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Neutral Endopeptidase Inhibition Enhances Substance P Mediated Inflammation Due to Hypomagnesemia

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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.

Keywords

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, TNF, histamine, PGE₂, & white blood cells [WBCs] [3,4,6], and cardiac tissue inflammation (WBC infiltration, elevated CD11b & 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 neprilysin) 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 LPO, can form covalent adducts with histidine and lysine residues of NEP [12,13]. 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 time-course data, and revealed that low Mg alone caused a > 7-fold increase in SP (Table 1). Phosphoramidon treatment of hypomagnesemic rats during week 1 led to further significant increases in SP levels at all examined times (days 3, 5, 6, and 7). Area integration of the time-course revealed that phosphoramidon-treated hypomagnesemic rats exhibited a greater than 2-fold increase in total SP (1.16 ng/ml/wk) vs the untreated low Mg group. Thus, NEP strongly influences SP bioavailability during early exposure to hypomagnesemia.

Phosphoramidon treatment of normomagnesemic rats (control) caused a moderate decline (26%) in circulating neutrophil NEP activity after 1 week, and 1 week of hypomagnesemia alone only resulted in a 15% loss (ns) of neutrophil NEP activity (Table 1). However, PMNs from phosphoramidon-treated hypomagnesemic rats exhibited a substantial reduction (50%, $p<0.01$) in neutrophil NEP activity after 1 week [14]. When the time of hypomagnesemia was increased to 3 weeks, PMNs displayed ~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, 1 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 3 weeks of low Mg diet. Although only a modest decrease in cardiac NEP was observed in 3 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 hypomagnesemic rats also exhibited heightened levels of inflammatory cell infiltration, and increased immunohistochemical staining for nitrotyrosine in perivascular 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, 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.

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LITERATURE CITED

1. Mantyh PW. Substance P & the inflammatory & immune response. *Ann NY Acad Sci.* 1991; 632:263–271. [PubMed: 1719871]
2. Bost KL, Pascual DW. Substance P: A late-acting B lymphocyte differentiation cofactor. *Am J Physiol Cell Physiol.* 1992; 262:C537–C545.
3. Weglicki WB, Phillips TM. Pathobiology of magnesium deficiency: a cytokine/neurogenic inflammation hypothesis. *Am J Physiol.* 1992; 263:R734–R737. [PubMed: 1384353]
4. Weglicki WB, Mak IT, Phillips TM. Blockade of cardiac inflammation in Mg-deficiency by substance P receptor inhibition. *Circ Res.* 1994; 24:1009–1013. [PubMed: 7512452]
5. Furness JB, Costa M, Papka RE, Della NG, Murphy R. Neuropeptides contained in peripheral cardiovascular nerves. *Clin Exp Hypertens [A].* 1984; 6(1–2):91–106.
6. Weglicki WB, Dickens BF, Wagner TL, Chmielinska JJ, Phillips TM. Immunoregulation by neuropeptides in MgD: *Ex vivo* effect of enhanced substance P production on circulating T cells from MgD mice. *Magne Res.* 1996; 9:3–11.
7. Mak IT, Kramer JH, Weglicki WB. Suppression of neutrophil and endothelial activation by substance P receptor blockade in the Mg-deficient rat. *Magne Res.* 2003; 16(2):91–97.
8. Scholzen TE, Steinhoff M, Bonaccorsi P, Klein R, Amadesi S, Geppetti P, Lu B, Gerard NP, Olerud JE, Luger TA, Bunnett NW, Grady EF, Armstrong CA, Ansel JC. Neutral endopeptidase terminates substance-P induced inflammation in allergic contact dermatitis. *J Immunol.* 2001; 166:1285–1291. [PubMed: 11145711]
9. Nadel, J. *Neurogen Inflamm.* CRC Press; 1996. Peptidase modulation of neurogenic inflammation; p. 115–127.
10. Iwamoto I, Kimura A, Ochiai K, Tomioka H, Yoshida S. Distribution of NEP activity in human blood leukocytes. *J Leuk Biol.* 1991; 49:116–125.
11. Lesser M, Fung K, Choi HS, Yoo OH, Cardozo C. Identification of two zinc metalloendopeptidases in alveolar macrophages of rats, guinea pigs, and human beings. *J Lab Clin Med.* 1992; 120:597–603. [PubMed: 1402335]
12. Lauderback CM, Hackett JM, Huang FF, Keller JN, Szweda LI, Butterfield DA. The glial glutamate transporter, GLT-1 is oxidatively modified by 4-HNA in the Alzheimer disease brain: the role of Aβeta 1. *J Neurochem.* 2001; 78:413–416. [PubMed: 11461977]
13. Wang DS, Iwata N, Hama E, Saido TC, Dickson DW. Oxidized neprilysin in aging & Alzheimer's disease brains. *Biochem Biophys Res Commun.* 2003; 310:236–241. [PubMed: 14511676]

14. Mak IT, Kramer JH, Chmielinska JJ, Khalid H, Landgraf K, Weglicki WB. Inhibition of neutral endopeptidase potentiates neutrophil activation during Mg-deficiency in the rat. *Inflamm Res*. 2008; 57(7):300–5. [PubMed: 18607539]
15. Kramer JH, Spurney C, Iantorno M, Tziros C, Mak I-T, Tejero-Taldo MI, Chmielinska JJ, Komarov AM, Weglicki WB. Neurogenic inflammation and cardiac dysfunction due to hypomagnesemia in a rodent model. *Am J Med Sci*. 2009 In Press.
16. Lu B, Gerard NP, Kolakowski LF Jr, Bozza M, Zurakowski D, Finco O, Carroll MC, Gerard C. Neutral endopeptidase modulation of septic shock. *J Exp Med*. 1995; 181:2271–2275. [PubMed: 7760013]

Figure 1.

Three weeks of hypomagnesemia decreases circulating neutrophil NEP activity (assayed using a fluorogenic peptide substrate) and increases basal superoxide anion generation (assayed by SOD-inhibitable cytochrome c reduction) in rats. Assay methods for can be found in reference 14. Values are means $\pm$ SE of 4–6 rats. * $p < 0.05$, ** $p < 0.01$ vs MgS control.

Figure 2A.

Figure 2B.

Figure 2.

(A) Immunohistochemical staining for NEP in heart (upper panels) and small intestine (lower) of 3 week MgS (left) & MgD (right) rats. Rabbit anti-NEP & FITC were used. (Mag. 20 $\times$). (B) Quantification (pixel number) of diminished NEP staining in the small intestines (A: lower panels) of 3 week MgD rats is evident (49 % decreased vs MgS, $p < 0.05$). Values are means $\pm$ SE of 5.

Figure 3.

NEP protein content in 3 and 5 week MgS and MgD rat cardiac ventricles. Homogenized tissue protein (70 μ g) was separated by SDS-page gel electrophoresis; transferred to PVDF membranes; probed with rabbit anti NEP antibody; incubated with donkey anti-rabbit; developed with ECL-plus. Values are means $\pm$ SE of 3–5. **: $p < 0.01$ vs MgS; †: $p = 0.0002$ for 5 week MgD vs 3 week MgD.

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 reference 3. The procedure for RBC glutathione measurements is described in reference 14. Values are means $\pm$ SE of 3–5 mice. ** $p < 0.01$, *** $p < 0.001$ vs MgS.

Figure 5.

Effect of NK-1 blockade on RBC glutathione levels in B6 [NEP(+/-) or wildtype] or NEP knockout [NEP(-/-)] mice on 1 week control (MgS) or low Mg (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 reference 14. Values are means $\pm$ SE of 4–5 mice. * $p < 0.05$ vs untreated MgS; + $p < 0.05$ vs untreated MgD.

Table 1

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

Parameter at dietary week one	Control	MgD (change vs cont)	MgD+PR (change vs cont)
Plasma SP	0.063 ± 0.01 (ng/ml/wk)	7.52 ± 0.82-fold Increase **	18.38 ± 1.74-fold Increase **
RBC GSH	6.78 ± 0.6 (nmol/mg Hgb)	7 ± 6 % decrease (ns)	21 ± 5% Decrease *
PGE ₂	281 ± 54 (pmol/ml)	1.6 ± 0.2-fold Increase *	2.67 ± 0.9-fold Increase *
PMN Superoxide Generation (basal)	133 ± 22 (pmol/min/10 ⁶ cells)	1.5 ± 0.3-fold Increase (ns)	3.9 ± 0.5-fold Increase **
PMN NEP Activity	4970 ± 538 (RFU/10 ⁶ cells)	15 ± 9% Decrease (ns)	50 ± 11 % Decrease *

Means ± SE of 4–6 rats which were placed on MgD (9 % RDA for Mg) or control 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 reference 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 method, 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 2

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

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

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 or 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.