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Regulatory responses of hepatocytes, macrophages and vascular endothelial cells to magnesium deficiency

Mei Shigematsu[a], Shozo Tomonaga[a], Fumie Shimokawa[b], Masaru Murakami[b], Toru Imamura[c], Tohru Matsui[a], Masayuki Funaba[a,*]

[a]Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
[b]Laboratory of Molecular Biology, Azabu University School of Veterinary Medicine, Sagamihara 252-5201, Japan
[c]Cell Regulation Laboratory, School of Bioscience and Biotechnology, Tokyo University of Technology, 1404-1 Katakura Hachioji, Tokyo 192-0982, Japan

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Abstract

The liver is the organ that responds to nutritional disturbances including magnesium deficiency. The present study evaluated cellular responses to magnesium deficiency using model cells of the liver, namely, HepG2 cells as hepatocytes, RAW264.7 cells as Kupffer cells and human umbilical vein endothelial cells (HUVECs) as vascular endothelial cells; we examined effects of culture with magnesium deficient medium on cell responses in individual types of cells as well as interactive responses among cells. Metabolomic analyses indicated that magnesium deficiency differentially affected the cellular content of metabolites among HepG2 cells, RAW264.7 cells and HUVECs. The cellular content of the metabolites in HepG2 cells and HUVECs was also affected by the conditioned medium from RAW264.7 cells cultured with the magnesium-deficient media. The changes in HUVECs partly resembled those of the livers of magnesium-deficient rats previously described. RNA-seq analyses indicated that magnesium deficiency modulated the expression levels of molecules related to the ubiquitin-proteasome pathway and oxidative stress/antioxidant response in HepG2 cells and RAW264.7 cells, respectively. Furthermore, when HUVECs were co-cultured with RAW264.7 cells, lipopolysaccharide-induced expression of interleukin (IL)-1β and IL-6 was enhanced by magnesium deficiency, depending on the presence of RAW264.7 cells. The present study reveals that magnesium deficiency affects cellular metabolism in HepG2 liver cells, RAW264.7 macrophages and HUVECs, and that the modulation of cellular responses to extracellular magnesium deficiency in HUVECs depends on the presence of RAW264.7 cells. The complex responses in individual cells and through cell interactions partly explain the regulatory reaction to magnesium deficiency in the liver.

Keywords: magnesium deficiency; liver; hepatocyte; macrophage; endothelial cell

1. Introduction

Magnesium acts as a co-factor of numerous enzymes and interacts with phosphorylated compounds such as ATP and nucleic acids. Magnesium acts as a natural calcium antagonist, and is involved in diverse biological processes including protein synthesis, muscle and nerve transmission, neuromuscular conduction, and regulation of blood glucose and blood pressure [1]. Magnesium deficiency induces various metabolic disorders; epidemiological studies indicate that a low magnesium status is associated with various pathological conditions such as atherosclerosis, asthma, hypertension, osteoporosis, diabetes mellitus, obesity, and colon and breast cancers [2–4]. Magnesium deficiency also affects metabolism in the liver by inducing oxidative stress, apoptosis, changes in glucose metabolism, and increases in the triglyceride and total cholesterol concentrations in the liver [5–8]. In addition, mast cells infiltrate the liver upon magnesium deficiency [9]. Furthermore, a metabolomic analysis has revealed fluctuations in the hepatic metabolite content in response to magnesium deficiency [10].

Parenchymal cells and non-parenchymal cells such as Kupffer cells (liver resident macrophages), vascular endothelial cells and hepatic stellate cells comprise the liver [11]. It is possible that magnesium deficiency individually affects cells that comprise the liver. In addition, the nutritional information may be integrated through interactions between the responding cells in the liver. The present study employed a cell culture system to evaluate cellular responses to extracellular magnesium deficiency, using HepG2 hepatocytes as a model of hepatocytes, RAW264.7 macrophages as a model of Kupffer cells, and human umbilical vein endothelial cells (HUVECs) as a model of vascular endothelial cells. Using these cells, the present study explored effect of extracellular magnesium deficiency from three aspects. First, we evaluated cell responses to extracellular magnesium deficiency in individual types of cells. It is possible that molecules secreted from Kupffer cells modulate cell metabolism in hepatocytes as well as vascular endothelial cells. Thus, we next evaluated the responses in HepG2 cells and HUVECs to the conditioned medium from magnesium-deficient RAW264.7 cells in HepG2 cells and HUVECs.

* Corresponding author at: Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan. Tel.: +81 75 753 6055; fax: +81 75 753 6344.
E-mail address: mfunaba@kais.kyoto-u.ac.jp (M. Funaba).

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Table 1
Oligonucleotide PCR primers for RT-qPCR

	Oligonucleotide	
	5'-primer	3'-primer
Human		
CCL20	5'-gctgctttgatgtcagtgct-3'	5'-gcagtcaaagttgcttgct-3'
CXCL2	5'-cccatggttaagaaaatcatcg-3'	5'-cttcaggaacagccaccaat-3'
CXCL3	5'-gaaaatcatcgaaaagatac-3'	5'-ggtaagggcagggaccac-3'
CXCL5	5'-ccttttctaaagaaagtcat-3'	5'-tgggttcagagacctccaga-3'
CX3CL1	5'-atctctgtcgtggctgctc-3'	5'-tgctgtctcgtctccaagc-3'
GAPDH	5'-agccacatcgctcagacac-3'	5'-gcccaatacgaccaaatcc-3'
HPRT1	5'-gttatgaccttgatttattttgca-3'	5'-cttcatcacatctcgagcaagacg-3'
IL-1β	5'-agctgatggcccctaaacag-3'	5'-gtagtggtggtcggagattcgt-3'
IL-6	5'-tgagtacaaaagtcctgatccagt-3'	5'-ttctgtgcctgcagcttcgtcagc-3'
PSMA1	5'-cagcacctccttgattctcag-3'	5'-caaacagtgacatcattgtcatactg-3'
PSMA5	5'-ttcactcatcatcctcaaacaag-3'	5'-caggctgcactgtggcta-3'
PSMA7	5'-tgagtgtgcgcttttgagag-3'	5'-gcgagaagacggtgatgg-3'
PSMB3	5'-tttaagcccttcatttgctctc-3'	5'-gagggactcacacattccgta-3'
PSMB6	5'-gaaggcatgaccaaggaaga-3'	5'-tctacccctgactctgcaatg-3'
PSMC3	5'-agtgacattgggggtttgg-3'	5'-ttgtggttcattggcaagac-3'
PSMC5	5'-gtgaatgataagagccaaaacctc-3'	5'-gcaataggcgaactttagcatt-3'
Mouse		
Ccl3	5'-ccatgacactctgcaaccaa-3'	5'-gtggaatcttccggctgtag-3'
Ccl4	5'-ttctgtgctccagggttctc-3'	5'-tgccgggaggtgtaagag-3'
Cox4l1	5'-tcactgcgctcgttctgat-3'	5'-cgatcgaaagtatgagggatg-3'
Cxcl2	5'-ccctggttcagaaaatcatcc-3'	5'-cttccgttgagggacagc-3'
Cxcl3	5'-ccccaggcttcagataatca-3'	5'-tctgatttagaatgcaggtc-3'
Hprt1	5'-gttatgacctagatttgttttgta-3'	5'-cttcatgacatgtcgagcaagtct-3'
Il-1β	5'-agttgacggaccccaaaag-3'	5'-gatgtgctgctgcgagatttga-3'
Il-6	5'-gagatacagaaactctaattcaca-3'	5'-ttctgtgactccagcttatctgtt-3'
Ndufb2	5'-tgactcggatgctgtgct-3'	5'-tgtcagtcctcgtcatcagg-3'
Nqo1	5'-agcgttcggtattacgatcc-3'	5'-agtacaatcagggctcttctcg-3'
Prdx1	5'-ccgctctgtggatgagattat-3'	5'-gtatcactgccaggtttccag-3'
Prdx2	5'-ggactctcagttcacccacct-3'	5'-atattcaggggcccaag-3'
Prdx5	5'-caccaggcagaaggcaag-3'	5'-gagacaccaaagaatcatccaa-3'
Sod1	5'-caggacctcattttaatcctcac-3'	5'-tgcccaggtctccaacat-3'
Sod2	5'-tgctctaatcaggacccattg-3'	5'-gtagtaagcgtgctcccacac-3'

Furthermore, lipopolysaccharide (LPS)-induced cytokine production is known to be modulated by magnesium deficiency [12]. We further examined effect of extracellular magnesium deficiency on cytokine expression induced by LPS in HepG2 cells and HUVECs in the presence of RAW264.7 cells.

2. Materials and methods

2.1. Cell culture

HepG2 human hepatoma cells and RAW264.7 mouse macrophage cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under a humidified 5% CO_2 atmosphere. HUVECs were cultured in M199 medium with 10% heat-inactivated FBS, 5 μg/mL heparin, 20 ng/mL FGF1 and 60 μg/mL kanamycin. The magnesium concentration in the control DMEM and M199 medium was 0.81 mM, which was comparable to the reference in human plasma (0.70 – 1.05 mM, [4]).

To evaluate the cellular content of metabolites, HepG2 cells and RAW264.7 cells were cultured in the control DMEM or magnesium-free DMEM in the absence of FBS for 24 hours. HUVECs were cultured in the control M199 or magnesium-free M199 with 10% heat-inactivated FBS, 5 μg/mL heparin, 20 ng/mL FGF1 and 60 μg/mL kanamycin for 24 hours. Because HUVECs were cultured in the presence of 10% FBS, some magnesium, i.e., one-tenth of magnesium concentration in normal plasma, was present.

To examine the effects of molecules released from RAW264.7 cells on the cellular content of the metabolites in HepG2 cells and HUVECs, the conditioned medium was prepared from RAW264.7 cells; RAW264.7 cells were cultured with the control DMEM or magnesium-free DMEM in the absence of FBS for 24 hours. After recovery of the culture medium, the culture supernatant was obtained by centrifugation at 3000 rpm for 3 min; the magnesium concentration in the culture supernatant from the magnesium-free medium was adjusted to 0.81 mM by addition of magnesium sulfate. HepG2 cells were cultured with either the control conditioned medium or the magnesium-free conditioned medium from the RAW264.7 cells in the absence of FBS for 24 hours. HUVECs were cultured with either of the conditioned media with 10% heat-inactivated FBS, 5 μg/mL heparin, 20 ng/mL FGF1 and 60 μg/mL kanamycin for 24 hours.

For the RNA-seq analyses, HepG2 cells and RAW264.7 cells were cultured in the control DMEM or magnesium-free DMEM for 36 hours and 16 hours, respectively. For the mixed culture, HepG2 cells (5×10^4 cells) and RAW264.7 cells (10×10^4 cells) were seeded in 12-well plates. After overnight culture, cells were cultured with control or magnesium-free DMEM supplemented with or without LPS from *Pseudomonas aeruginosa 10* (L8643, Sigma, St. Louis, MO, USA: 100 ng/mL) for 12 hours. HUVECs (6×10^4 cells) and RAW264.7 cells (10×10^4 cells) were seeded in 6-well plates. After overnight culture, cells were cultured with the control or magnesium-deficient medium for HUVEC/RAW264.7 cells supplemented with or without LPS (100 ng/mL) for 12 hours; the medium for HUVEC/RAW264.7 cells was the mixture of DMEM for RAW264.7 cells and M199 with 10% heat-inactivated FBS, 5 μg/mL heparin, 20 ng/mL FGF1 and 60 μg/mL kanamycin for HUVECs at a 1: 1 ratio. Additionally, cells were individually cultured and treated with magnesium-free (deficient) medium, LPS or both; HepG2 cells (10×10^4 cells) were seeded in 12-well dishes, and RAW264.7 cells (50×10^4 cells) or HUVECs (10×10^4 cells) were seeded in 6-well dishes.

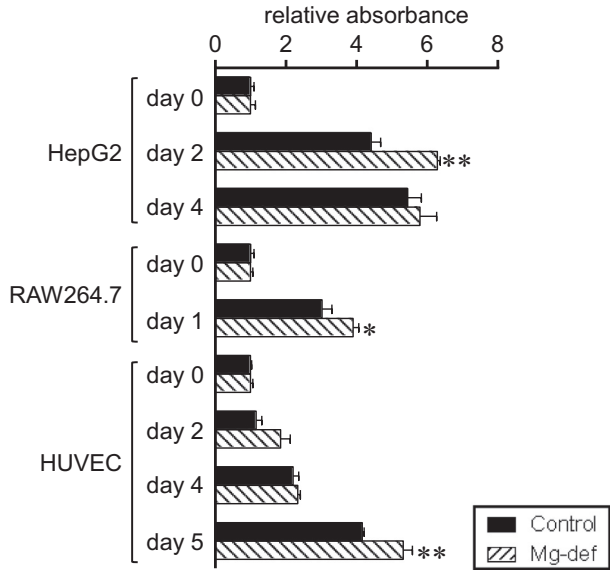

Fig. 1. Changes in uptake of MTS in response to extracellular magnesium deficiency in HepG2 cells, RAW264.7 cells and HUVECsHepG2 cells, RAW264.7 cells and HUVECs were cultured with control or magnesium-deficient medium. MTS assays were performed at the indicated day. The results are shown as the relative absorbance in cells on day 0 in each group (n = 4 for HUVEC or n = 5 for HepG2 and RAW264.7). Mean ± SE. * and **: $P < 0.05$ and $P < 0.01$, *vs.* the control group, respectively.

2.2. MTS assay

MTS assay based on tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] uptake and reduction was conducted by use of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI USA). HepG2 cells, RAW264.7 cells or HUVECs were seeded in 96-well plates (2×10^3 cells/well). At 17 hours after seeding (day 0), HepG2 cells and RAW264.7 cells were cultured with control or magnesium-free DMEM. Effect of extracellular magnesium restriction was evaluated in HUVECs in the presence of 10% heat-inactivated FBS, 5 μg/mL heparin, 20 ng/mL FGF1, because HUVECs did not grow in the absence of these reagents. Twenty μL of the reagent containing MTS and an electron coupling reagent (phenazine ethosulfate) was added on the indicated day 2 hours prior to measurement of absorbance. The plates were analyzed in a microplate reader at 490 nm with a reference wavelength of 655 nm. Absorbance in cells on day 0 in each group was set at 1.

2.3. Metabolomic analysis

Metabolomic analysis was performed by gas-chromatography mass-spectrometry (GC-MS) as previously described [13]. The soluble protein content in cells was also measured using the bicinchoninic acid method [14]. The peak area of each metabolite was measured, and the cellular content of the metabolite was calculated by dividing the peak area of the metabolite by the peak area of 2-isopropylmalic acid (internal standard) and the soluble protein content; the content of each metabolite in the control cells was set to 100. The pathways affected by the magnesium deficiency were detected using MetaboAnalyst [15]; metabolites with differences in cellular content that were more than 50% between control cells and magnesium-deficient cells were subjected to an Enrichment Analysis (http://www.metaboanalyst.ca/faces/docs/Tutorial.xhtml, accessed February 2017). To visualize changes in the cellular metabolite content in response to extracellular magnesium deficiency, a heat map was prepared, and a

hierarchical cluster analysis was performed as previously described [13]. The threshold for grouping was set to a correlation coefficient of 0.9 in the dendrogram, and the dendrogram was grouped to have as few clusters as possible.

2.4. RNA-seq

Total RNA isolation, the removal of ribosomal RNA, the construction of a whole transcriptome library, and the nucleotide sequence and subsequent bioinformatic analysis were performed as described by Jenjaroenpun et al. [16].

2.5. Functional category analysis

Functional categories enriched in the differentially expressed genes were identified by use of the database for annotation, visualization and integrated discovery (DAVID [17]). The probability that a Gene Ontology (GO) term was overrepresented was determined by a modified Fisher's exact test, comparing the proportion of genes in the whole genome that were part of the GO term to the proportion of the differentially expressed genes that were part of the same GO term, and the results were expressed as an EASE score. Clusters of overrepresented GO terms were generated based on the similarity between the differentially expressed genes assigned to each functional GO term. A summary of the description of each cluster was generated based on the constitutive GO term names of that cluster that achieved an EASE score < 0.05 following the Benjamini-Hotchberg correction of multiple comparisons.

2.6. Plasmids, RNA isolation and RT-quantitative PCR

Coding region of human and mouse interleukin (IL)-1β, IL-6 or HPRT1 was cloned into pcDNA3 by a PCR-based method. The appropriateness of expression plasmids was verified by nucleotide sequencing. Total RNA isolation, cDNA synthesis and real-time RT-quantitative PCR (RT-qPCR) were conducted as described by Asano et al. [18]. The oligonucleotide primers are shown in Table 1. The cycle of threshold (Ct) value was determined, and the abundance of gene transcripts was analyzed using the ΔΔCt method using GAPDH or HPRT1 (Hprt1) as the reference gene [19].

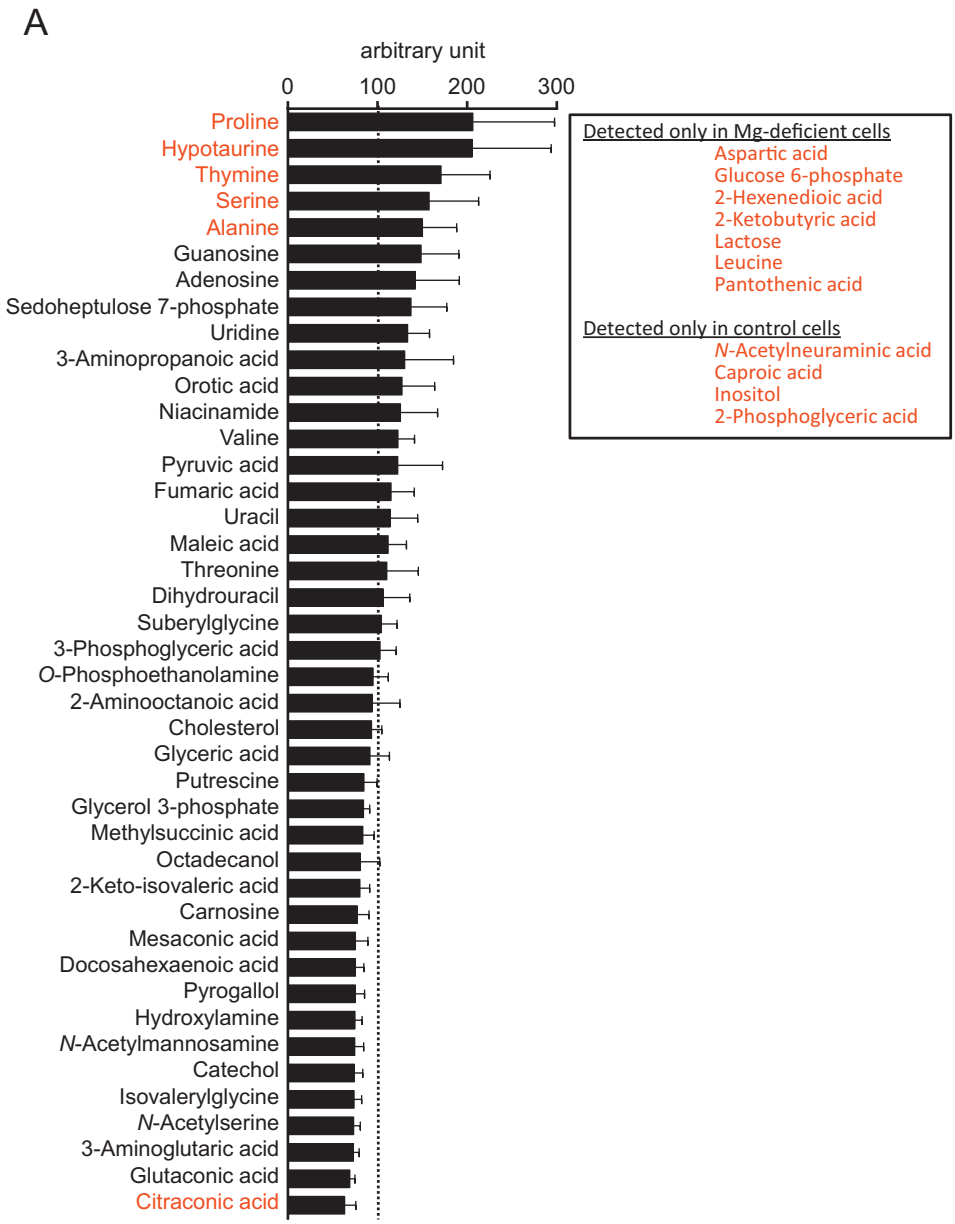

Fig. 2. Changes in the metabolite content in response to extracellular magnesium deficiency in HepG2 cells, RAW264.7 cells and HUVECs(A-C) HepG2 cells (A), RAW264.7 cells (B), and HUVECs (C) were cultured with control or magnesium-deficient medium for 24 h. The cellular content of the metabolites was comprehensively analyzed by metabolomic analyses. The results are shown as the relative content of the magnesium-deficient group (n = 6); the content in the control cells was set to 100. Metabolites with differences in cellular content that were higher than 50% between control cells and magnesium-deficient cells are shown in red. Metabolites detected only in either groups are also shown in red. Mean ± SE. * and **: $P < 0.05$ and $P < 0.01$, *vs.* the control group, respectively.

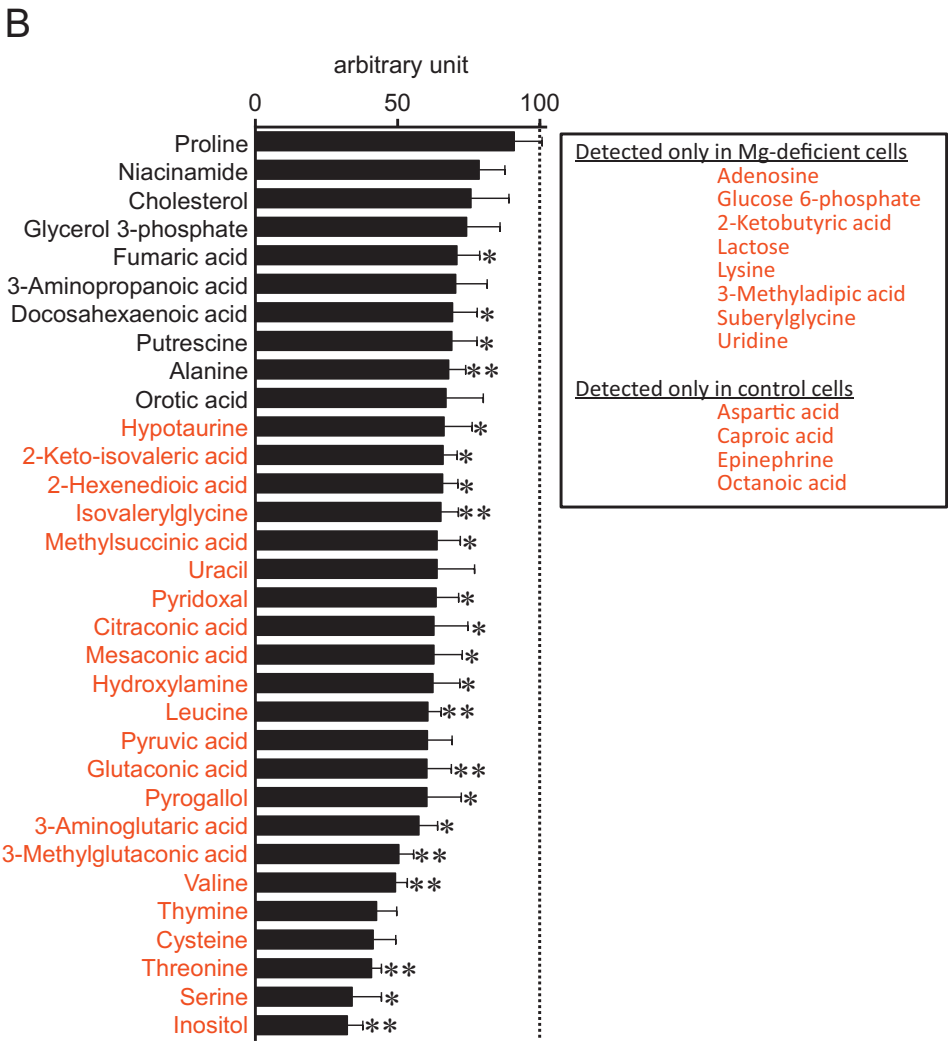

Fig. 2 (*continued*).

2.7. Statistical analysis

The data are expressed as the mean ± SEM. The data for gene expression were log-transformed to provide an approximation of a normal distribution before analyses. Differences between control cells and magnesium-deficient cells were examined by Student's *t*-test. When cells were treated with or without magnesium-deficient medium and LPS, differences between treatments were examined by the Tukey-Kramer test. $P < 0.05$ was considered significant.

3. Results

3.1. Extracellular magnesium deficiency differentially affects metabolite content in HepG2 cells, RAW264.7 cells and HUVECs

We first examined the effect of extracellular magnesium deficiency on cell viability in HepG2 cells, RAW264.7 cells and HUVECs individually. MTS assays to evaluate viable cell number revealed that culture with magnesium-deficient medium did not decrease uptake and reduction of MTS in all three cells; rather it was slightly increased on day 2 in HepG2 cells, day 1 in RAW264.7 cells and day 5 in HUVECs (Fig. 1). These results suggest that the magnesium-restriction medium does not induce cell growth inhibition.

The effect of extracellular magnesium deficiency on the cellular metabolite content was next evaluated in HepG2 cells, RAW264.7 cells and HUVECs (Fig. 2). A total of 53, 44 and 46 metabolites were detected in HepG2, RAW264.7 and HUVECs, respectively. In HepG2 cells, 7 metabolites were detected only in cells cultured with the magnesium-deficient medium, whereas 4 metabolites were detected only in the control cells (Fig. 2A). The magnesium deficiency did not significantly affect the metabolite content, but the cellular content of 5 metabolites (proline, hypotaurine, thymine, serine and alanine) and 1 metabolite (citraconic acid) were 50% higher and lower, respectively, in the magnesium-deficient cells relative to the control cells. As with the HepG2 cells, glucose 6-phosphate, 2-ketobutyric acid and lactose were detected in magnesium-deficient RAW264.7 cells but not in control RAW264.7 cells (Fig. 2B). Unlike the HepG2 cells, except for 8 metabolites that were detected only in magnesium-deficient RAW264.7 cells, the cellular metabolite content was generally decreased by magnesium deficiency in RAW264.7 cells (Fig. 2B). In HUVECs, the same metabolites were detected in both cell culturing conditions (Fig. 2C). The cellular content of 18 metabolites and 3 metabolites was 50% higher and lower, respectively, in magnesium-deficient cells relative to the control cells.

The detected metabolites partly overlapped among the HepG2 cells, RAW264.7 cells and HUVECs, but only 20 metabolites were detected in all three cell types (Fig. 3A). Of the 20 metabolites, 6 were not detected in the control cells or magnesium-deficient cells; using

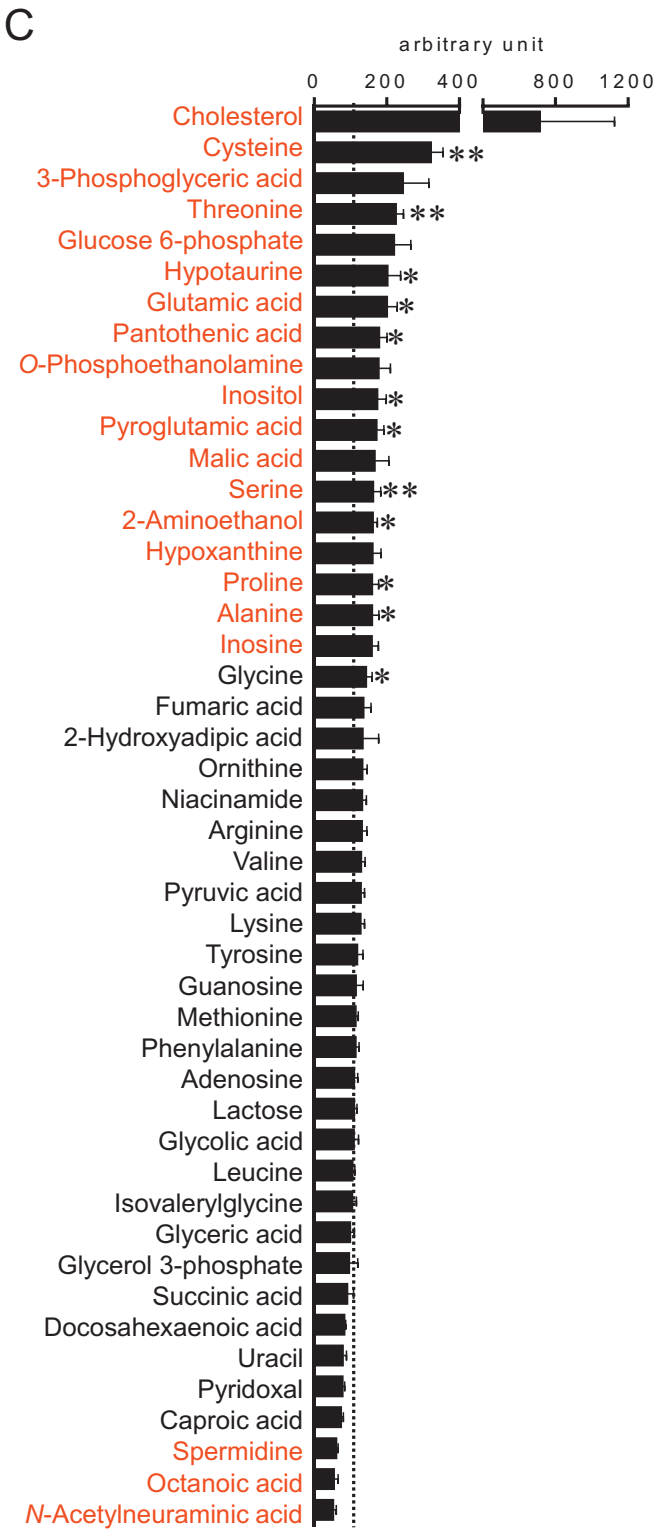

Fig. 2 (*continued*).

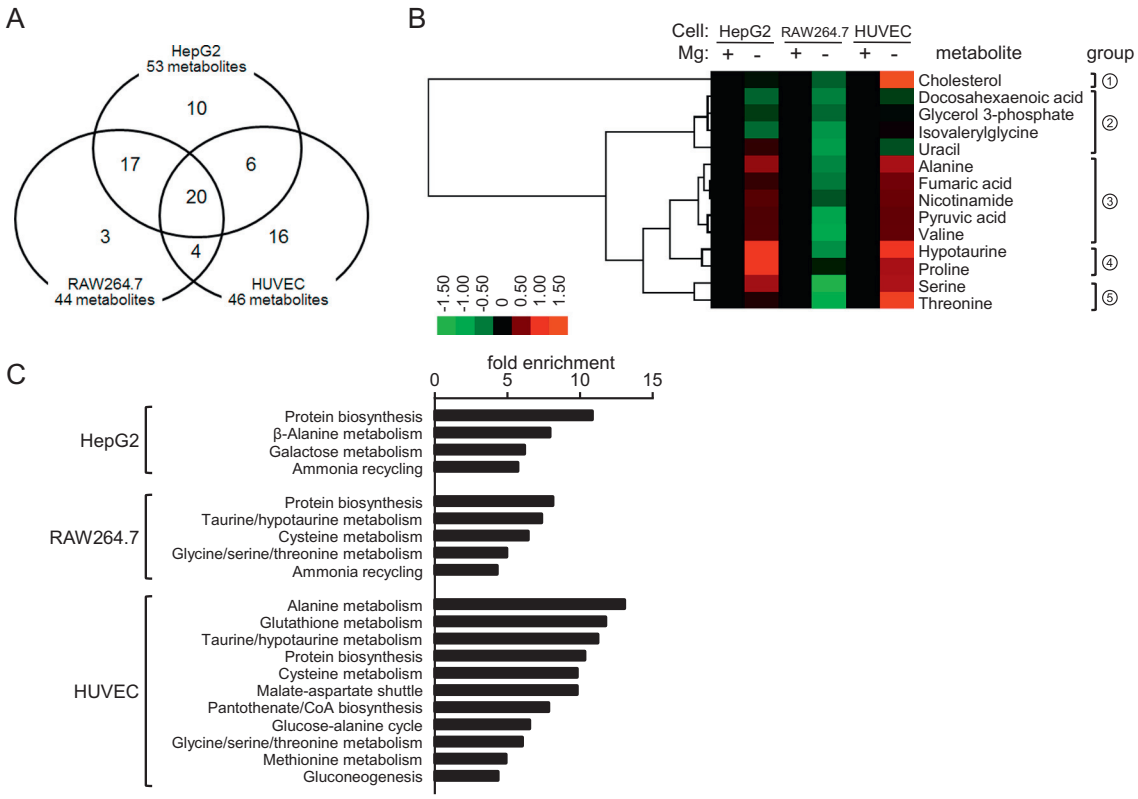

Fig. 3. Comparisons of the metabolites and metabolic pathways affected by extracellular magnesium deficiency among HepG2 cells, RAW264.7 cells and HUVECs(A) The number of metabolites detected in HepG2 cells, RAW264.7 cells and HUVECs is shown. (B) The relative abundances of 14 metabolites that were detected in all cells is shown as a heatmap. According to the hierarchical analyses, metabolites were categorized into 5 groups. (C) Metabolites shown in red in Fig. 1 were analyzed by MetaboAnalyst; significantly affected metabolic pathways by extracellular magnesium deficiency are shown.

the 14 metabolites that were detected in all cell types, a hierarchical analysis was performed to evaluate the effect of extracellular magnesium deficiency. On the basis of an r-value over 0.90, the metabolites were divided into 5 groups (Fig. 3B): an increase in HUVECs but not in HepG2 cells (group 1), decreases in all three cells (group 2), comparable increases in both HepG2 cells and HUVECs (group 3), a stronger increase in HepG2 cells than in HUVECs (group 4) and a stronger increase in HUVECs than in HepG2 cells (group 5).

MetaboAnalyst is a comprehensive web-based application for metabolomic data analysis and interpretation; it enables inferences of the metabolic pathways affected by the treatment [15]. The magnesium deficiency significantly affected 4 and 5 metabolic pathways in HepG2 cells and RAW264.7 cells, respectively. In contrast, the number of affected metabolic pathways was higher in HUVECs; 11 metabolic pathways were affected (Fig. 3C). In all 3 cell types, nitrogen metabolism including protein biosynthesis, amino acid metabolism and ammonia recycling was modified by extracellular magnesium deficiency. In addition, MetaboAnalyst highlighted glucose-associated metabolism including the glucose-alanine cycle and gluconeogenesis as the affected metabolic pathways in HUVECs.

We also evaluated pathways affected by extracellular magnesium deficiency by GO analysis of the RNA-seq data in HepG2 and RAW264.7

Table 2
Summary of the descriptive GO term names of the functional clusters affected by extracellular magnesium deficiency

Cell	Regulation	Category	Enrichment score
HepG2	Up-regulation		
		Ubiquitination	2.98
		Ubiquitin-proteasome system	2.52
		Protein binding	1.85
HepG2	Down-regulation		
		Translation	4.19
		Structural constituent of ribosome	1.98
		Blood coagulation	1.84
		Endoplasmic reticulum/Golgi	1.54
		Endoplasmic reticulum/Golgi	1.45
RAW264.7	Up-regulation		
		Ribosome	3.51
		Mitochondrion	2.09
		Oxidative stress	1.95
RAW264.7	Down-regulation		
		Translation	23.78

Using the DAVID functional annotation clustering analysis, overexpressed and underexpressed genes in cells cultured with magnesium-deficient medium were clustered according to GO terms. A summarized description of the significant clusters and enrichment score is shown.

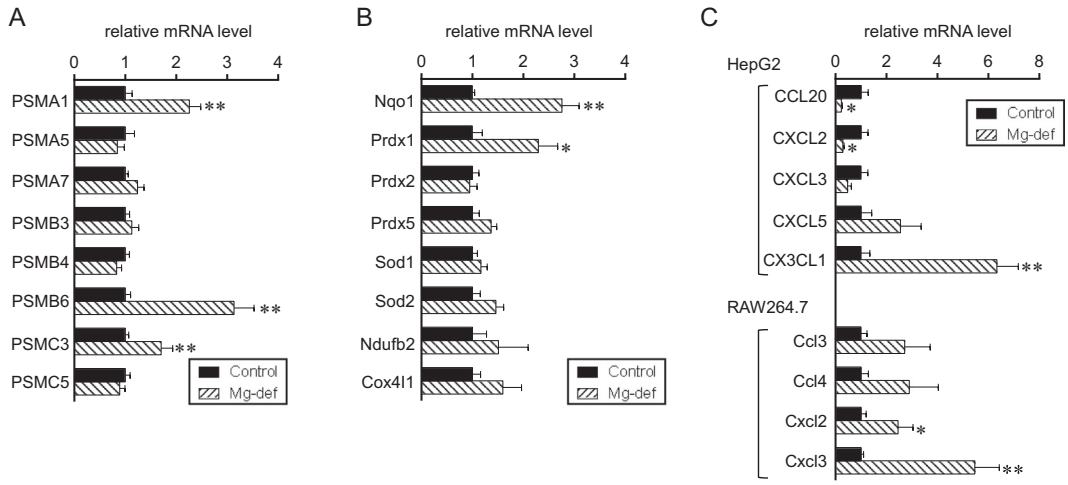

Fig. 4. Expression of genes affected by extracellular magnesium deficiency in HepG2 cells and RAW264.7 cellsHepG2 cells (A and C) and RAW264.7 cells (B and C) were cultured with the control medium or magnesium-deficient medium. (A) Expression of proteasome subunits was examined in HepG2 cells. (B) Genes related to the oxidative stress and antioxidant response were examined in RAW264.7 cells. (C) Chemokine expression was examined in HepG2 cells and RAW264.7 cells. The expression in HepG2 cells and RAW264.7 cells was normalized to the expression of GAPDH and HPRT1, respectively. The expression in the control group was set to 1. The data are shown as the mean ± SE (n = 4). * and **: $P < 0.05$ and $P < 0.01$, respectively.

cells (Table 2). The genes affected by extracellular magnesium deficiency were different between HepG2 and RAW264.7 cells; the expression of genes related to the ubiquitin-proteasome system was up-regulated by extracellular magnesium deficiency in HepG2 cells, whereas the expression levels of genes related to the mitochondrion and oxidative stress were increased in RAW264.7 cells.

RT-qPCR analyses indicated that the expression of several genes encoding proteasome subunits, i.e., PSMA1, PSMB6 and PSMC3, was significantly increased by extracellular magnesium deficiency in HepG2 cells (Fig. 4A). In RAW264.7 cells, the expression of genes related to oxidative stress, i.e., Nqo1 and Prdx1, was significantly increased by extracellular magnesium deficiency (Fig. 4B). We also noticed that the expression of chemokines was modulated by extracellular magnesium deficiency in both HepG2 and RAW264.7 cells (Fig. 4C); the expression of chemokines was generally increased by extracellular magnesium deficiency in RAW264.7 cells, whereas the expression of several cytokines such as CCL20 and CXCL2 was significantly decreased in HepG2 cells.

3.2. Cell interactions among HepG2 cells, RAW264.7 cells and HUVECs

The functions of the liver can be achieved through cellular communication between hepatocytes, Kupffer cells and vascular endothelial cells. Because macrophages are believed to patrol systemic conditions [20], Kupffer cells, which are liver resident macrophages, are likely to sense magnesium deficiency and react to the nutritional disturbance. We hypothesized that Kupffer cells secrete molecules in response to extracellular magnesium deficiency that affect metabolism in hepatocytes and vascular endothelial cells. Thus, HepG2 cells or HUVECs were treated with conditioned medium from RAW264.7 cells cultured with the control medium or magnesium-deficient medium; the magnesium concentration of the conditioned medium was equalized between the control conditioned medium and magnesium-deficient conditioned medium by the addition of magnesium sulfate.

Seventy-two metabolites and 59 metabolites were detected in HepG2 cells and HUVECs, respectively (Fig. 5). In HepG2 cells, the cellular content of only 3 metabolites was 50% higher or lower in cells treated with magnesium-deficient conditioned medium as compared to those treated with the control conditioned medium (Fig. 5A); treatment with the magnesium-deficient conditioned medium increased one metabolite (maleic acid) in HepG2 cells. In contrast, the conditioned medium affected the cellular content of 9 metabolites in HUVECs (Fig. 5B).

The detected 42 metabolites were overlapped in HepG2 cells and HUVECs (Fig. 6A), which corresponded to 58% and 71% of the detected metabolites in HepG2 cells and HUVECs, respectively. The hierarchical analysis indicated that the metabolites detected in both HepG2 cells and HUVECs were divided into 5 groups. Some metabolites were similarly affected by the conditioned medium from RAW264.7 cells between HepG2 cells and HUVECs, and others were differentially affected. The metabolites categorized as group 1 were increased by the magnesium-deficient conditioned medium in HUVECs but not in HepG2 cells, and the metabolites in group 5 were clearly decreased by the magnesium-deficient conditioned medium in HUVECs but not in HepG2 cells. The conditioned medium with the magnesium deficiency slightly and differentially affected the cellular content in group 4 between HepG2 cells and HUVECs. In contrast, the metabolites in groups 2 and 3 were increased by the conditioned medium with the magnesium deficiency in both cell types. The MetaboAnalyst analysis indicated that more metabolic pathways (16 pathways) were significantly affected by culturing with the magnesium-deficient conditioned medium in HUVECs, whereas only one pathway was affected in HepG2 cells (Fig. 6C).

The metabolomic analysis of the metabolites in cells treated with the conditioned medium highlighted the cell interactions in response to extracellular magnesium deficiency. Cytokine production in response to treatment with LPS is known to be enhanced by magnesium deficiency [12]. We examined expression of IL-1β, IL-6 and activin B; IL-1β that is expressed in Kupffer cells and activin B that is expressed in sinusoidal endothelial cells stimulates hepcidin expression in hepatocytes, indicating a cell interaction [21,22]. In addition, magnesium deficiency increases the plasma concentration of IL-6 [23]. Furthermore, expression of these proinflammatory cytokines is enhanced in response to LPS treatment [21,22,24]. Thus, we examined the regulatory expression of these cytokines in HepG2 cells and HUVECs in the presence of RAW264.7 cells and vice versa.

HepG2 cells and HUVECs are human-derived cells, whereas RAW264.7 cells are murine-derived cells. We designed PCR primers to discriminate human genes from mouse genes. We prepared plasmids encoding human or mouse IL-1β, IL-6, inhibin βB (a molecule consisting of activin B: [25]), and HPRT1 as the reference gene and performed PCR using the plasmid as the template (Fig. 7A, data not shown). PCR primers designed for the human genes recognized the

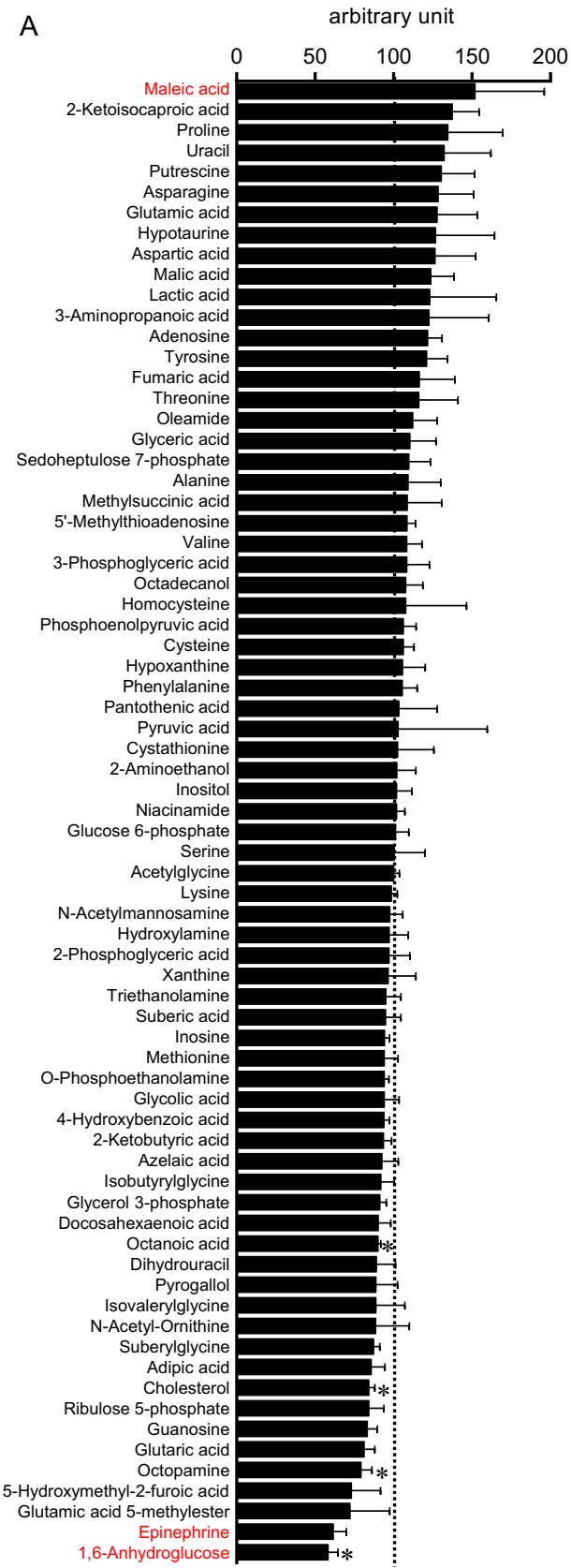

Fig. 5. Changes in the metabolite content in response to secreted molecules from magnesium-deficient RAW264.7 cells in HepG2 cells and HUVECsRAW264.7 cells were cultured with the control or magnesium-deficient medium for 24 h. HepG2 cells (A) and HUVECs (B) were cultured with the conditioned medium from RAW264.7 cells for 24 h; the magnesium concentration in the conditioned medium was adjusted to be equal between the groups by the addition of magnesium sulfate. The cellular content of the metabolites was comprehensively analyzed by metabolomic analyses. The results are shown as the relative content in cells treated with conditioned medium from RAW264.7 cells cultured with magnesium-deficient medium ($n = 6$); the content in the control cells was set to 100. Metabolites with differences in cellular content that were higher than 50% between control cells and magnesium-deficient cells are shown in red. Mean ± SE. * and **: $P < 0.05$ and $P < 0.01$, *vs.* the control group, respectively.

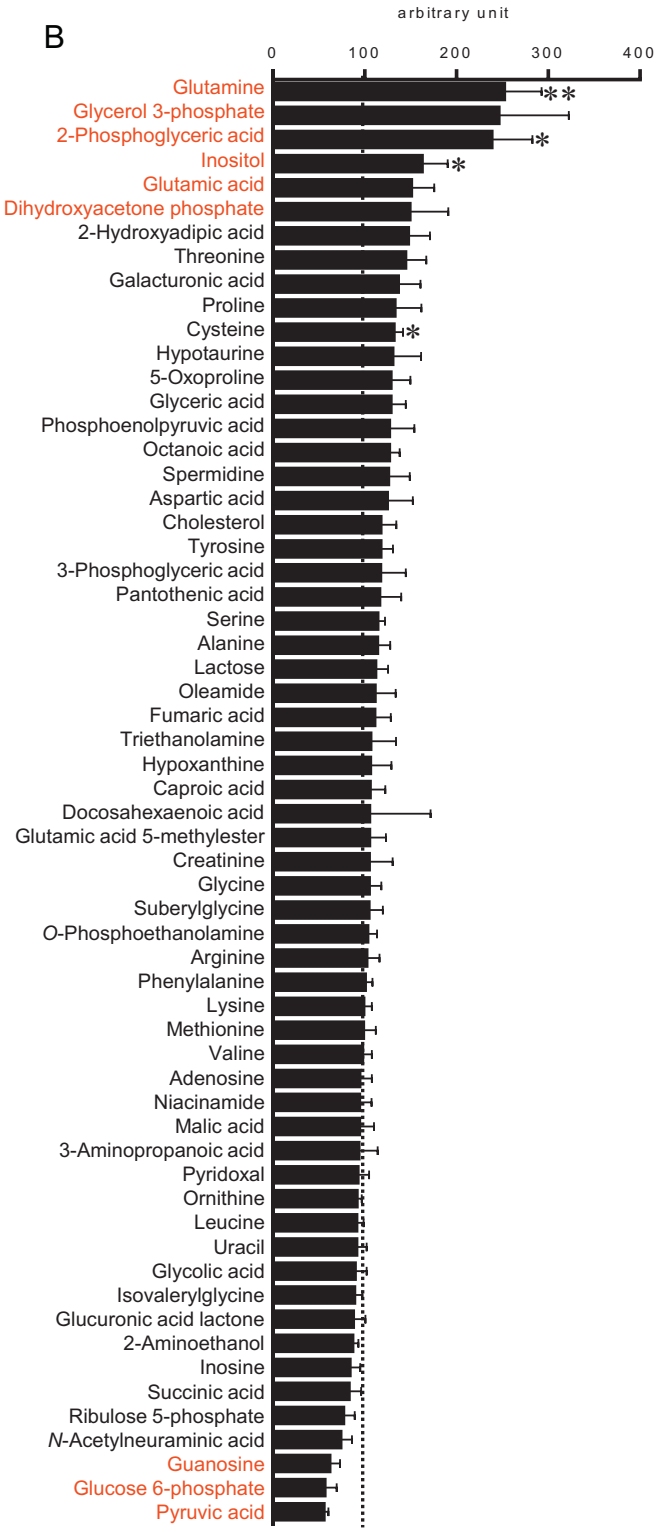

Fig. 5 (*continued*).

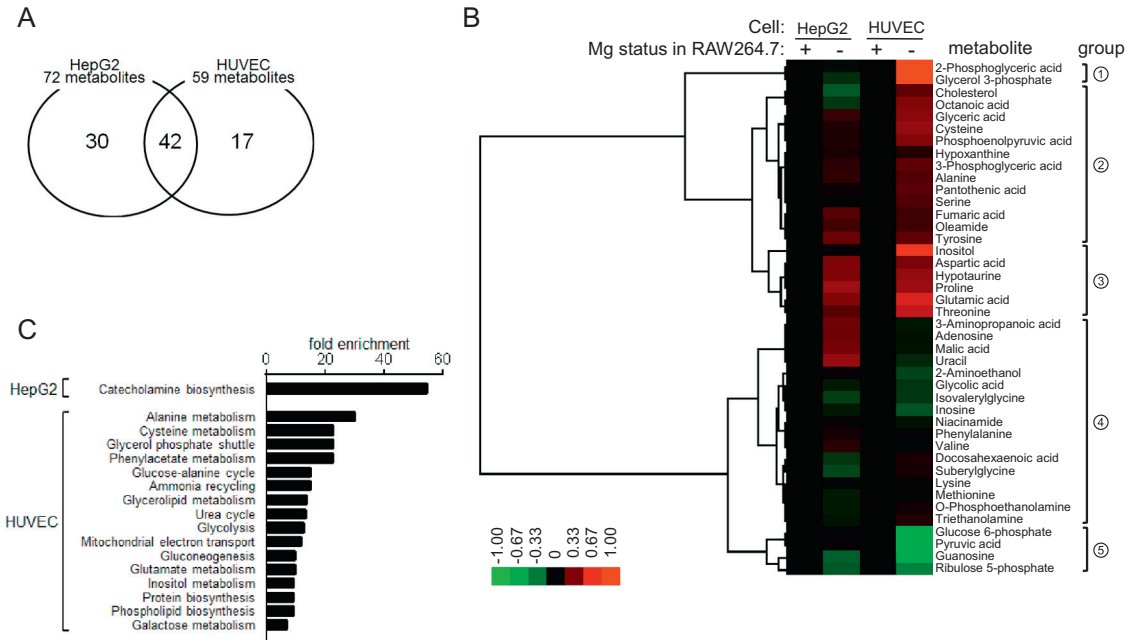

Fig. 6. Comparisons of the metabolites and metabolic pathways affected by conditioned medium from RAW264.7 cells cultured with magnesium-deficient medium in HepG2 cells and HUVECs(A) The number of metabolites detected in HepG2 cells and HUVECs is shown. (B) The relative abundance of 42 metabolites that were detected in both treatments in both cell types is shown as a heatmap. According to the hierarchical analyses, the metabolites were categorized into 5 groups. (C) The metabolites shown in red in Fig. 4 were analyzed by MetaboAnalyst; the metabolic pathways affected by extracellular magnesium deficiency are shown.

human plasmid but not the mouse plasmid, and mouse primers specifically recognized the mouse gene; the primer sets enabled discriminations in gene expression in HepG2 cells or HUVECs versus RAW264.7 cells. In the absence of RAW264.7 cells, expression of IL-1β was not affected by extracellular magnesium deficiency in HUVECs, regardless of LPS stimulation; LPS increased the expression of IL-1β (Fig. 7B). However, LPS-induced IL-1β expression in HUVECs was enhanced by extracellular magnesium deficiency in the presence of RAW264.7 cells, suggesting that the cell interaction upon extracellular magnesium deficiency and LPS stimulation depended on the RAW264.7 cells. Extracellular magnesium deficiency increased the expression of IL-1β in RAW264.7 in the absence of HUVECs, but in the presence of HUVECs, IL-1β expression was decreased by extracellular magnesium deficiency (Fig. 7C). The magnesium deficiency did not affect the expression of IL-6, regardless of LPS stimulation, when HUVECs and RAW264.7 cells were individually cultured (Fig. 7D and E). However, extracellular magnesium deficiency significantly enhanced LPS-induced IL-6 expression in HUVECs in the presence of RAW264.7 cells, although IL-6 expression in RAW264.7 cells was not affected by the presence of HUVECs. The synergistic effects of extracellular magnesium deficiency and LPS that were dependent on the mixed culture were not detected in the culture containing HepG2 cells and RAW264.7 cells (data not shown). In addition, the expression level of inhibin βB was near the detection limit in all three cell types, so the effects of extracellular magnesium deficiency and LPS could not be evaluated (data not shown).

4. Discussion

The present study examined the effect of extracellular magnesium deficiency on the responses in a cell culture system using HepG2 hepatoma cells, RAW264.7 macrophage cells and HUVECs; these cells were used as a model for hepatocytes, Kupffer cells and vascular endothelial cells, respectively. Here, we show that extracellular magnesium deficiency differentially affects the cellular content of multiple metabolites and gene expression among HepG2 cells, RAW264.7 cells and HUVECs. In addition, RAW264.7 cells secrete molecules in response to extracellular magnesium deficiency, which modify the metabolite content, especially in HUVECs. Furthermore, LPS-induced expression of IL-1β and IL-6 was enhanced in response to extracellular magnesium deficiency in HUVECs, depending on the presence of RAW264.7 cells. These complex responses may reflect protective reactions to harmful effects resulting in part from magnesium deficiency in the liver.

Liver is involved in a wide variety of physiological processes including integration of carbohydrate, protein, amino acid and lipid metabolism. These metabolic functions are carried out by hepatocytes. Kupffer cells, residential macrophages, are involved in innate immune response in liver. Vascular endothelial cells act as a constituent of blood vessels as well as a messenger of information of portal blood. Thus, three types of cells have differential roles in the liver. MetaboAnalyst indicated that the metabolic pathway affected commonly in three types of cells was “protein biosynthesis”, which was suggested because of changes in content of free amino acids in response to extracellular magnesium deficiency. It is known that magnesium is required for protein synthesis to proliferate cells [1,4]. However, the magnesium deficiency-induced changes were not uniform; culture in magnesium-deficient medium increased cellular content of several free amino acids in HepG2 cells and HUVECs. In contrast, some amino acid contents were rather decreased in RAW264.7 cells, suggesting differential regulation of “protein biosynthesis”. Comprehensive analyses of genes expressed in HepG2 cells indicated that the expression of genes related to ubiquitin-proteasome protein degradation pathway was generally up-regulated; in fact, expression of some proteasome subunits was increased by extracellular magnesium deficiency in HepG2 cells. Considering that the expression level of proteasome subunits reflects proteasome activity [26,27], protein degradation through stimulated proteasome activity is likely to be stimulated in response to extracellular magnesium deficiency in HepG2 cells and possibly in HUVECs.

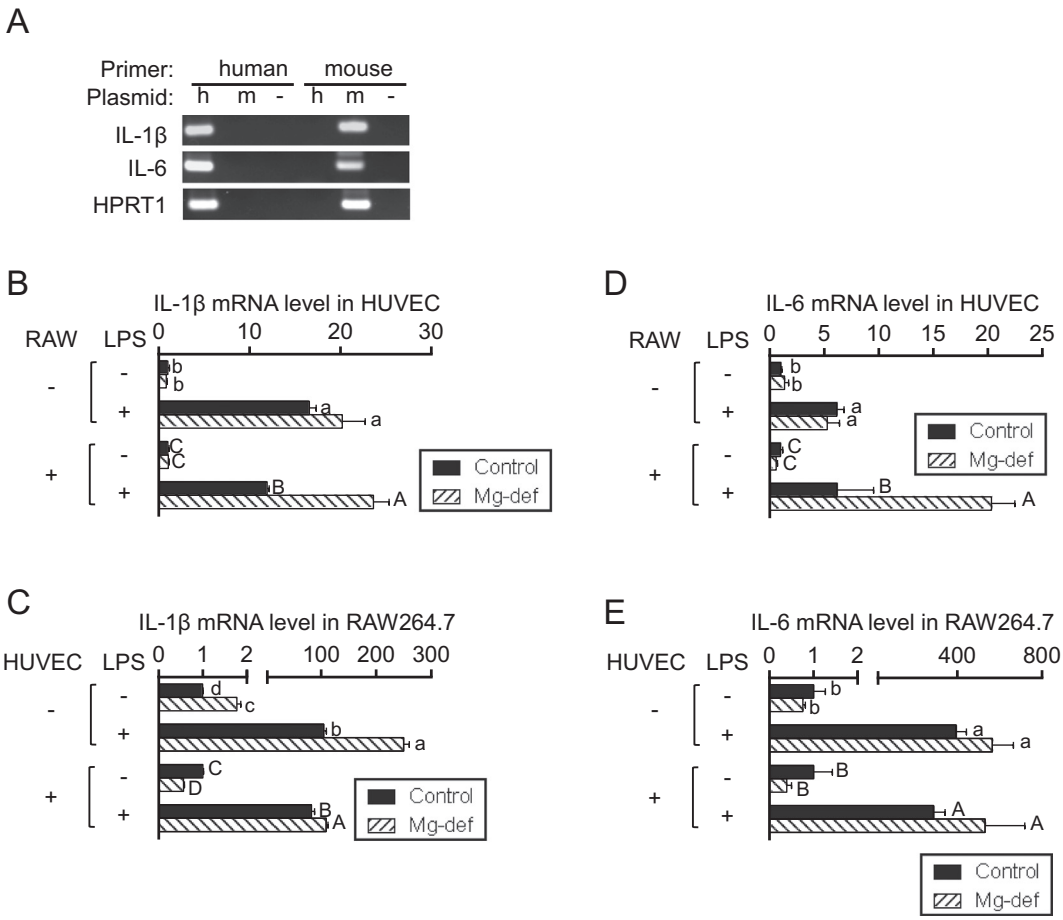

Fig. 7. Regulatory expression of IL-1β and IL-6 by extracellular magnesium deficiency and LPS in HUVECs and RAW264.7 cells(A) Human- or mouse-specific PCR primers were designed to detect IL-1β, IL-6 and HPRT1. One nanogram of plasmid encoding human- or mouse-derived IL-1β, IL-6 or HPRT1 was used as the template, and PCR was performed. After agarose gel electrophoresis of the PCR products, the gels were stained with ethidium bromide. h: plasmid expressing human-derived gene, m: plasmid expressing mouse-derived gene, -: distilled water as a negative control. (B-E) HUVECs were cultured with or without RAW264.7 cells in the presence of LPS or under the magnesium-deficient condition for 12 h. Expression of IL-1β (B and C) and IL-6 (D and E) was examined in HUVECs (B and D) and RAW264.7 cells (C and E). Expression was normalized to HPRT1 (Hprt1) expression. Expression in the control group for the HUVECs or RAW264.7 cells that were cultured alone or together was set to 1. The data are shown as the mean ± SE (n = 3). a, b, c, d: $P < 0.05$; A, B, C, D: $P < 0.05$.

Previous studies revealed that hepatic expression of proteasome subunits is modified by various factors; hepatic expression of PSMA2, PSMB5 and PSMC1 is lower in alcoholic patients than in healthy control individuals [26]. Infection with the hepatitis C virus increases the expression of PSMA6 in the liver [28]. Altered expression of proteasome subunits has also been shown in non-hepatic cells; inhibition of histone deacetylase down-regulates the expression of PSMA6, PSMB1, PSMB2 and PSMB5 but not PSMA4 and PSMB3 in Caco-2 intestinal epithelial cells [27]. In addition to these factors, the present results suggest the regulation of proteasome subunit expression by extracellular magnesium level.

We previously performed a metabolomic analysis of the livers of rats fed a magnesium-deficient diet for 8 weeks [10]; magnesium deficiency increased the free serine and threonine content in the livers of the rats. In contrast, the hepatic glucose 6-phosphate content was decreased by magnesium deficiency in rats. These results are consistent with the changes to the cellular metabolites in HUVECs in response to extracellular magnesium deficiency; the serine and threonine content was increased in HUVECs cultured with magnesium-deficient medium, and the conditioned medium from magnesium-deficient RAW264.7 cells decreased the cellular content of glucose 6-phosphate in HUVECs. In addition, the previous study suggested that taurine/hypotaurine metabolism, methionine metabolism, and glycine/serine/threonine metabolism were the affected metabolic pathways by magnesium deficiency in the liver [10]; these metabolic pathways were also detected in magnesium-deficient HUVECs (Fig. 3C). In contrast, these metabolites were unaffected or changed inconsistently upon extracellular magnesium deficiency in HepG2 cells; glucose 6-phosphate was detected only in HepG2 cells cultured with the magnesium-deficient medium. It is possible that changes in the cellular metabolite content in the liver in response to magnesium deficiency partly reflect modulated metabolic pathways in vascular endothelial cells rather than in hepatocytes. Hepatocytes occupy approximately 80% of the total liver volume, and non-parenchymal cells contribute only 6.5% to the liver volume [11]. However, the number of non-parenchymal cells accounts for 40% of total liver cells [11]; considering that the percentage of endothelial cells and Kupffer cells in the non-parenchymal cell population is 50% and 23%, respectively [29], these cells are estimated to comprise as much as 20% and 9.2%, respectively, of the total liver cell population. A similar distribution—hepatocytes (63%), endothelial cells (23%) and Kupffer cells (6%)—was also previously shown [30].

The present study revealed that culture with magnesium-deficient medium induced various cell responses in HepG2 cells, RAW264.7 cells and HUVECs. The rise in intracellular concentration of calcium possibly triggers the modulation of cell responses, because magnesium acts as a natural calcium antagonist, and reduction of extracellular magnesium induces elevation of intracellular calcium

[1,12]. Calcium ion acts as a second messenger of various signaling pathway [31], leading to various responses including oxidative stress [32] and inflammation [33]; in fact, magnesium deficiency induces oxidative stress in the liver [34]. The RNA-seq analysis in RAW264.7 cells indicated that the expression of genes related to oxidative stress was up-regulated by extracellular magnesium deficiency in RAW264.7 cells (Table 2). Specifically, extracellular magnesium deficiency increased the expression of Nqo1 and Prdx1, which are molecules that protect cells against oxidative stress [35,36], in RAW264.7 cells. It is possible that the gene induction is secondary to the onset of oxidative stress.

Extracellular magnesium deficiency modulated the expression of chemokines; expression of chemokines was generally increased in RAW264.7 cells, whereas expression of several chemokines was increased and that of others was decreased by extracellular magnesium deficiency in HepG2 cells. Chemokines are synthesized and secreted not only by leukocytes but also by various cell types [37]. Chemokines are mainly involved in leukocyte chemoattraction, but they have also been implicated in diverse cellular activities, including angiogenesis, fibrosis, proliferation, cytotoxicity and apoptosis [38]. The (patho)physiological significance of the modulated expression of chemokines in response to extracellular magnesium deficiency should be clarified in the future.

Magnesium deficiency is well-known to be related to the onset of inflammatory responses characterized by leukocyte activation as well as increased production of inflammatory cytokines including IL-1β and IL-6 [3]. An epidemiological study indicated a negative relationship between magnesium intake and the plasma IL-6 concentration in postmenopausal women [39]. Increased mortality due to infection with bacteria has been shown in magnesium-deficient rats [40]. Consistent with these results, enhancement of serum concentrations of IL-1β and IL-6 by LPS treatment is greater in magnesium-deficient rats than in control rats [41]. However, the increased concentrations of IL-1β and IL-6 cannot be explained by their expression in alveolar macrophages [41]. The present study revealed that LPS-induced expression of IL-1β and IL-6 was enhanced by extracellular magnesium deficiency in HUVECs, which was dependent on the presence of RAW264.7 cells. Cell interactions between vascular endothelial cells and Kupffer cells (macrophages) may enhance cytokine production, leading to exacerbated responses to microbial infection.

The present study used HepG2 cells, RAW264.7 cells and HUVECs as model cells of hepatocytes, Kupffer cells and endothelial cells, respectively. Considering that even characterization of residential Kupffer cells was different from that of infiltrated macrophages [42], these cell lines are just model cells for liver constituting cells. In addition, we examined effects of only one level of magnesium in the culture medium and one duration of magnesium deficiency. Thus, the present results may reflect only one aspect on cell responses in the magnesium-deficient liver. Nevertheless, the present study provides insights on regulatory responses in the liver in response to magnesium deficiency in a cell culture system, which would be useful for clarification of responses to the other nutritional aberration.

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