N-Methyl-d-aspartate Receptor Blockade Inhibits Cardiac Inflammation in the Mg\(^{2+}\)-Deficient Rat

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**ABSTRACT**

Elevated plasma levels of the neuropeptide substance P (SP) precede the perivascular inflammatory infiltrate seen in hearts of Mg\(^{2+}\)-deficient (MgD) animals. The N-methyl-d-aspartate (NMDA) receptor is found in neurons, and activation of this receptor participates in SP release; under normal circumstances, this release can be blocked by Mg\(^{2+}\). Therefore, we reasoned that blockade of the NMDA receptor with dizolcipine maleate (a noncompetitive NMDA receptor antagonist) would prevent SP release from C-fibers due to MgD. In this study, animals were implanted with slow-release pellets containing dizolcipine or placebo and were fed with diet sufficient in Mg\(^{2+}\) or deficient with only 9% of USDA-recommended Mg\(^{2+}\). SP immunostaining of dorsal root ganglia showed a time-dependent depletion of SP in the MgD animals, with a dramatic decrease of SP by week 2; this depletion was prevented by pretreatment with dizolcipine maleate. The significant increase in plasma prostaglandin E\(_2\) levels during MgD was prevented by dizolcipine, and the loss of total red blood cell glutathione content was significantly attenuated by NMDA blockade after 3 weeks of MgD (p < 0.01 versus controls). Immunohistochemical and Western blot analyses of ventricular tissue demonstrated that NMDA receptor blockade abolished MgD-related increase of endothelium adhesion molecule CD54 (weeks 1 and 2; \(p < 0.05\)), and of monocyte/macrophage surface protein CD11b expression (week 3; \(p < 0.05\)). We conclude that NMDA receptor blockade with dizolcipine maleate prevented SP depletion and reduced perivascular inflammatory infiltrates, thus decreasing cardiac injury due to Mg\(^{2+}\) deficiency.

The importance of clinical Mg\(^{2+}\) deficiency (MgD) has long been recognized (Kruse et al., 1932); the average American diet has been shown to be deficient in Mg\(^{2+}\) (Lichton, 1989). A recent National Research Council survey of mean daily Mg\(^{2+}\) consumption revealed that intake is only 68% of RDA for women and 80% for men (National Research Council, 1999). Epidemiological studies suggest that populations consuming low levels of Mg\(^{2+}\) are at greater risk of morbidity after a myocardial ischemic response episode than populations consuming enriched Mg\(^{2+}\) (Leary, 1986; Rylander et al., 1991). Hypomagnesemia is also common in hospitalized patients: 63% of intensive care patients exhibited hypomagnesemia (Altura et al., 1994), and 45% of patients with acute myocardial infarction had low Mg\(^{2+}\) levels (Vormann et al., 1983). Mg\(^{2+}\) wasting also occurs in other segments of the population, such as diabetics, alcoholics, human immunodeficiency virus-infected patients, leading to overt hypomagnesemia (Altura and Altura, 1985; Dubey and Soloman, 1989; Seelig, 1989). Whereas treatment of MgD in most instances can be successful with supplementation therapy, if left undiagnosed or allowed to coexist with other Mg\(^{2+}\)-wasting diseases, the severity of MgD can be profound.

Evidence from our laboratory suggests that severe dietary Mg\(^{2+}\) restriction causes the release of substance P (SP) from neural stores and that specific antagonists of this neuropeptide receptor can ameliorate most of the cardiovascular pathology associated with this reduction (Weglicki et al., 1994a). Furthermore, Richardson et al. (2003) recently suggested that SP presence in the heart is limited to extrinsic nerve fibers only, most likely originating from neurons located in cervical and upper thoracic dorsal root ganglia (DRG). Activation of the N-methyl-d-aspartic acid (NMDA) receptor has been associated with SP release from the central processes of primary afferent neurons (whose bodies are lo-

**ABBREVIATIONS**: MgD, Mg\(^{2+}\) deficiency; RDA, recommended daily allowance; DRG, dorsal root ganglion; NMDA, N-methyl-d-aspartate; PGE\(_2\), prostaglandin E\(_2\); RBC, red blood cell; ICAM, intercellular adhesion molecule.
cated in the DRG) in the dorsal horn of the spinal cord (Liu et al., 1997), and with SP and calcitonin gene-related peptide release from peripheral afferent nerve endings (McRoberts et al., 2001). Under normal conditions, Mg\textsuperscript{2+} exerts a voltage-dependent block on the NMDA receptor channel (Crunelli and Mayer, 1984; Mayer et al., 1984), and reduced extracellular Mg\textsuperscript{2+} lowers the threshold for its physiological activation (Nowak et al., 1984). This enhanced activation of the NMDA receptor could be responsible for triggering the neurogenic cardiovascular inflammation associated with dietary Mg\textsuperscript{2+} restriction. Thus, the increased release of neurotransmitters, including SP, would possibly exaggerate cellular responses, resulting in increased production of oxidative stress mediators, such as cytokines, oxy-radicals and nitric oxide, which could subsequently cause significant tissue injury. Still, the specific role of NMDA receptor activation in the inflammatory response seen in severe Mg\textsuperscript{2+} deficiency remains unclear. Thus, we propose that blockade of the NMDA receptor with the noncompetitive antagonist dizocilpine maleate in dorsal root ganglia neurons would abolish cardiac inflammation due to severe dietary deficiency of Mg\textsuperscript{2+} in the rat.

Materials and Methods

Experimental Protocol

All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (publication 85-23, revised 1996). Male Sprague-Dawley outbred rats (Harlan, Indianapolis, IN) about 7 weeks old were housed under 12-h light/dark cycle, with free access to bidistilled water and food. After 1-week adaptation, animals were randomly assigned to the different experimental groups. Dizocilpine maleate (also known as MK-801; Sigma-Aldrich, St. Louis, MO) was obtained as powder and then formulated as slow-release pellets (Innovative Research of America, Sarasota, FL), equivalent to a release dose of 0.5 mg/kg/day. Pellet matrix alone was used as placebo. Pellets were subcutaneously implanted on day 1 as described previously (Weglicki et al., 1994b). After the surgery, animals were fed with rat Chow containing 100 or 9% of RDA of Mg\textsuperscript{2+} content. Four treatment groups were examined in this study: animals implanted with placebo pellets were fed control diet (MgS; n = 15) or rat Chow with 9% of RDA Mg\textsuperscript{2+} (MgD; n = 15); and rats implanted with dizocilpine maleate pellets were fed control diet (S-Diz; n = 9) or rat Chow with 9% of RDA Mg\textsuperscript{2+} (D-Diz; n = 16). Treatment lasted 1, 2, or 3 weeks, when the animals were sacrificed with an overdose of ether. Of 55 animals used in the study, only one died during ether anesthesia. Once removed, hearts were dissected immediately: one-half were snap-frozen in liquid N\textsubscript{2} and the other half was embedded in OCT compound and frozen in methyl-butane cooled in dry ice. Upper thoracic and lower cervical DRG were removed and snap-frozen in liquid N\textsubscript{2}. All tissue samples were stored at −70°C until further use.

Plasma PGE\textsubscript{2} and Red Blood Cell (RBC) Glutathione

When under ether anesthesia, heparinized blood samples were withdrawn from vena cava. The RBCs were centrifuged within 20 min to be separated from the plasma. The plasma samples were quickly frozen and stored at −70°C for later analysis of PGE\textsubscript{2}. Total RBC glutathione was determined by the enzymatic recycling method as described previously (Muk et al., 1994, 1996). Red cell hemoglobin was determined by the cyanmethemoglobin method using Sigma Diagnostics hemoglobin reagent. Plasma PGE\textsubscript{2} levels were determined quantitatively by a PGE\textsubscript{2} enzyme-linked immunosorbsent assay kit (R&D Systems, Minneapolis, MN).

Immunohistochemistry

Immunofluorescence Assays. OCT-embedded cardiac and DRG tissues were sliced into 8- to 10-μm sections. In situ localization of SP and ICAM was accomplished with indirect fluorescent immunohistochemistry. Primary antibodies against SP (1:500; rabbit) and ICAM (1:50; mouse) were acquired from Chemicon International (Temecula, CA). Air-dried tissue sections were rinsed in Tris-buffered saline (pH 7.5) and incubated 45 min in blocking buffer (10% goat serum, 1% bovine serum albumin, 0.05% Triton-X100 in Tris-buffered saline). All antibodies were diluted in blocking buffer before addition to tissue sections. After overnight incubation with primary antibody at 4°C, tissue sections were washed and incubated at room temperature in a dark humidity chamber with secondary antibody conjugated with fluorescein isothiocyanate, Alexa 488, or Texas Red-Alexa 590 (1:500; Molecular Probes, Eugene, OR) for 1 h. Once washed and dried, the slides were mounted using Vectashield H-1000 (Vector, Cheshire, UK) as mounting medium. Samples were then examined under a fluorescence microscope (Olympus BX60; Olympus America Inc., Melville, NY) and multiple microphotographs taken with an Evolution Color MP camera (Media Cybernetics, Silver Spring, MD). Negative controls were included in all assays.

Immunoperoxidase Staining Protocol. Vectastain Elite ABC kit immunoperoxidase system (Vector Laboratories, Burlingame, CA) was used for CD11b localization on ventricular sections. Sections were always washed between steps. Frozen cardiac tissue sections (8 μm in thickness) were treated with 3% hydrogen peroxide for 3 min to quench endogenous peroxidase and incubated for 30 min with normal serum followed by overnight incubation with mouse anti-rat α-M Integrin (CD11b) antibody (1:200; Chemicon International). Next day, sections were incubated 10 min with biotinylated secondary antibody, followed by 5-min incubation with Vectastain Elite ABC reagent and finally submerged in peroxidase substrate solution to optimal stain development and counterstained with hematoxylin before mounting and examination under the microscope.

Western Blot Analyses of Ventricular Tissue

Snap-frozen heart tissue (n = 4–6/treatment group) was homogenized at 4°C with a tissue homogenizer in 5 volumes of radioimmunoprecipitation assay buffer, centrifuged at 33,000g for 15 min, and the pellets were discarded (Tejero-Taldo et al., 2002). Supernatant samples containing 50 or 75 μg of proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane as described previously (Tejero-Taldo et al., 2002). Membranes were probed overnight with specific antibodies against ICAM (1:100; mouse monoclonal; Serotec, Oxford, UK) and CD11b (1:200, goat; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by incubation with horseradish peroxidase-conjugated donkey anti-mouse (1:2000; Amersham Biosciences Inc.), followed by incubation with horseradish peroxidase-conjugated goat anti-rat (1:2000; Amersham Biosciences Inc.) antibodies. Membranes were developed using enhanced chemiluminescence (ECL Plus; Amersham Biosciences Inc.), and exposed to X-ray film. Optical densitometric evaluation was performed using a Personal Densitometer SI (Amersham Biosciences Inc.) and computerized analysis system (ImageQuant version 5.2; Amersham Biosciences Inc.). Arbitrary units calculated by the software for each band were normalized against background.

Statistical Analysis

Data are expressed as means ± S.E.M. Differences among treatment groups were analyzed with parametric one-way analysis of variance. When statistical significance was reached (p < 0.05), the analysis was completed with Bonferroni’s multiple comparison test. All statistical analyses were performed using GraphPad Prism (version 3.03; GraphPad Software Inc., San Diego, CA).
Results

Substance P Immunofluorescence Changes in Dorsal Root Ganglia with NMDA Receptor Blockade in the Mg\(^{2+}\)-Deficient Rat. Frozen sections (5–8 \(\mu\m) \(n=2–4/rat\) of lower cervical and upper thoracic DRG were immunostained with SP antibodies at the end of weeks 1, 2, and 3 of the experimental protocol. A constant and diffuse pattern of positive green fluorescence is appreciable in MgS animals at all times (Fig. 1). Severe MgD resulted in apparent decrease of fluorescence over time, with almost nonexisting green staining by the end of week 2 (MgD; Fig. 1). Concurrent treatment in vivo with dizolcipine maleate (NMDA receptor antagonist) did not cause any apparent changes on SP immunostaining of DRG (S-Diz; Fig. 1), importantly, it seemed to prevent the loss of fluorescent staining in DRG of MgD rats at weeks 2 and 3 of the experimental protocol (D-Diz; Fig. 1).

NMDA Blockade-Related Changes in Mg\(^{2+}\) Deficiency-Induced Systemic Stress. Total RBC glutathione and plasma PGE\(_2\) concentration were used as indexes of systemic stress and were measured at the end of week 3 of treatment. Severe MgD resulted in a significant depletion of RBC glutathione from 6.11 \(\pm\) 0.30 \(\mu\mol/g\ Hb\) in MgS controls to 3.09 \(\pm\) 0.22 \(p<0.001\). Concurrent treatment with dizolcipine maleate of animals on control diet (S-Diz) had no effect on total RBC glutathione levels (5.86 \(\pm\) 0.46 \(\mu\mol/g\ Hb\)), but it provided significant attenuation of RBC glutathione loss in the deficient animals (D-Diz: 4.19 \(\pm\) 0.35 \(\mu\mol/g\ Hb\), \(p<0.05\); Fig. 2A).

Plasma concentration of PGE\(_2\) was also measured in all animals at the end of week 3. Compared with MgS (336 \(\pm\) 44 pg/ml sample), PGE\(_2\) plasma concentration in the MgD animal reached almost 300% of control levels (930 \(\pm\) 122 pg/ml sample, \(p<0.001\); Fig. 2B). NMDA receptor blockade had no effect on plasma PGE\(_2\) levels (343 \(\pm\) 53 pg/ml sample) in control animals but completely prevented its elevation in the MgD animal (343 \(\pm\) 62 pg/ml sample, \(p<0.01\); Fig. 2B).

Changes in the Endothelial Adhesion Molecule ICAM Expression Levels in the Mg\(^{2+}\)-Deficient Rat. Adhesion molecules expressed on the surface of endothelium bind specifically to certain white blood cells, thus starting the extravasation process. ICAM (CD54) is an endothelial adhesion molecule that specifically binds cells of the monocyte/macrophage line, thus promoting their migration to extravascular spaces. Immunohistochemical analysis of ICAM presence in the ventricles of MgD animals showed a very apparent increase in ICAM immunostaining by the end of week 1, reaching peak staining intensity by the end of week 2 (Fig. 3). Western blot analyses of ventricular tissue showed a significant 1.5-fold increase in ICAM protein expression in the MgD animal by the end of week 1 (\(p<0.05\) versus MgS), increasing to 2-fold by the end of week 2 (\(p<0.05\) versus MgS; data not shown), and then returning to control levels by the end of week 3.

Concurrent treatment with dizolcipine maleate of control animals resulted in no appreciable changes in ICAM immunostaining of ventricular sections compared with matched controls, and throughout the length of the experimental protocol (Fig. 3). Also, no difference in ICAM protein expression was found on Western blot analyses between placebo- and dizolcipine-treated control animals (data not shown). Dizolcipine treatment of MgD animals, however, resulted in a marked decrease in ICAM immunostaining of the heart, compared with MgD placebo animals, at the end of weeks 1 and 2 (Fig. 3). Western blot analyses also revealed that dizolcipine prevented ICAM protein expression increase in MgD animals by the end of weeks 1 (\(p<0.05\) versus MgD) and 2 (\(p<0.05\) versus MgD; data not shown).

Changes in the Presence of Inflammatory Cells in the Heart of Mg\(^{2+}\)-Deficient Rats Treated with Dizolcipine Maleate. Western blot analyses and immunohistochemical localization on ventricular sections of the monocyte/macrophage cell surface marker CD11b (\(\alpha\)M-Integrin) were

![Fig. 1. SP immunostaining of the dorsal root ganglia. Cervical and upper thoracic DRG collected from placebo animals on control diet (MgS; 4–6) or on diet low in Mg\(^{2+}\) (MgD; 4–6) and from animals treated with dizolcipine both on control diet (S-Diz; 3) or on MgD diet (D-Diz; 4–6) were cut into 8-\(\mu\m) sections. DRG sections were indirectly immunostained for SP with antibody conjugated with Alexa 488 (green fluorescence). Microphotographs are arranged by treatment group (columns) and length of treatment (rows). Original magnification, 10\(\times\).]
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Fig. 2. Indexes of systemic stress. A, total RBC glutathione, measured at 3 weeks of treatment ($n = 3–6$), as explained under Materials and Methods. B, plasma concentration of PGE$_2$, measured at the end of week 3 ($n = 3–6$), as described under Materials and Methods. MgS, placebo animals on control diet (4–6); MgD, placebo-treated animals on MgD diet (4–6); S-Diz, rats on control diet treated with dizolcipine (3); D-Diz, dizolcipine-treated rats on MgD diet (4–6). *** $p < 0.001$ versus MgS; †† $p < 0.01$ versus MgD.

Discussion

We examined the role of NMDA receptor-mediated SP release on systemic stress and cardiac inflammatory disease secondary to severe MgD, by using the noncompetitive NMDA receptor antagonist dizolcipine maleate. MgD resulted in loss of SP immunofluorescence in DRG, decreased RBC glutathione content, and a significant elevation of plasma PGE$_2$. Concurrently, in the heart, MgD led to increased ICAM expression and CD11b+ cell infiltration. Dizolcipine blockade of NMDA receptors prevented SP immunofluorescence loss in DRG, ameliorated the decrease in RBC glutathione, and prevented plasma PGE$_2$ elevation. In the MgD heart, dizolcipine prevented ICAM expression increase and greatly reduced CD11b+ cell infiltration.

Prior studies from our laboratory identified SP as a key mediator in the pathological changes seen during severe MgD (Weglicki et al., 1994a,b). Early increase in SP plasma levels during severe MgD was accompanied by increased plasma calcitonin gene-related peptide, thus suggesting a neural origin for SP (Weglicki et al., 1994a). In the present study, we examined the presence of SP immunofluorescence in the DRG of MgD rats. We found a decrease in SP immunofluorescence already apparent by the end of week 1 that was further apparent at the end of week 2, with SP immunofluorescence being almost nonexistent by the end of week 3. However, taken alone, these data do not directly implicate neural SP in MgD pathology. Thus, based upon the fact that presynaptic NMDA receptors mediate, at least in part, SP release from neural terminals (Juranek and Lembeck, 1996; Liu et al., 1997; Marvizon et al., 1997), we treated MgD animals with the noncompetitive NMDA-receptor blocker dizolcipine maleate. Blockade of the NMDA receptor in MgD animals resulted in conservation of SP immunofluorescence in DRG of these animals throughout the length of the experiment. Furthermore, NMDA receptor blockade clearly ameliorated the inflammatory changes seen in MgD hearts, preventing ICAM expression increase and reducing CD11b+ positive cellular migration into the cardiac tissue. NMDA blockade also ameliorated the RBC glutathione loss and PGE$_2$ plasma concentration elevation.

RBC glutathione, a key index of systemic oxidative stress, was depleted significantly in the MgD rats probably due to excessive oxy-radical production by the activated inflammatory cells (Mak et al., 2003). In the present study, blockade of the NMDA receptor partially, but significantly, attenuated the glutathione loss, suggesting a direct link between NMDA activation and the inflammatory cascade leading to elevated cellular production of free radicals. In a previous study (Mak et al., 2003), we observed that the loss of the glutathione was effectively prevented by SP-receptor blockade. The combined data suggest that SP released from the neurons subsequent to NMDA receptor activation plays a critical role in promoting systemic oxidative stress. We have reported that the elevated PGE$_2$ during MgD is derived, in part, from the activated endothelium and that its induction is subjected to the similar cascade of events governed by SP (Mak et al., 2003). The

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The present study seems to suggest that such SP-governed cascade leading to PGE$_2$ elevation is completely linked to the upstream event of NMDA receptor activation.

During MgD, inflammatory cell infiltration of the myocardium is already apparent after only 1 week on MgD diet (Kurantsin-Mills et al., 1997). Because a change in vascular endothelium, with increased expression of adhesion molecules, usually precedes white blood cell migration into the heart (Steinhoff et al., 1991), we studied ICAM presence in ventricular tissue. We observed a significant increase in ICAM at 1 and 2 weeks, with a return to baseline levels by the end of week 3, in the MgD animal. Furthermore, we examined inflammatory cell infiltration of the ventricular tissue by monitoring CD11b$^+$ cells and found a steady increase in CD11b$^+$ cells in the MgD animal. Blocking SP release from neural stores with dizolcipine prevented the increase of ICAM expression in the heart and also greatly decreased the presence of CD11b$^+$ cells in the myocardium, suggesting a prominent role of neural SP in the pathogenesis of cardiac inflammation secondary to severe MgD.

![Fig. 3. Texas-Red indirect immunostaining of the adhesion molecule CD54 (ICAM) in ventricular myocardium. Cardiac ventricle sections were immunoprobed for CD54 (ICAM-Red) presence with antibody bound to Texas-Red. MgS, placebo animals on control diet (4–6); MgD, placebo-treated animals on MgD diet (4–6); S-Diz, rats on control diet treated with dizolcipine (3); D-Diz, dizolcipine-treated rats on MgD diet (4–6). Microphotographs are arranged by treatment group (columns) and length of treatment (rows). Original magnification, 20×.](image1)

![Fig. 4. Immunohistochemical localization and Western blot analyses of αM integrin (CD11b): expression in ventricular myocardium after 3 weeks of treatment. Tissue sections (8 μm) were indirectly probed for CD11b and visualized with an immunoperoxidase staining protocol (brown in microphotograph; see Materials and Methods) counterstained with hematoxylin (blue). Representative micrographs for MgS (A), MgD (B), S-Diz (C), and D-Diz (D) experimental groups are presented. E, Western blot analysis of CD11b protein expression in ventricular myocardium at the end of 3 weeks of treatment with representative bands. MgS, placebo animals on control diet (4–6); MgD, placebo-treated animals on MgD diet (4–6); S-Diz, rats on control diet treated with dizolcipine (3); D-Diz, dizolcipine-treated rats on MgD diet (4–6). ***p < 0.001 versus MgS. †p < 0.05 and ††p < 0.01 versus MgD.](image2)
In summary, blockade of the NMDA receptor with dizocilpine prevented the loss of SP immunofluorescence in the DRG of MgD animals and prevented the appearance of systemic oxidative stress and cardiac inflammation. We conclude that local cardiac inflammation and systemic stress in the Mg\textsuperscript{2+}-deficient rat are the result of SP release from nerve stores due to the decreased inhibition by Mg ions on the NMDA receptor.

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References


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