Low magnesium stimulated prostacyclin generation in cultured human endothelial cells

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Abstract. Prostacyclin, synthesized from arachidonic acid, is a strong vasodilator and the most powerful inhibitor known for platelet aggregation. Magnesium deficiency as a risk factor for cardiovascular diseases was related to imbalance of thromboxane and prostacyclin in the vasculature. In this study, we examined the effect of a low level of magnesium on prostacyclin generation in cultured human umbilical vein endothelial cells by measuring arachidonic acid release, 6-keto-prostaglandin F₁α (6-keto-PGF₁α) production, calcium (⁴⁵Ca²⁺) influx, and activity of phospholipase A₂ (PLA₂) and cyclooxygenases (COX), which are the two main enzymes that control the synthesis of prostacyclin. We found that lower levels of magnesium in the culture medium induced a time- and dose-dependent increase in arachidonic acid release. Low magnesium also enhanced 6-keto-PGF₁α production, activated PLA₂ and COX, enhanced ⁴⁵Ca²⁺ influx and decreased the remaining arachidonic acid in phospholipids. Our data indicate that the enhanced 6-keto-PGF₁α production could be due to (1) the stimulated ⁴⁵Ca²⁺ influx resulting in an activation of PLA₂, (2) the increased arachidonic acid liberation from the cell phospholipid, and (3) the activated COX activity. The increased prostacyclin production could provide protection against the cardiovascular effect of thromboxane which was increased by magnesium deficiency.

Key words: arachidonic acid, prostacyclin, phospholipase A₂, cyclooxygenases, low magnesium, cultured endothelial cells

Prostacyclin, a strong vasodilator and the most powerful inhibitor known for platelet aggregation, plays an important role in maintaining normal homeostasis. Prostacyclin, an unstable prostaglandin [1], is produced from arachidonic acid which is available in a small amount under normal physiological conditions. Arachidonic acid is liberated from the phospholipids by phospholipase A₂ (PLA₂) action when it is needed [2]. The cyclooxygenases (COX), the rate-limiting enzymes in the pathway of the synthesis of prostaglandins, then metabolize arachidonic acid to prostaglandin H₂, the common substrate for the synthesis of biologically active products such as thromboxane, prostacyclin and prostaglandin E₂ (PGE₂) [3]. The major product in endothelial cells is prostacyclin, best known as endothelium-derived anti-platelet and vasodilator factor [4].

Magnesium is the fourth most abundant cation in the body and the second most abundant intracellular cation [5]. It is involved in regulation of activity of enzymes critical to cellular metabolism and is essential in reactions involving ATP, which is required for the synthesis of fat, protein, nucleic acid and co-enzymes [6]. Magnesium is an important modulator of intracellular free Ca²⁺ concentration and pH,
both are major determinants of cell contraction, secretion, motility, and proliferation. A reduction of magnesium concentration markedly stimulated the response of vessels to angiotensin II and norepinephrine [7], elevated serum C-reactive protein [8] and increased thromboxane synthesis [9]. Magnesium deficiency, therefore, is not only associated with biochemical and clinical derangements [5, 6, 10] but may also be a key factor leading to cardiovascular diseases [7, 9].

Cardiovascular effects of magnesium deficiency are related to alterations in the biosynthesis of prostaglandins in the vasculature. More research is needed to evaluate the relationship between magnesium and prostacyclin production since the results of this relationship were inconsistent [11, 12]. Moreover, the activity of COX, one of the main enzymes involved in prostacyclin production, has not been examined in endothelial cells exposed to low magnesium medium. The purpose of the present study, therefore, was to determine how low magnesium would affect prostacyclin production by measuring PLA2 and COX activities, arachidonic acid release, 6-keto-prostaglandin F1α (6-keto-PGF1α) production, calcium (45Ca2+) influx and arachidonic acid concentration in phospholipid in the cultured endothelial cell.

**Materials and Methods**

**Materials**

L-α-1-palmitoyl-2-arachidonoyl-[arachidonyl-1-14C]-phosphatidylcholine (1.96 GBq/mmol, 53 mCi/mmol) was obtained from PerkinElmer (Boston, MA, USA). [Methyl-3H]choline chloride (85 Ci/mmol), arachidonic acid [5,6,8,9,11,12,14,15-3(N)] (200 Ci/mmol) and 45Ca2+ (5-30 Ci/g Ca) were from American Radio-labeled Chemicals, Inc. (St. Louis, MO, USA). COX activity assay kit was from Cayman Chemical Co. (Ann Arbor, MI, USA). Fetal bovine serum (FBS), Eagle’s minimum essential medium (MEM), 6-keto-PGF1α and other reagents were purchased from Sigma (St. Louis, MO, USA). Flasks and plates used for cell culture were from Corning Medical and Scientific Co. (Park Ridge, IL, USA).

**Cell culture**

Human umbilical endothelial cells (ECs) were obtained from ATCC (Rockville, MA, USA) and used at passages from 3 to 11. The cells were cultured in MEM supplemented with 10% FBS in 5% CO2 incubator at 37°C. When ECs grew to 70% confluence, they were cultured with [3H]arachidonic acid (0.2 μCi/mL) in MEM containing 5%FBS for 24 hours. After the cells were washed with MEM containing 0.125 mM BSA for 5 times, they were cultured in MEM-5%FBS containing magnesium at concentrations of 95, 190, 380 and 570 μM. A medium containing 949 μM magnesium was used as a control. The following experiments then were performed.

**Measurement of arachidonic acid release**

After the cells in 12-well plates were cultured in a medium containing magnesium at 95 μM for 2, 6, 16, 24, 40 and 56 hours or at 95, 190, 380 and 570 μM for 16 and 40 hours, the culture media were collected, centrifuged at 2000 x g for 10 minutes, and 200 μL of the supernatant counted for radioactivity [13]. The cells were digested in 1 mL of 0.1 N NaOH and the protein concentrations in the cells were determined (Bio-Rad, Hercules, CA, USA).

**Analysis of prostacyclin production**

The ECs in 6-well plates were cultured with magnesium at 95 μM for 16 and 40 hours. The culture media were then collected, and treated as follows [14]. In brief, the media were acidified to pH 3.5 with 1 M citric acid, and extracted twice with 2 volumes of ethyl acetate after addition of NaCl. Aliquots of the ethyl acetate extract were separated by TLC using ethyl acetate:acetic acid: 2,2,4-trimethyl pentane : H2O (110:20:50:100). The spots of 6-keto-PGF1α on the plates were collected and radioactivities in these spots were counted. The nonspecific activities obtained from some silica gel on blank spaces were subtracted from sample radioactivity. The cells were digested in 1 mL of 0.1 N NaOH and the protein concentrations in the cells were determined.

**Determination of the remaining [3H]arachidonic acid in phospholipid**

After 16 and 40 hours of culturing of the cells in 75 cm² flasks with magnesium at 95 μM, the media were aspirated and the cells were rinsed, trypsinized, collected and sonicated [15]. Aliquots were taken for protein assay and lipids were extracted [15]. The lipid extracts were separated by TLC using chloroform: methanol:acetic acid : water (25:15:4:2, v:v:v:v). The spots of phospholipid fractions on the plates were collected and radioactivities were counted. The nonspecific activities on silica gel plates and protein concentrations from the cells were obtained for calculation.
Assays of PLA₂ activity

For assays of PLA₂ activity, the method of Lupo et al. [16] was used with the following modifications. In brief, the cells in 75 cm² flasks were incubated in medium containing magnesium at 95 μM for 16 or 40 hours, washed with ice-cold HBSS without calcium and magnesium, scraped and transferred to 0.5 mL of homogenization buffer [16]. The homogenate was centrifuged at 1000 X g for 5 minutes and 50 μL of supernatant of the homogenate (200-300 protein) was added into 200 μL of 100 mM Tris-HCl buffer (pH 8.5) containing L-a-1-palmitoyl-2-arachidonoyl-[arachidonyl-1-14C]-phosphatidylcholine (200 μM, 211000 DPM/assay). The reaction was initiated by the addition of 50 μL of 30 mM calcium solution. The reaction mixture was incubated for 2 hours at 37°C and terminated by the addition of 1 mL of 0.1 M KCl and 1 mg/mL free arachidonic acid. The samples were vortexed and centrifuged. Arachidonic acid in the chloroform phase was separated by TLC using hexane: diethyl ether: acetic acid (80:20:1, v:v:v). The spots of free fatty acid were collected and radioactivities were calculated.

Study of COX activity

After the cells in 75 cm² flasks were cultured in a medium containing magnesium at 95 μM for 16 and 40 hours, the cells were scraped and collected by centrifugation. Then 500 μL of cold buffer (0.1 M Tris-HCl, pH 7.8 containing 1 mM EDTA) was added and the cells were sonicated and centrifuged at 10,000 x g for 15 min at 4°C. The COX activities in the supernatant were measured according to the method accompanied the COX activity assay kit.

Performance of ⁴⁵Ca²⁺ influx

The ECs in 12-well plates were cultured in a low magnesium medium containing 5% FBS for 16 or 40 hours and then incubated for 1 hour with 1 μCi of ⁴⁵Ca²⁺ in 1 mL MEM. The monolayers were washed thrice with ice-cold PBS containing 1 mM EGTA and were digested in 0.8 mL of 0.1 N NaOH [17]; 0.3 mL of the digested cells was used for measuring radioactivity and 0.2 mL for assay of protein.

Data analysis

Data were subjected to ANOVA and a Student-Newman-Keuls method or Student’s t-test. Differences with p < 0.05 were considered significant. All data are presented as mean ± SE.

Results

Arachidonic acid release

[³H]Arachidonic acid release was increased as the concentrations of magnesium was decrease in the culture medium (figure 1). When the magnesium concentration was equal or lower than 100 μM, the enhanced release was significant. The release was enhanced not only with the diminished magnesium, but also with a prolonged incubating time. A significant increase of [³H]Arachidonic acid release was observed after 16 hours in the cells cultured in a medium containing 95 μM of magnesium, compared with the control (figure 2). The greater changes of gradient of the release curve were found to be 16 to 40 hours after the incubation. We chose these incubating periods to measure prostacyclin production and enzyme activity.

Prostacyclin production

Since prostacyclin is quantitatively converted to 6-keto-PGF₁α and the later is chemically stable and biologically less active, 6-keto-PGF₁α was used as a measure of PGI₂ synthesis. We found that 6-keto-PGF₁α production was increased in the cells cultured

Figure 1. [³H]Arachidonic acid release from the endothelial cells cultured with magnesium at levels of 95, 190, 380, 570 or 949 μM for 16 and 40 hours. Results are expressed as mean ± SE of duplicate for each independent determination in eight experiments. Mean values with an asterisk are significantly different at a level of p < 0.05, compared with the control (949 μM) at the same period of incubation.
16 hours in low magnesium medium (figure 3). The increased production of PGI2 was continued as the incubation was prolonged to 40 hours (figure 3).

Enzyme activity
An alteration of PLA2 activity by low magnesium culturing was observed. A stimulative effect of low magnesium on PLA2 activity was observed after 16 hours of incubation and this effect continued up to 40 hours of incubation (figure 4). At the same time, COX activation was also observed under the low magnesium condition (figure 4).

45Ca2+ influx
Low magnesium affected 45Ca2+ influx into the cells (figure 5). A significant increase in 45Ca2+ influx was observed in the cells exposed to the medium containing 95 μM of magnesium after 16 and 40 hours of the incubation.

Remaining [3H]arachidonic acid in phospholipid
In the cells exposed to low levels of magnesium (95-570 μM), the concentration of [3H]arachidonic acid remaining in the phospholipid was significantly decreased after 16 hours of culture (figure 6). These reductions were continuous up to 40 hours of incubation (figure 6).

Discussion

The present study demonstrated that lower extracellular magnesium concentrations could lead to an increase in both arachidonic acid release and prostaglandin biosynthesis in cultured vascular endothelial cells. Our data extended previous findings that the outflows of prostaglandins, such as 6-keto-PGF1α and PGE2, and thromboxane from the perfused mesenteric arterial bed of magnesium-deficient rats were significantly increased [12]. However, the data we obtained differed from that reported by Satake et al [18]. They found a decreased trend of prostacyclin production when the extracellular magnesium was reduced from 1 mM to 0.3 mM in the absence of histamine, although this decrease was not significant. We consider these inconsistent results might be from different experimental conditions, such as cell passage number, culture medium, incubating period with the medium containing lower levels of magnesium and so on.
The enhanced arachidonic acid release caused by low magnesium treatment could result from the activated PLA₂. Prostaglandin production is controlled by the availability of free arachidonic acid released from membrane phospholipid by the action of PLA₂. Under normal conditions, mammalian tissue contains no significant amounts of preformed prostaglandins [19] or the free precursor, arachidonic acid [20]. When prostaglandins are needed, esterified arachidonic acid is hydrolyzed from tissue lipids [21]. During this process, PLA₂ catalyze the rate-limiting step in prostaglandin in most tissues.

The calcium influx was significantly enhanced in the cells cultured in the medium containing a low concentration of magnesium. Our previous data also showed that low magnesium treatment not only stimulated calcium influx, but also enhanced the total intracellular free calcium concentration [17]. All of these data indicated that magnesium deficiency affected calcium balance at the cellular level [17]. Since many phospholipases are calcium dependent [22], increased concentration of cytoplasmic calcium by low magnesium could stimulate phospholipase activity, resulting in increased free arachidonic acid which would then be converted rapidly to prostacyclin. For example, Ca²⁺-stimulated lipolysis was accompanied by a significant increase in prostaglandin E₂ production [23]. Although data on the source of calcium in endothelial cells are not consistent [24, 25], the enhanced calcium influx in this study provided evidence that extracellular calcium was involved in the regulation of arachidonic acid release. That stimulation of PGI₂ production by ionophore A23187 occurred only when the endothelial cells were incubated in the buffer which contained extra calcium [25], also indicating that arachidonic acid...
Acid release was directly related to calcium influx rather than to calcium mobilization.

The remaining arachidonic acid in the phospholipid was lower in the cells exposed to magnesium at a lower than normal level. The decrease could not be explained by inhibited loading of [3H]arachidonic acid to the cells by low magnesium because the loading was performed prior to the culturing of the cells to low magnesium. Two hypotheses could be formulated to account for these data. First, low magnesium might reduce the activity of one or more of the enzymes that are required for reacylation of fatty acids because magnesium loss was associated with disturbances of lipid metabolism [12, 26, 27]. Second, low magnesium treatment might increase the rate at which membrane fatty acids are turned over, which was proven by our data that the activity of PLA2 was stimulated and the release of arachidonic acid was accelerated by low magnesium treatment. We therefore determined that culturing at a low magnesium concentration increased the liberation of arachidonic acid.

The report that anti-inflammatory drugs could inhibit COX, including both COX-1 and COX-2 [28], indicated that COX participated in the inflammatory process. COX-2 was found to be over-expressed during the inflammatory processes [29]. A relationship between magnesium deficiency and inflammation in experimental magnesium deficiency is known to have a profound effect on the process of inflammation [30]. That is, magnesium deficiency increased the levels of proinflammatory cytokines (IL-6, TNF-α) in animals [31]. People who consumed less magnesium than the recommended daily allowance were more likely to have elevated C-reactive protein than people who consumed at least the recommended daily allowance [32]. Plasma prostaglandin E2, indicative of systemic inflammation, rose signifi-

**Figure 6.** Remaining [3H]arachidonic acid in phospholipids of the endothelial cells cultured with magnesium at levels of 95, 190, 380, 570 or 949 μM for 16 and 40 hours. Results are expressed as mean ± SE of duplicate for each independent determination in eight experiments. Mean values with an asterisk are significantly different at a level of p < 0.05, compared with the control (949 μM) at the same period of incubation.
cantly in rats on a magnesium-deficient diet [33]. The increased appearance of COX products in the perfusate was significant when the total tissue content of magnesium was decreased [34, 35]. Moreover, substance-P is known to have multiple pro-inflammatory properties. Substance P receptor blockade significantly blocked the stimulated-production of PGL₂ in the magnesium-deficient rat [36]. Based on these reports, it is possible that COX was activated in the cells treated with low magnesium medium. Though not designed to be investigated in this study, whether the increased activity of COX is only from a stimulated enzymatic activity or also from increased quantity of the enzyme needs to be further explored.

Conclusion

Low magnesium resulted in an increase of prostacyclin production by activating PLA₂ and COX. It indicated that vascular PLA₂ and COX contributed to the anti-atherogenic and anti-thrombotic effects, mainly via prostacyclin production. Since magnesium deficiency causes an increase of thromboxane production [36], the stimulated biosynthesis of PGL₂ may counteract the cardiovascular effect of thromboxane.

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