Effects of magnesium sulphate on leptin-dependent platelet aggregation: an *ex vivo* study

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Abstract. Magnesium sulphate has well known antiplatelet properties. Its effect on leptin-dependent platelet aggregation has not been studied previously. Thus, we performed this *ex vivo* study to investigate whether magnesium sulphate is able to inhibit leptin-dependent aggregation of human platelets. We obtained platelet rich plasma (PRP) from venous blood samples of 16 healthy male volunteers, and we measured ADP-induced platelet aggregation in the presence of leptin alone (5-500 ng/mL) or leptin and magnesium sulphate (0.25-8 mM). Platelet pre-incubation with leptin led to a significant and dose-dependent increase in ADP-induced platelet aggregation. Magnesium sulphate was able to inhibit the pro-aggregating effect of leptin in a dose-dependent manner. The inhibitory effect was apparent at 1 mM of magnesium sulphate concentration (% maximal aggregation=38.1±12.2) and reached its maximum at 8 mM (% maximal aggregation=20.0±7.8). Our results demonstrate that leptin-dependent platelet aggregation is inhibited by magnesium sulphate in a dose-dependent manner. It seems conceivable that the blocking of hydrolysis of phosphoinositide and of intracellular calcium mobilization by magnesium sulphate may be involved in these findings.

Key words: platelet aggregation, leptin, magnesium sulphate

Introduction

Clinical findings suggest that magnesium depletion has a role in various disorders, especially in cardiovascular diseases [1, 2]. Platelet magnesium depletion has been previously demonstrated in patients with hypertension, diabetes and obesity [3-5]. Furthermore, magnesium depletion and its association with platelet hyperreactivity are well recognized in a variety of diseases including acute myocardial infarction [6], preeclampsia [7], and diabetes mellitus [8]. Magnesium has important antithrombotic properties acting on platelets, coagulation, fibrinolysis, and endothelial mediators with vasodilating and antithrombotic qualities [9]. Recently, it has been demonstrated that magnesium sulphate antiplatelet activity involves changes in membrane fluidity with resulting interference of fibrinogen binding to the GPIIb/IIIa complex, inhibition of phosphoinositide breakdown and thromboxane A₂ formation, and of intracellular Ca²⁺ mobilization [10, 11].

Leptin, the product of the *ob* gene [12], is a protein mainly secreted by the adipose tissue that signals the size of energy stores to the central nervous system.
Although leptin’s major role seems to be in the regulation of body weight and energy metabolism, some data suggest that this hormone could be involved in other pathophysiological mechanisms [14]. Recent studies demonstrate that leptin is able to accelerate arterial thrombosis [15, 16], and to promote the aggregation of human platelets through the activation of its receptor on platelet membrane, with the consequent post-receptor phosphorylation of STAT proteins [17]. This finding has been confirmed in ob/ob mice [15] and healthy subjects [18]. We hypothesized that phospholipase C (PLC), protein kinase C (PKC), calcium, and phospholipase A2 (PLA2) could be involved in leptin-dependent platelet aggregation [19], and we recently reported on the putative role of these cytosolic enzymes in the proaggregating activity of leptin [20, 21].

To our knowledge, no study has been performed to evaluate the possibility that magnesium may interfere with leptin-enhanced aggregation of human platelets. Therefore, the aim of this ex vivo study was to investigate whether magnesium sulphate is able to inhibit leptin-dependent aggregation of human platelets.

**Methods**

After obtaining their written informed consent, 16 healthy normal-weight males (age 29.7±3.6 years; body mass index 23.2±1.2 kg/m²) subjects were enrolled in the study. All subjects were selected from the staff of the University Hospital of Messina, Italy, and underwent a complete clinical and laboratory assessment to exclude major health problems. To participate in the study all subjects were requested not to take drugs in the 4 weeks before sampling.

In all subjects, after overnight fasting, a venous blood sample was obtained in the supine position from the cubital vein of the arm using a cannula that had been placed 30 minutes before sampling to avoid the stress of venipuncture, and maintained by slow infusion of saline solution (0.5 mL/min). For platelet study, blood samples (25 mL) were collected in a conical polipropylene tube containing 3.33 mL/L of ACD (consisting of 64 mmol/L citric acid, 85 mmol/L sodium citrate and 111 mmol/L dextrose). An aliquot (5 mL) was separately collected in ACD, and plasma obtained by centrifugation was used for the assay of plasma leptin (Human Leptin RIA kit, Linco Research Inc., St. Charles, MO, USA) concentrations. Only subjects with normal plasma leptin concentrations were enrolled in the study (plasma leptin levels 4.0±1.3 ng/mL).

Platelet rich plasma (PRP) was obtained by centrifugation at 800 g for 20 minutes at 4 °C. The PRP was centrifugated at 2000 g at 4 °C to form a soft platelet pellet, resuspended in HEPES Tyrode’s buffer (HBS) consisting of 145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L NaH₂PO₄, 1 mmol/L MgSO₄, 5.5 mmol/L glucose, 3.0 mg/L BSA, 10 mmol/L HEPES, pH 7.4, and washed once. Centrifugation was performed at 4 °C in order to minimize spontaneous platelet activation [22]. Measurements were successively carried out at room temperature.

Platelets were counted by a Coulter counter (Coulter International Corporation, Miami, FL, USA) and resuspended in HBS buffer at a concentration of 0.5-1 x 10⁸ platelets/mL. Aliquots (0.4 mL) containing 1 mM CaCl₂ were dispensed into aggregometer cuvettes. Aliquot of HBS was used as a reference to indicate 100% in optical aggregation experiments. Aggregation response to agonist was optically monitored with a platelet aggregometer (PACKS4, Helena Laboratories, Beaumont, TX, USA). Light transmission was continuously compared with that from the HBS. Platelet aggregation was expressed as % maximal aggregation (i.e. highest percentage of platelet aggregation in the least time).

To study leptin effects on agonist-induced aggregation, 0.4 mL of platelet suspension was preincubated with leptin 5-500 ng/mL (Linco Research Inc., St. Charles, MO, USA) for 5 minutes, and then stimulated by the addition of ADP (Sigma Chemical Co, St. Louis, MO, USA) 2 µM.

In order to investigate the effects of magnesium sulphate on leptin-dependent aggregation, platelets were preincubated with magnesium sulphate (0.25, 0.5, 1, 2, 4, and 8 mM) (Bioindustria S.p.A., Novi Ligure, AL, Italia) for 3 minutes at 37 °C, and then stimulated with leptin (50 or 100 ng/mL) and ADP.

Statistical analysis was performed using ANOVA one way test with Scheffe post-hoc test for multiple comparisons. Data were expressed as mean±SD. Two-tailed values of p<0.05 were considered statistically significant. All statistical procedures were performed using SPSS V 10.0 statistical software package (SPSS Inc, Chicago, IL, USA).

**Results**

The effects of leptin on ADP-induced platelet aggregation are reported in figure 1. The stimulation of platelets with ADP alone did not produce any change in % maximal aggregation with respect to aggregation measure obtained with buffer alone (18±5.2, p=n.s.). Similar results were obtained with both 100
ng/mL leptin alone (20.1±6.1, p=n.s.), and 8 mM magnesium sulphate alone (19.8±5.7, p=n.s.). Platelet pre-incubation with leptin led to a significant and dose-dependent increase in ADP-induced platelet aggregation: % maximal aggregation rose from 16.0±4.8 with ADP alone to 76.1±13.3 with leptin 100 ng/mL. Higher concentrations of leptin did not produce any further increase in platelet aggregation response.

To investigate the effect of magnesium sulphate on leptin-enhanced platelet aggregation, we measured ADP-induced platelet aggregation after incubation with leptin alone or in combination with rising concentrations of magnesium sulphate, and we found that the effect of leptin on platelet aggregation was inhibited in a dose-dependent manner. The inhibitory effect was apparent at 1 mM of magnesium sulphate concentration (% maximal aggregation=38.1±12.2) and reached its maximum at 8 mM (% maximal aggregation=20.0±7.8) (figure 2).

Discussion

The results from the present study add to present knowledge by demonstrating for the first time the inhibitory effect of magnesium sulphate on leptin-enhanced platelet aggregation. Magnesium is the second most abundant intracellular divalent cation, and is an obligatory cofactor of several enzymes [23]. The association between magnesium depletion and platelet hyperaggregability has been recognized in high-risk clinical conditions [6-8]. Furthermore, magnesium has been shown to reduce platelet aggregation in both in vitro and ex vivo investigations [24-26].

Magnesium sulphate has been reported to interfere with several pathways involved in the regulation of platelet function: in particular, it inhibits agonist-induced GPIIb/IIIa complex exposure, phosphoinositide breakdown, and calcium mobilization in human platelets [10, 11]. The inhibitory effect of magnesium sulphate on the hydrolysis of phosphoinositide may help to explain our findings. Indeed, the pro-aggregating action of leptin has been found to be strongly reduced by the U73122, an inhibitor of PLC [20, 21], and the activation of PLC is a key early component in platelet activation [27-29], leading to the hydrolysis of phosphatidylinositol 4,5 diphosphate which, in turn, results in the formation of 1,2-diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP$_3$) [27, 28]. DAG stimulates protein kinase C (PKC) and phospholipase A$_2$ (PLA$_2$) [30, 31], while IP$_3$ is
able to mobilize intracellular calcium [27]. PKC, PLA₂, and IP₃ act synergistically to induce platelet activation including aggregation, secretion of alpha-and dense-granule contents, formation of thromboxane A₂ (TxA₂), and expression of adhesive receptors [27, 28, 30]. Thus, the block of the phosphoinositide breakdown exerted by magnesium sulphate may represent an important mechanism in the inhibition of leptin-dependent platelet aggregation.

Our results are also in agreement with findings demonstrating that leptin is able to mobilize intracellular calcium in human platelets [20, 21]. Indeed, magnesium sulfate may inhibit the activation of protein kinase C, followed by inhibition of phosphoinositide breakdown and intracellular calcium mobilization. On the other hand, magnesium sulfate inhibits the Na+/H+ exchanger, leading to reduced intracellular calcium mobilization, and ultimately to inhibition of platelet aggregation and the ATP-release reaction [11]. Thus, although platelet calcium was not measured in the present study, it is conceivable that the reduction of calcium mobilization may also represent a pathophysiological mechanism involved in the inhibitory action exerted by magnesium sulphate on leptin-dependent platelet aggregation.

Our study also confirms that leptin is able to promote the aggregation of human platelets [15, 17, 18, 20, 21], and by demonstrating the inhibitory effect of magnesium sulphate, further suggests that the pro-aggregating action of leptin may depend upon the activation of intracellular pathways other than Janus kinase (JAK) and signal transducer and activator of transcription (STAT) protein tyrosine phosphorylation, which was formerly thought to be the unique second messenger system affected by the hormone [19-21].

**Conclusions**

Our preliminary study confirms that leptin is able to enhance ADP-induced aggregation of human platelets. Furthermore, it demonstrates that leptin-dependent platelet aggregation is inhibited by magnesium sulphate in a dose-dependent manner. Although the mechanisms by which magnesium sulphate is able to inhibit leptin-enhanced platelet aggregation need to be investigated further, it seems conceivable that blocking of the hydrolysis of phosphoinositide and of intracellular calcium mobilization may represent the most important inhibitory pathways. Future studies in this field may provide further insight into the mechanism of leptin-dependent platelet aggregation, and could be of potential interest in the search of putative specific pharmacological targets to reduce the risk of thrombotic complications in obese patients.

**References**


