Rapid Communication

Blockade of Cardiac Inflammation in Mg$^{2+}$ Deficiency by Substance P Receptor Inhibition

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Abstract In previous work we reported the elevation of circulating inflammatory cytokines in rodents maintained on a Mg$^{2+}$-deficient diet. Within the first week of Mg$^{2+}$ deficiency, significant elevation of the neuropeptides substance P (SP) and calcitonin gene--related peptide (CGRP) occurs. The present study was designed to assess the effects of SP receptor blockade by CP-96,945 and its inactive enantiomer CP-96,344 on tissue cytokine levels and in vivo oxidative indexes. CP-96,345 had no significant effect on circulating levels of SP or CGRP; however, at the tissue level, a significant decrease ($P<.01$) in myocardial accumulation of SP occurred; the inactive enantiomer was only slightly effective. In addition, CP-96,345 significantly reduced (by 53%) the accumulation of tumor necrosis factor-α (TNF-α) (but not interleukin-1 and interleukin-6) within the lesions; the effect of the enantiomer was insignificant. We conclude that treatment with CP-96,345 inhibits SP and TNF-α tissue levels in cardiac lesions, indicating a linkage between this neuropeptide and TNF-α. Both SP and TNF-α can trigger free radical production; plasma thiobarbituric acid--reactive materials were elevated 2.5-fold and red blood cell reduced glutathione was reduced 55% during Mg$^{2+}$ deficiency. In the presence of CP-96,345, both indexes of in vivo oxidation were significantly attenuated; the enantiomer was ineffective. These latter observations point to a neuropeptide/TNF-α/free radical--triggered mechanism that may be the major pathway of systemic oxidative injury inducing the cardiomyopathic lesions seen during Mg$^{2+}$ deficiency. (Circ Res. 1994;74:1009-1013.)

Key Words • neuropeptides • cytokines • glutathione • Mg$^{2+}$ deficiency • free radicals • CP-96,345 • CP-96,344

We have studied the effects of Mg$^{2+}$ deficiency on the formation of focal inflammatory lesions in the heart in a rodent model.1-4 During the progression of Mg$^{2+}$ deficiency, we observed dramatic increases in circulating levels of inflammatory cytokines (interleukin-1 [IL-1], IL-6, and tumor necrosis factor-α [TNF-α]).5,6 Time-course studies demonstrated that these cytokine elevations occurred beginning at day 12 and reached their highest levels by day 21. Before these events, elevations of circulating levels of substance P (SP) occurred after only 5 days on the diet;7 this neuropeptide is known to stimulate the production of several mediators of inflammation, such as inflammatory cytokines, histamines, and prostaglandin E2.8,9 We postulated that neurogenic inflammation may be the central mechanism of injury in this model.6,7 Recently, we developed a microimmunochemical technique for measuring the levels of cytokines and neuropeptides in cardiac tissue. This manuscript presents our observations on the effects of SP receptor blockade by CP-96,345 and its inactive enantiomer CP-96,344 on the accumulation of cytokines and neuropeptides in cardiac tissue during the progression of Mg$^{2+}$ deficiency.

Materials and Methods

Male Sprague-Dawley rats (100 to 120 g) were housed in groups of five animals per cage and were kept under a 12/12-hour light/dark cycle with food and deionized water given ad libitum. Animals were fed either a modified diet containing low (<1 mmol/kg) MgCl$_2$ (Mg$^{2+}$-deficient group) or the same diet supplemented with 100 mmol/kg MgCl$_2$ (Mg$^{2+}$-sufficient group). Both diets were obtained from Teklad Inc. The two agents, CP-96,345 and CP-96,344 (Pfizer Inc.), were formulated into sustained-release pellets (3.5 mg drug each) by Innovative Research and were implanted subcutaneously at the onset of the dietary protocol. At 1, 2, and 3 weeks on the diets, blood and cardiac tissue were obtained after anesthesia with ether.

Microdissection of Cardiac Biopsy Sections

Five serial transverse cryostat sections (10 μm thick) were prepared from liquid nitrogen--snap-frozen myocardium and placed on cold (~5°C) Mylar coverslips. Each section was briefly stained in 0.01% aqueous cotton blue (to aid in the morphological identification of the tissue) before being placed into a precooled microincubation chamber (Narishige). Defined morphological areas, either inflammatory lesions (defined by a dense perivascular infiltration of inflammatory host cells associated with morphological irregularities in the surrounding myocytes) or normal myocardial tissue (5 mm from the lesion area), were morphologically identified and microdissected from the surrounding tissue with an M-155 glass needle micromanipulator (Narishige). After microdissection, each area of interest was flooded with 25 μL of warm (22°C) buffer (100 mmol/L phosphate buffer, pH 7.0, containing 0.2% Nonidet P-40) administered with an M-6 microinjection system (Narishige). Recovery of the injected fluid was performed with the same instrument and clarified by centrifugal ultrafiltration through a 30-kD filter (10 000g for 10 minutes in a Beckman Airfuge), and the filtrate was analyzed by high-performance capillary electrophoresis (HPCE). Before HPCE analysis, the total protein concentration of each sample was measured by direct spectrophotometry at 280/260 nm and normalized to 10 μg protein/mL.

HPCE Analysis of Microdissected Samples

All separations were performed on an ISCO 3140 capillary electrophoresis system using the integrated method and analy-
sis program supplied by the manufacturer. Moderately hydrophobic internal surface fused-silica capillaries (CE100; outer diameter, 375 μm; inner diameter, 75 μm) were obtained from IScO and cut to a standard length of 75 cm (50 cm to the detector cell). HPCE separations were performed at controlled capillary chamber temperatures of 15°C; integrations of the isolated peaks were analyzed by the data management program provided with the instrument. Chromatograms were stored as ASCII files and plotted using INPLOT 4.03 (GraphPad Software, Inc) and a Hewlett-Packard I1P laser-jet printer.

Samples (20 nL) were introduced by vacuum injection into the capillary, which had previously been filled with 100 mmol/L phosphate buffer; the sample components were separated at a 150-μA constant current. The migration of the separated components was monitored by on-line UV detection at 200 nm, and the resulting electropherogram was read directly into a computerized recording system.

Fraction Collection of HPCE-Separated Peaks

Separated peaks were recorded by collecting the migration time of each peak and then calculating its elution time (t_e) from the following formula:

\[ t_e = \frac{L}{L_d} t_i \]

where L is the total length of the capillary, L_d is the capillary length to the detector, and t_i is the time when the peak passes the detector.

Fractions were collected by interrupting the current just before the peak elution time and placing the detector end of the capillary and the electrode into a 75-μL vial containing 10 μL of running buffer, which was placed under 10 μL of mineral oil. The current was reapplied, and the peak was forced to electromigrate into the collection vial. This process was repeated for each peak.

Chemiluminescence-Enhanced Immunoassay of HPCE-Isolated Cytokines

Cytokines and neuropeptide concentrations in fractions collected from each HPCE separation were measured by chemiluminescence-enhanced enzyme-linked immunosassay (CHEM-ELISA) by use of specific antibodies directed against each cytokine (R&D Systems) or neuropeptides (Chemicon International, Inc). Each IgG fraction of these antibodies was labeled with alkaline phosphatase (Sigma Chemical Co) before use. The CHEM-ELISA was performed in the following manner: the contents of each fraction were biotinylated with hydroxysuccinimide-biotin (Pierce) and immobilized on avidin-coated microtiter strips. Each strip was incubated with a 1:1000 dilution of an alkaline phosphatase-labeled specific antibody for 2 hours at room temperature. The strips were washed five times in 100 mmol/L phosphate buffer (pH 7.2) and 0.2% Tween 20, and the bound antibodies were detected after their reaction with 50 μL of 0.25 mmol/L AMPPD, an alkaline phosphatase chemiluminescence substrate (Tropix). The CHEM-ELISA results were analyzed by the ANELISA-R software package (Man-Tech Associates).

Determinations of Peroxides and Glutathione Levels

Total red blood cell glutathione was determined by the enzymatic “recyclic method” of Griffith,10 as described previously.11,12 Plasma peroxide levels were measured by the thiobarbituric acid method as described previously.11,13

Results

Fig 1 depicts the time course of increases of SP (panel A) and CGRP (panel B) in cardiac tissue at the end of the first, second, and third weeks on the Mg2+-deficient diet; both lesion levels and nonlesion (normal) adjacent tissue levels are depicted. Microdissection of the Mg2+-deficient hearts allowed us to obtain tissue samples several millimeters away from lesion-containing areas and to compare their cytokine and neuropeptide concentrations with those found in lesion-containing areas. By use of HPCE to isolate the individual peptides before quantitative measurement by CHEM-ELISA, five lesion areas were microdissected from each of the five serial ventricular myocardium sections obtained from each animal (total of 25 lesions per animal). In addition, 25 identical nonlesion (normal myocardium) areas were examined from the same sections. Microdissection followed by HPCE recovery of both neuropeptides and cytokines was estimated to be between 90% and 95% of the total materials when compared with CHEM-ELISA analysis of the residual materials present in the microdissected areas after the initial extraction. The precision of the technique, tested by running replicate samples (n=5) on both the same and three alternate days, gave coefficients of variation for within-run precision at 3.32% and for between-run precision at 4.05%. Using this technique, we have demonstrated that the calcitonin gene–related peptide (CGRP) levels in the lesion areas were substantially higher (64.9±11.5 pg/μg protein) than the SP levels (8.1±3.2 pg/μg protein) at the end of the first week; this pattern continued through the end of the second week, when the CGRP levels were even higher (115±14.5 pg/μg protein), whereas the SP levels were only 19.5±7.7 pg/μg protein. This pattern was reversed at the end of the third week, when the levels of SP in the lesion areas were dramatically increased (Fig 1A) but only moderate elevations of CGRP (Fig 1B) levels persisted. The Mg2+-deficient tissue revealed only trace amounts of these neuropeptides (<5 pg/μg protein) during the entire 3-week period.
whereas CP-96,344 produced only a moderate, though significant, decrease from 61.3±6.5 to 42.0±3.3 pg/μg protein; CP-96,344–treated animals exhibited an intermediate response (21.4±1.2 pg/μg protein).

Fig 3 shows the effects of CP-96,345 and CP-96,344 on TNF-α, IL-1, and IL-6 levels in the cardiac lesions. The most dramatic effect was seen on TNF-α (Fig 3A), which fell from 56.8±1.9 to 28.4±2.1 pg/μg protein with CP-96,345; a moderate but significant inhibiting effect on IL-1 was produced by CP-96,345. CP-96,344 did not alter TNF-α, IL-1, or IL-6 levels.

The effects of CP-96,345 and CP-96,344 on changes in plasma thiobarbituric acid–reactive (TBAR) materials and red blood cell glutathione levels during Mg 2+ deficiency are shown in Fig 4. The TBAR contents in the Mg 2+-sufficient rats were maintained unchanged at 0.83±0.03 nmol/mL plasma. At 21 days, the TBAR values of the Mg 2+-deficient rats rose significantly (P<.001) to 2.28±0.4 nmol/mL; however, the increase in TBAR level due to Mg 2+ deficiency was significantly (P<.01) reduced by CP-96,345 but not by CP-96,344 (Fig 4A). In association with the observed elevated plasma peroxide accumulation, red blood cell glutathione of the Mg 2+-deficient animals decreased 55% (from 1.60±0.22 to 0.74±0.15 nmol reduced glutathione per milliliter packed red blood cells, P<.001 in the Mg 2+-sufficient samples). Fig 4B also indicates that the loss of red blood cell reduced glu-

Discussion

Our previous work using CP-96,345 revealed that circulating levels of SP and CGRP were not affected by CP-96,345 but not by its enantiomer, CP-96,344.

**Fig 2.** Bar graphs showing effects of CP-96,344 and CP-96,345 on levels of substance P (A) and calcitonin gene–related peptide (B) in cardiac lesions from Mg 2+-deficient rats at the end of 21 days on the deficient diet. Other conditions are as described in Fig 1. **P<.01 vs Mg 2+-deficient placebo group.

**Fig 3.** Bar graphs showing effects of CP-96,344 and CP-96,345 on tumor necrosis factor-α (A), interleukin-1 (B), and interleu-
in-6 (C) levels in cardiac lesions of the deficient rats. Other conditions are as described in Fig 2. **P<.01 vs Mg 2+-deficient placebo group.

**Fig 4.** Bar graphs showing effects of CP-96,344 and CP-96,345 on Mg 2+-deficiency–induced increase in plasma thiobarbituric acid–reactive (TBAR) materials (A) and loss of red blood cell (RBC) total glutathione (B). Tissue samples were obtained from the rats fed the diets for 21 days. **P<.01 vs Mg 2+-deficient placebo group.
vascular pro-oxidant processes would also be suppressed. CP-96,345, also indirectly from SP receptors, isomer to CP-96,344 (provided by Pfizer) was estimated to be 85%, the possibility remains that the small effect produced by the inactive isomer was due to racemic contamination.

The present study also supports the importance of assessing tissue levels of neuropeptides and cytokines rather than circulating levels. Indeed, there was a discordance when one compares the effects of CP-96,345 on the circulating levels of neuropeptides with levels in the myocardial tissue, particularly in the lesion areas. These findings support the notion that circulating cytokines represent the “tip of the iceberg,” which was emphasized by Cavallion et al. The sensitivity provided by HPEC separation of microdissected tissue samples allowed the quantification of both neuropeptides and cytokines directly in these localized areas of inflammation. By this unique approach, we are now able to discriminate tissue-nonspecific effects (eg, calcium channel blockade) from those due to SP receptor blockade in this model. Our data indicate that the inactive enantiomer (CP-96,344), which has calcium channel-blocking ability, has only a slight effect on cytokines, SP, and CGRP levels in cardiac tissue. Neuropeptide receptors are widely distributed in most tissues, especially on vascular and perivascular cells (eg, endothelial cells, mast cells, and leukocytes). In the present study, accumulation of SP was detected in the cardiac vascular and perivascular sites. Although the detailed cellular events leading to increased tissue SP level remain to be determined, the data suggest that increased SP accumulation could arise directly from upregulation of SP receptors in the target cells during Mg deficiency and indirectly from an increased number of infiltrated inflammatory cells with bound SP. In the presence of CP-96,345, the increase in the receptor-trapped SP levels due to both processes would presumably be suppressed. These data also support the argument that activation of SP receptors initiates pro-oxidant events during Mg deficiency, since CP-96,345, but not the inactive enantiomer CP-96,344, produced major attenuating effects on parameters of free radical stress (TBAR materials and red blood cell glutathione levels).

Our present findings support the following mechanism and time frame of injury: During the first week on the Mg deficient diet, substantial elevations of circulating SP and CGRP are probably due to a release of stored forms of these peptides from C fibers within the heart and other tissues. The circulating levels are not affected by CP-96,345; however, significant inhibition of receptor-mediated accumulation of tissue neuropeptides and cytokines occurs. In addition, circulating blood levels of TBA materials and glutathione are sensitive indicators of drug effects. Conceivably, SP can directly stimulate the production of inflammatory cytokines and free radical generation from macrophages and neutrophils. The inflammatory cytokines, particularly IL-1 and TNF-α, are also known to trigger superoxide formation in cells with receptors for these cytokines. Finally, since blockade with CP-96,345 was very effective in reducing parameters of oxidative stress, we propose that neurogenic inflammation is a major contributor to the cardiomyopathy of Mg deficiency.

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