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Short-term magnesium deficiency upregulates ceramide synthase in cardiovascular tissues and cells: cross-talk between cytokines, Mg^{2+},NF-kB and de novo ceramide

Burton M. Altura, [1,2,3,4,5] **Nilank C. Shah,**[1] **Gatha Shah,**[1] **Aimin Zhang,**[1] **Wenyan Li,**[1] **Tao Zheng,**[1] **Jose Luis Perez-Albela,**[6] **and Bella T. Altura**[1,3,4,5]

Departments of [1] *Physiology and Pharmacology,* [2] *Medicine,* [3] *The Center for Cardiovascular and Muscle Research, and* [4] *The School of Graduate Studies Program in Molecular and Cellular Science, State University of New York Downstate Medical Center, Brooklyn, New York,* [5] *Bio-Defense Systems ,Inc, Rockville Centre, New York , and* [6] Instituto Bien de Salud, Lima, Peru

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Send all correspondence to: Dr. B.M. Altura, Box 31
SUNY Downstate Medical Center
450 Clarkson Ave.
Brooklyn, NY 11203
Email:baltura@downstate.edu

ABSTRACT

Altura BM, Shah NC, Shah G, Zhang A, Li W, Zheng T, Perez-Albela JL, Altura BT. Short-term magnesium deficiency upregulates ceramide synthase in cardiovascular tissues and cells: cross-talk between cytokines ,Mg^{2+} ,NF-kB and de novo ceramide. *Am J Physiol Heart Circ Physiol 000: 000-000, 2011.* The present study tested the hypotheses that 1) short-term dietary deficiency(MgD) of magnesium(21 days) would result in the upregulation of ceramide synthase(CS) in left ventricular**(LV)**, right ventricular, atrial, and aortic smooth muscle(AM), as well as induce a synthesis/release of select cytokines and chemokines into **the** LV,AM and serum , **2)**exposure of primary cultured vascular smooth muscle cells(VSMCs) to low extracellular Mg concentration would lead to the synthesis/release of select cytokines/chemokines , activation of NF-kB and the de novo synthesis of ceramide , **3)**inhibition of CS by fumonisin B1(FB1) or inhibition of N-sphingomyelinase(N-SMase) by scyphostatinSCY) in VSMCs exposed to low Mg would result in reductions in the levels of the cytokines/chemokines and lowered levels of ceramide concomitant with inhibition of NF-kB activation. The data indicated that short-term MgD(10 % normal dietary intake) resulted in the upregulation of CS in ventricular, atrial and aortic smooth muscles coupled to the synthesis/release of 12 different cytokines/chemokines as well as activation of NF-kB in the LV, AM ,and sera; even very low levels of water-borne Mg(e.g., 15 mg/l/day) either prevented or ameliorated the upregulation and synthesis of the cytokines/chemokines . Our experiments also showed that VSMCs exposed to low extracellular Mg resulted in the synthesis of 12 different cytokines and chemokines concomitant with synthesis/release of ceramide. However, inhibition of the synthesis and release of ceramide by either FB1 or SCY attenuated, markedly , the generation of ceramide ,release of the cytokines/chemokines ,and activation of NF-kB (as measured by activated p65 & cRel).

cardiac muscle ; vascular muscle ; p65; cRel; N-SMase; water- borne magnesium

Improper nutrition, high cholesterol intake and fatty diets are known to promote lipid deposition and accelerate growth and transformation of smooth muscle cells (SMCs) in the vascular wall (20, 37 , 39). Over the past five decades, an accumulation of epidemiological and experimental data have indicated that a reduction in the dietary intake of Mg , as well as low Mg content in drinking water , are risk factors for the development of hypertension, atherosclerosis , vasospasm ,sudden cardiac death ,stroke , and inflammatory conditions by ill-defined mechanisms(e.g., see Ref.1,4-6, 17-19,27-29, 35,38,6,48, 49, 63,64,65,67, 72). Hypermagnesemic diets have been shown to ameliorate hypertension and atherogenesis (3, 5, 7,8,18, 23, 67). At present, the average dietary intake of Mg has declined from~450-485 mg/day in 1900 to ~185-235 mg/day for large segments of the North American population (4, 30, 56). Furthermore, the myocardial level of Mg has consistently been observed to be lower in subjects dying from ischemic heart disease in soft-water areas than in subjects living in hard-water areas (4,6,27-29,46,48,63-65,67,72).

Using sensitive ion-specific Mg^{2+}-selective electrodes, it has been found that patients with hypertension, ischemic heart disease, stroke, atherosclerosis and certain inflammatory conditions exhibit a significant depletion of serum/plasma ionized, but not total, Mg(5,7,17,19,35,64,67).Dietary deficiency of Mg in rats and rabbits has been demonstrated to cause vascular remodeling concomitant with hypertension and atherosclerosis(i.e., arteriolar wall hypertrophy and alterations in the matrices) of unknown origin(7,8,18,23, 45).

As early as 15 years ago, using cerebral and peripheral vascular smooth muscle cells (VSMCs) in primary cultures, it was demonstrated that variation in free $[Mg^{2+}]_0$ causes sustained alterations in membrane phospholipids and second messengers as well as activation of several signal transcription molecules, identical to those mentioned above(13, 54,55). Such paradigms, using variations in Mg^{2+}, also cause membrane oxidation, truncation of membrane fatty acids , and the activation of apoptotic pathways(i.e., caspase-3,apoptotic protease activation factor-1, and release of mitochondrial cytochrome c) concomitant with

the significant activation of neutral sphingomyelinase (N-SMase), and alterations in membrane sphingomyelin(SM),leading to the release of ceramides in cultured VSMCs(14-16, 68). Very recently, using a short-term(21 days)rat model of dietary Mg deficiency ,we noted decreased levels of serum SM, lipid peroxidation, and fragmentation of DNA coupled with generation of the tumor suppressor-transcription factor p53 in the left and right ventricular muscles as well as in atrial muscle and VSM(14,16). These alterations (including the change in serum SM) were highly correlated ($P<0.01$) with the levels of serum ionized Mg levels (14, 16, 68).

The *de novo* synthesis of SM is brought about via the action of serine palmitoyl-CoA transferase (SPT), 3-ketosphinganine reductase, ceramide synthase(CS) ,dihydroceramide desaturase, and SM synthase(SMS)(74). SMS requires phosphatidylcholine(PC) and ceramide as substrates to manufacture SM and diacylglycerol(DAG; see Ref. 74). This reaction directly affects SM, PC, and ceramide as well as DAG levels. Three of us have previously noted, using primary cerebral and peripheral VSMCs in culture, that a variation in $[Mg^{2+}]_0$ influences the cellular levels of SM, PC, DAG, and ceramide (55). Ceramide , either released as a consequence of SMase acting on SM and/or activation of either SPT , SMS ,or activation of CS, is now thought to play important roles in fundamental patho-physiologic processes such as cell proliferation, membrane receptor functions , angiogenesis, microcirculatory functions, immune inflammatory responses, cell adhesion, atherogenesis, senescence, programmed cell death(12,21,22,24,31,33,36,44, 58,59,73, 79, 82,83) as well as cellular membrane transport and distribution of Mg^{2+} in VSMCs(82). Although the activation of N-SMase, SPT-1 and SPT-2 as well as SMS by low $[Mg^{2+}]_0$ results in (and ensures) ceramide production in cardiovascular tissues (14-16), the activation of CS by low $[Mg^{2+}]_0$ could result in additional levels of *de novo* ceramide. No direct information exists, however, on the potential latter pathway of activation by low $[Mg^{2+}]_0$ in cardiovascular tissues and/or cells. But, using primary cerebral and peripheral VSMCs in culture, exposed to low$[Mg]_0$, we have found that a specific (and selective) inhibitor of CS, namely fumonisin B1, resulted in a marked reduction in *de novo* synthesis of ceramide (15), presumably suggestive of an

upregulation of CS. Whether a dietary deficiency of Mg results in upregulation of CS is not known. Recently, ceramide synthases have been characterized by a family of at least six different isoforms (69). Initially, they were named Longevity Assurance (LASS) genes (62). Since CSs appear to be localized in the endoplasmic reticulum (69), it can effect links to cell membrane structures and affect many cell functions. We hypothesized that short-term Mg deficiency in: 1) intact rats would upregulate CS activities in cardiac and vascular smooth muscles; and 2) imbibing low levels of a water- soluble Mg salt in drinking water would inhibit or reverse the predicted effects of dietary deficiency upregulation of CS.

Leukocytes and endothelial cells as well as VSMCs can modulate inflammatory conditions via the elaboration and release of cytokines and chemokines (37, 43,70, 71).Mg deficiency (MgD) has been shown in rats to result in upregulation of interleukin-1(IL-1alpha) , IL-6 and TNF-alpha in serum and endothelial cells(92). These pleiotropic cytokines have been implicated in atherogenesis, hypertension and numerous immune-inflammatory pathways (37, 71). Certain cytokines and chemokines, however, are anti-inflammatory and vital in wound healing [e.g., interferon-gamma (IFN-gamma), IL-4, IL-8, IL-10, Il-12, IL-13, granulocyte – macrophage colony stimulating factor (GM-CSF), transforming growth factor-beta (TGF-beta), and RANTES(regulated upon activation, normal T expressed and secreted), among others] (37, 39, 71). Although cytokine secretion has been, and is, a widely studied process, specific mechanisms for regulation of cytokine (and chemokine) release still remain to be determined (37,71).Interestingly, cytokines have recently been implicated in the generation/release of ceramide(32,59) as well as the activation of SMases (41,59). We hypothesized that: 1) *de novo* generation of ceramide in short-term MgD(14) would upregulate **and** release several cytokines and chemokines into sera of such animals and in primary cultured VSMCs, 2) imbibing low levels of a water-soluble Mg salt in drinking water would inhibit or reverse the predicted effects of dietary deficiency of Mg on the upregulation **and** release of the cytokines and chemokines; 3) blockage of *de novo* synthesis (with a specific inhibitor of CS) in primary cultured VSMCs would result in an attenuation of the generation of select cytokines/chemokines in these vascular cells; and4) blockage of low $[Mg^{2+}]_0$- induced activation of N-SMase

would result in reduced levels of ceramide and an attenuation of the generation of select cytokines/chemokines.

Nuclear factor-kappa B (NF-kB) is now known to be a prime regulator of growth processes, differentiation, cell migration, and cell death (for review, see 39). NF-kB is a transcription factor and a pleiotrophic regulator of numerous genes involved in inflammatory processes (37). NF-kB is thought to be a pivotal transcription factor in atherogenesis and hypertension (26, 53, 71). It is not clear as to what factor(s) initiates expression of these molecular and cellular events. Recently, we have reported in preliminary experiments that short-term exposure of cerebral and peripheral VSMCs to low $[Mg^{2+}]_0$ results in an upregulation of several DNA-binding proteins involved in activation of NF-kB (11,13). Some studies also suggest that NF-kB activation may be triggered by a release/generation of ceramide (66). However, there is also some evidence, in certain cells, that ceramide may not be necessary for NF-kB activation (32).Moreover, several lines of evidence suggest that ceramides are important in cytokine generation and in cytokine-induced apoptosis (39, 71). We designed experiments with primary culture of VSMCs to determine whether upregulation of CS (and *de novo* synthesis of ceramide)(e.g., 16), and activation of N-SMase and generation/release of ceramide(14-16),induced by low $[Mg^{2+}]_0$, is associated with activation of NF-kB and release/generation of cytokines and/or chemokines in the vascular cells.

MATERIALS AND METHODS

Animals, diets,sera, and organ-tissue collections. Mature male and female rats (200+/-65 g) were used for all experiments. All experiments were approved by the Animal Use and Care Committee of the State University of New York Downstate Medical Center. Equal numbers of paired male and female animals were used for all nutrition experiments. Control (600 ppm Mg) and MgD (60 ppm Mg) pellet diets were obtained from DYETS (Bethlehem, PA; AIN -93 G diets). All animals were given their respective diets for 21 days as previously described (14,16). MgD animals were allowed to drink triply distilled water ($Mg^{2+} < 10^{-6}$ M) containing one of four different levels of Mg aspartate –HCl (0, 15, 40, or 100 mg/l Mg, Verla Pharm, Tutzing, Germany). All control animals received a normal Mg-containing diet (i.e., 600 ppm) as well as triply- distilled water to drink. On the 22^{nd} day, sera and tissues (the left and right ventricles, atria, abdominal aorta between the superior mesenteric arteries, and renal arteries, cleaned of all connective tissues) were collected quickly after anesthesia (45 mg/kg im pentobarbital sodium). Tissues were stored rapidly under liquid nitrogen (-85^0 C) until use. Whole blood was collected under anaerobic conditions in red-stoppered (no anticoagulant present) tubes, allowed to clot under anaerobic conditions, and then centrifuged under anaerobic conditions in capped vacutainer tubes. The sera were then collected into additional red-stoppered tubes under anaerobic conditions for processing shortly thereafter, similar to previously described methods (14, 17). Serum samples were then analyzed within 2 h after collection, as previously described (14, 17). Total Mg levels were measured by standard techniques in our laboratory (Kodak DT-60 Analyzer, Ektachem Colorimetric Instruments, Rochester, NY). The method compares favorably with atomic absorption techniques for total Mg (17). A Mg^{2+}-selective electrode with a novel neutral carrier-based membrane (NOVA 8 Analyzer, NOVA Biomedical Instruments, Waltham, MA) was used to measure the free divalent cation in the sera (17). The ion-selective electrode was used in accordance with established procedures developed in our laboratory having an accuracy and precision of 3 % (17).

Biochemical measurements of ceramide synthase (CS) in tissues and sera . For the direct ELISA CS assay employing a goat anti-rat LASS3 polyclonal antibody-unconjugated (Abcam, Inc. Cambridge UK), multiple steps were required as follows. Before generating a lysate, the tissues (stored under liquid N_2) were first cut into small cubes, then transferred into a hand homogenizer using 3 ml ice-cold RIPA buffer/gram of tissue (RIPA buffer consisted of: 20 mM Tris-HCl-ph 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton x-100, 1 % sodium deoxycholate, 0.1 % SDS with freshly added PMSF, and with freshly added aprotinin and leupeptin to 5 ug/ml just before use). The tissues were kept in the RIPA buffer on ice for 10 min before homogenizing by then pushing the piston slowly into the mixture via a continuous twisting. The tissues were kept submerged in the ice during the homogenization process. The procedure was repeated until the tissues were liquefied. The liquefied tissue samples were then divided into 1.5 ml tubes and centrifuged for 3-min at 4^0 C. The clear supernatants were next transferred into new tubes, removing approximately 20 ul for protein determinations (via BCA Protein Determination Kits). The lysates were then brought to 5 mg/ml by adding ice-cold RIPA buffer, and then stored in liquid N_2 until use.

The antigen was diluted to a final concentration of 20 ug/ml in PBS buffer (1.16 g Na_2HPO_4, 0.1 g KCl , 0.1 g K_3PO_4, 4.0 g NaCl, pH 7.4). The wells of a PVC microliter plate were coated with the antigen. **A** polyclonal LASS3 antibody, diluted to the optimal concentration (Abcam, Cambridge, UK) in blocking buffer(1 % BSA, serum, non-fat dry milk, casein , gelatin in PBS), was added immediately before use. The CS was analyzed according to the procedures outlined in the ELISA CS assay kit (Abcam, Inc). The absorbance (optical density) of each well was read with a plate reader. Standard curves were used to measure the concentrations of the enzyme.

Assay of cytokines and chemokines . Sera and tissues were harvested from the control and MgD animals, as described above, and kept frozen until biochemical analysis. Multi-Analyte ELISArray Kits (SA Biosciences Corporation, Frederick, MD) designed for rats were used for the quantitative measurement of 12 different cytokines and chemokines (i.e., IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFN-gamma, TNF-alpha, GM-CSF, and RANTES). **W**e read the absorbance**s** at 450 nm

within 30 min of stopping the reaction. Standards were used; standard curves were plotted, and the experimental cytokine/chemokine values were calculated.

Isolation of vascular muscle and primary culture of aortic VSMCs. Rat aortic VSMCs were isolated according to established methods (13,81) in our laboratory (n=10-12 animals/group) and cultured in DMEM containing 1.2 mmol/l $[Mg^{2+}]_0$, FCS , and antibiotics at 37^0 C in a humidified atmosphere composed of 95 % air-5 % CO_2 (55,81). After confluence had been reached, VSMCs were placed in media containing either 0.3**,0.6** or 1.2 mmol/l $[Mg^{2+}]_0$, for varying periods of time (2 or 24 h). It should be stressed that the experiments using cell cultures and those below on primary VSMCs in culture were never part of the whole animal nutritional experiments (described above); these experiments and others were separate from the nutritional experiments.

Influence of an inhibitor (fumonisin B1) of CS on the de novo synthesis of ceramide , select cytokines and chemokines. Before the cells were radiolabeled (as a measure of *de novo* synthesis of ceramide)(16), some cultured VSMCs were treated with 75 uM fumonisin B1 (Sigma-Aldrich, St.Louis MO) for 3 h, as previously in normal Krebs-Ringer solution buffered with 5 % CO_2 (NKRB) (16). After the treatment of VSMCs with the inhibitor(and controls without fumonisin), VSMCs were labeled with [^{3}H]palmitic acid (4-20 uCi/ml) at 37^0C for 18 h, rinsed with fresh NKRB solution and transferred to NKRB solutions (with and without fumonisin B1) containing 0.3,0.6, or 1.2 mmol/l $[Mg^{2+}]_0$. Radioactivity was counted in a scintillation counter (LS-6500, Beckman).

Where appropriate, the select cytokine (i.e., IL-1beta, IL-6, IL-8, TNF-alpha) and chemokine(MCP-1) concentrations were measured in the VSMCs (with and without fumonisin B1)using ELISA with specific antibodies and methods we have recently reported for other cell types(10). Some of the experiments using the primary VSMCs examined the changes in cellular monocyte chemoattractant protein-1(MCP-1) as this particular chemokine has been demonstrated to play major roles in monocyte recruitment and is implicated in atherogenesis(37). The major cells in the vascular walls (i.e., endothelial, VSMCs, and macrophages) all

have been shown to contribute to overexpression of MCP-1 in atherosclerotic tissues.

NF-kB expression in cardiovascular tissues and primary cultured VSMCs Briefly, for the primary aortic smooth muscle cells, we utilized electrophoretic mobility supershift assays(EMSA) similar to those we performed in our laboratory previously(11). Nuclear protein extracts were separated by SDS/PAGE on 7.5% (wt/vol) polyacrylamide gels made in a buffer containing a TRIS/boric acid/EDTA mixture(11). Electrophoresis was performed at a constant voltage of 100 V for 90 min at 4^0C. For the supershift assays , nuclear proteins were incubated with antibodies specific for the known NF-kB components(p50,p52,p65,RelB, and cRel; Santa Cruz Biotechnology, CA) before gamma-^{32}P-labeled(11). NF-kB oligonucleotides were added as described(11). To verify that activation of NF-kB was in fact the result of incubation in low $[Mg^{2+}]_0$(with/without inhibitors), separate groups of VSMCs were exposed to the prototypical NF-kB inhibitor, pyrrollolidine dithiocarbamate(PDTC; 0.1 uM; 11,39).

For measurement of NF-kB in ventricular and aortic smooth muscle obtained from MgD animals, we utilized a highly-sensitive ELISA kit recently developed for numerous high-through-put sampling(TransAM NF-kB Family Transcription Factor Assay Kit; Active Motif North America, Carlsbads,CA). As in the case for the primary cultured aortic smooth muscle cells, this kit assays for p50,p52,p65, RelB, and cRel subunits.

Measurement of IkB degradation and NF-kB activation in primary cultured aortic smooth muscle cells exposed to low $[Mg^{2+}]_0$, PDTCand funonisin B1. To determine whether IkB phosphorylation (and degradation) is stimulated by low concentrations of$[Mg^{2+}]_0$ (as a possible consequence of CS activation), the VSMCs were incubated for 18h and IkB breakdown assayed using a highly-specific rabbit IkB antibody(Santa Crux Biotechnology, CA)(11).

Influence of an inhibitor (scyphostatin) of N-SMase on ceramide levels, select cytokines and chemokines, and NF-kB expression in VSMCs exposed to low $[Mg^{2+}]_0$. Before exposure of cultured VSMCs to low $[Mg^{2+}]_0$ with scyphostatin, the cells were exposed for 2h in NKRB solutions containing different concentrations of

$[Mg^{2+}]_0$ (either 1.2 or 0.3 mM). We then exposed the cells to the different concentrations of $[Mg^{2+}]_0$ with or without 75 uM scyphostatin(Sigma-Aldrich, St.Louis, MO) for 18 h. We extracted the lipids in the cells as we have detailed elsewhere (54, 55). The ceramide was next converted into ceramide-1-[^{32}P] phosphate by *Escherichia coli DAG kinase* and the lipids separated on high performance TLC plates as described elsewhere (16, 58). After autoradiography, spots corresponding to ceramide-1- phosphate were carefully scraped into vials, and the radioactivity was counted in a scintillation counter (LS-6500, Beckman). Quantitation of ceramide levels and results as picomoles per 10^8 cells were determined (16).

Where appropriate, the select cytokine and chemokine concentrations were measured in the VSMCs (with and without scyphostatin) as above.

Expression of NF-kB levels (with and without scyphostatin), using the p65 and cRel subunit**s**, were measured as per the methods described above.

Statistical analyses. Where appropriate, means and means +/- SE were calculated. Differences between means were assessed for statistical significance by Student's t-tests and ANOVA followed by a Newman-Keuls test. In some cases correlation coefficients were calculated by the method of least squares. P values of <0.05 were considered significant.

RESULTS

Influence of diet on water consumption and food intake and overall physiological condition. As shown recently, using an identical dietary regimen of Mg in controls and MgD animals(14), there were no significant differences in either water consumption or food intake between the diverse subgroups of rats(i.e., controls=600 ppm Mg, Mg-deficient-MgD, MgD+15 mg/L Mg/day, MgD+40 mg/L Mg/day, or 100 mg/L Mg/day). All of the MgD subgroups(n=18-30 animals per group), irrespective of the amount of Mg in the diets or in the drinking water, showed no loss in gait or any other outward signs of pathology or behavior.

Serum total and ionized Mg levels. Feeding the animals the synthetic AIN-93G MgD pellet diet(n=18-30/group) resulted in a total serum ***Mg*** *level* ***of 1.00*** *mM* ***/L,*** whereas the animals receiving the MgD diet exhibited a serum *total Mg level of* 0.38+/-0.004 mM/L($p<0.01$). The serum level of ionized Mg in the normal, control *group was* 0.60+/-0.002 mM/L,whereas in the MgD group the serum ionized level was reduced to 0.30+/-0.003 mM/L($p<0.01$).

Feeding the MgD animals various levels of Mg in their drinking water (as seen previously, ref. 14,15) resulted in concentration-dependent rises in both the total and ionized levels of serum Mg. 100 mg/L/day of Mg^{2+} elevated the total Mg level *to normal, i.e*., 0.98 +/- 0.004mM/L, whereas feeding 15 and 40 mg/L/day of Mg in the drinking water raised the total Mg levels to 67**(**0.66+/-0.003mM/L**)** and 83%**(**0.81+/-0.006 mM/L**)**, respectively, of normal (n=18-26, $p<0.05$). With respect to the serum ionized levels, feeding the animals 100 mg/L/day of Mg restored the level of ionized Mg to normal while feeding 15 and 40 mg/L/day of Mg to the rats raised the *serum ionized levels to* 60%**(**0.38+/-0.004 mM/L**)** and 65%**(**0.42+/-0.004 mM/L**)**, respectively, of normal(n=18-26,$p<0.05$).

Influence of dietary Mg intake on CS levels in cardiac and vascular smooth muscles: relationship to serum ionized Mg. Figure 1 shows that feeding rats a MgD for 21 days resulted in an almost 300% rise CS enzymatic activity in LV and aortic smooth muscle ,and over a 200% rise in CS activity in RV and atrial muscle.

Interestingly , feeding the MgD animals as little as 15 mg/L Mg^{2+} in drinking water in LV, RV and aortic smooth muscle reduced the rises in CS levels produced in animals fed the MgD diets, whereas 40 mg/L Mg^{2+} in drinking water prevented the rises in CS activities in cardiovascular tissues. Although not shown, we found high degrees of correlation between the falls in serum ionized Mg and the rises in the enzymatic activities of CS; the lower the serum Mg^{2+}, the greater the elevation in activities of CS in all of the cardiovascular tissues studied($r=0.78-0.96, p<0.01$). There were either no ($p>0.05$) or weak correlations (e.g., 0.26-0.37) between total serum Mg and CS (data not shown).

*Influence of dietary Mg deficiency with and without Mg supplementation on ceramide synthase activity in sera .*Table 1 demonstrates that 21 days of short-term MgD results in a 100% increase in serum CS activity. Although 15 mg/L of Mg added to the drinking water did not prevent the rise in CS activity 40 mg/L of Mg in the drinking water completely prevented the rise in CS caused by the MgD.

Influence of dietary Mg intake on serum cytokine and chemokine levels: relationship to serum ionized Mg. Figure 2 demonstrate**s** that feeding rats the MgD diet for 21 days resulted in four- to 16- fold rises in the serum levels of the cytokines and chemokines, with the greatest rises in activities observed in IL-1A(approx. 16-fold),RANTES(approx.13-fold), TNF-alpha(approx. 9-fold) , GM-CSF(approx. 9-fold), and IL-10(approx. 8-fold). Feeding the MgD animals 15-40 mg/L Mg^{2+} in the drinking water largely prevented the rises in serum levels of all 12 cytokines and chemokines(Fig. 2). Linear regression analyses demonstrated strong correlations between the reduction in serum ionized Mg and cytokine (and chemokine) levels(r=0.85-0.96;e.g., see Fig. 3). However, there were either no or weak (e.g., r=0.28-0.42) correlations of total serum Mg to serum cytokine and chemokine levels (data not shown).

Influence of dietary Mg intake on cytokine and chemokine levels in left ventricular and aortic muscle. Figures 4 and 5 demonstrate that feeding rats the MgD diet for 21 days resulted in 3-10 –fold rises in the ventricular and aortic smooth muscle levels of the 12 different cytokines and chemokines, with the greatest increases seen with IL-1A, IL-1B,and TNF-alpha. Like that observed in the

sera,feeding the MgD animals 15-40 mg/L Mg in the drinking water largely prevented the rises in tissue levels of all 12cytokines and chemokines.

Influence of an inhibitor (fumonisn B1) of CS on the de novo synthesis of ceramide, select cytokines and chemokines and NF-kB expression in primary rat aortic smooth muscle cells exposed to low $[Mg^{2+}]_0$. The results shown in Table **2** demonstrate that exposure of aortic smooth muscle cells to low $[Mg^{2+}]_0$ produced concentration-dependent increases in *de novo* synthesis of ceramide as shown previously(16); the lower the $[Mg^{2+}]_0$, the greater the increase in the *de novo* synthesis of ceramide. Pretreatment of the VSMCs with the CS inhibitor, resulted in a marked reduction in the *de novo* synthesis of ceramide(Table **2**). Interestingly, exposure of the VSMCs to the CS inhibitor also resulted in a marked reduction in the expression of the p65 and cRel element**s** of NF-kB (Fig.**6**) as well as cytokine levels in the VSMCs (Table **3**). Interestingly, neither the p50, p52 nor the RelB subunits of NF-kB were significantly altered by either exposure to low Mg^{2+} or the CS inhibitor(data not shown;P>0.05).

The data illustrated in Table **3** indicate that exposure of primary aortic SMCs to low $[Mg^{2+}]_0$ resulted in approximately 2-10 fold increases in the levels of the cytokines and MCP-1. Pretreatment of the VSMCs with fumonisin B1 attenuated the production of the cytokines and MCP-1 in cells exposed to low $[Mg^{2+}]_0$.

*Influence of an inhibitor (scyphostatin) of N-SMase on ceramide levels , select cytokines and NF-kB expression in primary VSMCs exposed to low $[Mg^{2+}]_0$.*Pretreatment of primary VSMCs with the N-SMase inhibitor resulted in a reduction in the generation of ceramide ,on exposure to low $\{Mg^{2+}]_0$(Table 4). Use of the N-SMase inhibitor also resulted in a reduction in the generation of the p65 and cRel subunit**s** of NF-kB(Fig. 6) as well as cytokine levels(Table 5).

The data shown in Table 5 indicate that primary aortic SMCs pretreated with scyphostatin(in the presence of low $[Mg^{2+}]_0$ result in an attenuation of the production of the cytokines and MCP-1.

Influence of MgD diet on NF-kB subunits in left ventricle and aorta. The data in Figures 7 and 8 indicate that feeding animals the MgD diet for 21 days resulted

in marked activation of the p65 and cRel NF-kB subunits but not either the p50,p52 , or RelB subunits in both the left ventricle and aorta,thus similar to what we found for the primary cultured aortic smooth muscle cells. Interestingly, feeding the MgD animals as little as 15 mg/L Mg^{2+} in the drinking water in both the left ventricles and aortas(obtained from the intact animals) reduced the expression of both p65 and cRel subunits of NF-kB(Figs. 7 and 8).

Influence of PDTC on IkB degradation and p65 activation in the nuclei of low $[Mg^{2+}]_0$ – treated primary cultured aortic VSMCs with and without exposure to fumonisin B1. The data shown in Table 6 indicate that treatment of VSMCs with PDTC did indeed inhibit low $[Mg^{2+}]_0$ – induced IkB breakdown. Addition of fumonisn to the VSMCs exposed to low $[Mg^{2+}]_0$ also attenuated the breakdown of IkB, but to a lesser extent than PDTC.

Multiple regression analyses and correlations of serum CS activity, serum ionized Mg , with ventricular (and aortic muscle) cytokines and chemokines obtained from MgD animals. The data summarized in Fig. 9 using multiple regression analysis indicates high degrees of correlation of serum CS activity ,serum ionized Mg ,and ventricular and aortic muscle cytokines/chemokines($P<0.001$).

DISCUSSION

The results reported, herein, are the first demonstration that short-term dietary deficiency of Mg in an intact mammal results in activation of CS in diverse cardiovascular tissues and cells. To our knowledge, this is the first time anyone has shown an upregulation of CS by Mg deficiency in any cell type in any species. The *de novo* pathway of sphingolipid synthesis has gained considerable attention over the past decade (34, 47, 52, 61). Ceramide synthases are important proteins of the endoplasmic reticulum (69). The molecular pathways via which ceramides are synthesized are known to be highly conserved between yeast and mammals (69). Six homologs of CS are known to exist in mammals (69), which were initially termed Lass (Longevity Assurance) genes, but recently changed to ceramide synthase(CS) . CS catalyzes the formation of ceramide from the precursor, sphinganine , in the sphingolipid pathway. The mycotoxin ,fumonisin B1, used in the present studies inhibits this major step in the generation of ceramide(75). We show in the present study that inhibition of CS by fumonisin B1 results in a marked reduction in the *de novo* synthesis of ceramide(as measured by the uptake of[^{3}H]palmitic acid) in primary aortic smooth muscle cells.

Our experiments confirm and add further support to the concept that lowered levels of Mg can lead to the formation of ceramide in cardiovascular tissues and cells (55). We also confirm that ceramide synthesis in cardiovascular tissues and cells is, at least, in part, a result of activation of CS and N-SMase(14-16, 55). When taken together with previous findings from our labs, it becomes clear that the *de novo* synthesis and generation of ceramides in cardiovascular tissues and cells, exposed to low $[Mg^{2+}]_0$ environments, is due to the action of at least four enzymes, namely, CS, N-SMase, SPT, and SMS.

It is now, generally, accepted that ceramides can be produced in many types of cells and tissues when they are exposed to ultraviolet radiation, endotoxins, retinoic acid, ionizing radiation, balloon injury of arterial vessels, phorbol esters, serum deprivation, daunorubicin , apoptotic signals, as well as cytokines, among

other agents(21, 24, 33, 36, 44, 59, 73). Many of these agents, including cytokines, are known to activate SPT, SMS, SMases, and CS to produce ceramides in many cell types (31, 69, 71). The present findings support our previous suggestion (14-16) that Mg deficiency should be added to the list of stimuli known to activate CS and N-SMase pathways, at least in rat cardiovascular tissues and VSMCs. One of the most important, key pathways leading to ceramide generation is via the CS homologs which act to acylate sphinganine to produce ceramide(69). Previously, we have shown that the production of low-$[Mg^{2+}]_0$ environments, either in vivo(e.g., identical model of Mg deficiency used here)(15,16) or in primary cultured vascular cells(15, 55),results in the activation of SMase, SMS ,and SPT(SPT-1 and SPT-2) and the production of ceramides. Thus, the present findings ,when taken together with the latter studies, indicates that ceramide is most likely generated in cardiovascular tissues and cells in low $[Mg^{2+}]_0$ by four major enzymes in the sphingolipid pathway.

We believe the new experiments shown, herein, support our previous suggestions that the exposure of cardiovascular tissues to low $[Mg^{2+}]_0$ environments can result in sizable quantities of ceramides and could be responsible, in large measure, for activation of apoptotic events seen in MgD animals(14) as well as many of the structural ,cellular and lipid dysfunctional changes noted in atherogenesis and hypertension. It is of interest to note that the cytokines (e.g., IL-1beta, IL-6,IL-8,and TNF-alpha, among others) documented to play important roles in apoptotic events are shown, herein, to be generated by at least two key enzymes (i.e., CS and N-SMase) needed for the **synthesis** of ceramides. In addition, it should be pointed out that many of the cytokines/chemokines found to be released by low Mg, herein, are known to be activated/generated in atherogenesis(37, 39, 71). It is now accepted that apoptotic events play major roles in the development of atherogenesis and hypertension. However, up until our recent studies, it has not been possible to determine whether MgD- induced pathophysiological changes in the cardiovascular system are linked to alterations in sphingolipid metabolism.

A key function of ceramide's role in pathophysiological actions is its ability to induce cell differentiation and transformation (33, 36, 58, 73). Abnormal cell

differentiation, transformation, and growth are pivotal events in the development of both atherogenesis (20, 37,40) and hypertension (40, 57). Hyperplasia and cardiovascular hypertrophy are common events in hypertension and key elements in target organ damage. But, the precise mechanisms regulating alterations in tissue mass, transformation of VSMC phenotypes, plaque formation in vascular walls, and lipid deposition are not completely understood. Cytokines/chemokines are now known to be generated/released in these cellular events along with activation of NF-kB DNA- binding proteins, as well as activation of the tumor suppressor protein p53 (37, 71). We believe it is more than coincidental that short-term MgD results in generation/release of at least 12 different, key cytokines and chemokines along with activation of the key NF-kB DNA –binding protein**s**,p65 and cRel(present work) and activation of p53 shown recently(16). The latter protein is known to play critical roles in cell transformation, growth, and apoptotic events (50, 51). It is important to note ,here, that ceramide, p53, and activation of NF-kB can induce cell cycle arrest(and senescence), induce programmed cell death , and are associated with DNA damage(genotoxic events). It has been demonstrated that Mg deficiency can produce all three of these pathophysiological events in several cell types, including cardiac and VSMCs (Ref. 6, 11, 13, 14-16, 68, 81).Our present study suggests that MgD environments drive ceramide synthesis, at least in VSMCs, via the activation of two major enzymes in the sphingolipid pathway: CS and N-SMase.

Some additional discussion of the potential relevance of the present findings, with the p65 and cRel protein**s,** and the NF-kB family, to atherogenesis are in order. The NF-kB family of proteins is composed of five different and related transcription factors: c-Rel, RelB, p50, p52, and p65 (for recent review, see 39). Homo-and heterodimers are formed from these transcription factors, which share an N-terminal DNA-binding dimerization domain known as the Rel homology domain. These NF-kB dimers can bind to a number of target DNA sequences termed kB sites (which contain transcription activation domains [TADs]) that allow co-activator recruitment and target gene expression (for review, see 39). Since p50 and p52 **(**not however activated by low Mg^{2+} environmemnts in the present work) are major players in the non-canonical pathway but lack TADs, they

activate transcription by forming heterodimers with the p65, c-Rel or RelB proteins (for review, see 39). NF-kB plays a major role in the transcription of the genes encoding many pro-inflammatory cytokines and chemokines , analyzed herein, which also regulate the expression of adhesion molecules important in atherogenesis , an inflammatory syndrome(37, 39, 43,70). It is important to point out here that the cytokines (i.e.,IL-1beta, IL-6,IL-8, TNF-alpha)and MCP-1, found to be elevated, herein, in VSMCs exposed to low$[Mg^{2+}]_0$,are identical to those found in VSMCs, T-cells, monocytes/macrophages and endothelial cells in atherosclerotic plaques(for review, see 39). We do not believe this is a coincidence. Activation of the p65 and cRel protein**s** found in the present study ,to be induced by low $[Mg^{2+}]_0$,**are** pivotal in recruitment of leukocytes(39) and in several arms of the innate and adaptive immune systems in atherogenesis(37, 39). Since Mg deficiency has been shown to result in accelerated atherogenesis in rabbits, which was shown to be associated with increased levels of leukocytes and p53 in the thickened atherosclerotic plaques (18), we hypothesize that p65 and cRel were first ,more than likely, activated, at least in part , by synthesis **and** release of ceramides(via CS, SMases,SPT-1 and 2, and SMS) in the Mg-deficient environment, which would have then acted to activate **and** release cytokines/chemokines and growth factors. These classes of protein molecules could then be expected to activate NF-kB family members and MAP signaling pathways. It is of interest to note, here, that we have found that low $[Mg^{2+}]_o$ environments have been shown to activate/synthesize all of these pathways(for review see 6; also refs. 14-16,68). Interestingly, activated forms of NF-kB have been reported to be present in VSMCs, macrophages and endothelial cells of human atherosclerotic lesions (39, 71).

Likewise, we hypothesize that many of the structural and adaptive vascular wall and blood flow disturbances seen in the arterioles and arteries in Mg-deficiency – induced hypertension (7, 8,45) are also, in large measure, a consequence of synthesis **and** release of sphingolipids and activation of NF-kB. Even though it has been repeatedly demonstrated that prolonged administration of Mg^{2+}(oral and intravenous) can lower arterial blood pressure in both experimental and clinical forms of hypertension(4-6, 23, 45, 64, 67,80), the

precise mechanism(s) is not known. It has been suggested, often, that Mg^{2+} lowers blood pressure by promoting vasodilation and decreasing work load on the myocardium via direct actions on Ca^{2+} channels(and cellular redistribution) in vascular and cardiac muscle cells(2,3, 5, 7, 8,23, 64, 67). In view of our present study and those recently published(14-16, 54, 55), we believe that Mg's effects on ceramide and sphingolipid metabolism must now be taken into consideration in helping to explain the blood pressure-lowering actions of this divalent cation.

Our present findings which demonstrate activation of CS in left and right ventricular as well as atrial muscle coupled with elevation in serum levels of 12 different cytokines and chemokines ,obtained from MgD animals, may have direct relevance to heart failure and sudden-cardiac death syndromes. The potential roles of proinflammatory cytokines in these syndromes is gaining acceptance (25, 37, 43,70, 71). Interestingly, intravenous administration of either IL-1beta or TNF-alpha(two cytokines shown here to be elevated by low Mg) produce a profile of cardiac-hemodynamic failure; left ventricular ejection and LV pressure volume indices drop precipitously(25, 71). Several of the cytokines shown, in the present work, to be elevated in sera from MgD animals (e.g., IL-1alpha, Il-1beta, IL-2, IL-6. TNF-alpha) have been found to be associated with heart failure in humans (71). Furthermore, the presence of rising plasma levels of IL-6 has been suggested to be a harbinger of impending morbity/mortality in human subjects diagnosed with heart failure (71). Recently, secretory SMase was reported to be upregulated in chronic heart failure patients (for review, see 60). Extensive clinical studies over the past three decades has identified magnesium deficient states in thousands of patients in congestive heart failure, acute ischemic heart diseases, acute myocardial infarction, angina, and sudden –cardiac death(for reviews, see 1, 4,6, 38, 67). In vitro studies from other laboratories on perfused working rat hearts obtained from MgD animals (77) and in vitro studies from our labs on perfused working rat hearts (9, 78) clearly demonstrate that even short-term MgD results in reduction in a variety of hemodynamic cardiac functions. Overall, these in vitro studies on perfused rat hearts demonstrate that short-term magnesium deficiency results in falls in cardiac output, coronary flow, stroke volume,

developed pressures, and ischemia concomitant with a lowering of high-energy phosphate.

We believe it is important to point out here that while our experiments investigated various and numerous biochemical and molecular analytes in the left and right ventricles and atria, these tissues are composed of not only myocytes(approx, 60%),but fibroblasts(approx.27%), VSMCs(approx. 10%), and endothelial cells(approx. 7%). Thus, although the bulk of the tissue masses are composed of muscle cells, it will not be clear what the exact contribution of each cell type is (in terms of the quantitative results) until further experiments are carried –out.

Over the past several decades, experimental and clinical evidence has been brought forth that suggests a striking linkage between dietary deficiency of Mg and diverse types of cardiovascular maladies, e.g., atherogenesis, hypertension, coronary artery disease, congestive heart failure, irregular heart rhythms, vasospasm, peripheral arterial diseases, myocardial infarction, diabetic-related vascular diseases, dyslipidemias, strokes, and sudden cardiac death(e.g., see 4, 6-8,14-16, 23, 29, 38,45, 45,48,49, 63, 64,67,72,77). More than 50 years ago, Kobayashi (42) showed in an epidemiological study that when the hardness of drinking water was elevated, the rate of death from cardiovascular diseases decreased. This concept has gained credibility over the past five decades from a large number of studies from different parts of our planet (4-6, 27-29,46, 48,63, 67, 72); the death rates by sudden cardiac death are lower in hard water areas than in soft water areas. Despite the fact that the hardness of water is due to the concentration of Ca^{2+} and/or Mg^{2+}, the overwhelming evidence , to date, supports the idea that it is the Mg content that is responsible for most of the protective effects of hard water(28,29,38,46,48,63). More than 20 years ago, it was suggested that as little as 15-30 mg/l/day of Mg^{2+} in drinking water should be cardioprotective (46,48). Recently, using the same model of dietary deficiency of Mg as in the present study(21 days of MgD), we showed , for the first time, in well-controlled experiments that as little as 15 mg/L/day of Mg^{2+}, in drinking water, either prevented or ameliorated the formation of reactive oxygen species(ROS), DNA fragmentation, caspase-3 activation,p53,mitochondrial release of cytochrome c, lipid peroxidation, activation of apoptosis, hydrolysis of

sphingomyelin, upregulation of SPT-1 and SPT-2, and activation of SMS(14-16). Although the present work indicates that as little as 15 mg/L/day of Mg^{2+} in water can prevent/ameliorate the upregulation of CS in cardiac and vascular muscles, something between 15 and 40 mg/L/day of Mg^{2+} in water must be imbibed to prevent the synthesis/release of most of the cytokines and chemokines into the blood stream. From our present, and previously published data (14-16), we hypothesize that between 15 and 40 mg/L/day of water-borne Mg^{2+} should be both cardioprotective and vascular protective.

While the activation of CS and N-SMase and synthesis/release of cytokines and chemokines most likely play important roles in the biological synthesis of ceramide and activation of the NF-kB family of transcription factors in Mg deficiency, this could be one of many ways in which Mg deficiency is a cardiovascular risk factor. A few additional words with respect to the activation of CS by low $[Mg^{2+}]_0$ in the atria appear to be in order. The atria were the only organ-tissues in which **only** high levels of $[Mg^{2+}]_0$ (i.e., 100 mg/l) added to the drinking water **were able** to prevent high degrees of CS stimulation(see Fig. 1). This finding is quite surprising and different from what is observed for activation of SPT 1, SPT 2, and SMS by low vs. high $[Mg^{2+}]_0$ in the atria(15,16). Dysfunction of the cardiac atria is well-known to be a high risk factor for induction of strokes. We believe it is, thus, possible that unabated generation of ceramides(acting to induce apoptosis and greater than normal relaxation) in atrial muscle via activation of CS by low $[Mg^{2+}]_0$,despite eventual repletion of tissue Mg, may not overcome a prior activation of CS and be a risk factor for strokes. These findings and their potential clinical relevance merit future study.

We believe, at the very least, that this study, when viewed in light of previous recent studies(14-16,68), adds considerable support for the hypothesis suggested more than two decades ago(46,48), that water intake (e.g., from tap waters, well waters, bottled waters, and beverages using tap/well/spring waters) in humans varying between 1 and 2 L/day, with Mg^{2+} intakes varying from <5 to> 100 mg/l, may , as we have suggested recently(14-16, 68), represent an excellent way to overcome and control marginal intakes of Mg obtained with most Western diets. Moreover, in view of our previous findings and those presented here, it is

probably propitious to suggest that all desalinated-purified recovered/recycled waters, harvested rainwaters, well waters, tap waters, and all bottled waters given to humans should be supplemented with bioavailable Mg^{2+} to ameliorate/prevent the induction of cardiovascular risk factors and disease processes worldwide.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Table 1. *Influence of MgD on serum ceramide synthase with and without Mg supplementation*

Group	Ceramide synthase activity(U/ml)
Controls	356.8+/- 17
MgD	796 +/-22.5
MgD + 15	664 +/-39
MgD + 40	385.9 +/- 26
MgD +100	345.7 +/- 17

N=8-10 each. Values are means +/- SE. All values except MgD +15 are significantly different from MgD alone($P<0.01$,ANOVA).

Table 2. *Influence of fumonisin B1 on the de novo synthesis of ceramide in aortic smooth muscle as a function of $[Mg^{2+}]_0$ and $[^3H]$palmitic acid incorporation*

$[Mg^{2+}]_0$,mM/L	cpm x 10^3/mg wet wt
Controls, 1.2 mM/L	0.59 +/- 0.05
0.6	0.91 +/-0.07*
0.3	1.16 +/-0.06**
With fumonisin-$[Mg^{2+}]_0$	
0.6	0.74 +/- 0.06
0.3	0.89 +/-0.05

N=8-10 different animals per group. *P<0.05 compared to controls (1.2 mM/L $[Mg^{2+}]_0$. **P<0.01 compared to all other mean values(ANOVA). All mean values in the fumonisin groups are significantly different from their respective control values in the paired Mg^{2+} concentrations (P<0.05).

Table 3. ***Influence of fumonisin B1 on cytokine/chemokine levels released from aortic smooth muscle cells exposed to low*** $[Mg^{2+}]_0$

$[Mg^{2+}]_0$, mM/L	IL-1B (pg/ml)	IL-6 (pg/ml)	IL-8 (ng/ml)	TNF (pg/ml)	MCP-1 (ng/ml)
Controls, 1.2 mM/L	6.2 +/-0.8	302+/-65	0.34 +/-0.08	8.8 +/-1.4	0.36 +/-0.08
0.6	32.4 +/-3.8*	524+/-55*	1.82 +/-0.16*	18.2 +/-3.4*	1.05 +/-0.12*
0.3	48.6 +/- 4.2*	732 +/-68*	3.02 +/-0.28*	28.4+/-4.2*	3.35 +/-0.32*
With fumonisin					
0.6	22 +/- 1.8	408+/-36	0.98+/-0.22	12.2+/-1.4	0.74+/-0.08
0.3	34+/- 2.4	610+/-48	1.96+/-0.26	20+/- 1.8	2.1+/-0.22

N=10-12 different animals per group. *P<0.05 compared to controls (1.2 mM $\{Mg^{2+}]_0$. All mean values in the fumonisin groups are significantly different from their respective control values in the the paired Mg^{2+} concentrations (P<0.05, ANOVA)>

Table 4. Influence of scyphostatin on ceramide levels in aortic smooth muscle cells exposed to low $[Mg^{2+}]_0$

Group,$[Mg^{2+}]_0$, mM/l	Ceramide(pmol/10^8 cells)
Controls, 1.2 mM/L Mg^2	32+/- 4.2
0.3	78+/- 7.8*
With scyphostatin	
0.3	52+/-6.6**

N=8-10 different animals per group. *P<0.01 compared to controls 1.2 mM/L Mg^{2+}. **P<0.01 compared to all other groups (ANOVA).

T

Table 5.*Influence of scyphostatin on cytokine/chemokine levels released from aortic smooth muscle cells exposed to low $[Mg^{2+}]_0$*

$[Mg^{2+}]_{0,}$ mM/L	IL-B (pg/ml)	IL-6 (pg/ml)	IL-8 (ng/ml)	TNF (pg/ml)	MCP-1 (ng/ml)
Controls, 1.2 mM	5.8+/-0.6	252+/-48	0.26+/-0.06	6.8+/-1.2	0.28+/-0.06
0.3	42.4+/-3.8*	668+/-56*	2.88+/-0.26*	24.2+/3.8*	3.12+/-0.3*
With scyphostatin					
0.3	21.2+/-1.8**	440+/-38**	1.08+/-0.22**	14.4+/-1.2**	1.64+/-0.4**

N=8-10 animals per group. *P<0.01 compared to controls (1.2 mM Mg^{2+}. **P<0.01 compared to all other mean values(ANOVA**).**All mean values in the scyphostatin groups are significantly different from their respective control values in the paired Mg^{2+} concentrations (P<0.05, ANOVA).

Table 6. Inhibition by PDTC of IkB degradation and NF-kB(p65) activation in the nuclei of low $[Mg^{2+}]_0$ –treated primary aortic VSMCs with/without fumonisin B1

$[Mg^{2+}]_0$, mM/L	% Change from baseline	
	IkB-alpha	p65
Controls, 0.6 mM Mg^{2+}	-38 +/- 4.2 *	182 +/-22*
0.3 mM Mg^{2+}	-58 +/-6.6*	376 +/- 32*
With PDTC- $[Mg^{2+}]_0$		
0.6	102 +/-8.4	-9.2 +/- 3.4
0.3	126 +/- 9.6	-14 +/- 3.6
With Fumonisin- $[Mg^{2+}]_0$		
0.6	-20 +/- 1.3 **	110 +/- 6.6**
0.3	-32 +/- 1.6**	222 +/- 14**

N=8-10 animals per group. *P<0.05 compared to normal 1.2 mM/L $[Mg^{2+}]_0$.
**P<0.05 compared to controls and PDTC(ANOVA).

FIGURE LEGENDS

Fig.1. Ceramide synthase (CS) levels in left ventricle (LV), right ventricle (RV), atria and aortic smooth muscles in normal and Mg-deficient rats with and without Mg^{2+} added to their drinking water. Concentrations of Mg^{2+} per liter added to drinking water as follows: 15 mg/l (MgD + 15), 40 mg/l (MgD + 40), and 100 mg/l (MgD + 100). All values are means +/- SE; n=10-14 animals/group. MgD values were highly significantly different from controls (Cont; $P < 0.001$); MgD + 15 , MgD + 40, and MgD + 100 values were all significantly different from MgD values ($P < 0.01$ by ANOVA)**,**except for the atria. In the atria only the MgD + 100 values were statistically significant from the MgD animals($P<0.01$).

Fig.2.Pg/ml change in IL-1A, IL-1B, IL-2**,** IL-4,IL-6, IL-10, IL-12, IL-13, IFN-gamma, TNF-alpha, GM-CSF, and RANTES levels in sera measured in normal and MgD rats with and without Mg^{2+} added to their drinking water. Mean values +/- SE for MgD animals were highly significantly different from controls for all **of the** cytokines ($P< 0.001$). All Mg subgroup mean values (Mg + 15, 40, and 100) are significantly different from MgD mean values($P < 0.01$) by ANOVA.

Fig.**3**. Linear correlation between serum cytokine/chemokine levels and serum ionized Mg levels in normal and MgD rats with and without Mg added to the drinking water. N=10-12 animals per group. Bars are SEs. RANTES= dashed line with square ( ) (linear regression equation : y=-1534.5x + 973.87; r=0.853), IL-1B= dotted line with square( ) (linear regression equation : y=-1852.7x + 1410.4; r= 0.96), IL-2= dotted line with triangle( )(linear regression equation : y=-415.32x + 1410.4; r= 0.90), IL-12=solid line with square( )(linear regression equation : y=-2011.5x + 1314.8; r=0.92), IL-6=solid dashed line with square( )(linear regression equation : y=-289.53 + 214.94; r=0.80), and IL-13=dotted line with solid circle( )(linear regression equation : y=-1110.6x + 775.05; r=0.96).

Fig.4. Influence of dietary Mg intake on cytokine and chemokine levels in left ventricular with and without Mg added to the drinking water. N=10-12 animals

each. Mean values +/- SE for MgD animals were all highly significantly different from control values for all of the cytokines/chemokines($P<0.001$).

Fig.5. Influence of dietary Mg intake on cytokine and chemokine levels in aortic smooth muscle with and without Mg added to the drinking water, N=10-12 animals each. Mean values +/- SE for MgD animals were all highly significantly different from control values for all of the cytokines/chemokines($P<0.001$).

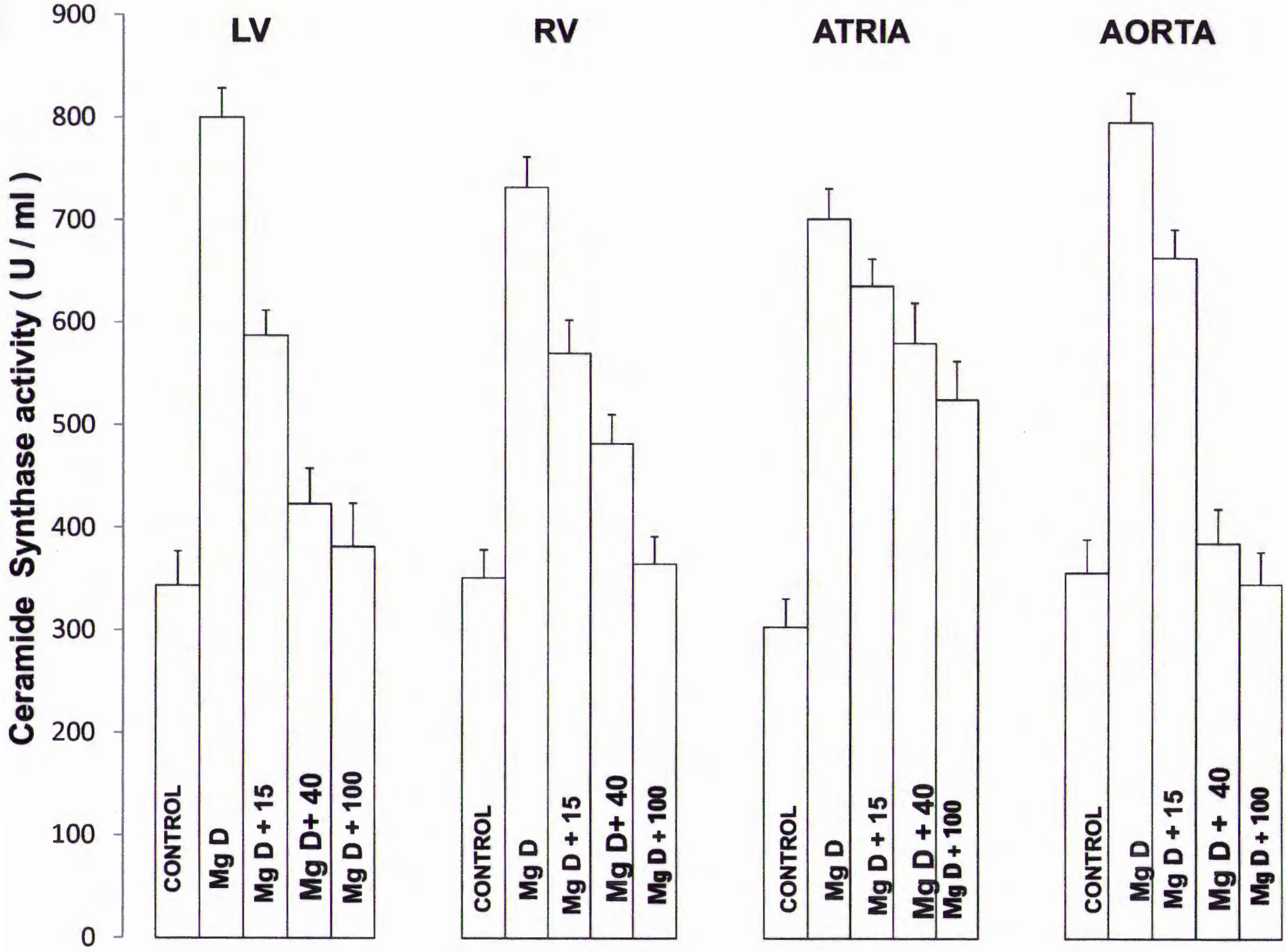
Fig.**6**. Influence of fumonisin B1 and scyphostatin on the expression of p65 and cRel in aortic smooth muscle cells exposed to low $[Mg^{2+}]_0$. Values are means +/-SE obtained by measuring the % change from control radiolabeled blots. All low $[Mg^{2+}]_0$ mean values in 0.3 and 0.6 mM/l are significantly different from control($P<0.01$). All mean values with fumonisin or scyphostatin in 0.3 and 0.6 mM/L Mg^{2+} are significantly different from values without fumonisin/scyphostatin in low Mg^{2+}($P<0.01$).

Fig.7. Influence of MgD diet and supplementation with Mg^{2+} added to the drinking water on activation of NF-kB subunits in left ventricles.Designations for diets similar to Fig. 1. N=10-14 animals per group**.** Single asterisk indicates mean values which are significantly different from all other mean values($P<0.01$, ANOVA). Dagger indicates mean values which are significantly different from all other mean values($P<0.01$,ANOVA). Double asterisk indicates mean values which are significantly different from all other values($P<0.01$, ANOVA).

Fig.8. Influence of MgD diet and supplementation with Mg^{2+} added to the drinking water on activation of NF-kB subunits in aortae.Designations for diets similar toFig. 1. N=10-14 animals per group**.** Asterisks and daggers signify significant differences between other mean values as in Fig. 7.

Fig.9. Multiple regression analysis of serum and LV CS with serum ionized Mg ,serum cytokine/chemokine levels with serum ionized Mg, and aorta and LV cytokine/chemokine levels with ionized Mg. Regression equations with r-values are as follows:1) serum IL-1a vs. Mg2+:$y=-2083x + 1319$; $r=0.88$ 2) serum IL-1b vs. Mg2+: $y=-1853x + 1410$; $r= 0.96$;3)serum IL-2 vs. Mg2+: $y=-415.3x + 285.8$; $r=0.90$; 4)serum IL-4 vs. Mg2+ : $y=-1106x + 705.3$; $r= 0.88$; 5) serum IL-6 vs. Mg2+: y=-

289.5x + 214.9; r=0.80 ; 6)serum IL-10 vs. Mg2+: y= -1225x + 904.5 ; r=0.75; 7)serum IL-12 vs.Mg2+: y=-2012x + 1315 ; r=0.92; 8)serum IL-13 vs. Mg2+: y=-1111x + 775; r=0.96 ;9)serum IFN-g vs. Mg2+ : y=-2089x + 1545; r= 0.93; 10)serum TN-F -alpha vs. Mg2+: y=-1709x + 1092; r=0.83; 11)serum GM-CSF vs. Mg2+: y=-2318x + 1568; r= 0.96; 12)serum RANTES vs. Mg2+: y=-1534x +973.9 : r=0.85. For LV cytokines/ chemokines vs. Mg2+: 1) IL-1a: y=-2502x + 1554;r=0.80;2) IL-1b: y=-1844x +1486; r=0.95; 3) IL-2: y==676x + 450.5; r=0.86; 4)IL-4: y=-1134x =748 ; r=0.93; 5)IL-6: y=-572x +395 ; r= 0.82; 6) IL-10: -1324x + 925; r=0.53;7) IL-12: y= -2138x + 1398; r=0.88; 8)IL-13: y=-1142x + 842; r=0.88; 9) IFN-g: y-01866x + 1404; r=0.95; 10) TNF-alpha: -1798x + 1147; r=0.82; 11) GM-CSF: y=-2105x + 1414x ; r=0.88; 12) RANTES:-1569x + 994;r=0.68. For aorta cytokines/chemokines vs . Mg2+: 1) IL-1a: y=-2195x + 1360; r=0.87; 2) Il-1b: y=-1911x + 1270; r=0.91; 3) IL-2: y=-530x + 369; r=0.76; 4)IL-4: y=-821x + 558; r= 0.96; 5) IL-6: y= -525x + 371; r= 0.77; 6)IL-10: y=-897x+673;r=0.53;7)IL-12:y=-1911x+1270;r=0.91:8)IL-13:y=-1142x+842.43;r=0.88;9)IFN-g:y=-1866x+1404;r=0.95;10)TNF-alpha:y=-1798x+1147;r=0.82 11)GM-CSF:y=-1543x+1097;r=0.89;12)RANTES:y=-1191x+784;r=0.82 For serum CS y=-384x+689;r=0.69 For LV CS:y=-1232x+1083;r=0.86


Ceramide Synthase activity (U / ml)
900
800
700
600
500
400
300
200
100
0
LV
RV
ATRIA
AORTA
CONTROL
Mg D
Mg D + 15
Mg D+ 40
Mg D + 100

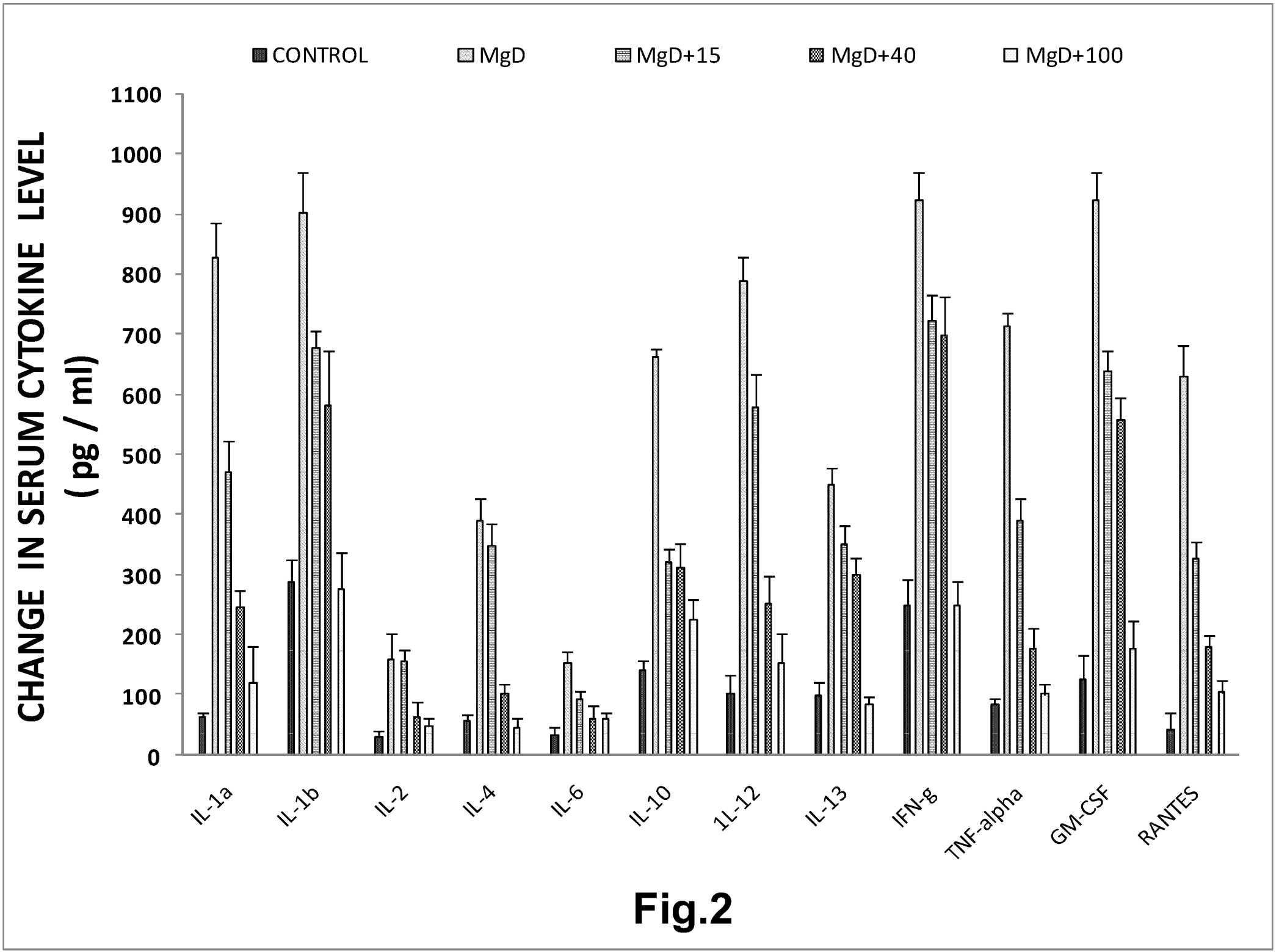

CONTROL
MgD
MgD+15
MgD+40
MgD+100
CHANGE IN SERUM CYTOKINE LEVEL
(pg / ml)
1100
1000
900
800
700
600
500
400
300
200
100
0
IL-1a
IL-1b
IL-2
IL-4
IL-6
IL-10
1L-12
IL-13
IFN-g
TNF-alpha
GM-CSF
RANTES

Fig.2

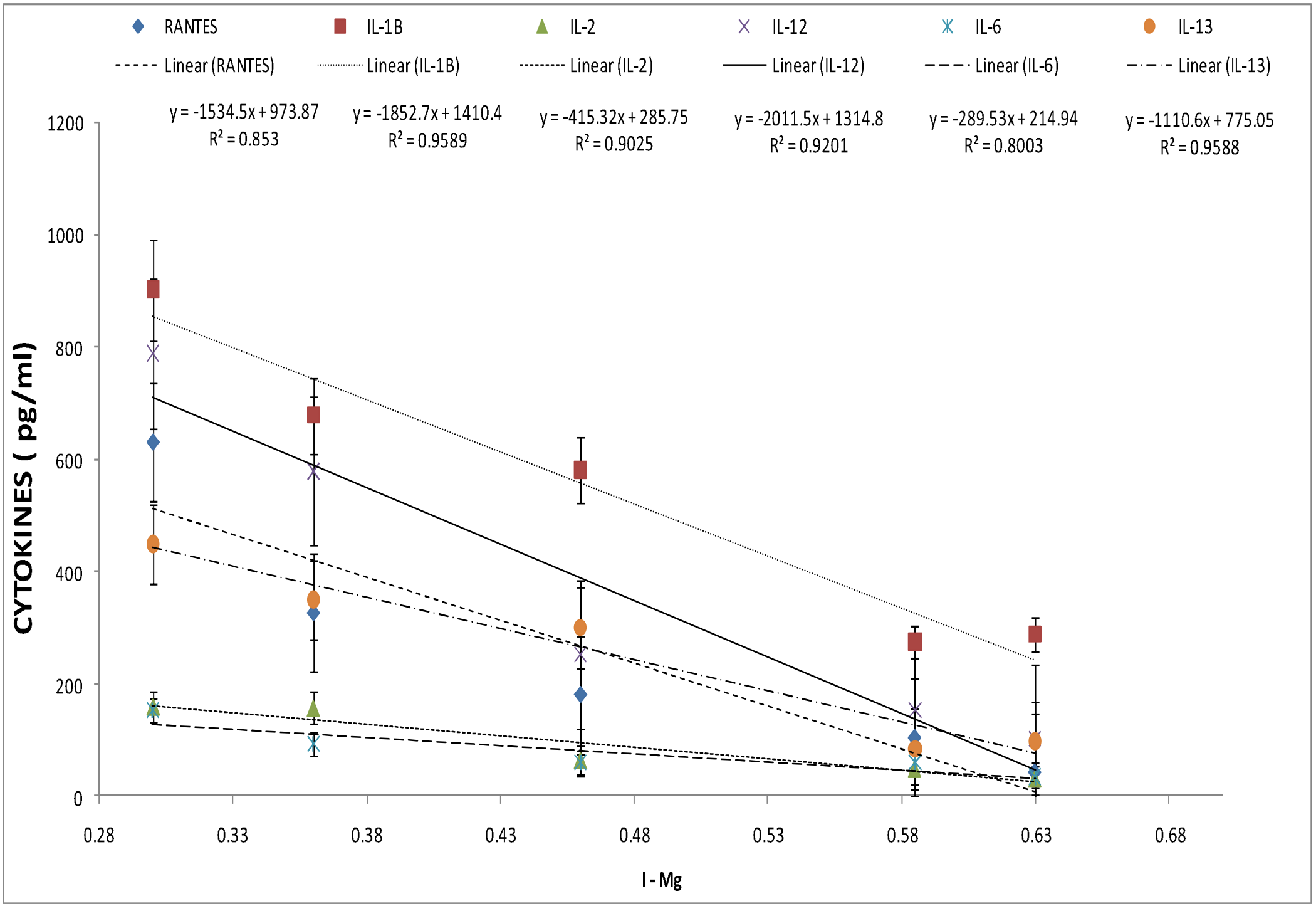

RANTES
IL-1B
IL-2
IL-12
IL-6
IL-13
Linear (RANTES)
Linear (IL-1B)
Linear (IL-2)
Linear (IL-12)
Linear (IL-6)
Linear (IL-13)
y = -1534.5x + 973.87
R² = 0.853
y = -1852.7x + 1410.4
R² = 0.9589
y = -415.32x + 285.75
R² = 0.9025
y = -2011.5x + 1314.8
R² = 0.9201
y = -289.53x + 214.94
R² = 0.8003
y = -1110.6x + 775.05
R² = 0.9588
CYTOKINES (pg/ml)
1200
1000
800
600
400
200
0
0.28
0.33
0.38
0.43
0.48
0.53
0.58
0.63
0.68
I - Mg

Fig. 3

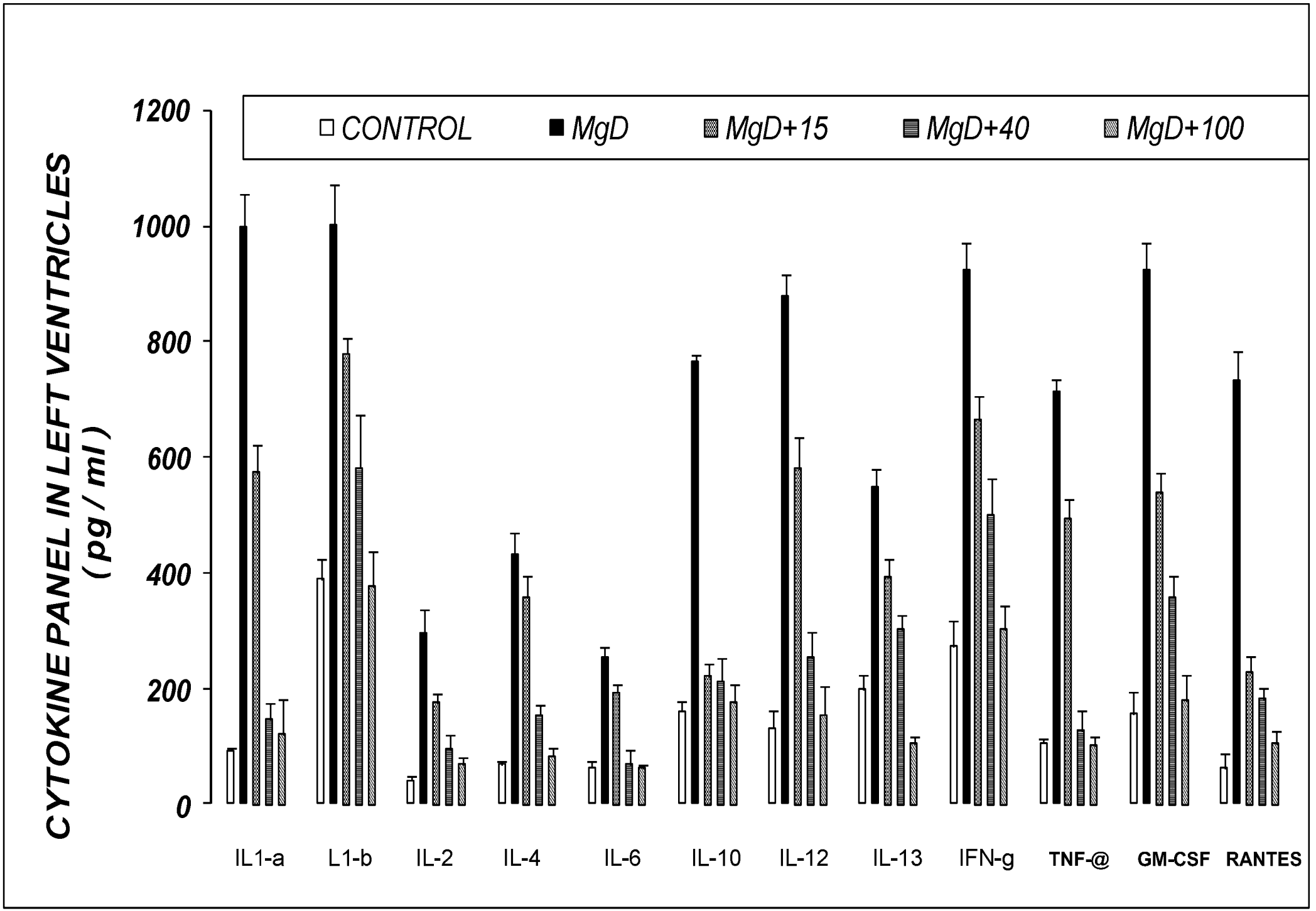

CONTROL
MgD
MgD+15
MgD+40
MgD+100
CYTOKINE PANEL IN LEFT VENTRICLES
(pg / ml)
1200
1000
800
600
400
200
0
IL1-a
L1-b
IL-2
IL-4
IL-6
IL-10
IL-12
IL-13
IFN-g
TNF-@
GM-CSF
RANTES

Fig.4

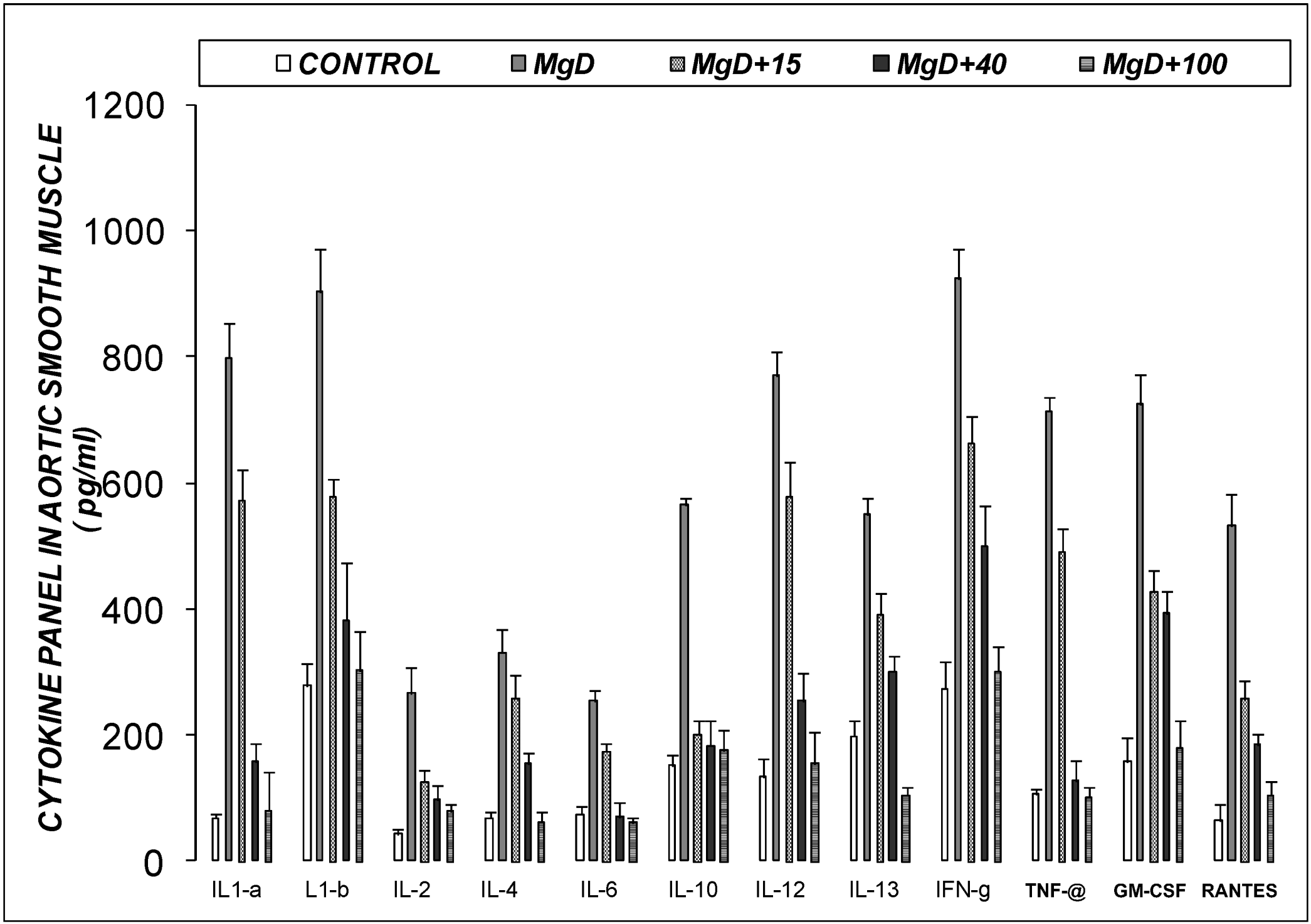

CONTROL
MgD
MgD+15
MgD+40
MgD+100
CYTOKINE PANEL IN AORTIC SMOOTH MUSCLE
(pg/ml)
1200
1000
800
600
400
200
0
IL1-a
L1-b
IL-2
IL-4
IL-6
IL-10
IL-12
IL-13
IFN-g
TNF-@
GM-CSF
RANTES

Fig.5

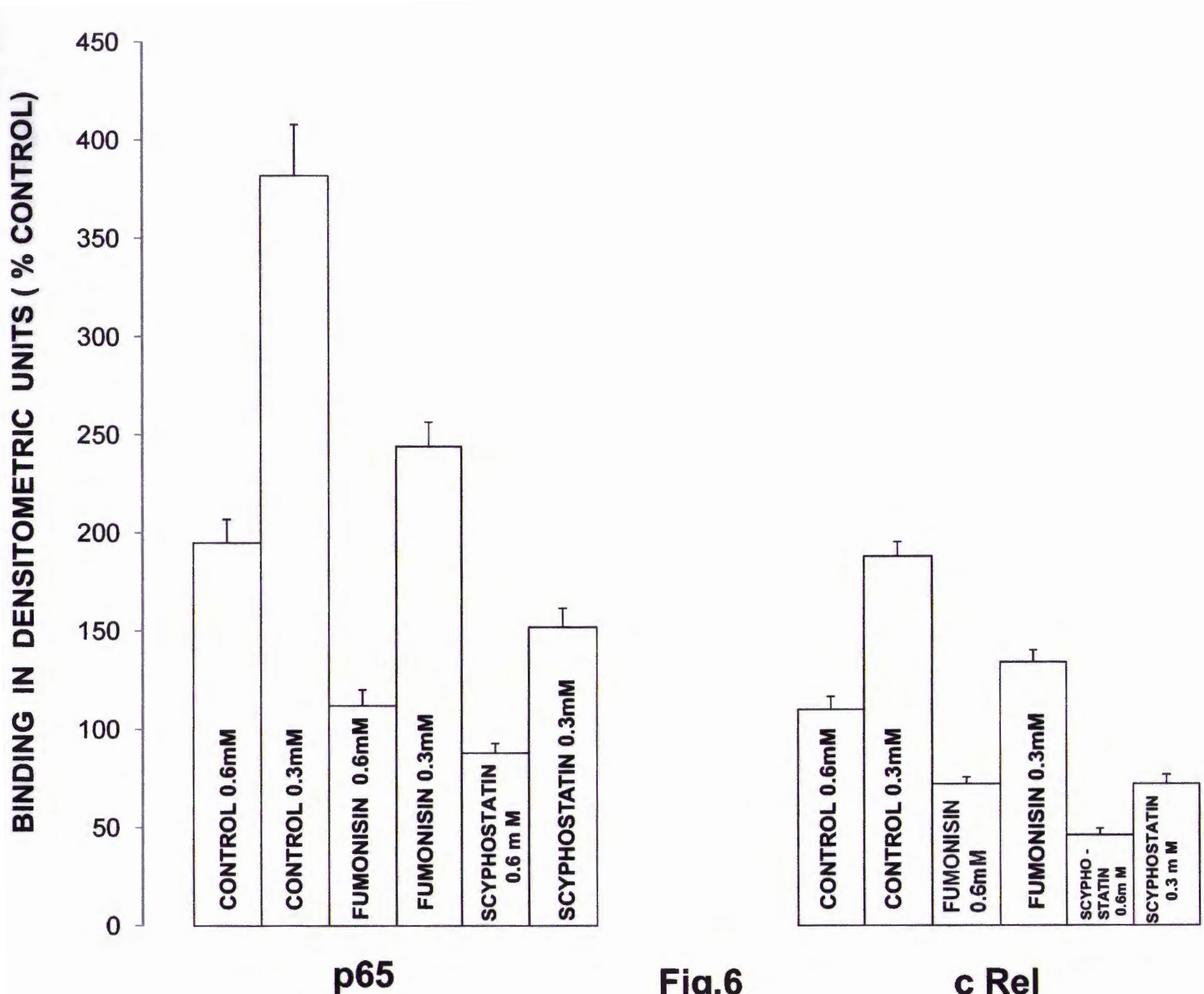

BINDING IN DENSITOMETRIC UNITS (% CONTROL)
450
400
350
300
250
200
150
100
50
0
CONTROL 0.6mM
CONTROL 0.3mM
FUMONISIN 0.6mM
FUMONISIN 0.3mM
SCYPHOSTATIN 0.6 m M
SCYPHOSTATIN 0.3mM
p65
CONTROL 0.6mM
CONTROL 0.3mM
FUMONISIN 0.6mM
FUMONISIN 0.3mM
SCYPHO - STATIN 0.6m M
SCYPHOSTATIN 0.3 m M
c Rel

Fig.6

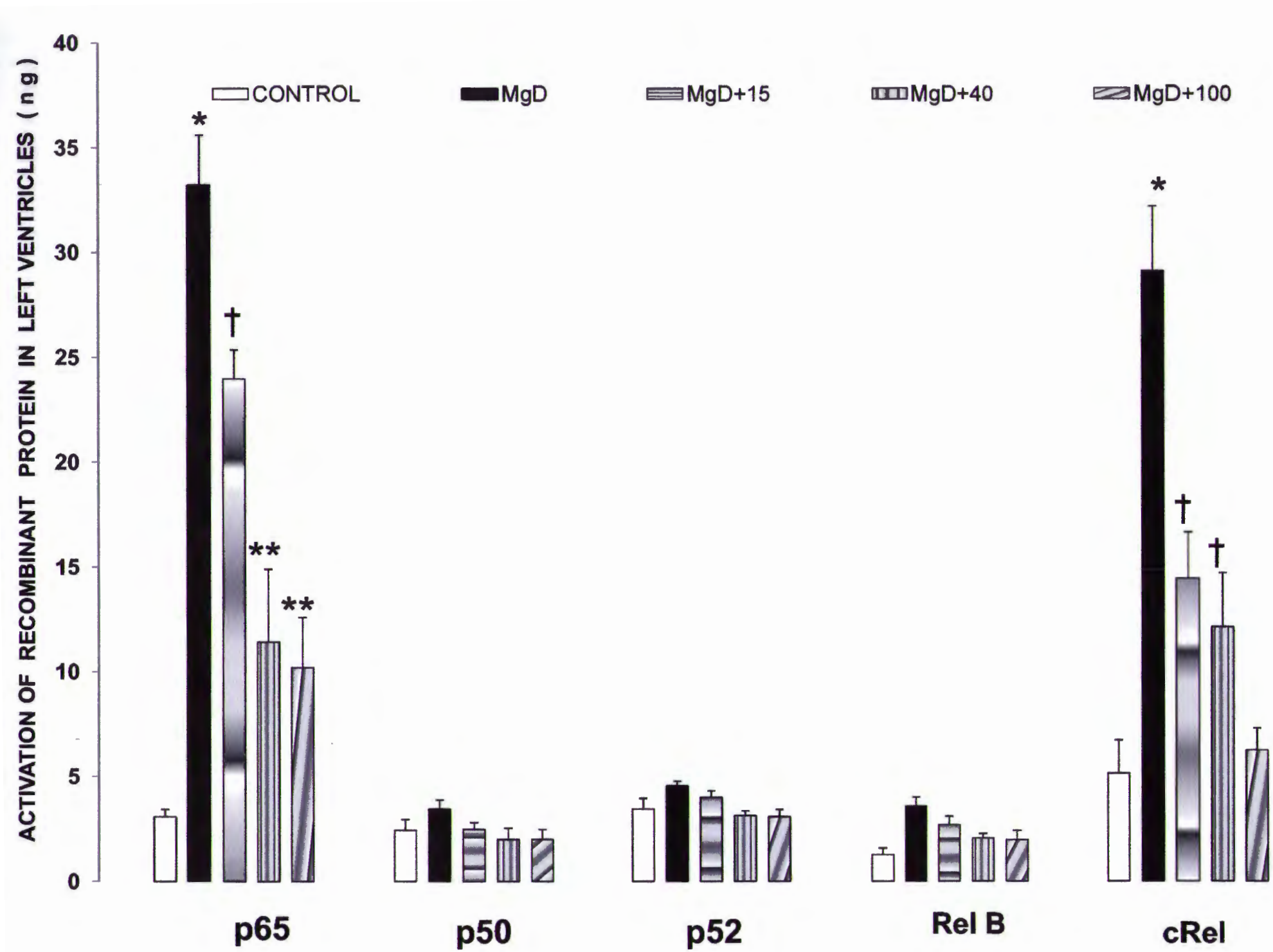

ACTIVATION OF RECOMBINANT PROTEIN IN LEFT VENTRICLES (n g)
40
35
30
25
20
15
10
5
0
CONTROL
MgD
MgD+15
MgD+40
MgD+100
*
†
**
**
*
†
†
p65
p50
p52
Rel B
cRel

Fig.7

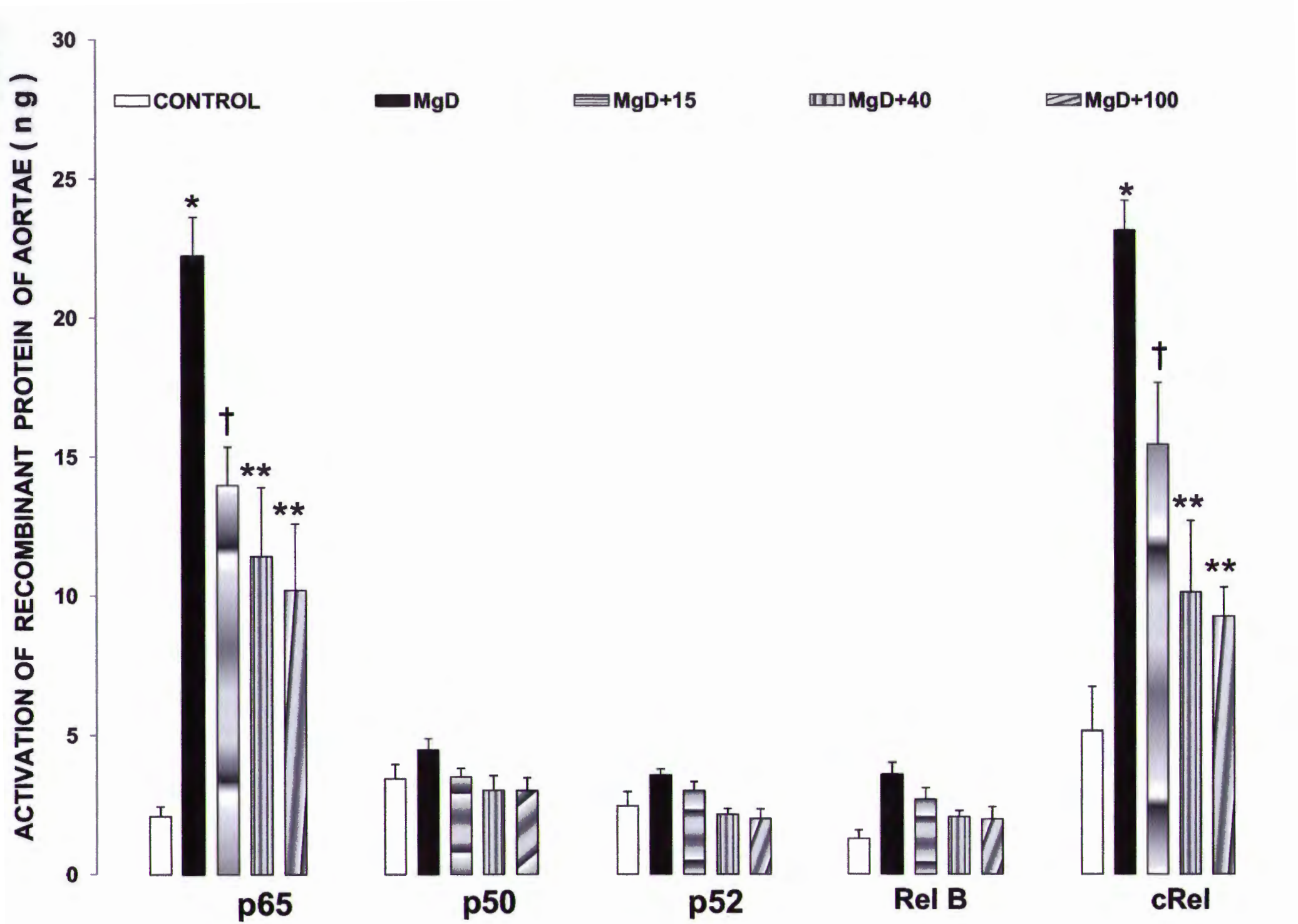
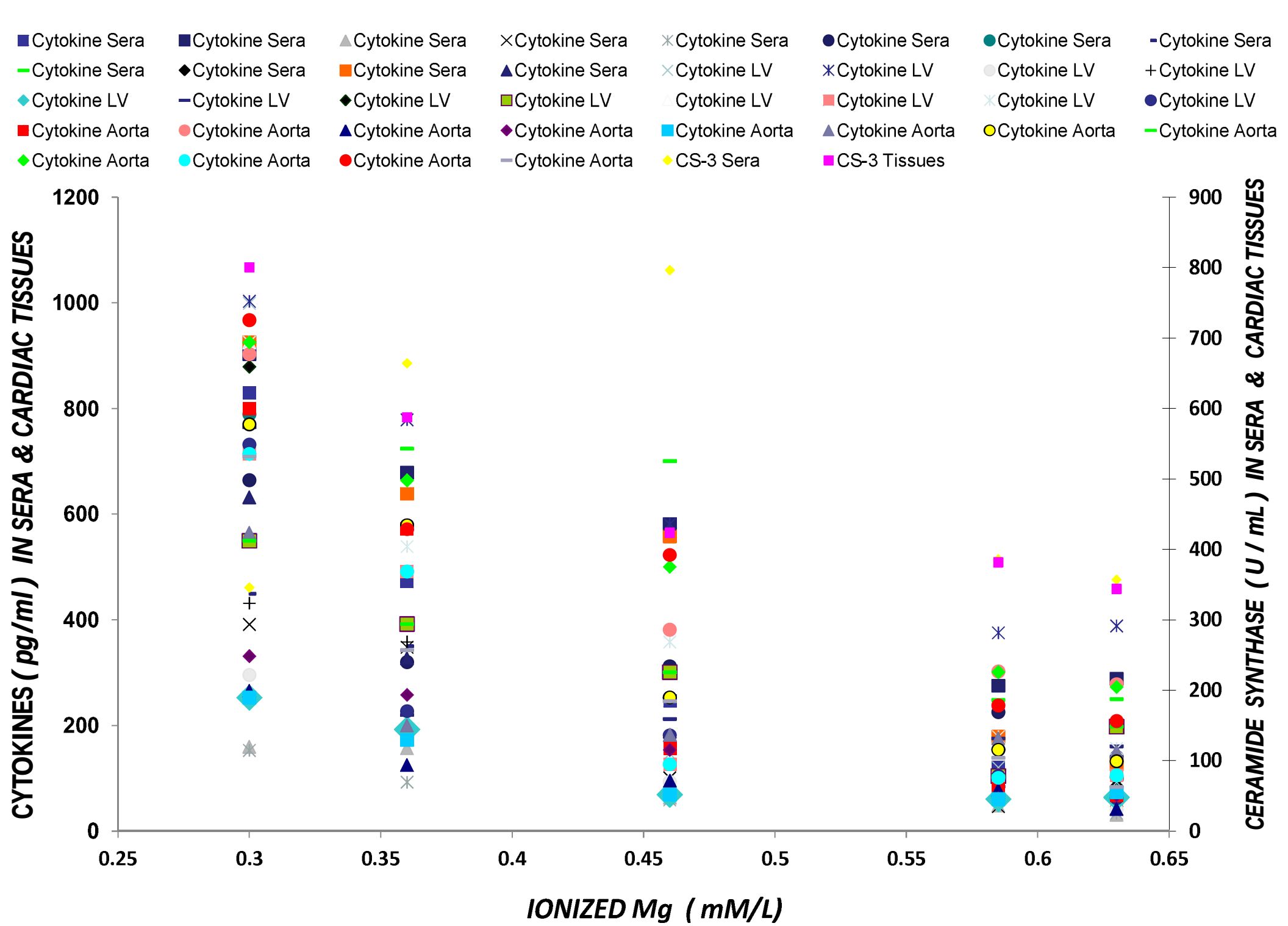

ACTIVATION OF RECOMBINANT PROTEIN OF AORTAE (n g)
30
25
20
15
10
5
0
CONTROL
MgD
MgD+15
MgD+40
MgD+100
*
†
**
**
*
†
**
**
p65
p50
p52
Rel B
cRel

Fig.8


Cytokine Sera
Cytokine Sera
Cytokine Sera
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Cytokine Sera
Cytokine Sera
Cytokine Sera
Cytokine Sera
Cytokine LV
Cytokine LV
Cytokine LV
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Cytokine LV
Cytokine LV
Cytokine LV
Cytokine LV
Cytokine LV
Cytokine LV
Cytokine LV
Cytokine LV
Cytokine Aorta
Cytokine Aorta
Cytokine Aorta
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Cytokine Aorta
Cytokine Aorta
Cytokine Aorta
Cytokine Aorta
Cytokine Aorta
Cytokine Aorta
Cytokine Aorta
CS-3 Sera
CS-3 Tissues
CYTOKINES (pg/ml) IN SERA & CARDIAC TISSUES
CERAMIDE SYNTHASE (U / mL) IN SERA & CARDIAC TISSUES
0
200
400
600
800
1000
1200
0
100
200
300
400
500
600
700
800
900
0.25
0.3
0.35
0.4
0.45
0.5
0.55
0.6
0.65
IONIZED Mg (mM/L)

Fig.9