ORIGINAL ARTICLE

Effect of magnesium supplementation and depletion on the onset and course of acute experimental pancreatitis

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ABSTRACT

Background and objective High calcium concentrations are an established risk factor for pancreatitis. We have investigated whether increasing magnesium concentrations affect pathological calcium signals and premature protease activation in pancreatic acini, and whether dietary or intraperitoneal magnesium administration affects the onset and course of experimental pancreatitis.

Methods Pancreatic acini were incubated with up to 10 mM magnesium; $[Ca^{2+}]_i$ (fura-2AM) and intracellular protease activation (fluorogenic substrates) were determined over 60 min. Wistar rats received chow either supplemented or depleted for magnesium (<300 ppm to 30 000 ppm) over two weeks before pancreatitis induction (intravenous caerulein 10 μg/kg/h/4 h); controls received 1 μg/kg/h caerulein or saline. C57BL6/J mice received four intraperitoneal doses of magnesium (NaCl, Mg^{2+} 55 192 or 384 mg/kg bodyweight) over 72 h, then pancreatitis was induced by up to eight hourly supramaximal caerulein applications. Pancreatic enzyme activities, protease activation, morphological changes and the immune response were investigated.

Results Increasing extracellular Mg^{2+} concentration significantly reduced $[Ca^{2+}]_i$ peaks and frequency of $[Ca^{2+}]_i$ oscillations as well as intracellular trypsin and elastase activity. Magnesium administration reduced pancreatic enzyme activities, oedema, tissue necrosis and inflammation and somewhat increased Foxp3-positiv T-cells during experimental pancreatitis. Protease activation was found in animals fed magnesium-deficient chow—even with low caerulein concentrations that normally cause no damage.

Conclusions Magnesium supplementation significantly reduces premature protease activation and the severity of pancreatitis, and antagonises pathological $[Ca^{2+}]_i$ signals. Nutritional magnesium deficiency increases the susceptibility of the pancreas towards pathological stimuli. These data have prompted two clinical trials on the use of magnesium in patients at risk for pancreatitis.

Significance of this study

What is already known on this subject?

- Premature intracellular protease activation is critical for the onset of pancreatitis and parallels acinar cell injury.
- Protease activation in pancreatic acinar cells depends on the apical release of very high (millimolar) concentrations of calcium from intracellular stores.
- Synthetic calcium chelators have been found to reduce intracellular protease activation and acinar cell injury in vitro and in vivo.
- Intracellular magnesium can replace intracellular calcium and may therefore act as a natural and physiological calcium antagonist.

What are the new findings?

- In isolated pancreatic acini an increase in extracellular magnesium reduces the peak $[Ca^{2+}]_i$ response to pathological secretagogue stimulation, the frequency of $[Ca^{2+}]_i$ oscillations, and intracellular protease activation as well as cell injury.
- Feeding a magnesium enriched diet to rats over 2 weeks or administering intraperitoneal magnesium to mice over 72 h reduces the severity of subsequent experimental pancreatitis.
- Feeding a magnesium-deficient diet to rats leads to pancreatic damage and intrapancreatic protease activation, even when submaximal secretagogue concentrations, which normally do not induce pancreatitis, were used.

How might it impact on clinical practice in the foreseeable future?

- These data indicate that magnesium represents a physiological, cheap, orally available and well tolerated calcium antagonist that could be used to treat patients with pancreatitis or at risk of pancreatitis.
- As a consequence of these results, two international randomised clinical trials (ISRCTN00142233 and ISRCTN46556454) have been launched to test the therapeutic potential of magnesium in patients at risk for developing pancreatitis.

INTRODUCTION

Acute pancreatitis, a fatal disease for 20% of severely affected patients, has long been considered a disorder of pancreatic self-digestion, in which premature and intracellular activation of digestive proteases induces tissue injury.[1–5] Hypercalcaemia

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is a well known risk factor for acute pancreatitis,[6–8] and it has been shown that elevated concentrations of acinar cytosolic calcium are an important trigger of the disease.[9] In experimental models of acute pancreatitis, dramatic changes in intracellular free calcium concentrations have been detected.[10 11] On secretagogue stimulation, intracellular protease activation is primarily observed at the apical pole of acinar cells at the site where the stimulus-induced calcium release takes place.[10] Under experimental conditions the removal of calcium from the incubation media or chelation of intracellular calcium prevents cellular injury.[12 13] These data support the hypothesis that control of intracellular calcium levels can prevent premature intrapancreatic zymogen activation and thereby reduce local and systemic damage associated with pancreatitis.

Magnesium, a critical cofactor for multiple enzymatic reactions in almost all eukaryotic systems, is decreased in the serum of patients with acute pancreatitis,[14–16] and a substantial magnesium deficiency has been reported in individuals with chronic pancreatitis.[17] Previously, we have shown in isolated pancreatic acinar cells that increased intracellular magnesium concentrations directly influence the frequency and amplitude of calcium oscillations in response to cholecystokinin (CCK) or the acetylcholine analogue carbachol.[18] Characterisation of the spatial and temporal distribution of intra-acinar magnesium concentrations has indicated that magnesium could antagonise calcium signals.[18]

We therefore suggest that magnesium represents a natural calcium antagonist in acinar cells and thus may have a beneficial effect in pancreatitis. The aim of the present study was to investigate the effect of magnesium on premature enzyme activation in vitro as well as on pancreatic inflammation in vivo.

MATERIALS AND METHODS

Materials

Caerulein was provided by Farmitalia Carlo Erba (Freiburg, Germany). Magnesium-enriched and magnesium-depleted diets were formulated by Altrumin (Lage, Germany). A trypsinogen activation peptide (TAP) enzyme immunoassay was obtained from Biotrin International (Sinsheim-Reihen, Germany). Magnesium-L-aspartate-hydrochloride and magnesium-sulfate-heptahydrate were obtained from Verla (Tutzing, Germany) and Sigma (Munich), respectively. The trypsin substrate $(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$-rhodamin110 and the elastase substrate $(CBZ\text{-}Ala_4)_2$-rhodamin110 were obtained from Invitrogen (Eugene, Oregon, USA). All other chemicals were of the highest purity commercially available and were purchased from either Sigma (Deisenhofen, Germany) or Merck (Hannover, Germany).

Cell isolation procedure

Pancreatic acini were prepared according to a modified collagenase protocol as previously reported.[3 19] In brief, after a 12-h fast the animals were killed and the pancreas was rapidly removed, minced into small pieces and placed into buffer (pH 7.4) containing NaCl (130 mM), KCl (5 mM), HEPES (10 mM), KH_2PO_4 (1.2 mM), $CaCl_2$ (1 mM), $MgSO_4$ (1 mM), glucose (10 mM) and collagenase type V (100 U/mL, Sigma, Deisenhofen, Germany). After 10 min of incubation at 37°C under continuous shaking (120 cycles/min), the digested tissue was washed three times in 10 mL buffer without the presence of collagenase and again shaken 10 times to dissociate the acini. Acini were then filtered through muslin gauze, centrifuged at 400 rpm for 3 min and washed twice more in buffer solution containing 4% bovine serum albumin (BSA). After the second wash, acini were suspended in 8 mL of buffer containing soybean trypsin inhibitor (0.1 mg/mL) and BSA (0.2% w/v). A stock suspension of acini was kept on ice for up to 4 h without significant reduction in cell viability (>95%) as assessed by trypan blue exclusion.

Measurement of intracellular free calcium concentrations

For microfluorometric studies of intracellular free calcium ($[Ca^{2+}]_i$) dynamics, rat acini were loaded with the AM-ester of fura-2 (5 μM) at room temperature for 20 min. Fura-2-loaded cells were plated onto glass cover slips coated with Cell-Tak (1 μL/cm^2) and kept at room temperature for 30 min before they were mounted in a perfusion chamber with an internal volume of 0.28 mL and placed on the stage of a Nikon Diaphot-TMD inverted microscope equipped with a Fluor X 100 oil immersion objective. Acini were continuously perfused with medium at a flow rate of 3 mL/min at room temperature. Fluorochrome excitation (340 and 380 nm for fura-2) was achieved using a xenon lamp in series with two chopper-linked monochromators that were coupled to the microscope via fibre optics. Emitted light was collected behind a band-pass filter (509 nm for fura-2) by a photomultiplier. In unloaded controls background fluorescence was found to be negligible.

Ratios obtained from the dual excitation wavelengths probe fura-2 were converted to calcium concentrations as previously reported[16] using the formula

$$(Ca^{2+})_i = (R\text{-}R_{min})/(R_{max}\text{-}R) \times K_d \times (I_{ion\text{-}free}/I_{ion\text{-}saturated})\ 380\ nm,$$

in which R_{min} and R_{max} are the minimal and maximal ratios obtained in either ion-free or ion-saturated solutions, respectively. K_d is the dissociation constant of fura-2 for calcium, and I is the fluorescence intensity at the 380 nm wavelength of the free dye divided by the saturated ion–dye complex.[20 21] All calibration parameters were obtained in separate experiments.

Protease activity in isolated acini

Acinar cells were prepared by collagenase digestion, maintained and stimulated in DMEM medium containing 2% BSA and 10 mM HEPES. Acinar cells were stimulated with either physiological (0.001 mM) or supramaximal (10 mM) concentrations of CCK.[22–24] Intracellular trypsin (substrate bis-(CBZ-Ile-Pro-Arg)-rhodamine110 from Invitrogen) and elastase (substrate bis-(CBZ-Ala4)-rhodamine110) activity were measured as previously described.[22 23]

ATPase activity and kinase activity in isolated acini

Acinar cells were prepared by collagenase digestion as described above, then incubated with or without 5 mM Mg^{2+} for 30 min and subsequently stimulated with supramaximal concentrations of CCK (10^{-7} M). ATPase activity was detected using a colorimetric assay (Innova Biosciences; product code 601-0120) according to the manufacturer's protocol but without adding Mg^{2+} to the measurement buffer. The reaction is started by purified Pi-free ATP and detected by PiColorLock Gold reagent (an improved malachite green formulation). Kinase activity was determined employing a fluorometric assay from Bioquest (product code 31001) according to the manufacturer's instructions (biological replicates, n>3; technical replicates, n=3). The assay is based on monitoring ADP formation, which is directly proportional to enzyme phosphotransferase activity and is measured fluorimetrically. It is characterised by a high sensitivity (<0.3 μM ADP) and broad ATP tolerance (1–300 μM).

Animals and magnesium diets

Male Wistar rats were obtained from Harlan Winkelmann Breeding Laboratories (Borchen, Germany). They were housed in Nalgene shoebox cages under a 12-h light/dark cycle with unlimited access to water. All rats were adjusted to laboratory conditions over the course of 1 week prior to the experiments. Rats weighing 140–160 g were divided into four groups (n=15/group) and were pair-fed with diets containing the following concentrations of magnesium over 14 days:

1. magnesium low (<300 ppm),
2. magnesium normal (450 ppm),
3. magnesium high (1950 ppm),
4. magnesium very high (30 000 ppm).

Male 14-week-old C56BL6/J mice were obtained from Charles River (Sulzbach).

For urine collection and subsequent magnesium measurements, these were housed in metabolic cages under a 12-h light/dark cycle with unlimited access to water. All mice were adjusted to laboratory conditions over the course of 1 week prior to the experiments. Mice weighing 25–30 g were divided into four groups (n=10/group) and were pretreated over a period of 3 days with daily intraperitoneal $MgSO_4$ at a concentration of 55.5, 192 or 384 mg/kg/bodyweight dissolved in 300 µL of NaCl 0.9%. Control animals received 300 µL NaCl 0.9% alone. The dose of 384 mg/kg bodyweight parallels the LD25% for intraperitoneal application, and 192 mg/kg bodyweight has previously been shown to be biologically effective in vivo via intraperitoneal administration.[24]

All animal experiments were started after an overnight fast and were conducted according to the guidelines of the local animal use and care committee and the guiding principles of the American Physiological Society.

Experimental pancreatitis

Rats were equipped with jugular vein catheters under pentobarbital anaesthesia (60 mg/kg intraperitoneally) after 14 days on the different magnesium diets, and allowed to recover for 12 h with tap water ad libitum but no chow. They were then randomly allocated to one of the following groups:

1. infusion of saline (NaCl 0.9%) for 4 h,
2. infusion of submaximal caerulein concentrations, 1 µg/kg/h for 4 h,
3. supramaximal stimulation with caerulein 10 µg/kg/h for 4 h.

At the end of the infusion periods, the rats were killed under ether anaesthesia by bleeding of the abdominal aorta. Blood samples were centrifuged at 4°C (3000g, 10 min), and serum was stored at −20°C for further studies. The entire pancreas was immediately removed in a standardised fashion, trimmed of fat and divided into portions for the following assays.

Mice received up to eight hourly intraperitoneal injections of caerulein (50 µg/kg bodyweight) dissolved in 200 µL NaCl 0.9% to induce a pancreatitis of greater severity.[25]

Caerulein was injected 1 h after administration of the last magnesium dose. All animals were fasted overnight with access to water ad libitum prior to induction of pancreatitis.

The mice were sacrificed at different time points after the first caerulein injection. Blood samples were centrifuged at 4°C (3000g, 10 min) and serum was stored at −20°C for further studies. The entire pancreas was immediately removed in a standardised fashion and divided into portions for the following assays. The entire spleen was immediately removed in a standardised fashion for subsequent FACS analysis.

Morphology

For morphological analysis, small pieces of the pancreas were fixed in 4% neutral phosphate buffered formalin (4°C) and embedded in paraffin. Sections (2 µm) were stained with H&E as previously described[26] and were evaluated by an observer unaware of the treatment groups. Histomorphological evaluation of the specimens included the quantification of areas of necrosis as previously reported.[22]

For transmission electron microscopy, pancreatic specimens no larger than 2 mm were immersed in iced 2% glutaraldehyde/2% formaldehyde solution at pH 7.4 with 0.1 M cacodylate buffer. Blocks were postfixed in 1% osmium tetroxide and embedded in Epon. Silver thin sections were contrasted with uranyl and examined on an electron microscope (Philips EM 10, Eindhoven, The Netherlands).[27]

Evaluation of pancreatic oedema

Pancreatic oedema was evaluated as pancreatic water content and was calculated by the wet-weight/dry-weight ratio: the initial weight (wet weight) was divided by the weight after desiccation at 160°C for 12 h (dry weight).

Ion measurements

Free serum calcium and magnesium concentrations were determined with an ion- specific electrode (Electrolyte Analyzer, Graze, Austria).

Total magnesium content in pancreatic tissue was determined by atomic absorption spectroscopy (AAS) as described by Kotz *et al.*[21] Briefly, 300 mg of pancreatic tissue was solubilised with 3.5 mL aqua dest. and 1.5 mL nitric acid over 8 h in ascending temperatures from 110 to 155°C. After solution in caesium chloride–lanthanum chloride buffer a calibration was prepared, and AAS measurements were performed under standardised conditions.

Biochemical assays

Amylase and lipase activity

Serum amylase and lipase activity were measured photometrically with a CobasBio automated analyser from Roche (Basel, Switzerland). Calibrator, control serum, amylase and lipase reagents were purchased from Sigma.

Trypsinogen activation peptide

For measurement of TAP, urine was collected shortly before killing the rats and stored in an end-concentration of 10 mM EDTA at −20°C until assayed. Blood samples were collected in 0.2 mol/L EDTA. After centrifugation (3000g, 20 min, 4°C), the supernatant was stored at −20°C. For measurements of pancreatic TAP levels, a 250-mg tissue portion of the pancreas was immersed in 0.2 M Tris-HCl buffer (pH 7.5) containing 0.02 M EDTA and 1% Triton X-100, and immediately boiled (at 100°C) for 15 min to denature the remaining proteases and stop additional TAP generation. Afterwards, the samples were homogenised in a Braun polytron (Braun Melsungen, Melsungen, Germany) for 1 min. After subsequent centrifugation (1500g, 10 min, 4°C), the supernatants were stored at −80°C until assayed. TAP was quantified with the Biotrin TAP enzyme immunoassay, which operates on the basis of competition between free and immobilised peptide for binding to an anti-TAP antibody.

Tissue trypsin and elastase activity

To determine the activity of elastase and trypsin in pancreatic tissue, 250-mg portions were frozen in fluid nitrite oxide and stored at −80°C until assayed. The pancreatic tissue was homogenised for 1 min in a buffer containing 100 mM HEPES, 300 mM EDTA, 20% glycerin, 0.2% Triton X-100, pH 7.5. Afterwards, the protein concentration was measured photometrically (LKB Biochrom, Cambridge, UK) using a Bio-Rad assay.

All samples were adjusted to the same protein concentration with phosphate buffered saline, and enzyme activity was determined over a time period of 60 min at 37°C. For intercellular trypsin activity we used the substrate bis-(CBZ-Ile-Pro-Arg)-rhodamine110 (Invitrogen), and for elastase, bis-(CBZ-Ala_4)-rhodamine110. In acinar cell experiments, the measurement of only intracellular protease activity was ensured by using phenylmethanesulfonylfluoride (PMSF) and aprotinin as inhibitors in the extracellular buffer.[28]

Myeloperoxidase activity

Myeloperoxidase (MPO) activity measurement was performed as previously described.[26] Briefly, pancreatic tissue was homogenised on ice in 20 mM potassium phosphate buffer (pH 7.4) and centrifuged. The pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% cetyltrimethylammoniumbromide. The suspension was frozen-thawed in cycles, sonicated and centrifuged at 20 000 g. MPO activity was assayed in 50 mM potassium-phosphate buffer (pH 6) containing 0.53 mM O-dianisidine and 0.15 mM H_2O_2. The initial increase in absorbance was measured at room temperature with a Spectramax spectrophotometer. The results are expressed in units of MPO activity. Bars indicate mean values in mU MPO activity per mg pancreatic protein±SEM from at least six animals per time-point.[23]

Flow cytometric analysis

To analyse subtypes of inflammatory cells, splenic single-cell suspensions from treated mice and controls were prepared. A total of 1×10^6 leucocytes were stained with directly-conjugated antibodies anti-mouse CD3 APC (17A2) anti-mouse CD4 PerCp-Cy5.5 (RM4-5,), anti-mouse CD62L APC-Cy7 (MEL-14,), CD25 Brilliant Violet 421 (PC-61, all Biolegend, Fell, Germany). Data were recorded using the BD LSR II system (Becton Dickinson, NJ, USA). Intracellular staining was performed with the intracellular staining kit and anti-mouse/human FoxP3 APC (3G3, Miltenyi Biotec, Bergisch Gladbach, Germany) as per manufacturer's instructions and cells were analyzed using FlowJo software.[30] To determine serum concentrations of cytokines, a cytometric bead array (Mouse Inflammation Kit, Becton Dickinson, San Diego, California, USA) was performed according to the manufacturer's instructions.

Statistical analysis

All values in the figures represent means±SEM from at least four separate experiments unless otherwise indicated. Differences between groups were compared using analysis of variance with Bonferroni correction for multiple comparisons or t tests where applicable. Differences considered statistically significant were those with a p value <0.05.

RESULTS

Incubation of isolated rat pancreatic acinar cells stimulated with supramaximal concentration of CCK (10^{-7} M) in the presence of different concentrations of extracellular magnesium (ranging from 0.2 to 10 mM) resulted in a dose dependent alteration of intracellular calcium transients (figure 1A) as well as a dose dependent change in the frequency of calcium oscillations (figure 1B). Under these conditions high extracellular magnesium levels (10 mM) resulted in a decrease in CCK-induced calcium transients, whereas low magnesium (0.2 mM) enhanced calcium transients and the frequency of calcium oscillations (figure 1A,B). These data indicated that magnesium alters the availability of $[Ca^{2+}]_i$ and could indeed be regarded as a physiological calcium antagonist in acinar cells. Next we tested whether this change in calcium transients affected Ca^{2+} dependent intracellular protease activation. Incubating isolated rat pancreatic acinar cells with supramaximal doses of CCK (10^{-7} M) resulted in the intracellular activation of trypsin and elastase (figure 1C,D). The maximum of trypsin activity (figure 1C) was reached after an incubation period of about 30 min followed by a slow decrease in activity over the next 30 min. The same experiment in the presence of 5 mM magnesium resulted in a significant decrease in intracellular trypsin (figure 1C) and elastase activity (figure 1D). To rule out alternative effects of magnesium we studied its effect on ATPases in acini and found that in Mg-free medium, neither the addition of Mg^{2+} nor CCK alone affected ATPase activity, but the combination of both increased it (figure 1E). The overall kinase activity, on the other hand remained unchanged (data not shown). The effect of intracellular calcium mobilisation in pancreatic acinar cells was reversed by increasing extracellular magnesium concentration and the reduced calcium signals paralleled the effect of magnesium on premature intracellular enzyme activation.

To test whether the effect of magnesium on the cellular level translates to a protective effect of magnesium on pancreatitis severity in vivo, we fed male Wistar rats with chow containing different Mg^{2+} concentrations for 2 weeks before subjecting them to a physiological (caerulein 1 µg/kg bodyweight/h) or pathological (caerulein 10 µg/kg bodyweight/h) stimulus.

As shown in figure 2A, 2 weeks on the respective magnesium diets resulted in concentration-dependent differences of the serum magnesium levels of the animals: in those fed a low magnesium diet (<300 ppm magnesium) we measured serum magnesium levels of 0.25±0.01 mM, whereas in those receiving the highest magnesium-containing diet (30 000 ppm) we detected magnesium levels of 0.9±0.03 mM (figure 2A). Tissue magnesium levels increased in parallel but the increase did not reach significance (figure 2B). From baseline levels serum calcium increased under the magnesium-deficient diet and decreased under the diet high in magnesium (figure 2C). Neither stool consistency nor animal behaviour appeared to be affected by the magnesium content of the diet.

After induction of acute pancreatitis by intravenous infusion of supramaximal caerulein concentrations via the jugular vein (10 µg/kg/h), and in line with previous reports, there was no significant change in serum Mg^{2+} levels (figure 2D), while the decrease in serum calcium level was most pronounced in animals fed the high magnesium chow (figure 2E). Animals on high and very high magnesium chow had a significantly smaller decrease in serum calcium levels.

Acute pancreatitis is characterised by increased activities of the pancreatic enzymes amylase and lipase in serum. Induction of acute pancreatitis after supramaximal intravenous caerulein stimulation (10 µg/kg/h) induced the characteristic increase in both serum amylase and serum lipase activity (figure 3A,B). The most pronounced increase was detected not in the saline group

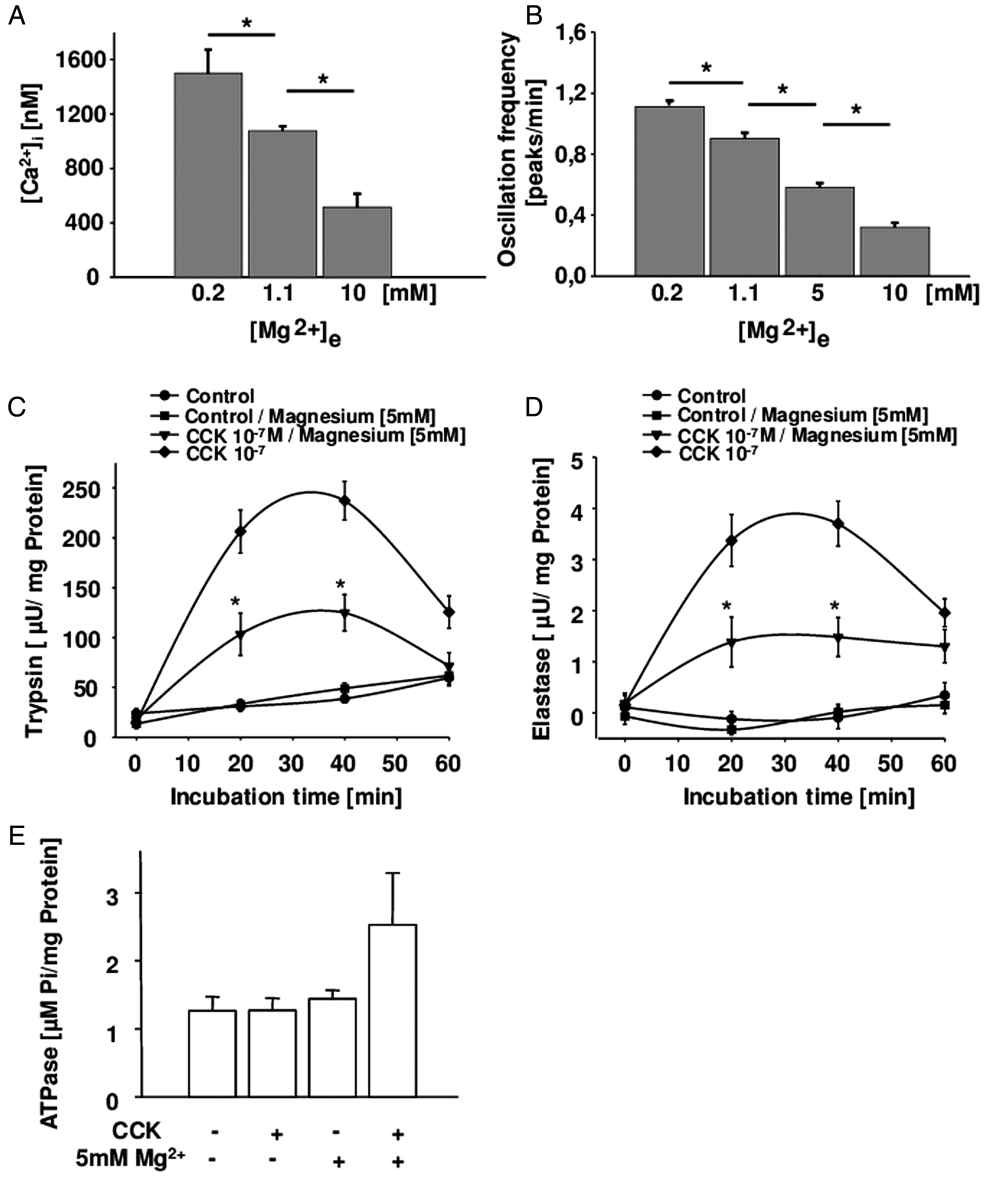

Figure 1 Cholecystokinin (CCK) (10^{-7} M)-induced intracellular calcium mobilisation in isolated rat pancreatic acinar cells is dependent on extracellular magnesium concentrations (A). Furthermore, the oscillation frequency of intracellular calcium oscillations after CCK stimulation (20 pM) is inversely related to the extracellular magnesium concentration (B). Activation of trypsin (C) and elastase (D) in isolated pancreatic acini cells after supramaximal stimulation with CCK (10^{-7} M) decreases in the presence of magnesium (5 mM). Magnesium (5 mM) increases CCK (10^{-7} M)-induced ATPase activity in isolated pancreatic acini. Asterisks indicate significant differences ($p<0.05$).

but in the magnesium-deficient rats. Both amylase and lipase activities decreased with increasing dietary magnesium concentrations. Submaximal doses of caerulein (1 µg/kg bodyweight/h) were not sufficient to induce an increase in amylase and lipase levels in the animals on the high magnesium diet, whereas the same stimulus evoked a significant increase in both parameters in the animals on the low magnesium diet (figure 3A,B).

Caerulein-induced pancreatitis in rats is characterised by an extensive oedema, which can be quantified by the wet/dry ratio and studied on histological examination. Control animals injected with sodium chloride solution had a wet/dry ratio in the range of about 2.6–3.0 (figure 3C). Submaximal stimulation with caerulein (1 µg/kg bodyweight/h) induced a significant increase of the wet/dry ratio only in the animals fed the low magnesium diet. Supramaximal stimulation with caerulein (10 µg/kg bodyweight/h) induced an increase in the wet/dry ratio in all animals. However, the increase was most prominent in the animals fed the low magnesium diet and decreased with increasing magnesium concentrations in the diet (figure 3A,D). In these animals, light as well as electron microscopy of the pancreas revealed greater local damage and severity of pancreatitis characterised by dilatation of the endoplasmatic reticulum, damage to intracellular membranes, inflammatory cell infiltration and disturbed polarity of secretory vesicles. Quantification of intracellular vacuole formation showed that after supramaximal caerulein stimulation the number of vacuoles increased most prominently in the magnesium-deficient group (figure 3D). However, even submaximal stimulation led to an increase in vacuole formation in the magnesium-deficient animals. This can easily be seen in the electron micrographs of normal tissue architecture of unstimulated acini, that include polarised acinar cells with abundant zymogen granules and condensing vesicles clustered to the apical pole of the cells and no visible vacuoles (figure 4A). Interestingly, low magnesium chow

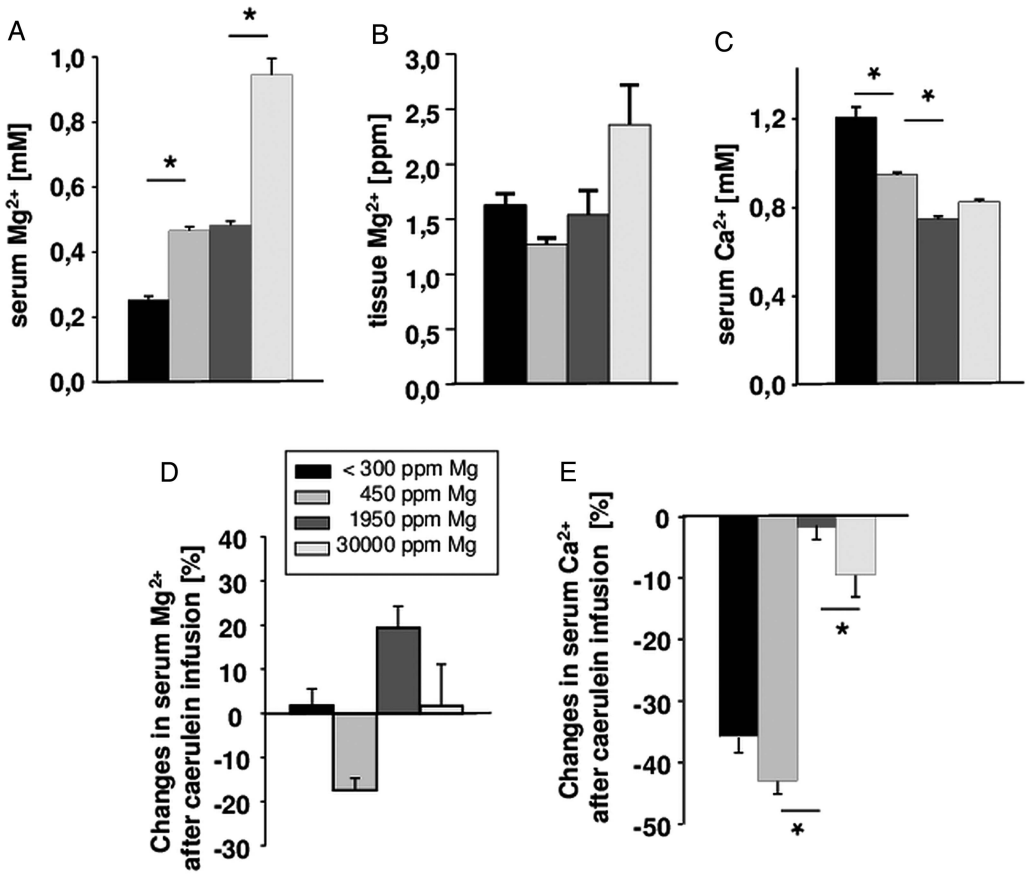

Figure 2 Serum Mg^{2+} concentration in rats fed with different magnesium diets for 2 weeks (A). Tissue Mg^{2+} concentrations in rats fed with different magnesium diets for 2 weeks (B). Serum Ca^{2+} concentrations in rats fed with different magnesium diets for 2 weeks (C). Changes in serum Mg^{2+} levels after induction of pancreatitis (caerulein infusion 10 μg/kg/h over 4 h) in rats fed with different magnesium diets (D). Changes in serum Ca^{2+} levels after induction of pancreatitis (caerulein infusion 10 μg/kg/h over 4 h) in rats fed with different magnesium diets. Asterisks indicate significant differences ($p<0.05$) compared to animals fed with the next lower magnesium diet.

in itself did not cause morphological changes (figure 4B). However, submaximal stimulation in low magnesium diet-fed animals (figure 4D), in contrast to high magnesium diet-fed animals (figure 4C), did lead to pancreatitis, indicating an increased susceptibility to injury of animals even after submaximal secretagogue stimulation.

After supramaximal stimulation with caerulein, the extent of interstitial oedema and the size and number of intracellular vacuoles was found to be highest in animals on the low magnesium diet (figure 4F) when compared to high magnesium-fed animals (figure 4E). In animals fed the highest magnesium diet, only minor subcellular changes were visible on micrographs (figure 4E). Vacuole formation after supramaximal caerulein infusion decreased with increasing magnesium concentration of the diet. Moreover, stimulation with submaximal caerulein doses (1 μg/kg/h) had no effect in the high-magnesium-fed rats (figure 4C) while it was sufficient to induce vacuole formation in magnesium-deficient rats (figure 4D). H&E staining of paraffin sections from caerulein-induced pancreatitis in the mouse (seven hourly caerulein injections of 50 μg/kg) after pretreatment with 384 mg/kg magnesium for 72 h indicated reduced areas of necrosis and a less pronounced inflammatory infiltrates at 24 h after induction of pancreatitis (figure 4I) when compared to saline treatment (figure 4H) or untreated controls (figure 4G). In a next step, we investigated the concentration of TAP, a marker for the severity of pancreatitis and an indicator for intrapancreatic trypsin activation, in pancreatic tissue and in urine. Submaximal concentrations of caerulein (1 μg/kg/h) effectively induced pancreatic TAP formation only in magnesium-deficient rats (figure 5A). After supramaximal stimulation with caerulein (10 μg/kg/h), pancreatic TAP formation could be found in all animals. However, those on a high magnesium diet showed a significantly (approx. 50%) lower increase in pancreatic TAP formation than the magnesium-deficient animals (figure 5A). These results were confirmed when urinary TAP concentrations were measured (figure 5B): after submaximal caerulein stimulation (1 μg/kg/h), TAP was significantly increased in magnesium-deficient rats and in those on a normal diet. TAP concentrations in urine also rose after supramaximal doses of caerulein (10 μg/kg/h). In animals on the high magnesium diets, the caerulein-induced increase in urinary TAP was markedly attenuated (by approx. 80% compared to magnesium-deficient animals; figure 5B).

To confirm the above data, we investigated the effect of dietary magnesium on premature intrapancreatic zymogen activation in pancreatic homogenates treated with caerulein to induce pancreatitis. Here again trypsin activity was highest in the magnesium-deficient group (figure 5C). Elastase activity also decreased in parallel with increasing magnesium content of the diet (figure 5B,D). Submaximal doses of caerulein were effective in inducing elastase activation only in magnesium-deficient animals (figure 5D). These data clearly show that, not only in isolated acini, but also in vivo magnesium can prevent premature digestive zymogen activation in

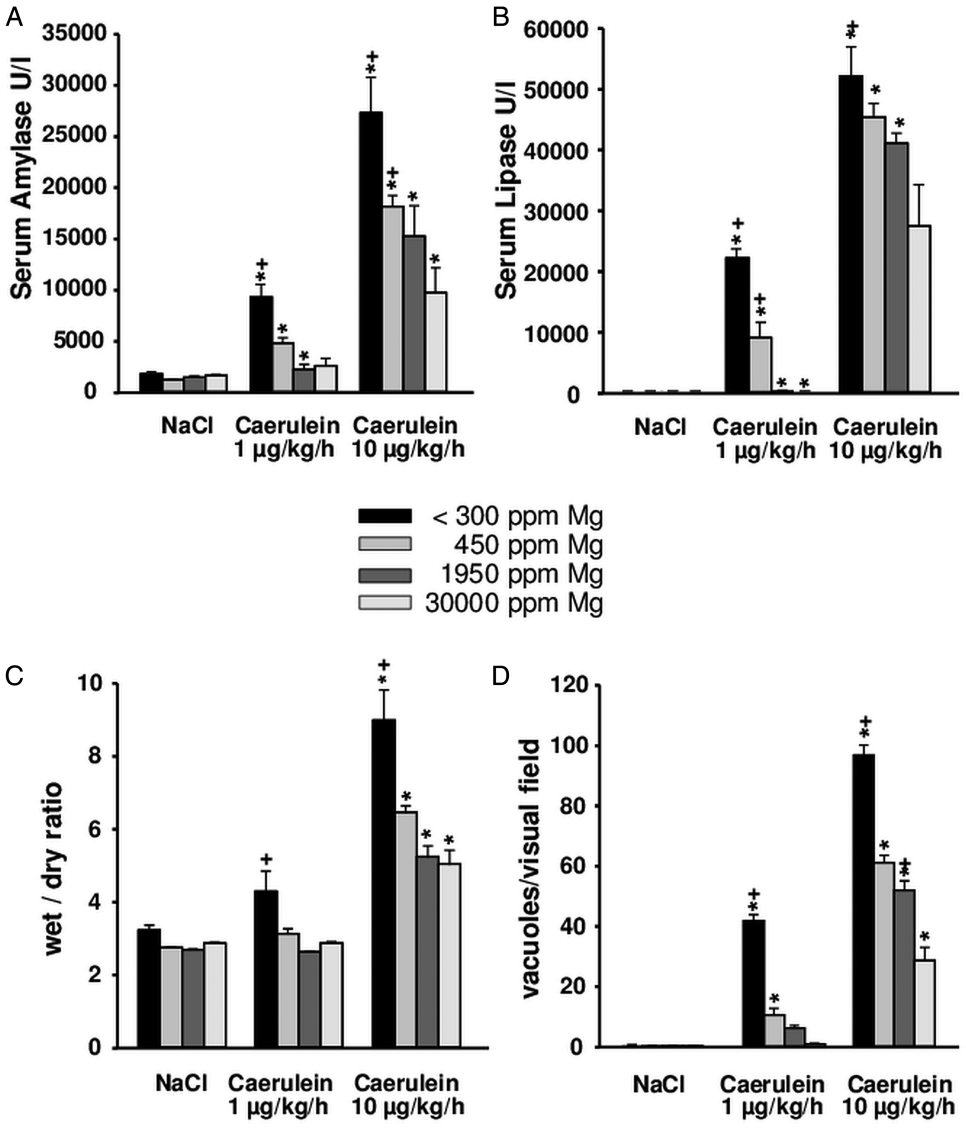

Figure 3 Effect of different magnesium diets on the release of pancreatic enzymes into the circulation in rats, as indicated by serum amylase concentrations (A) and serum lipase concentrations (B), after either submaximal (1 µg/kg bodyweight/h) or supramaximal (10 µg/kg/h) caerulein stimulation. Effect of different magnesium diets on pancreatic oedema, as indicated by the wet/dry weight ratio (C) and intracellular vacuoles per visual field (D), after either submaximal (1 µg/kg/h) or supramaximal (10 µg/kg/h) caerulein stimulation.

the pancreas, and dietary magnesium deficiency greatly lowers the pancreatic threshold level for injury from exogenous stimuli.

In order to study the effect of magnesium in a more severe model of pancreatitis involving tissue necrosis, we used C57BL6/J mice for up to eight hourly supramaximal intraperitoneal caerulein injections (50 µg/kg bodyweight). These mice were pretreated with three different concentrations of magnesium (55.5, 192 and 384 mg/kg bodyweight) intraperitoneally over a period of 72 h prior to the experiment, and this resulted in an increase in the concentration of urinary Mg^{2+} (figure 6A). Serum Mg^{2+} levels were also increased compared to saline controls (figure 6B). Pretreatment with magnesium concentrations above 55 mg/kg bodyweight resulted in reduced serum lipase levels at 1 h and 24 h of pancreatitis (figure 6C). Histomorphological analysis suggested reduced areas of necrosis and less inflammatory infiltrates at 24 h after induction of pancreatitis (figures 4I and 6D) when compared to saline treatment (figure 4H) or untreated controls.

Pancreatic MPO levels appeared marginally reduced in animals pretreated with magnesium doses above 55 mg/kg bodyweight (figure 6E). Interestingly, an expansion of FoxP3 positive T-cells suggested an effect of magnesium on the immune system, possibly reducing a pro-inflammatory immune response (figure 6F). In line with this finding, magnesium pretreatment resulted, already at baseline, in somewhat decreased levels of the pro-inflammatory cytokine tumour necrosis factor (TNF)α (figure 6G). Other changes regarding the systemic inflammatory response were either not detected in this model of acute pancreatitis or were so minor that differences were not biologically meaningful, suggesting that ultimate confirmation will probably require an even more severe and sepsis-associated model of pancreatitis.

DISCUSSION

Magnesium is a well known modulator and second messenger of intracellular signalling processes in a number of organs and cells types.[30] We and others have shown that magnesium

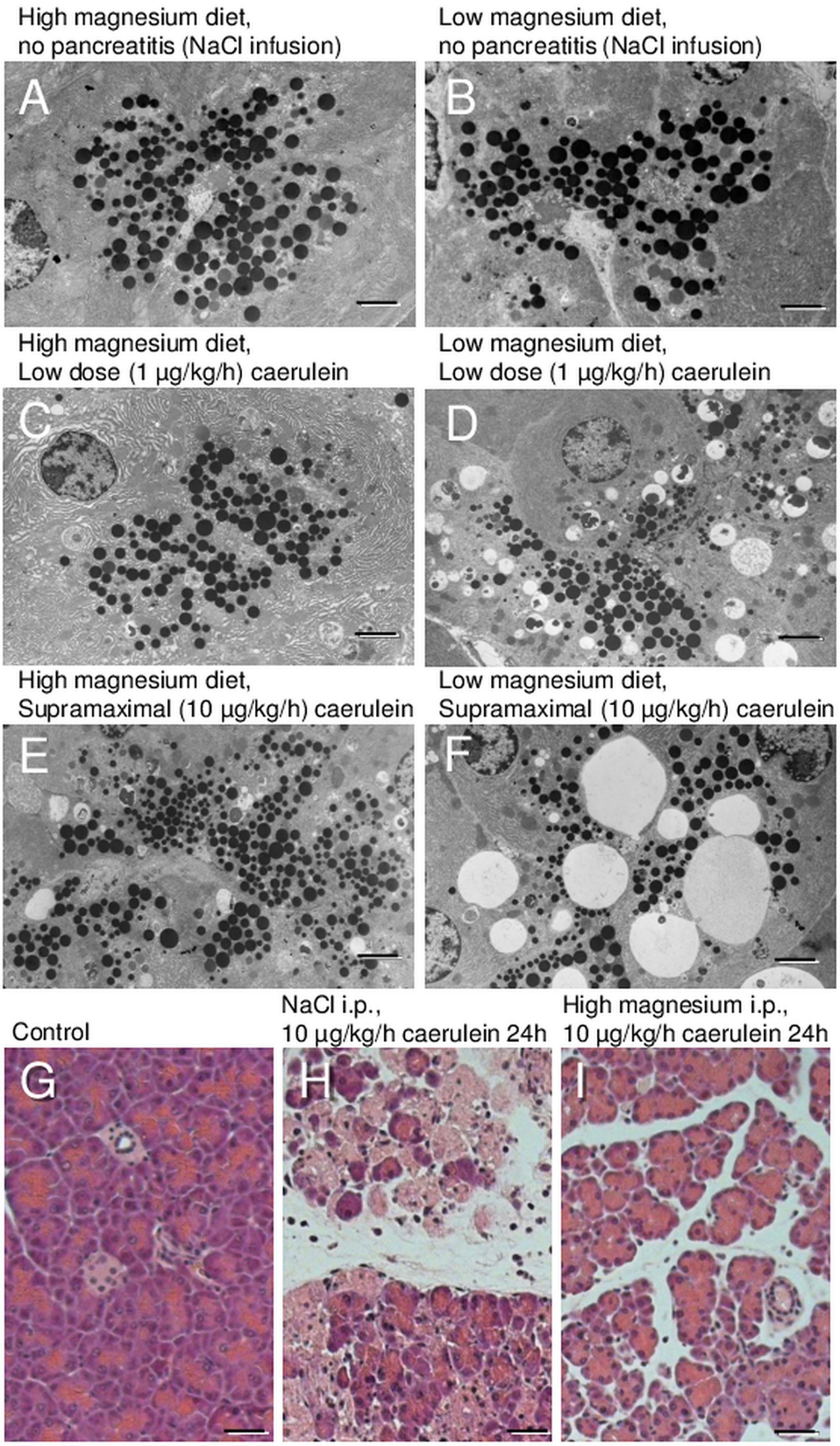

Figure 4 Representative electron micrographs of the pancreas from rats fed a low or high magnesium diet. (A, C and E) the high magnesium diet group; (B, D and F) the lowest magnesium-fed group. In the low magnesium group in (D), signs of acute pancreatitis with significantly more vacuoles and a dilated endoplasmatic reticulum are apparent. Bars correspond to 1 µm. Submaximal caerulein application in low magnesium fed animals evoked changes characteristic of with pancreatitis. H&E staining of micrographs taken at 0 h (G) and 24 h after the first caerulein injection and pretreated with saline (H) or 384 mg/kg bodyweight Mg^{2+} (I) in mice. Note the decrease in areas of necrosis and the diminished inflammatory infiltrate as a result of Mg^{2+} administration. Bars in (G, H and I) correspond to 100 µm.

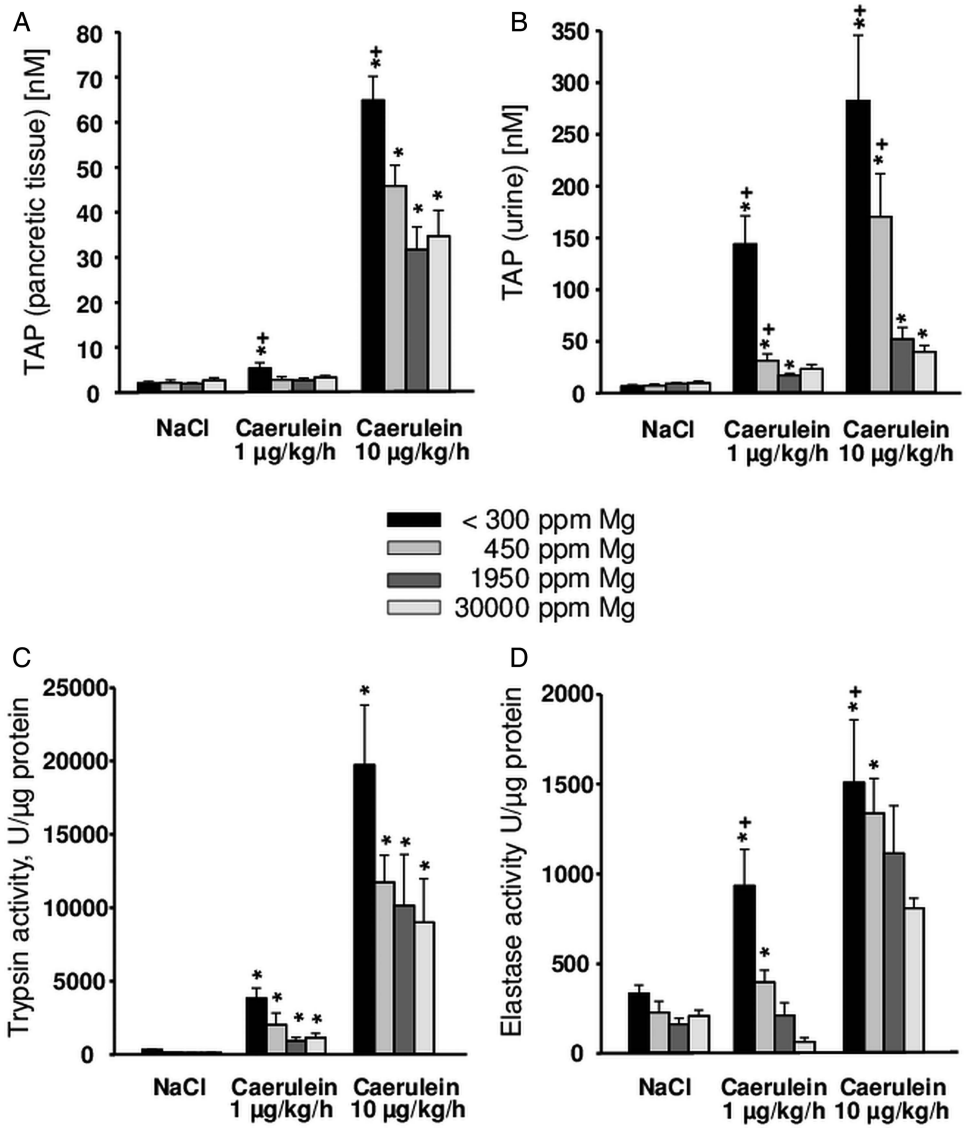

Figure 5 Effect of different magnesium diets on the formation of trypsinogen activation peptide (TAP) in rats, as indicated by TAP concentrations in pancreatic tissue (A) and TAP excretion in urine (B), after caerulein stimulation of either submaximal (1 µg/kg/h) or supramaximal (10 µg/kg/h) doses. Effect of different magnesium diets on the premature intracellular protease activation, trypsin (C) and elastase (D), after caerulein stimulation of either submaximal (1 µg/kg/h) or supramaximal (10 µg/kg/h) concentrations. Asterisks indicate significant differences ($p<0.05$) compared to control animals (NaCl infusion) on the same magnesium diet, while+indicates significant differences ($p<0.05$) compared to animals stimulated with the same caerulein concentration but fed with the next higher magnesium diet.

predominantly interferes with calcium signalling[31] and primarily acts on the cellular calcium influx in acinar cells, thereby controlling cellular enzyme secretion in pancreatic acinar cells.[12 32] In the present study we could demonstrate that magnesium not only regulates physiological mechanisms but also affects pathophysiological pathways. Stimulation of acinar cells with supramaximal doses of CCK results in premature intracellular activation of digestive proteases followed by tissue injury.[4] Our in vitro data show that CCK-induced formation of proteolytic activity inside isolated acinar cells can be effectively reduced by either chelating calcium with BAPTA-AM[12] or adding extracellular magnesium. The magnesium action on premature intracellular enzyme activation can thus be explained by its function as a calcium antagonist, since increases in cytosolic calcium are an established precondition for the induction of trypsinogen activation.[10 33] These data would suggest that the cell biological effect of magnesium in acinar cells is direct because calcium is replaced by magnesium in intracellular stores[17] and predominantly involves calcium-dependent signalling events. Furthermore, magnesium can act as a natural calcium channel blocker that has a direct role in protecting mitochondria from calcium overload, improving their function and increasing the potential of ATP synthesis.[34] Cellular stress would usually result in an attenuation of the Na^+, K^+ATPase activity, disturbing metabolic energy generation.[35] In our setting, magnesium in combination with CCK somewhat increased ATPase activity while we could exclude an effect of magnesium on overall kinase activity. Our data from animals fed different magnesium-containing diets confirm the relevance of the in vitro observations.

Elevated magnesium levels achieved by feeding a magnesium-enriched diet greatly reduced premature intracellular activation of digestive zymogens in response to supramaximal caerulein stimulation in vivo and significantly attenuated the severity of experimental pancreatitis.

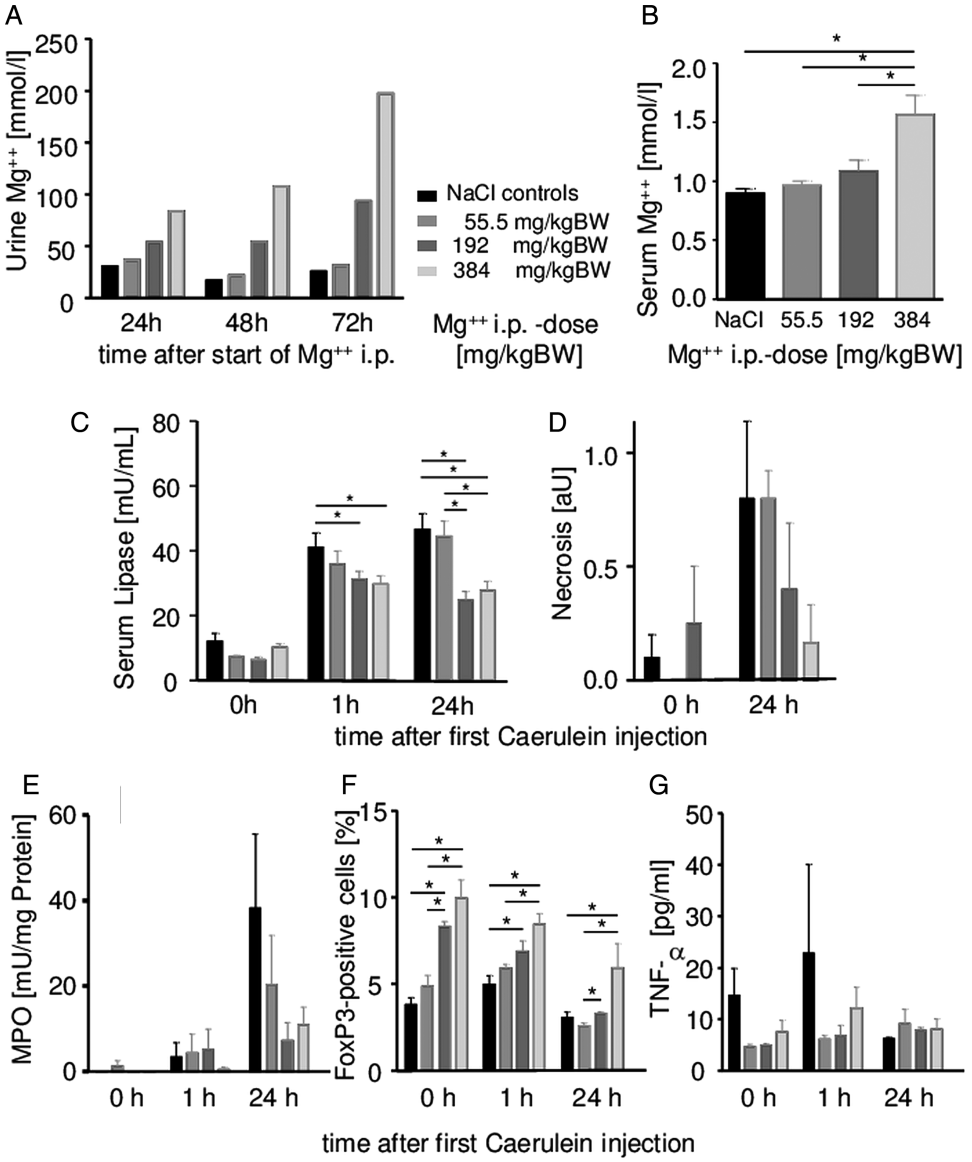

Figure 6 Cumulative urinary Mg^{2+} concentrations after intraperitoneal magnesium injection over 72 h (A). Serum Mg^{2+} concentrations at baseline after intraperitoneal pretreatment with varying concentrations of magnesium for 72 h (B). Effect of Mg^{2+} pretreatment on serum lipase levels after supramaximal caerulein stimulation (50 μg/kg bodyweight, C). Semiquantitative analysis of pancreatic necrosis after Mg^{2+} pretreatment after supramaximal caerulein stimulation (50 μg/kg bodyweight; D). Effect of Mg^{2+} pretreatment on pancreatic myeloperoxidase (MPO) levels during the course of pancreatitis (E). Mg^{2+} pretreatment resulted in an expansion of T-cells (CD4+FoxP3+) (F), already present before pancreatitis induction. Mg^{2+} pretreatment somewhat lowered basal TNFα levels as well as the TNFα increase during caerulein-induced pancreatitis (G).

In line with clinical data showing low serum magnesium concentrations in patients with pancreatitis,[14–16] our experimental data suggest that magnesium deficiency is a risk factor for the disease. As detected by electron microscopy, supramaximal stimulation with caerulein was barely effective in inducing damage in acinar cells of animals on a high magnesium diet. In magnesium-deficient animals, on the other hand, even submaximal stimulation evoked changes consistent with acute pancreatitis. One reason for this finding might be the inverse relationship between magnesium and calcium levels after the 2-week feeding period with different magnesium diets. Such an inverse correlation has been reported previously.[36] Low magnesium levels enhanced the availability of calcium while, at the same time, the protective role of magnesium in stress responses was found to decline.[37] Cellular calcium entry is therefore facilitated, resulting in either enhanced bioavailability as a second messenger involved in cell metabolism or, if overwhelming, permitting pathophysiological events such as premature enzyme activation to occur.

The role of hypercalcaemia in inducing acute pancreatitis is well known in humans and has been confirmed in the hyperstimulation model of pancreatitis in rodents.[8] Zhou *et al* demonstrated that submaximal doses of caerulein were effective in inducing acute pancreatitis in hypercalcaemic animals.[8]

Our data are also in line with the findings of an observational study by Papazachariou *et al* in chronic pancreatitis patients, which demonstrated that magnesium deficiency is more frequent in patients than in controls.[17] Magnesium deficiency therefore seems to be a risk factor that predisposes to pancreatitis even under conditions of sub-threshold stimuli.

Only limited clinical data are available on the effects of magnesium levels on the clinical outcome of acute pancreatitis. In

an earlier study, Holtmeier reported three patients with chronic pancreatitis, hypomagnesaemia and abdominal pain.[38] Intravenous magnesium therapy produced a rapid remission of symptoms.[38] Ryzen and Rude measured magnesium levels in serum and in peripheral blood mononuclear cells in 29 normocalcaemic or hypocalcaemic patients with acute pancreatitis and concluded that patients are frequently magnesium deficient.[16]

While our data show that magnesium plays a role at an early step of the pathophysiological cascade in acute pancreatitis, namely on the intracellular calcium transients and premature enzyme activation, other cellular targets for magnesium as well as indirect effects cannot be excluded. Such events might include the activity of lysosomal enzymes in the pancreas,[33] and the interaction of acinar cells with surrounding stellate cells[34] or with inflammatory cells.[25] The molecular identification of multiple Mg^{2+} transporters and their biophysical characterisation in recent years has improved our understanding of Mg^{2+} homeostasis in the immune system. By now it is understood that high serum levels of magnesium can suppress the secretion of pro-inflammatory cytokines as observed here for TNFα[40] and can therefore prevent an overwhelming immune response. In line with this observation, high dose magnesium treatment resulted in an expansion of FoxP3 positive T-cells at baseline in our study, which was further increased during the course of pancreatitis. Conversely, experimental magnesium deficiency in rats has been reported to induce a clinical inflammatory syndrome after only a few days, characterised by leucocyte and macrophage activation, release of inflammatory cytokines and acute phase proteins, and excessive production of free radicals. Increases in extracellular magnesium concentrations of a magnitude as achieved in our study significantly decreased the inflammatory response.[40] It needs to be pointed out, however, that even the more severe model of pancreatitis in the mouse we used here in addition to rats, is transient and animals recover completely. The minor inflammatory changes of pancreatitis, and the marginal beneficial effect of Mg^{2+} on inflammation, may not indicate that magnesium can mitigate pancreatitis-associated sepsis or mortality in humans, rather than preventing pancreatitis in at-risk patients, which our study suggests.

Another important target of magnesium is the generation of free radicals which have been implicated in the pathogenesis of acute pancreatitis and which can alter intracellular calcium concentrations, the formation of which is enhanced by magnesium deficiency.[41] Lipid membrane oxidation, an indirect sign of radical action, was more rapid and pronounced in magnesium-deficient endothelial cells as shown by DCFA (dichlorofluoresceine-acetate) at the single cell level. In contrast, high magnesium levels can suppress formation of free radicals and thereby prevent many of their deleterious vascular side effects.[42]

In summary, this study indicates that nutritional magnesium deficiency predisposes to acute pancreatitis, while high nutritional or parenteral magnesium can limit the morphological as well as the biochemical severity of pancreatitis. The effect of magnesium suggests that magnesium could serve as a natural, inexpensive, orally bioavailable and well tolerated calcium antagonist, suitable for the treatment of pancreatitis, or even the prevention of pancreatitis in at-risk patients. As a direct consequence of the study presented here, two international, randomised clinical trials have been initiated and are currently recruiting patients. One (EUOPAC2, clinical trials gov. no. NCT, ISRCTN 00142233) recruits patients with recurrent episodes of idiopathic or hereditary pancreatitis in order to test whether nutritional oral magnesium supplementation can reduce future recurrences. The MagPEP trial (clinical trials gov. no. ISRCTN 46556454) attempts to reduce the rate of ERCP-induced pancreatitis by intravenous magnesium infusion. If the results of these trials are positive, they will lead to immediate changes in clinical practice.

Contributors VS, FCM, ST, GOC, JS, BK, CvdB, JAS, AO, MS, TS and MML conducted experiments for the study; JM, GF, WD, JS, MS and MML wrote and corrected the manuscript. MML and FCM conceived the study and developed the hypothesis. MML, JM and WD provided funding. All authors have seen and approved the final version of the manuscript.

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Effect of magnesium supplementation and depletion on the onset and course of acute experimental pancreatitis

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