Neutral endopeptidase inhibition enhances substance P mediated inflammation due to hypomagnesemia

William B. Weglicki1,2, Joanna J. Chmielinska1, Isabel Tejero-Taldo1, Jay H. Kramer1, Christopher F. Spurney3, Kandan Viswalingham2, Bao Lu4, I. Tong Mak1

1 Departments of Biochemistry & Molecular Biology; 2 Medicine, The George Washington University Medical Center, Washington; 3 the Division of Cardiology, Children’s National Medical Center, Washington; 4 Children’s Hospital, Harvard Medical School, Boston, USA

Correspondence: W.B. Weglicki, M.D., The Department of Biochemistry & Molecular Biology, 439 Ross Hall, The George Washington University Medical Center, 2300 Eye ST., N.W., Washington, D.C. 20037, USA
<phywbw@gwumc.edu>

Abstract. During dietary deficiency of magnesium neurogenic inflammation is mediated, primarily, by elevated levels of substance P (SP). The enzyme most specific for degrading this neuropeptide is neutral endopeptidase (NEP). In recent studies we found that pharmacological inhibition of NEP by phosphoramidon resulted in elevated plasma levels of SP and greater oxidative stress. We also observed that hypomagnesemia reduced cardiac and intestinal expression of NEP. In these magnesium-deficient rats increased intestinal permeability and impaired cardiac contractility occurred. In our colony of genetically-engineered NEP knockout mice that have reduced ability to degrade SP, we found increased oxidative stress that was prevented by SP (neurokinin-1) receptor blockade. Thus, we submit that inhibition of NEP by pharmacological, genetic and dietary approaches (magnesium restriction), causes greater neurogenic inflammation that may result in increased intestinal and cardiac dysfunction.

Key words: hypomagnesemia, NEP inhibition, substance P, neutrophil activation, oxidative/nitrosative stress, NEP knockout mice

In earlier studies we discovered that circulating levels of substance P (SP) [1, 2], were significantly elevated in hypomagnesemic rodents receiving Mg-deficient (MgD) diets [3, 4]. Calcitonin gene-related peptide (CGRP) was also elevated, and both neuropeptides probably emanated from sensory-motor neuron fibers [5]. Significant elevations of SP preceded significant increases in other inflammatory parameters (circulating IL-1, IL-6, TNFα, histamine, PGE2, and white blood cells [WBCs] [3, 4, 6], and cardiac tissue inflammation (WBC infiltration, elevated CD11b and ICAM). SP elevations also preceded significant changes in indices of oxidative/nitrosative stress, including: lipid peroxidation products, endogenous antioxidant depletion, and nitric oxide oxidation products (nitrite+nitrate). SP receptor (neurokinin-1 or SPR)-blockade in vivo attenuated multiple parameters of oxidative/nitrosative stress including circulating neutrophil superoxide production and circulating nitrite+nitrate levels [15]. Thus, this neuropeptide may be triggering many of the events which eventually promote cardiomyopathy and dysfunction.

Neutral endopeptidase (NEP or nephrin) is the principal proteolytic SP-degrading enzyme [8]. NEP is expressed by various tissues and cells, including heart, small intestine, kidney, brain, airway epithelium, vascular endothelium [9], neutrophils [10] and macrophages [11]. NEP inactivation may lead to enhanced SP-mediated systemic inflammation. NEP can also be a target of oxidative damage; in particular, 4-hydroxynonenal (4-HNE), a by-product of lipid peroxidation, can form covalent adducts with histidine and lysine residues of NEP.
We reasoned that NEP inactivation may occur in hypomagnesemia, and may partly account for the rise in SP during hypomagnesemia. The NEP inhibitor, phosphoramidon, may prevent breakdown of SP leading to excess of this neuropeptide during hypomagnesemia. To pursue this pharmacological intervention we decided to discern its potential proinflammatory effects in hypomagnesemic rats.

Using the NEP inhibitor, phosphoramidon (5 mg/kg/day, s.c. pellet), we investigated the influence of NEP inhibition during hypomagnesemia on circulating SP levels in conjunction with changes in oxidative stress (circulating PGE2, red blood cell glutathione [RBC GSH] loss, and PMN activation) [14]. Total circulating SP levels were obtained by area integration of the 1 week time-course, RBC GSH was determined by an enzymatic recycling methods, PGE2 by measuring its stable breakdown metabolite (PGEM ELISA kit, Cayman Chemical, Ann Arbor, MI), PMN superoxide by an SOD-inhibitable cytochrome c reduction method, and NEP activity by a fluorimetric assay. *p < 0.05, **p < 0.01 vs control.

Means ± SE of 4-6 rats which were placed on Mg-deficient (MgD, containing 2.25 mmol MgO/kg diet or 9% of control Mg) or control Mg-sufficient (MgS, containing 25 mmol MgO/kg diet) diets for 1 week with or without concurrent phosphoramidon (PR; 5 mg/kg/day, s.c. pellet). Methods for all parameters can be found in [14]. Plasma SP was measured using a colorimetric ELISA kit from R&D Systems (Minneapolis, MN) and total values were obtained by area integration of the 1 week time-course. RBC GSH was determined by an enzymatic recycling methods, PGE2 by measuring its stable breakdown metabolite (PGEM EIA kit, Cayman Chemical, Ann Arbor, MI), PMN superoxide by an SOD-inhibitable cytochrome c reduction method, and NEP activity by a fluorimetric assay. *p < 0.05, **p < 0.01 vs control.

Table 1. Effect of neutral endopeptidase (NEP) inhibition on inflammatory/oxidative parameters during 1 week of diet-induced hypomagnesemia in rats.

<table>
<thead>
<tr>
<th>Parameter at dietary week one</th>
<th>Control</th>
<th>MgD (change vs control)</th>
<th>MgD+PR (change vs control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma SP</td>
<td>0.063 ± 0.01 (ng/mL/wk)</td>
<td>7.52 ± 0.82-fold Increase**</td>
<td>18.38 ± 1.74-fold Increase**</td>
</tr>
<tr>
<td>RBC GSH</td>
<td>6.78 ± 0.6 (nmol/mg Hb)</td>
<td>7 ± 6% Decrease (ns)</td>
<td>21 ± 5% Decrease*</td>
</tr>
<tr>
<td>PGE2</td>
<td>281 ± 54 (pmol/mL)</td>
<td>1.6 ± 0.2-fold Increase*</td>
<td>2.67 ± 0.9-fold Increase*</td>
</tr>
<tr>
<td>PMN Superoxide Generation (basal)</td>
<td>133 ± 22 (pmol/min/10^6 cells)</td>
<td>1.5 ± 0.3-fold Increase (ns)</td>
<td>3.9 ± 0.5-fold Increase**</td>
</tr>
<tr>
<td>PMN NEP Activity</td>
<td>4970 ± 538 (RFU/10^6 cells)</td>
<td>15 ± 9% Decrease (ns)</td>
<td>50 ± 11% Decrease*</td>
</tr>
</tbody>
</table>

Mean ± SE of 4-6 rats which were placed on Mg-deficient (MgD, containing 2.25 mmol MgO/kg diet or 9% of control Mg) or control Mg-sufficient (MgS, containing 25 mmol MgO/kg diet) diets for 1 week with or without concurrent phosphoramidon (PR; 5 mg/kg/day, s.c. pellet). Methods for all parameters can be found in [14]. Plasma SP was measured using a colorimetric ELISA kit from R&D Systems (Minneapolis, MN) and total values were obtained by area integration of the 1 week time-course. RBC GSH was determined by an enzymatic recycling methods, PGE2 by measuring its stable breakdown metabolite (PGEM EIA kit, Cayman Chemical, Ann Arbor, MI), PMN superoxide by an SOD-inhibitable cytochrome c reduction method, and NEP activity by a fluorimetric assay. *p < 0.05, **p < 0.01 vs control.

Figure 1. Three weeks of Mg-deficiency (MgD) decreased circulating neutrophil NEP activity (assayed using a fluorogenic peptide substrate) and increased basal superoxide anion generation (assayed by SOD-inhibitable cytochrome c reduction) in rats. Assay methods can be found in [14]. Values are means ± SE of 4-6 rats. *p < 0.05, **p < 0.01 vs Mg-sufficient (MgS) control.
Figure 2. A) Immunohistochemical staining for NEP in heart (upper panels) and small intestine (lower panels) of 3 week Mg-sufficient (MgS, left) and Mg-deficient (MgD, right) rats. Rabbit anti-rat NEP primary antibody (Millipore Co., Chemicon, Billerica, Ma, USA) and Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Invitrogen Corp., Molecular Probes, Carlsbad, CA, USA) were used. Magnification 20 x. B) Quantification (pixel number) of NEP staining in the small intestine (A lower panels) of 3 week MgD rats shows 49% (p < 0.05) decrease in comparison with NEP staining of 3 week MgS animals. Values are means ± SE, n = 5.
played ~50% (p < 0.01) lower NEP enzymatic activity (figure 1). Thus, prolonged hypomagnesemia alone resulted in a substantial loss of NEP activity in the PMNs.

SP receptor blockade (L-703,606 at a low dose of 0.5 mg/kg/day) partially attenuated the loss of PMN NEP activity, possibly due to reduction of oxidative stress when the SPR-blocker was present. In association, we showed that the basal superoxide generating activity of PMNs from hypomagnesemic rats was significantly higher at 3 weeks (figure 1), and was substantially attenuated by SPR-blockade [7].

In the absence of in vitro stimulation, neutrophils isolated from control rats displayed only low levels of basal superoxide producing activity. PMN activity was markedly and significantly elevated 3.9-fold in the phosphoramidon-treated 1 week Low Mg group (table 1: p < 0.01 vs controls; p < 0.025 vs MgD alone). We also assessed the effect of phosphoramidon on rat plasma levels of PGE2 metabolites (PGEM) after 7 days of low Mg diet: low Mg diet alone resulted in a moderate, but significant increase (1.6-fold higher than control) in PGEM content, and phosphoramidon-treated hypomagnesemic rats exhibited a further elevation (2.67-fold higher, table 1). Hypomagnesemia caused depletion of RBC GSH during the second and third weeks of the diet, yet the loss of GSH was insignificant (7%) during week 1 (table 1). However, one week of phosphoramidon treatment enhanced the loss of GSH to 21%, which was significantly (p < 0.01) lower than that of the control or hypomagnesemic groups. Thus, modulating plasma SP levels during hypomagnesemia by inhibiting SP degradation can influence in vivo oxidative stress.

Recent studies using immunochemical staining for NEP have shown modest decreases (30%, n = 3, ns) in cardiac ventricular tissue NEP from 3 week hypomagnesemic rats. By contrast, substantially less staining for NEP in the small intestine of 3 week hypomagnesemic rats was observed (figure 2A), with 49% lower intensity (pixel count, p < 0.05, n = 5) vs controls (figure 2B). These findings show the differing degrees of NEP loss in cardiac and intestinal tissues after three weeks of low Mg diet. Although only a modest decrease in cardiac NEP was observed in three week hypomagnesemic rats, extending the dietary period to 5 weeks revealed substantial quantitative differences in cardiac NEP protein content (western blot analysis). Hearts from the hypomagnesemic group exhibited a 45% decrease in NEP protein compared to control (figure 3). Ventricular tissue from 5 wk hypomag-

nesemic rats also exhibited heightened levels of inflammatory cell infiltration, and increased immunohistochemical staining for nitrotyrosine in peri-vascular and endothelial regions. This suggests that 5 weeks of hypomagnesemia induces substantial nitrosative stress (peroxynitrite derived from nitric oxide) in cardiac tissue.

We determined the effect of extending low Mg diet to 5 wks on circulating SP levels in the rat. After the initial transient rise in SP during week 1 of hypomagnesemia, subsequent elevations were observed at week 3 (7.2-fold higher vs controls) and week 5 (6.5-fold). This heightened circulating SP was associated with enhanced oxidative stress,
Figure 4. Circulating substance P (SP) (A) and RBC glutathione (B) levels in hypomagnesemic and control mice at dietary week 2. SP measurements were made by chem.-ELISA and data is modified from [3]. The procedure for RBC glutathione measurements is described in [14]. Values are means ± SE of 3-5 mice. ** p < 0.01, *** p < 0.001 vs MgS.

Figure 5. Effect of SP-receptor blockade (SPB) by L-703-606 on RBC glutathione levels in wildtype B6 [NEP\(^{+/+}\)] or NEP knockout [NEP\(^{−/−}\)] mice on 1 week control (MgS) or MgD diets. Mice received L-703,606 (1.5 mg/kg/day s.c. pellet) or placebo; the procedure for RBC glutathione measurements is described in [14]. Values are means ± SE of 4-5 mice. * p < 0.05 vs untreated MgS; + p < 0.05 vs untreated MgD.
as indicated by the 40-55% decline in RBC GSH and elevations in plasma lipid peroxidation product (8-isoprostane) levels, which were 80% higher (p < 0.05) in week 3 and 203% higher in week 5 low Mg rats vs controls [15]. These data suggest that lipid peroxidation in vivo was actively occurring during late phase hypomagnesemia. After 5 weeks of hypomagnesemia, circulating PMNs isolated from these rats exhibited a 4-5 fold higher basal superoxide generating activity compared to those from controls [15], suggesting endogenous basal activation. We also found the NEP activity remained low (40-50% of controls) in hypomagnesemic PMNs at 5 weeks. This reduced capacity to degrade SP may partly contribute to the enhanced oxidative stress as well as both the diastolic and systolic dysfunction observed by echocardiography at 5 weeks of hypomagnesemia [15].

In other studies, we verified the presence of neurogenic inflammation and associated oxidative stress in a hypomagnesemic murine model [3, 6]. Mice were placed on the same dietary regimen (9 vs 100% RDA for Mg) used for rats, and blood samples were collected after 2 weeks for assessment of circulating SP, RBC glutathione, and plasma Mg levels. Plasma Mg dropped to ~30% of control levels after 2 weeks, and was associated with >11-fold increase in plasma SP content and a nearly 50% loss in RBC glutathione levels (figure 4). Thus, the impact of hypomagnesemia observed in the rat, was also seen in our mouse model.

We used NEP knockout mice (from homozygous breeding pairs) [16] to assess the role of NEP in modulating SP-mediated oxidative stress in early hypomagnesemia. At 1 week, low Mg already induced a significant 25% (p < 0.05) loss of RBC total GSH in NEP knockout mice (figure 5). By contrast, hypomagnesemia only induced a non-significant 5% decrease of RBC GSH in the wildtype control mice. This early response from hypomagnesemic NEP knockout mice is similar to that observed for hypomagnesemic rats treated with the NEP inhibitor, phosphoramidon [14]. Loss of GSH in hypomagnesemic NEP knockout mice was completely prevented by SP-receptor blockade, indicating heightened SP-induced systemic oxidative stress. Cardiac function aberrations (echocardiography) in NEP knockout mice were not observed when these mice were maintained on the normal Mg diet (table 2). However, it is anticipated that diet-induced hypomagnesemia will convey an earlier onset of contractile dysfunction in this knockout strain compared to its wildtype control, in response to the further enhancement of neurogenic inflammation.

In summary, we obtained clear evidence for inhibition of NEP in hypomagnesemic rats; its activity in PMNs, cardiac and intestinal tissues decreased with the progressive duration of Mg deficiency. In addition, phosphoramidon-induced inhibition of NEP activity during Mg deficiency resulted in further increases in circulating SP levels and a corresponding enhancement of oxidative stress and inflammation; SP receptor blockade significantly attenuated this enhanced stress and inflammation. A similar pattern of increased oxidative stress in NEP(-/-) knockout mice during hypomagnesemia was observed and was also blunted by SP receptor blockade. Collectively, our data support the central role of NEP in modifying neurogenic inflammation and the subsequent oxidative/inflammatory events during hypomagnesemia.

**Acknowledgments**

We dedicate this manuscript to the memory of our esteemed colleague, Dr. Kandan Viswalingham.

**Table 2.** Comparison of echocardiographic parameters for B6 control and NEP-knockout mice on the Mg-normal diet.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>B6 Control</th>
<th>NEP-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVPWd,mm</td>
<td>0.94 ± 0.06</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>LVDs,mm</td>
<td>2.18 ± 0.12</td>
<td>2.28 ± 0.19</td>
</tr>
<tr>
<td>LVPWs,mm</td>
<td>1.20 ± 0.09</td>
<td>1.22 ± 0.04</td>
</tr>
<tr>
<td>LVEF</td>
<td>0.77 ± 0.03</td>
<td>0.84 ± 0.03</td>
</tr>
<tr>
<td>%FS</td>
<td>40.0 ± 3.04</td>
<td>46.0 ± 3.02</td>
</tr>
<tr>
<td>HR, BPM</td>
<td>451 ± 19.5</td>
<td>413 ± 20.2</td>
</tr>
<tr>
<td>E/A</td>
<td>2.05 ± 0.08</td>
<td>2.04 ± 0.21</td>
</tr>
</tbody>
</table>

Means ± SE of 3-4 mice. Echo was performed using a GE VingMed System Five with a 10 MHz pediatric probe. LVDs: LV chamber diameter in systole; LVPWd & LVPWs: LV posterior wall in diastole and systole; LVEF: LV ejection fraction; %FS: % fractional shortening; HR: heart rate; and E/A: mitral E/A wave ratio. No significant differences were noted for any of the described parameters in these mouse strains.

**References**


