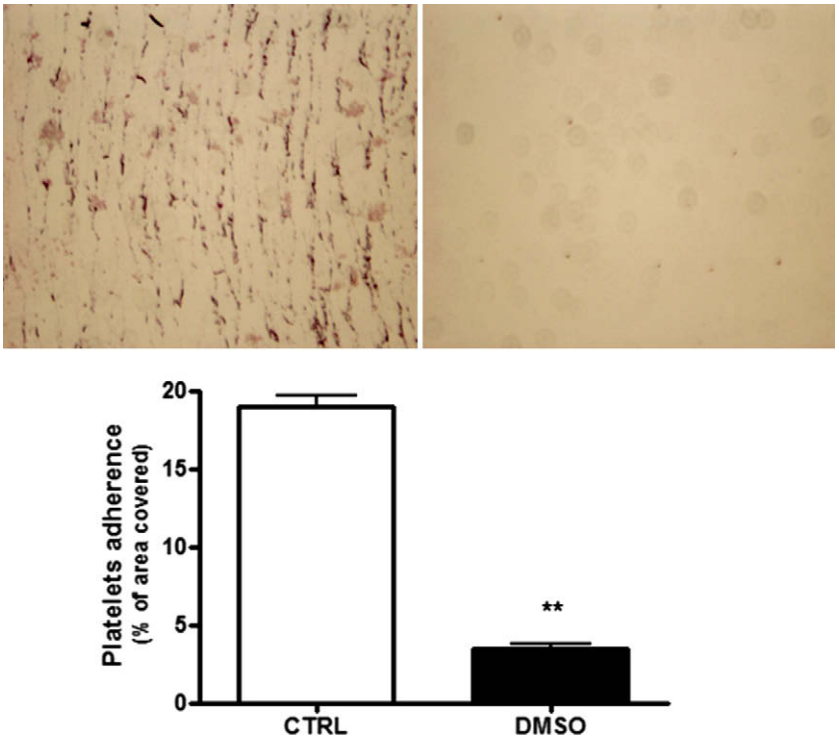

Fig. 1. DMSO inhibits shear stress-induced platelet adherence. Upper panel: shear stress induces platelet activation and adherence to the polystyrene surface of the cell culture dish (left). Treatment with DMSO (right) strongly inhibits adherence of activated platelets. Adhering platelets are visualized by May–Grünwald staining. Lower panel: histogram showing quantification of adhering platelets without (clear bar) and with (black bar) DMSO treatment, $n = 5$, $P < 0.0001$ versus control.

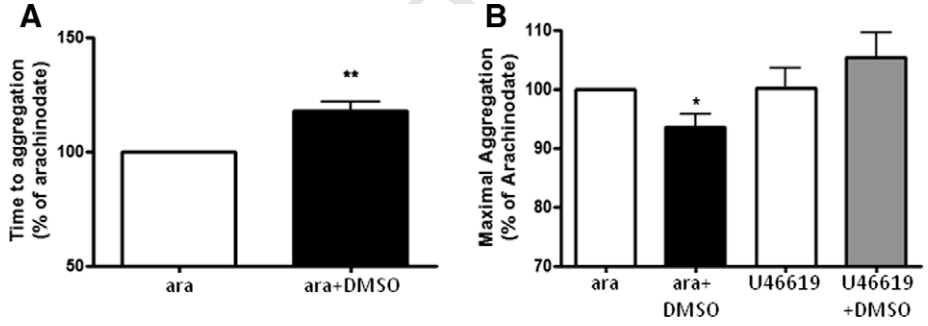

Fig. 2. DMSO delays platelet activation and decreases maximal platelet aggregation. (A) DMSO pretreatment (black bar) significantly increased time to aggregation (lag phase) in arachidonate-stimulated platelets, $n = 9$, $P = 0.0004$ versus control. (B) Arachidonate(ara)-induced platelet aggregation was blunted by DMSO treatment and U46619 stimulated comparable aggregation to ara. Co-incubation of U46619 with DMSO prevented the inhibitory effect of DMSO, $n = 6$, $P =$ NS versus ara.

Table 1
Lag phase and maximal aggregation in response to other agonists than arachidonate is unaffected by DMSO.

	Collagen	Ristocetin	ADP	Epinephrine
Mean lag phase (s, ±SEM; n = 10)				
CTRL	83 ± 5.1	36 ± 2.9	26 ± 1	39.5 ± 3.2
DMSO	88 ± 4.6	38 ± 2.5	33 ± 4.3	45 ± 1.8
Mean maximal aggregation (±SEM; n = 10)				
CTRL	76.52 ± 3.4	81.92 ± 1.7	68.8 ± 1.4	41.4 ± 10.5
DMSO	77.28 ± 2	81.3 ± 2.9	69.8 ± 2.1	44.1 ± 15.1

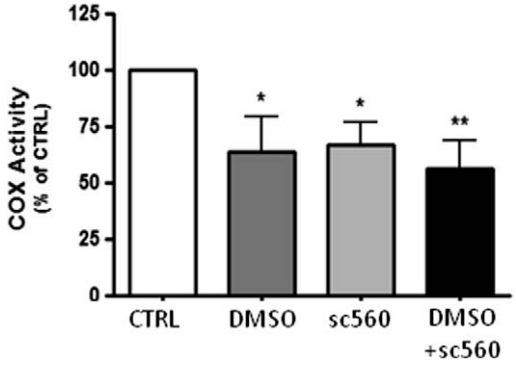

Fig. 3. DMSO inhibits COX-1 activity. DMSO 0.5% significantly decreased total COX activity ($n = 5$, $P = 0.0455$) compared to control. Similarly, COX-1 inhibitor SC-560 caused a comparable inhibition of total COX activity ($n = 5$, $P = 0.0107$) compared to control. Co-incubation of DMSO and SC-560 caused no further decrease in COX activity ($n = 5$, $P =$ NS) compared to DMSO alone.

molecular insights [6,8]. In this study, we for the first time can clearly demonstrate that DMSO inhibits platelet adherence and platelet aggregation which are essential steps for the physiological and pathophysiological formation of a stable occlusive thrombus [24]. Thrombus formation is the key process in stent thrombosis,

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a rare but serious complication observed following DES deployment [30,31]. Furthermore, platelet activation is also a relevant contributing factor in the regulation of tissue factor (TF) expression [7] – the key regulator of the coagulation cascade. In fact, platelets were shown to not only lead to the formation of the primary plug, but also suggested to contribute to the triggering of thrombin generation, fibrin deposition and clot consolidation [21]. Findings from this study reinforce the concept that DMSO is an interesting alternative for the coating of DES where currently employed drugs do not completely address the risks of stent thrombosis [4,5,14,15,17,20,31,34] and, in addition, were shown to induce the expression of TF [29,32,38].

Formation of an occlusive thrombus is a dynamic processes which requires a coordinated series of events involving the coagulation system, the fibrinolytic system and platelet activation [26]. Thus, delaying platelet activation can impair thrombus formation by disturbing the timing required for the coordinated action of the above cited systems [11]. Besides inhibiting arachidonate-induced maximal platelet aggregation, DMSO also prolonged lag phase. This finding could offer an elegant additional explanation to previous observations where DMSO-treated mice showed a permanent dynamic state of thrombus formation and lysis but failed to develop stable occlusive thrombi following laser injury [6].

The exogenous thromboxane A2 analogue U46619 prevented the inhibitory effect of DMSO on platelet aggregation implying that DMSO alters the release of prothrombotic thromboxane A2. Thromboxane A2 is a potent platelet activating eicosanoid synthesised from COX-dependent endoperoxides [2] and its inhibition has been proposed as a novel approach to prevent stent thrombosis [37]. To identify the possible mediator of altered thromboxane A2 levels, total COX activity was assayed. COX-1, but not COX-2 activity was shown to be significantly decreased by DMSO treatment hence revealing that DMSO impairs arachidonate-induced platelet aggregation via inhibition of COX-1.

In conclusion, this study offers new evidence for the suitability of DMSO as a potential new drug to be used in DES. Presently available DES have efficiently addressed the problem of VSMC-mediated restenosis; however, stent-thrombosis remains a serious concern [12,30,31]. Accordingly, current clinical guidelines recommend prolonged periods of systemic dual anti-platelet therapy with obviously increased risks of bleeding complications. As a platelet inhibitor DMSO eluting stents could allow a simplified local antiaggregant treatment protocol in DES patients thereby reducing systemic treatment associated risks and costs [20,35]. Through its dual mechanism of action DMSO may represent an interesting novel agent for DES capable of preventing both restenosis and thrombotic complications simultaneously.

Acknowledgments

This work was supported by the Swiss National Science Foundation (Grant # 3200B0-102232/1 to F.C.T. and Grant # 3100-068118.02/1 to T.F.L.), the Swiss Heart Foundation (to G.G.C.), the Hartmann–Müller Foundation, the Herzog–Egli Foundation, the Olga Mayenfisch Foundation and an unrestricted grant of the MERCATOR Foundation, Switzerland.

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