Levosimendan has an inhibitory effect on platelet function

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Levosimendan enhances cardiac contractility by increasing myocyte sensitivity to calcium, and induces vasodilatation. Although studies have evaluated the efficacy of levosimendan in heart failure, it is not clear whether it might produce functional influence on platelet response. In this study, the effect of levosimendan on platelet aggregation was investigated. Platelet function tests were performed in 12 healthy male volunteers. Three concentrations of levosimendan solution were prepared that would result in 10, 25, and 45 ng/ml levosimendan concentrations in the blood similar to that observed after clinical therapeutic intravenous application of 0.05–0.1 μg/kg/min. Each concentration of levosimendan solution and a control diluent without levosimendan were incubated with whole blood at 37°C. After incubation for 15 min, aggregation responses were evaluated with adenosine diphosphate (ADP) (5 and 10 μM) and collagen (2 and 5 μg/ml) in platelet-rich plasma. Preincubation with all dilutions of levosimendan inhibited aggregation of platelets induced by ADP and collagen significantly. Levosimendan also inhibited significantly the secondary wave of platelet aggregation induced by ADP. The results showed that there was a relationship between levosimendan concentration and inhibition of platelet aggregation. In conclusion, this study with an in vitro model showed that levosimendan had a significant inhibitory effect on platelets in clinically relevant doses. Am. J. Hematol. 83:46–49, 2008. © 2007 Wiley-Liss, Inc.

Introduction

Levosimendan is a novel drug developed for the treatment of heart failure that increases the contractile force of the myocardium by enhancing the sensitivity of myofilaments to calcium [1–4]. Levosimendan also opens adenosine triphosphate (ATP)-dependent potassium channels [5]. Several studies have evaluated the efficacy of levosimendan and have shown favorable effects on the hemodynamics and outcome of patients with decompensated heart failure [6–9].

The patients with heart failure generally received concomitant medications (angiotensin-converting enzyme inhibitors, vasodilators, digoxin, diuretics, beta-blockers, aspirin, and low-molecular weight heparin) that have possible effects on platelet function. However, there is no data on the effects of this novel drug on platelet functions. Although there is no reported case with a bleeding problem in levosimendan-treated patients, it is important to clarify this situation because levosimendan and other medications taken by patients may have possible additive effects on platelet function. For this reason, the present trial was designed to study the effects of levosimendan on platelet function. Although it was impossible to solely use levosimendan for patients with advanced heart failure because of their need of other medications, the trial was designed to study platelet function after incubation with levosimendan in the healthy volunteers.

Materials and Methods

Tests for platelet function were performed in 12 healthy male volunteers (mean age 28 years, range 24–36 years). It was ascertained that no drugs had been taken within 2 weeks before testing. Written consent was obtained from all healthy volunteers. Subjects were excluded if they had an abnormal platelet count, a history of thrombosis or abnormal bleeding, active neoplasia, or active inflammatory disease. Venous blood samples were drawn without a tourniquet from the antecubital vein. These samples were anticoagulated with 0.129 molar sodium citrate solution (blood to anticoagulant ratio: 9/1). Whole blood counts were done by standard laboratory technique using an Abbott Cell-Dyn 4000 cell counter device (Abbott Park, IL). Blood samples were kept at room temperature and tested within 30 min.

It is known that intravenous application of 0.05 and 0.1 μg/kg/min levosimendan results in 15 ± 4 ng/ml and 35 ± 9 ng/ml concentration in the plasma, respectively [10]. For this reason, levosimendan solutions in three different concentrations, which would yield 10, 25, and 45 ng/ml levosimendan concentrations in the plasma similar to that observed after clinical therapeutic intravenous application of 0.05–0.1 μg/kg/min, were prepared. Levosimendan was supplied commercially as 2.5 mg/ml, 5 ml preparation (Simdax®, Orion Corporation, Orion Pharma, Espoo, Finland) and test solutions (10, 25, and 45 ng/μl) were prepared by using 5% dextrose solution and levosimendan. After blood samples were drawn, the hematocrit was determined and the blood sample was divided into four equal parts. According to the amount of plasma (derived from the hematocrit), the calculated volume of levosimendan solution and the diluent without levosimendan as control were added to blood samples (1-μl solution for 1 ml of plasma), which yielded 0, 10, 25, and 45 ng/ml levosimendan concentrations in the plasma. Each aggregation study was performed with freshly prepared levosimendan solution and the diluent (dextrose 5%). Each concentration of levosimendan and control solutions was incubated with whole blood at 37°C. After incubation for 15 min, blood samples were centrifuged (100*g*, 10 min) to isolate platelet-rich plasma (PRP) from supernatant. The remainder of the blood was centrifuged again (2,400*g*, 20 min) to prepare platelet-poor plasma (PPP). The PRP was diluted with the PPP to yield test PRP with a final platelet count of 250 ± 50 × 10^9/L. Turbidometric aggregation was performed using a Whole Blood Lumi-Ionized Calcium Aggregometer (Chrono-log Corporation, Model 560-Ca Havertown, PA)

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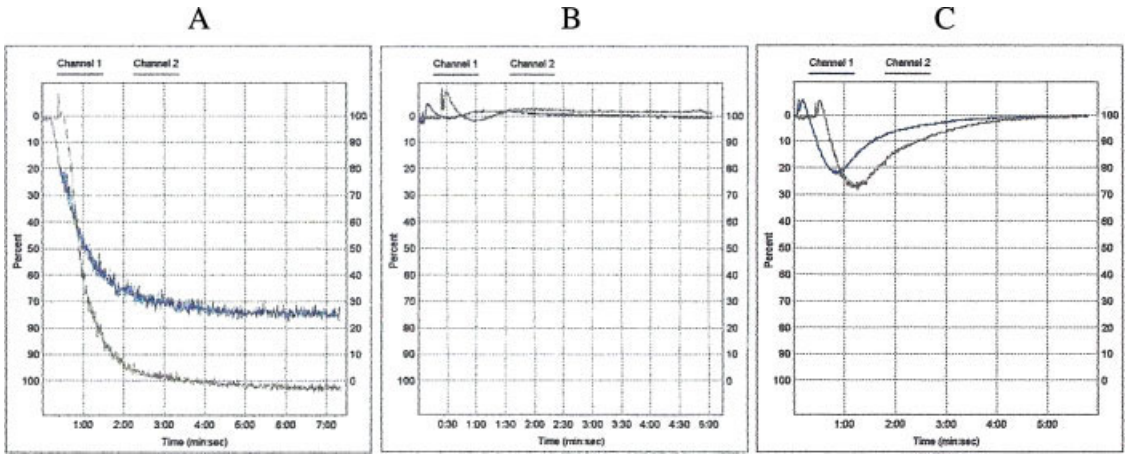

Figure 1. (A) Normal platelet aggregation pattern from control group. (B) After incubation with 45 ng levosimendan/ml, a case had "no platelet aggregation" response to ADP. (C) After incubation with 25 ng levosimendan/ml, a case achieved primary response to ADP, but had "no secondary wave". (Blue line, 5-μM ADP; black line, 10-μM ADP). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Results of Percentage of Platelet Aggregation Response After Levosimendan Incubation (n = 12)

	Percentage of maximum aggregation (%) (Mean ± S.E.)			
	Control	L10 (ng/ml)	L25 (ng/ml)	L45 (ng/ml)
ADP 5 μM	62.33 ± 6.23	36.67 ± 7.70	24.58 ± 4.81	14.17 ± 5.99
ADP 10 μM	86.92 ± 5.86	65.08 ± 9.52	50.25 ± 8.57	24.33 ± 9.86
Collagen 2 μg/ml	74.17 ± 5.51	54.42 ± 9.50	43.67 ± 8.11	26.75 ± 7.80
Collagen 5 μg/ml	92.17 ± 5.73	76.67 ± 9.58	72.50 ± 10.82	54.25 ± 11.07

L, Levosimendan.

*$P < 0.04$ for all comparisons except L25 ng/ml × control for collagen 5 μg/ml, L10 ng/ml × L25 ng/ml for ADP 5 μM and collagen. A Wilcoxon test was used to determine differences between paired data.

**$P < 0.001$, there is difference between control, L10 ng/ml, L25 ng/ml, and L45 ng/ml for ADP and collagen. Friedman repeated measures analysis of variance was used for these comparisons.

according to the protocol of Chrono-log Corporation. Platelet aggregation responses were evaluated with adenosine diphosphate (ADP) (5 and 10 μM final concentrations) and collagen (2 and 5 μg/ml final concentrations). The investigators were blinded to the sequences by which different dilutions were studied in the aggregation. Aggregating reagents were obtained from Chrono-log Corporation. Platelet aggregation curves were calculated automatically by the device and maximal aggregation (%) was obtained from the aggregation curve.

"No platelet aggregation" was defined as no change in light transmission in a 5-min period after the addition of an aggregating reagent (Fig. 1). If only a first phase of aggregation was observed and there was no visible additional aggregation (second phase of aggregation) after the addition of ADP, it was defined as "no secondary wave" (Fig. 1).

Statistics

Aggregation data presented as mean ± S.E. Friedman repeated measures analysis of variance was used to compare platelet aggregation response to ADP or collagen between control and each levosimendan solution. A Wilcoxon test was used to determine differences between paired data. A Pearson χ^2 test was used to compare secondary platelet aggregation wave response to ADP between control and each levosimendan solution. A Fisher's Exact χ^2 test was used to compare secondary platelet aggregation wave response to ADP between paired data. $P \leq 0.05$ was considered significant.

Results

Twelve healthy volunteers were evaluated. All of them were males and had a normal platelet count. As shown in Table I, a significant inhibition of the platelet aggregation response to 5–10 μM ADP and 2–5 μg/ml collagen after incubation with all concentrations of levosimendan compared with control was observed. Incubation with 45 ng levosimendan/ml had maximum inhibitory effect on platelet aggregation response to ADP and collagen compared with 10 and 25 ng levosimendan/ml. In the same way, incubation with 25 ng levosimendan/ml compared with 10 ng levosimendan/ml lead to higher inhibition of aggregation response to ADP and collagen. These results suggested that there was a relationship between levosimendan concentrations (10, 25, and 45 ng/ml) and inhibition of platelet aggregation responses in dose dependent manner ($P < 0.001$ for 5 and 10 μM ADP; $P < 0.001$ for 2 μg/ml collagen; $P \leq 0.001$ for 5 μg/ml collagen).

Examples of platelet aggregation tracings observed in this study are shown in Fig. 1.

Levosimendan also inhibited secondary wave of platelet aggregation induced by ADP. In all cases that had no secondary wave, disaggregation occurred after the primary wave was observed (Fig. 1C). As seen in Table II, all concentrations of levosimendan compared with control significantly impaired secondary wave of platelet aggregation response to ADP ($P < 0.04$ for all comparisons except levosimendan 10 ng/ml × control for 10 μM ADP). Our results also suggested that there was a relationship between levosimendan concentrations (10, 25, and 45 ng/ml) and impairment of secondary wave of platelet aggregation response to ADP ($P < 0.001$ for 5 μM ADP; $P < 0.007$ for 10 μM ADP).

TABLE II. Distribution of Cases With Abnormal Platelet Aggregation ($n = 12$)

	Number of cases (%) with abnormal platelet aggregation			
	Control	L10 (ng/ml)	L25 (ng/m)l	L45 (ng/ml)
ADP 5 μM	0 (0%)	6[a] (50%)	9[a] (75%)	11[b] (92%)
ADP 10 μM	0 (0%)	4[a] (33%)	7[a] (58%)	10[c] (83%)

L, Levosimendan.
[a]One of these had no platelet aggregation, the rest had absent second wave.
[b]Six of these had no platelet aggregation, the rest had absent second wave.
[c]Five of these had no platelet aggregation, the rest had absent second wave.

Discussion

The drug-related platelet dysfunction is one of the most common platelet abnormalities. The clinical challenge in evaluating acquired disorders of platelet function is to determine whether observed derangements in platelet function pose a threat to the patient. Although several studies have evaluated the efficacy of levosimendan in patients with heart failure, we do not know whether or not it might produce functional influence on platelet response.

Levosimendan is a novel drug developed for the treatment of heart failure. It differs from other cardiotonic agents in that it utilizes a unique dual mechanism of action. It sensitizes troponin C to calcium in a calcium concentration-dependent manner. It increases the contractile force of the myocardium by enhancing the sensitivity of myofilaments to calcium [1,2,4]. The effects of calcium on cardiac myofilaments during systole are thus enhanced, improving contraction at low energy cost [11]. Levosimendan also opens ATP-dependent potassium channels in vascular smooth muscle, thus inducing vasodilatation [5,12,13]. Activation of ATP-sensitive potassium channels, both on sarcolemma and mitochondria, may protect against myocardial ischemia, and decreased levels of cytokines may prevent the development of further myocardial remodelling [14].

In our study, levosimendan has been shown to blunt the platelet aggregatory response to ADP and collagen. There was also a relationship between levosimendan concentrations (10, 25, and 45 ng/ml) and inhibition of platelet aggregation responses in dose-dependent manner ($P < 0.001$ for 5 and 10 μM ADP; $P < 0.001$ for 2 μg/ml collagen; $P \leq 0.001$ for 5 μg/ml collagen). Platelet membrane receptors and their downstream signaling elements are potential targets for drugs [15]. Because of the enhanced sensitivity of myofilaments to calcium, one might speculate that the platelet inhibitory effect of levosimendan was related to calcium and levosimendan might affect calcium homeostasis.

Soluble platelet agonists interact with their specific receptors on the cell surface, and this interaction triggers platelet activation. The increase in cytosolic free calcium represents a pivotal step during platelet activation. Calcium is released from the dense tubular system and enters the cytosol from extracellular fluid across the platelet membrane via specific calcium channels. Some of the platelet inhibitory agonists stimulate the pumping of calcium from the cytoplasm back to the dense tubular system [16]. For these reasons, the control of calcium signaling is critical in mechanism of platelet activation and inhibition, and platelet calcium signaling is strictly controlled in space, time, and amplitude [17].

Inhibition of calcium influx into platelets and translocation from intracellular storage sites have been suggested as one of the antiaggregatory mechanisms [15,18,19]. It may be that levosimendan alters calcium homeostasis; it may suppress the increase in intracellular calcium by inhibiting calcium influx from the extracellular space or by reducing calcium mobilization from intraplatelet storage pools. It is also possible that levosimendan resulted in depletion of calcium stores. And also, levosimendan may interact with calcium binding sites of calmodulin. Thus, the increased ability of calmodulin to bind calcium might be the basis for the inhibition of platelets in the presence of the levosimendan. Although we do not know now, levosimendan may also involve mechanisms other than calcium. In fact, biochemical analysis is required to clarify these possible causes of inhibitory effect of levosimendan on platelet function.

Our study results also showed that levosimendan inhibited significantly the secondary wave of platelet aggregation induced by ADP ($P < 0.04$ for all comparisons except levosimendan 10 ng/ml $\times$ control for 10-μM ADP). Furthermore, there was a relationship between levosimendan concentrations and impairment of the secondary wave of platelet aggregation response to ADP ($P < 0.001$ for 5-μM ADP; $P < 0.007$ for 10-μM ADP). It is known that primary aggregation is a direct consequence of stimulation with an agonist, whereas secondary aggregation is mainly dependent on the release of platelet granule contents [20]. After incubation with levosimendan, the amount of stimulus (ADP) was insufficient to fully activate the platelet and induce platelet secretion of storage granules containing ADP, etc. If these granule contents are exhausted by inducers of the release reaction such as levosimendan, it could give rise to the impairment of the secondary wave of platelet aggregation. Circulating "exhausted" platelets simulating storage pool diseases can be observed in clinical scenarios where there is ongoing in vivo platelet activation, such as cardiopulmonary bypass, disseminated intravascular coagulation, and thrombotic thrombocytopenic purpura or hemolytic uremic syndrome [21]. The secondary wave inhibition in our study may be related to exhausted platelets following their in vitro exposure to levosimendan and induction of the release reaction. Platelet release defects can also be seen with defects of platelet signal transduction, including G protein activation, phospholipase C activation, calcium mobilization, and tyrosine phosphorylation [22]. Similarly, the cause of impairment of the secondary wave of platelet aggregation may be inhibition of platelet signal transduction by levosimendan. Drugs, such as aspirin, that inhibit thromboxane A_2 formation prevent the secondary phase of ADP-induced aggregation [20]. This may be another possible effect of levosimendan on platelets.

Conclusions

Our results show that levosimendan can diminish the responsiveness of normal platelets to ADP and collagen in vitro, although the mechanism of action of the agent is not known. Although the clinical significance of the observed effects is not yet known, clinicians should be aware of the potential impairment of platelet function by levosimendan. Adjuvant drugs used in the heart failure may have antiplatelet effects, and levosimendan may potentiate these effects. It can be speculated that levosimendan-induced impairment of platelet function might be clinically significant in patients with preexisting qualitative or quantitative platelet defects. It may be required to be careful while using levosimendan in situation where optimal hemostasis is critical such as trauma, post coronary artery bypass grafting surgery, and the presence of additional hemostatic defects. On the other hand, there is no reported case of bleeding problems in levosimendan-treated patients, and it is possible that these in vitro findings have no clinical significance. And lastly, it could be speculated that the antiplatelet effect of levosimendan could

be beneficial in patients with ischemic heart disease and in patients at risk for thromboembolic complications.

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