A study of magnesium deficiency in human and experimental pulmonary hypertension

Marie-Camille Chaumais¹,³,⁴, Florence Lecerf⁶, Laurent Savale¹,²,³, Sven Günther¹,²,³, Alice Huertas¹,³, David Montani¹,²,³, Fréderic Perros¹,³, Marc Humbert¹,²,³, Michèle German-Fattal⁵,³

¹ Université Paris-Sud 11, Faculté de Médecine, 94276 Kremlin-Bicêtre, France ² AP-HP, Centre de Référence de l’Hypertension Pulmonaire Sévère, Service de Pneumologie et Réanimation Respiratoire, Hôpital Antoine Béclère, 92140 Clamart, France ³ Inserm U999, IPSIT, LabEx LERMIT, Centre Chirurgical Marie-Lannelongue, 92350 Le Plessis-Robinson, France ⁴ AP-HP, Service de Pharmacie, Hôpital Antoine Béclère, 92140 Clamart, France ⁵ Université Paris-Sud 11, Faculté de Pharmacie, 92290 Châtenay-Malabry, France ⁶ Laboratoire d’Analyses Biomédiqales, Centre Chirurgical Marie-Lannelongue, 92350 Le Plessis-Robinson, France

Correspondence: Michèle German-Fattal, Inserm U999, Université Paris-Sud 11, Centre Chirurgical Marie-Lannelongue, 133, avenue de la Résistance, 92350 Le Plessis Robinson, France.

Abstract. Pulmonary hypertension (PH) is defined as an increase in mean pulmonary arterial pressure above 25 mmHg. Pulmonary vasoconstriction, cellular proliferation, inflammation, and oxidative stress are involved in the pathophysiology of PH. Since hypomagnesemia was reported to promote endothelial cell dysfunction leading to inflammation and oxidative stress, we investigated the potential involvement of magnesium (Mg) deficiency in experimental and human PH. Our results indicate that Mg deficiency has no impact on hypoxia-induced PH development or severity, and that no reduction in Mg plasma concentration was observed in patients with severe pulmonary arterial hypertension. Thus, hypomagnesemia does not appear to play a role in the pathophysiology of experimental and human pulmonary hypertension.

Key words: magnesium, pulmonary hypertension, hypoxia, endothelial cell dysfunction and proliferation [2]. Inflammatory mechanisms are known to play a key role in both human and experimental PH [3-10]. Inflammation could also increase oxidative stress (OS), which has been demonstrated in pulmonary vascular lesions of PAH patients [11]. Similarly, OS activates vascular inflammation by inducing nuclear factor-xB transcription leading to endothelial cell (EC) dysfunction [12, 13]. Magnesium (Mg) is the most abundant, intracellular, divalent cation in humans and plays an essential role as cofactor for nucleic acids and numerous enzymes [14-16]. Mg is also known to be a
physiological calcium antagonist and an endothelial cell integrity agent [17, 18]. In vitro Mg deficiency promotes EC dysfunction followed by inflammation and OS [19-25]. Mg deficiency is also reported to alter the endothelial-dependant vasorelaxation in the systemic circulation [26, 27]. In animal models, hypomagnesemia induced by a Mg-deficient diet is linked to inflammatory and oxidative responses, although data are controversial [28-32]. We therefore hypothesized that Mg deficiency could contribute to the pathophysiology of PAH. This was further supported by the demonstration that the Mg supply may attenuate experimental and human PH. Indeed, Mg sulphate decreases the severity of experimental PH by blockage of voltage-dependant calcium channels leading to decreased mPAP and cardiac output improvement [33-35]. Mg sulphate was also shown to improve persistent PH of the newborn [36, 37]. However, the role of Mg deficiency in the development of PH remains unclear. The aim of our study was therefore to investigate the potential involvement of Mg deficiency in the pathophysiology of PH in an experimental model of hypoxia-induced PH in mice, and in patients with PAH.

Materials and methods

Animals

Female C57Bl/6J five-week-old mice (Janvier, Le Genest-St-Isle, France) were housed under temperature and light controlled conditions with free access to food and water prior to the experiments. All animal experiments were performed in accordance with institutional guidelines.

Experimental models and study design

Forty mice were exposed to normobaric hypoxia (10% FiO₂) or normoxia (20.9% FiO₂) for three weeks after randomization into four groups (n=10): normal Mg-containing diet (1,400 mg Mg/kg, Harlan, Gannat, France) and normal water under normoxia (N-C); synthetic Mg-deficient diet (50 mg Mg/kg) prepared in the laboratory and deionized water to avoid consumption of Mg from drinking normal water under normoxia (N-D); normal Mg-containing diet and normal water under hypoxia (H-C); synthetic Mg-deficient diet and deionized water under hypoxia (H-D). Before exposure to chronic hypoxia or normoxia, Mg-deficient diet groups were fed with the Mg-deficient diet for two weeks in order to obtain hypomagnesemia at the time of experiment. The chamber environment was monitored by an oxygen analyzer. Carbon dioxide was removed by soda lime granules, and excess humidity was prevented by cooling the recirculation circuit. The chamber was unsealed for less than 30 min twice a week to supply food, replace CO₂ absorbent and clean the cages.

Hemodynamics

Mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (60 mg/kg) and xylazine (8 mg/kg). Blood samples were collected from the retro-orbital plexus using capillary tubes for hematocrit measurement. Then, a 24-gauge needle (Medex Medical Ltd., Great Britain, 4033) connected to a pressure transducer CardioMax III (Columbus instruments. Columbus, USA), was inserted into the right ventricle via the right jugular vein, and the right ventricular systolic pressure (RVSP) was immediately recorded.

Tissue preparation

After undergoing hemodynamic measurements, all anesthetized animals were euthanized by exsanguination via the abdominal aorta. Post-mortem explanted lungs were distended by intra-tracheal infusion of OCT diluted in phosphate buffered saline (PBS) (1:1), to preserve lung morphology. After a quick freeze in isopentane on dry ice, lungs were stored at -80 °C.

The right ventricle was dissected from the left ventricle plus septum (LV+S), and these dissected samples were weighed to obtain the right ventricle-to-left ventricle plus septum ratio [RV/(LV+S)].

Pulmonary vascular morphometry

The arteriolar muscularization percentage was measured on 7 μm-thick sections of frozen lung tissue by immunofluorescence staining. Sections were double-stained with monoclonal fluorescein isothiocyanate-conjugated anti-α-smooth muscle-actin (Sigma-Aldrich, St Louis, USA), and polyclonal, anti-human von Willebrand Factor antibodies (Dako, Glostrup, Denmark). The proportion of arteries accompanying alveolar duct
showing immunoreactivity for α-smooth muscle actin antibody (as an index of muscularization) was expressed as the percentage of total vessels counted for each animal by a blinded observer. At least 100 vessels were analyzed per animal (n=5 per group).

**Patients**

The Mg concentration of PAH patients was investigated. Only patients with idiopathic, heritable or anorexigen-induced PAH were analyzed in order to avoid magnesemia modifications due to other pathologies. Patients with excessive alcohol consumption, which could have interfered with magnesemia, were also excluded from the study. Moreover, only patients with a recent diagnosis were enrolled in order to eliminate any potential effect of PAH-specific treatments on Mg concentrations. Treatments initiated before the diagnosis of PAH were systematically recorded for each patient to elucidate their magnesemia. Consent was obtained for each patient.

**Magnesium determination**

Plasma Mg was quantified using a xylidyl blue complexometric method and the routine procedure (Laboratoire d’Analyses Biomédicales, Centre Chirurgical Marie-Lannelongue, Le Plessis-Robinson, France), in a clinical chemistry analyzer (AU400, Olympus, Rungis, France). Results were expressed in mmol/L.

**Statistical analysis**

Quantitative variables were presented as mean values ± SEM. Comparisons for all parameters were analyzed using the nonparametric Kruskal Wallis test (PRISM software, GraphPad, San Diego, CA, USA). Statistical significance was defined as p<0.05.

**Results and discussion**

Plasma Mg concentration in mice decreased dramatically after five weeks of a Mg-deficient diet (Table 1). The Mg-deficient diet had no impact on body weight or hematocrit under normoxia or hypoxia conditions. Exposure to chronic hypoxia induced a significant decrease in weight and a significant elevation of the hematocrit compared to N-C group (Table 1). Three-week hypoxia exposure resulted in a significant increase in RVSP (20±0.8 mmHg versus 29.5±1.0 mmHg, P<0.01) and in right ventricular hypertrophy (0.235±0.008 versus 0.311±0.009 P<0.05), compared to normoxia (figure 1). The Mg-deficient diet given two weeks before hypoxia and during the three weeks of hypoxia exposure had no significant effect on the hypoxia-induced increase in RVSP and right ventricular hypertrophy (figure 1). The percentage of arteriolar muscularization (51.8±7.1%) in the lung sections was significantly higher under chronic hypoxia (P<0.01 versus N-C group) (figure 2). The Mg-deficient diet did not affect the degree of arteriolar muscularization under normoxia or under hypoxia (figure 2A). Thus, Mg deficiency had no impact on hypoxic PH development or severity in this model.

Mg deficiency has been reported in a variety of vascular diseases, including systemic hypertension [38]. This prompted us to test whether hypomagnesemia could be identified in PAH patients. Ten patients (two males and eight females) with a recent diagnosis of PAH were enrolled for plasma Mg quantification. The mean patient age was 67.3±4.1 years. At the time of diagnosis, five patients had systemic hypertension, two had diabetes mellitus and one had dyslipidemia. Seven PAH patients had idiopathic PAH, one had heritable PAH and two displayed anorexigen-induced PAH. At the time of diagnosis, eight patients were in the New York Heart Association (NYHA)
Figure 1. Right ventricular systolic pressure (RVSP) (A) and right cardiac remodeling expressed as RV/LV+S (B) in the four groups of mice. N-C group: mice with normal Mg-containing diet and normal water under normoxia. N-D group: mice with synthetic Mg-deficient diet (50 mg Mg/kg) and deionized water under normoxia. H-C group: mice with normal Mg-containing diet and normal water under hypoxia. H-D group: mice with synthetic Mg-deficient diet (50) and deionized water under hypoxia. (n=10). * p < 0.05 vs N-C, ** p < 0.01 vs N-C.

Figure 2. A) Percentage of arteriolar muscularization of mice lung sections. N-C group: mice with normal Mg-containing diet and normal water under normoxia. N-D group: mice with synthetic Mg-deficient diet (50 mg Mg/kg) and deionized water under normoxia. H-C group: mice with normal Mg-containing diet and normal water under hypoxia. H-D group: mice with synthetic Mg-deficient diet (50 mg Mg/kg) and deionized water under hypoxia. *p < 0.01 vs N-C (n=5), **p < 0.01 vs N-C (n=5). B) Arteriolar muscularization of lung sections in N-C (a) and H-C (b) mice. Green: α-smooth muscle actin staining; red: von Willebrand factor (vWF) staining indicating endothelial cells; blue: DAPI staining showing the nuclei.
Table 2. Plasma Mg concentration, treatments and clinical characteristics of the 10 PAH patients.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Diagnostic</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>plasmatic Mg level (mmol/L)</th>
<th>NYHA</th>
<th>mPAP (mmHg)</th>
<th>6-MWD (m)</th>
<th>Chronic treatment before diagnosis</th>
<th>PAH specific treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 F</td>
<td>i PAH</td>
<td>78</td>
<td>38.6</td>
<td>0.79</td>
<td>3</td>
<td>53</td>
<td></td>
<td>furosemide/trimetazidine irbesartan/esomeprazol allopurinol/acetylleucine paracetamol/insulin</td>
<td>tadalafil</td>
</tr>
<tr>
<td>2 F</td>
<td>i PAH</td>
<td>64</td>
<td>31.1</td>
<td>0.74</td>
<td>2</td>
<td>44</td>
<td>281</td>
<td>furosemide/zolpidem cyamemazin/levothyroxin clonazepam/pravastatin</td>
<td>bosantan</td>
</tr>
<tr>
<td>3 M</td>
<td>i PAH</td>
<td>78</td>
<td>23.7</td>
<td>0.79</td>
<td>3</td>
<td>42</td>
<td>245</td>
<td>furosemide/tamsulosine acetylysine/levocetirizine allopurinol/tiotropium/ramipril</td>
<td>bosantan</td>
</tr>
<tr>
<td>4 F</td>
<td>i PAH</td>
<td>48</td>
<td>17.7</td>
<td>0.78</td>
<td>3</td>
<td>45</td>
<td>233</td>
<td>furosemide/digoxine buprenorphine/tiotropium bromide</td>
<td>sildenafil</td>
</tr>
<tr>
<td>5 F</td>
<td>i PAH</td>
<td>69</td>
<td>34.5</td>
<td>0.78</td>
<td>3</td>
<td>62</td>
<td>130</td>
<td>furosemide/zolpidem spironolactone/fluindione ramipril/propranolol plaquenil/lercanidipin rosvastatin/esomeprazol</td>
<td>AMBITION protocol</td>
</tr>
<tr>
<td>6 F</td>
<td>i PAH</td>
<td>79</td>
<td>29.7</td>
<td>0.93</td>
<td>3</td>
<td>49</td>
<td>210</td>
<td>furosemide/acetylysine spironolactone/clopindogrel ivabradine/esomeprazole/ramipril</td>
<td>tadalafil</td>
</tr>
<tr>
<td>7 M</td>
<td>i PAH</td>
<td>83</td>
<td>30.5</td>
<td>1.1</td>
<td>3</td>
<td>50</td>
<td>215</td>
<td>furosemide/amlodipine simvastatin/allopurinol bisoprolol/acetyllysine perindopril/tamsulosine/potassium</td>
<td>bosantan</td>
</tr>
<tr>
<td>8 F</td>
<td>anorex PAH</td>
<td>69</td>
<td>24.8</td>
<td>0.89</td>
<td>3</td>
<td>25</td>
<td>430</td>
<td>furosemide/clopindogrel alprazolam/repaglinin fluoxetine/bisoprolol zolpidem/quinapril/metformine</td>
<td>bosantan</td>
</tr>
<tr>
<td>9 F</td>
<td>anorex PAH</td>
<td>59</td>
<td>35.9</td>
<td>0.7</td>
<td>4</td>
<td>61</td>
<td>90</td>
<td>furosemide/clopindogrel alprazolam/repaglinin fluoxetine/bisoprolol zolpidem/quinapril/metformine</td>
<td>bosantan</td>
</tr>
<tr>
<td>10 F</td>
<td>h PAH</td>
<td>46</td>
<td>30.7</td>
<td>0.85</td>
<td>3</td>
<td>58</td>
<td>350</td>
<td>furosemide/fluidione potassium</td>
<td>bosantan/sildenafil epoprostenol</td>
</tr>
</tbody>
</table>

F: female; M: male; NYHA: New York Heart Association; mPAP: mean pulmonary arterial pressure; 6-MWD: 6-min-walk distance, i PAH: idiopathic PAH; anorex PAH: anorexigen-induced PAH; h PAH: heritable PAH
functional class III, one in NYHA II and one in NYHA IV. The mean 6-minute-walk distance was 243 ± 35 meters and the mean PAP was 49 ± 3.4 mmHg. A specific treatment was started for nine out of the 10 patients. Nine patients had long-course treatments before the diagnosis of PAH (table 2). Among those, the diuretic drug furosemide was always reported. Anti-hypertensive treatments were the most frequent medications in the population (six patients). Coagulation inhibitors, central nervous system and statin treatments were recorded in four, three and three patients, respectively. Whereas furosemide is known to reduce plasma Mg concentrations by way of an increase in Mg renal excretion [39], the other drugs reported in table 2 have no impact on plasma Mg concentrations. Despite treatment with furosemide, all PAH patients displayed normal plasma Mg concentrations except one patient with a moderately elevated Mg concentration. Although Mg is found mainly at the intracellular level, and plasma magnesium is not fully representative of the general magnesium status, it is the first parameter which decreases in case of Mg deficiency. Our results indicate therefore a well-balanced homeostasis, and do not support the hypothesis that Mg deficiency contributes to the pathophysiology of idiopathic, heritable or anorexigen-induced PAH. In addition to a variety of drug therapies, magnesemia could be influenced by many factors including comorbidities, excessive alcohol consumption, and diet. One of the limits of this study could be the single measurement of magnesemia. However, magnesemia was measured at the time of PAH diagnosis and therefore represents the magnesium status of PAH patients at this time point. Moreover, since specific treatments for PAH are prescribed within a few days of diagnosis, repeated Mg determinations cannot be performed. Another limit is the small number of patients enrolled for plasma Mg measurements. However, since PAH is a rare disease, with a prevalence of around 15 patients per million [40], not many patients could be included.

Conclusion

Our results do not demonstrate a role for hypomagnesemia in the pathophysiology of experimental and human PH. These results support the fact that the beneficial effect obtained by Mg sulphate in the treatment of PAH is due only to the physiological calcium antagonism, which is a feature of magnesium.

References


