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Rebamipide, a Gastroprotective Drug, Inhibits Indomethacin-Induced Apoptosis in Cultured Rat Gastric Mucosal Cells: Association with the Inhibition of Growth Arrest and DNA Damage-Induced 45α Expression

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Rebamipide, a gastromucosal protective drug, suppresses indomethacin-induced gastropathy in humans and rodents. Effects of rebamipide on gene expression in indomethacin-treated gastric mucosal cells (RGM1) were investigated using high-density oligonucleotide arrays. Indomethacin induced apoptosis in RGM1 cells in a dose-dependent manner. Rebamipide pretreatment significantly reduced indomethacin-induced apoptosis. We used gene expression profiling on high-density oligonucleotide probe arrays to characterize the transcriptional response of RGM1 cells to indomethacin treatment for 6 hr. Of the 8,799 probes examined, 717 (8.1%) were induced (400 probes) or repressed (317 probes) at least 1.5-fold. Among the 158 genes that were induced by indomethacin at least 2.0-fold, four genes that were down-regulated by rebamipide at least 2.0-fold are listed: growth arrest and DNA-damage-inducible 45α (GADD45α), golgi SNAP receptor complex member 1, iodothyronine deiodinases, and transcription factor 8. Real time-PCR confirmed GADD45α expression and its inhibition by rebamipide. Inhibition of apoptosis-related genes is possibly important for the cytoprotective effect of rebamipide against indomethacin-induced gastric mucosal cell injury.

KEY WORDS: apoptosis; DNA microarray; GADD45; indomethacin; rebamipide.

Rebamipide (2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinone-4-yl] propionic acid), a gastroprotective agent, is widely used clinically as an anti-ulcer and anti-gastritis drug (1–3). Both in vivo and in vitro experiments have demonstrated that rebamipide prevents gastric mucosal injury induced by a variety of stimuli. Rebamipide induces the production of prostaglandins in the gastric mucosa and increases gastric mucus synthesis (2). Previous

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studies have demonstrated that the antioxidative properties of rebamipide are also implicated in the protection of gastric mucosal injury against ischemia-reperfusion (4), indomethacin administration (5), and *Helicobacter pylori* infection (6–8). We have demonstrated the direct scavenging action of rebamipide against hydroxyl radicals by an electron spin resonance assay (9, 10), and the mechanism of its scavenging action was confirmed by a recent study (11).

In addition to antioxidative action, the underlying mechanisms by which rebamipide exerts its cytoprotective effect in the damaged gastric mucosal cells are, in part, mediated by the induction of several genes, including those coding heat shock protein 70 (HSP70) (12, 13), cyclooxygenase-2 (COX-2) (14, 15), and prostaglandin EP4 receptor (EP4) (16). The mechanisms of the cytoprotective effects of rebamipide on gastric mucosal cells, however, have not been fully addressed. In order to characterize the cytoprotective effects of rebamipide on nonsteroidal anti-inflammatory drug (NSAID)-induced gastropathy, we stimulated cultured gastric mucosal cells with indomethacin and measured comprehensive changes in mRNA expression using a high-density oligonucleotide array in the absence and presence of rebamipide.

METHODS

Gastric Epithelial Cell Line. The rat gastric mucosal cell line RGM1 (RCB-0876 at Riken Cell Bank, Tsukuba, Japan), established by Matsui and Ohno, was used (17). RGM1 cells have the characteristics of gastric mucous-producing cells and carry prostaglandin EP4 receptors (17, 18). Furthermore, they do not express transcripts of the gastrin receptor, the H_2 receptor, histidine decarboxylase, somatostatin, or pepsinogen 1 (18). RGM1 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 mg/ml amphotericin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. RGM1 cells were separated by trypsinization and seeded into 96-well culture plates or 8-well chamber glass slides. Experiments were performed when the cells were confluent.

Cell Viability. Cell viability was assessed by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) assay (Cell Counting Kit, Dojindo, Kumamoto, Japan) as previously described (19). Based on the manufacturer's protocol, WST-1 solution was added to 96-well plates and then incubated in a CO_2 incubator for 2 hr. The plates were read at 450 nm in a Microplate Reader (MPR-A4I, Tosoh, Tokyo) to quantify the number of living cells.

Microscopic Analysis for Apoptotic Cells. The effect of indomethacin on apoptosis and necrosis was determined morphologically by fluorescent microscopy after labeling with Hoechst 33342 (HO342) and propidium iodide (PI), as described previously (20). Twenty four hours after exposure to indomethacin, cells were incubated with 10 μg/ml HO342 dye for 15 min at 37°C and with 10 μg/ml PI for 10 min at 37°C. Floating and attached cells were collected by trypsinization, washed in Hank's balanced salt solution (HBSS), and centrifuged. The pellet was resuspended in 200 μl of HBSS. This suspension was placed on a microscope slide, coverslipped, and examined under a dry objective using a combination of epillumination and filters.

Preparation of Biotin-Labelled Complementary RNA (cRNA) and Hybridization to DNA Chips. Total RNA was extracted using a Qiagen RNeasy kit (Qiagen, Valencia, CA) and treated with DNase1 (DNase1 kit, Qiagen, Valencia, CA) to remove any residual genomic DNA. Preparation of cRNA and target hybridization was performed according to the Affymetrix GeneChip technical protocol. In brief, double-stranded cDNA was prepared from 5 μg of total RNA using Life Technologies Superscript Choice system (Life Technologies, Inc., Gaitherburg, MD, USA) and an oligo-(dT)24 anchored T7 primer. Biotinylated RNA was synthesized from the double-stranded cDNA by in vitro transcription using a 3′-Amplification Reagents for IVT Labeling kit (Affymetrix, Santa Clara, CA, USA). Transcription products were purified using a Qiagen RNeasy column (Qiagen, Valencia, CA). After biotinylation, the in vitro transcription products were fragmented for 35 min at 94°C in a buffer composed of 200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, and 150 mM magnesium acetate. Affymetrix GeneChip arrays (Rat Genome U34A array, Affymetrix) were hybridized with the biotinylated products (0.05 μg/ml/chip) for 16 hr at 45°C using the manufacturer's hybridization buffer. After washing the arrays, the hybridized RNA was detected by staining with streptavidin-phycoerythrin (6× SSPE, 0.01% Tween-20, pH 7.6, 2 mg/ml acetylated bovine serum albumin, and 10 μg/ml streptavidin-phycoerythrin from Molecular Probes). The DNA chips were scanned using a specially designed confocal scanner (GeneChip Scanner 3000, Affymetrix).

Real Time-PCR. After 6 hr of incubation with indomethacin, total RNA was isolated with the acid guanidinium phenol chloroform method using an Isogen kit (Nippon Gene, Tokyo, Japan). The concentration of RNA was determined by absorbance at 260 nm in relation to absorbance at 280 nm. RNA was stored at −70°C until reverse transcription was performed. An aliquot (1 μg) of extract RNA was reverse-transcribed into first-strand complementary DNA (cDNA) at 42°C for 40 min, using 100 U/ml reverse-transcriptase (Takara Biochemicals, Shiga, Japan) and 0.1 μM of oligo (dT)-adapter primer (Takara) in a 50 μl reaction mixture. Real-time polymerase chain reaction (PCR) was carried out with a 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) using the DNA-binding dye SYBER Green I for the detection of PCR products. The reaction mixture (RT-PCR kit, Code RRO43A, Takara) contained 12.5 μl Premix Ex Taq, 2.5 μl SYBER Green I, custom-synthesized primers, ROX reference dye, cDNA (equivalent to 20 ng total RNA) to give a final reaction volume of 25 μl Primers were as follows: for GADD45a, sense 5′-GCAGAGCAGAAGATCGAAAGGA-3′ and antisense 5′-TCGTACACGCCGACAGTTATG-3′; for GADD153, sense 5′-GTCTCTGCCTTTCGCCTTTG-3′ and antisense 5′-AGGTGCCCCCAATTTCATCT-3′; and for β-actin, sense 5′-GAGCAAACATCCCCCAAAGTT-3′, and antisense 5′-GCCGTGGATACTTGGAGTGACT-3′. The PCR settings were as follows: initial denaturation of 10 s at 95°C was followed by 40 cycle of amplification for 5 s at 95°C and 31 s at 60°C. The PCR products were quantified using standard DNA for each purified by PCR products of reverse-transcribed RNA.

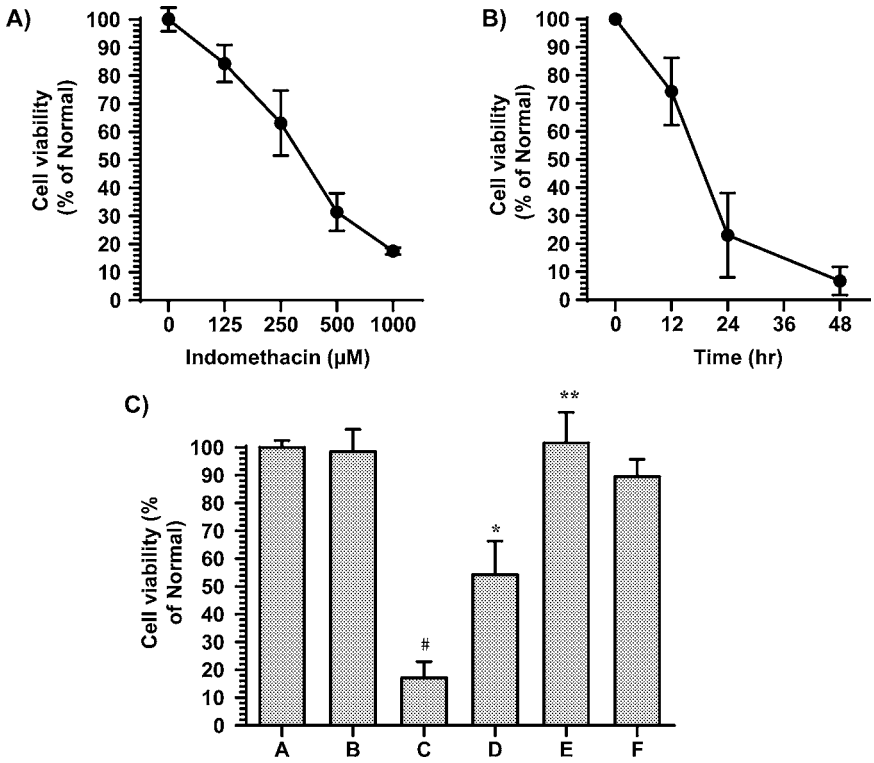

Fig 1. The induction of cell death by indomethacin treatment in RGM1 cells. (A) Concentration dependence of viability of RGM1 cells 24 hr after indomethacin treatment. (B) Time course of cell viability after a 500 μM indomethacin treatment. (C) Effect of pretreatment with rebamipide (1 and 10 μM) for 6 hr on indomethacin-induced cytotoxicity. Cell viability was measured 24 hr after indomethacin exposure. A, normal cells; B, dimethylsulfoxide (0.5%) alone; C, indomethacin (500 μM); D, rebamipide (1 μM) pretreatment + indomethacin; E, rebamipide (10 μM) pretreatment + indomethacin; F, rebamipide (10 μM) pretreatment alone. The results are given as percentage variation of O.D. versus non-stimulated RGM1 cells and are expressed as mean ± SEM of three separate observations performed in triplicate. $^{\#}p < 0.01$ compared with group B (dimethylsulfoxide alone), and $^{*}p < 0.05$ and $^{*}p < 0.01$ compared with group C (indomethacin).

The relative expression was then calculated as the density of the product of the respective target gene divided by that for β-actin from the same cDNA.

Statistical Analysis. The results of the cell viability test and real time-PCR analysis are expressed as mean ± SEM of three separate observations performed in triplicate. The Student's *t* test was used in statistical analysis. A *P* value of 0.05 or less was considered statistically significant.

Array data analysis was carried out using Affymetrix GeneChip Operating Software (GCOS) version 1.0. GCOS analyzes image data and computes an intensity value for each probe cell. Briefly, mismatch probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals. To determine the quantitative RNA abundance, the average difference values (i.e., gene expression levels) representing the perfect match–mismatch for each gene-specific probe family is calculated, and the fold changes in average difference values were determined according to Affymetrix algorithms and procedures. For the pathway analysis, gene probe set ID numbers were imported into the Ingenuity Pathway Analysis software (Ingenuity Systems, Mountain View, CA). The identified genes were mapped to genetic networks available in the Ingenuity database and were then ranked by a score. The score is the probability that a collection of genes is equal to or greater than the number in a network that could be achieved by chance alone. A score of 3 indicates that there is a 1/1000 chance (significance = 0.001) that the focus genes are in a network randomly. Therefore, gene sets with scores of 3 or higher have a 99.9% confidence of not being randomly generated. This score was used as the cut-off for identifying gene networks significantly affected by indomethacin.

RESULTS AND DISCUSSION

Effects of Rebamipide on Indomethacin-Induced Apoptosis. Recently, it has been demonstrated that indomethacin-induced gastric mucosal injury involves apoptosis in vivo (21, 22) and in vitro (23–25). Previous findings showed that cytoprotection by gastroprotective drugs such as sucralfate and polaprezinc against gastric mucosal injury caused by indomethacin involves the suppression of apoptotic events propagated by caspase-3-like

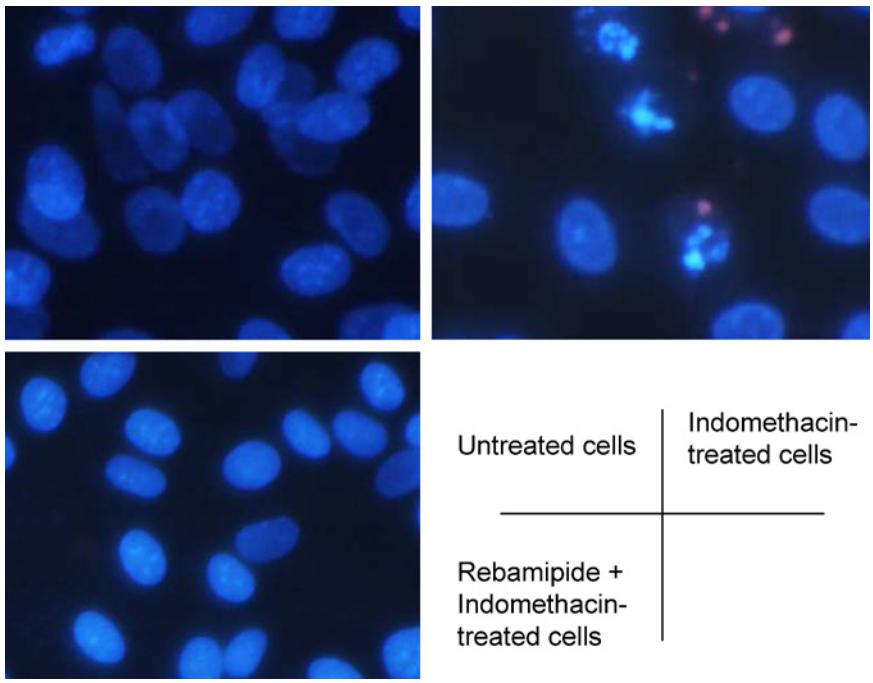

Fig 2. Fluorescence microscopic view of indomethacin-exposed RGM1 cells stained with Hoechst33342 (HO342) and propidium iodide (PI). Untreated cells show viable cells that excluded PI while the nuclei of the cells were stained with HO342 dye. Treatment of cells with indomethacin (500 μM) clearly induced chromatin condensation, which was not apparent in cells pretreated with rebamipide (10 μM).

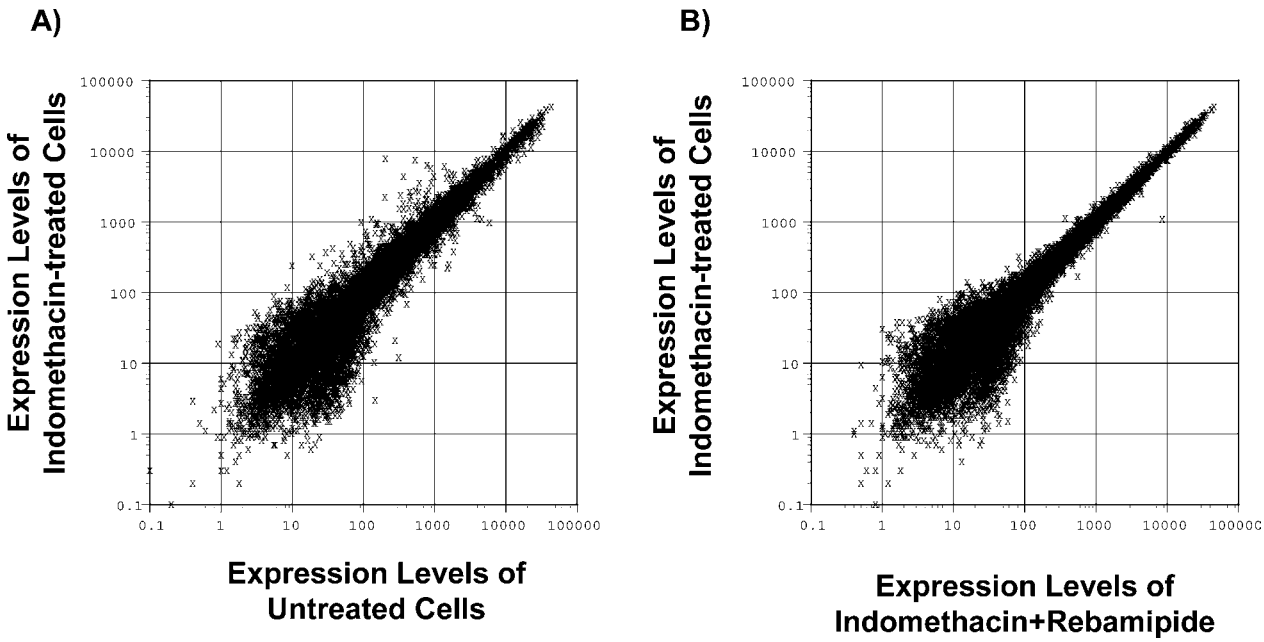

Fig 3. Global comparison of the differences in gene expression levels. (A) Scatter plot of the expression levels of indomethacin-treated and vehicle-treated RGM1 cells. (B) Scatter plot of the expression levels of indomethacin-treated and rebamipide-pretreatment plus indomethacin-treated RGM1 cells.

proteinase (21, 25). Fujii *et al.* (25) first demonstrated caspase-3-like proteinase activation and mitochondrial cytochrome *c* release during indomethacin-induced apoptosis in RGM1 cells. In the present study, we confirmed that indomethacin is indeed able to induce cell death in RGM1 cells in a dose- and time-dependent fashion (Fig. 1A and B). As our previous studies showed that rebamipide, a gastroprotective drug, significantly inhibited the indomethacin-induced gastric mucosal injury in rats (5) and humans (26), the effect of rebamipide on indomethacin-induced apoptosis in RGM1 cells was investigated in the present study. As shown in Fig. 1C, pretreatment with rebamipide for 6 hr significantly reversed the decrease in cell viability in RGM1 cells after a 24-hr exposure to indomethacin (500 μM). Since previous reports demonstrated that treatment of gastric mucosal cells with indomethacin induces both necrosis and apoptosis, we carried out double-staining experiments with PI and HO342 to distinguish the types of cell death. PI staining causes pink nuclear staining in necrotic cells and late apoptotic cells because necrotic cells lose their membrane integrity, whereas living cells and early apoptotic cells are not stained with PI. By staining with HO342, morphological evidence of apoptosis can be seen. Fluorescence microscopy results showed that 24-hr treatment of cells with indomethacin (500 μM) decreased in number of living cells and clearly induced chromatin condensation and nuclear fragmentation with blue spot (early phase of apoptosis) and red spot (late phase of apoptosis) (Fig. 2). On the other hand, these changes were not apparent after 6-hr pretreatment of cells with rebamipide (10 μM) prior to indomethacin exposure. These data indicate that rebamipide demonstrates cytoprotective action against indomethacin toxicity by inhibiting the apoptotic pathway.

GeneChip Analysis for mRNA Expression. In the present study, we used the high-density oligonucleotide microarray technique for mRNA expression profile of RGM1 cells in order to investigate the mechanism of cell death under the conditions of indomethacin exposure in vitro. We used the Rat Genome GeneChip U34A array (Affymetrix), which contained 8,799 probes representing approximately 7,000 full-length sequences and approximately 1,000 EST clusters selected from the UniGene database. This array is a powerful tool for detecting changes in mRNA expression due to a toxic or stress-related response. Comparison of the expression profiles from the vehicle-treated cells and indomethacin-treated cells (Fig. 3A) and from the indomethacin-treated cells and rebamipide plus indomethacin-treated cells (Fig. 3B) enabled the identification of differentially regulated genes associated with indomethacin toxicity and the pharmacological action of rebamipide, respectively. The scatter plot for untreated cells and indomethacin-treated cells indicated a considerable deviation from the identity line, which suggests discordance in the patterns of gene expression between untreated cells and indomethacin-treated cells. Our goal is to identify the subset of genes that are primarily responsible for such discordance.

The present study showed that of the 8,799 probes examined, 717 (8.1%) were up-regulated (400 probes) or down-regulated (317 probes) at least 1.5-fold after a 6 hr-exposure to indomethacin (500 μM) in comparison with the vehicle-treated cells. To further refine the list of indomethacin-affected genes, our next investigation was to identify the genes that are known to interact biologically. To this end, we used the Pathway Analysis tool (Ingenuity Systems) to carry out analysis on the 717 genes mentioned earlier. Table 1 shows 10 genetic networks affected

TABLE 1. GLOBAL CARONICAL PATHWAY AFFECTED BY INDOMETHACIN IN RGM1 CELLS

Caronical pathway	*Significance*	*Genes*
p38 MAPK Signaling	9.99 × 10-4	**DDIT3**, DUSP1*, HSPB1*, **IL-1R1***, MAPK14, **MAX, MYC***, **PLA2G4A**, TGFB3*, **TNFRSF1A**, **TNFRSF6**
ERK/MAPK Signaling	1.43 × 10-2	BAD, DUSP1*, **EIF4EBP1***, HSPB1*, **MYC***, MYCN, PIK3R2*, **PLA2G4A**, PPP1CC, PPP2R2B, PRKAR2B, **PPKCE**, PKT2B*, YWHAH*
Synthesis/Degradation of Ketones	1.57 × 10-2	BDH, **HMGCL**, HMGCS2*
Cell Cycle: G1/S Checkpoint Regulation	1.85 × 10-2	CCND1*, CCND2, CCND3*, **GSK3B**, **MYC***, **RBL2**, TGFB3
VEGF Signaling	1.90 × 10-2	ACTB*, ACTC, ACTN4, BAD, **EIF2B3***, FIGF, **HIF1A**, PIK3R2*, **VEGF***
Sterol Biosynthesis	1.91 × 10-2	**FDFT1***, FDPS, **HMGCR***, **NQO1**
Amyloid Peocessing	2.03 × 10-2	CAPN2, **GSK3B**, MAPK14, PRKAR2B, **PRKCE**, PSEN2
Glutamate metabolism	3.26 × 10-2	**GCLC**, **GCLM**, GLUL, **GSR**, **PPAT**
Insulin Receptor Signaling	5.01 × 10-2	BAD, **EIF2B3***, **EIF4EBP1***, **GSK3B**, **JAK2**, **PDE3B**, PIK3R2*, PPP1CC, PRKAR2B, PTPN1, **SGK**
Fatty Acid Metabolim	5.40 × 10-2	**ADH7**, **ALDH3A1**, CPT1A, **CYP1A1***, **CYP1B1**, **CYP2C19**, ECH1, **FACL3**, **FACL4***, IVD

Notes. Gene/protein identifiers that made the user-defined cutoff and map to the Global Molecular Network are displayed in Column 3; bold, up-regulated: non-bold, down-regulated. *: Duplicates. Gene/protein IDs marked with an asterisk indicate that multiple identifiers from your input list mapped to a single gene in the Global Molecular Network.

TABLE 2. GENES UP-REGULATED AT LEAST 5.0-FOLD IN RGM-1 CELLS EXPOSED TO INDOMETHACIN

Probe Set ID	*Description*	*Average Difference**		
		Indomethacin	*Sham*	*Fold Change†*
rc_H31287_g_at	tribbles homolog 3 (Drosophila)	7882.5	203.0	27.86
E00778cds_s_at	cytochrome P450, family 1, subfamily a, polypeptide 1	238.0	9.9	22.63
U30186_at	DNA-damage inducible transcript 3	7530.7	530.4	14.93
AB015432_s_at	tumor-associated protein 1	4371.4	363.9	13.93
AF000899_at	nucleoporin p58	116.9	8.5	11.31
M32167_at	vascular endothelial growth factor	421.7	36.8	11.31
rc_AI070295_at	growth arrest and DNA-damage-inducible 45 alpha	323.7	18.9	10.56
E00717UTR#1_s_at	cytochrome P450, family 1, subfamily a, polypeptide 1	984.3	92.5	8.00
X89225cds_s_at	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	6329.2	778.8	8.00
AB015433_s_at	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	5714.9	874.5	6.96
rc_AA957917_s_at	solute carrier family 7 (cationic amino acid transporter, y + system), member 1	1089.5	129.2	6.96
rc_AI008677_s_at	3-phosphoglycerate dehydrogenase	484.1	64.7	6.50
U09540_at	cytochrome P450, family 1, subfamily b, polypeptide 1	2230.7	199.3	6.50
U09540_g_at	cytochrome P450, family 1, subfamily b, polypeptide 1	2374.4	329.3	6.06
U70476_at	solute carrier family 7 (cationic amino acid transporter, y + system), member 1	418.8	133.3	6.06
Y00396mRNA_g_at	v-myc avian myelocytomatosis viral oncogene homolog	734.5	106.9	6.06
rc_AI070295_g_at	growth arrest and DNA-damage-inducible 45 alpha	921.7	151.4	5.66
AF020618_at	myeloid differentiation primary response gene 116	357.5	49.9	5.66
AF020618_g_at	myeloid differentiation primary response gene 116	1510.3	366.6	5.66
L32591mRNA_at	growth arrest and DNA-damage-inducible 45 alpha	2750.4	537.3	5.28
rc_AI014163_at	interferon-related developmental regulator 1	933.5	159.0	5.28
U01348_at	N-Acetyltransferase-2	102.5	17.3	5.28
rc_AA850734_at	vascular endothelial growth factor	975.0	152.8	5.28

*Average difference indicates expression level of the gene.
†Fold chages in average difference values were calculated using Affymetrix software algolithm (GCOS ver. 1.0).

by indomethacin as defined by the Pathway Analysis tool. These networks describe functional relationships between gene products based on known interactions reported in the literature. The tool then associates these networks with known biological pathways. These networks shown in Table 1 were associated with cell cycle, apoptosis, proliferation, and metabolism. In particular, the p38 mitogen-activating protein kinases (MAPK) signaling pathway was found to be highly significant since a greater number of identified genes were present in it than would be expected by chance. p38 MAPK is reported to be activated by indomethacin (27–29) and is associated with the induction of apoptosis. As activation of p38 leads to a down-regulation of nuclear factor-κB (NF-κB) activity, the apoptosis may have been mediated, in part, through inhibition of NF-κB.

Genes demonstrating greater than 5-fold alterations are listed in Table 2, which includes many genes associated with the apoptotic pathways. TRB3 (rc_H31287_g_at), a highly up-regulated gene, is a mammalian homolog of *Drosophila tribbles* and binds directly to the serine-threonine kinase Akt/PKB and can block activation of the kinase (30). Akt/PKB is a central player in the signal transduction pathways that are activated in response to growth factors or insulin and is believed to contribute to several cellular functions, including nutrient metabolism, cell growth, and apoptosis (31). Therefore, the present data indicates the possibility that up-regulation of TRB3 resulting in interference with Akt/PKB activation may contribute to the apoptotic process after indomethacin exposure. Tumor-associated protein 1 (AB015432_s_at) is a rat homolog of the L-type amino acid transporter 1 (LAT1). LAT1 is highly expressed in cultured cells and malignant tumors, presumably to support their continuous growth (32). The enhanced expression of LAT1 in cells exposed to indomethacin, a toxic substance, may be an adaptive response to survive against cell death signaling. The growth arrest and DNA damage-inducible (GADD) gene is a member of a group of genes induced by agents that damage DNA and/or cause growth arrest. As shown in Table 2, GADD153 homolog (DNA-damage inducible transcript 3, U30186_at) and GADD45α (rc_AI070295_at, rc_AI070295_g_at, L32591mRNA_at) were up-regulated at least 5.0-fold after 6-hr exposure to indomethacin (500 μM) in comparison with the vehicle-treated cells. Increased GADD45 gene expression has been detected in many mammalian cell types and has been implicated in terminal differentiation (33), growth suppression (34), and

TABLE 3. GENES UP-REGULATED AT LEAST 2.0-FOLD IN INDOMETHACIN TREATMENT AND DOWN-REGULATED AT LEAST 2.0-FOLD IN REBAMIPIDE TREATMENT

		*Average difference**			*Fold change†*	
Probe Set ID	*Description*	*Vehicle*	*Indomethacin*	*Indomethacin + Rebamipide*	*Indomethacin/ vehicle*	*Indomethacin + Rebamipide/indomethacin*
rc_AI070295_at	growth arrest and DNA-damage-inducible 45 alpha	18.9	323.7	82	10.56	0.33
rc_AI177943_s_at	golgi SNAP receptor complex member 1	75.5	266.2	128.2	2	0.76
AB011068_g_at	type II iodothyronine deiodinase	82.3	268.8	125.5	2	0.71
U51583_at	transcription factor 8	42.7	90.2	40	2.14	0.44

*Average difference indicates expression level of the gene.
†Fold chages in average difference values were calculated using Affymetrix software algolithm (GCOS ver. 1.0).

apoptosis (35). Although microinjection of the exogenous GADD45 expression vector into human fibroblast has been shown to cause G2/M arrest (36), it is still unclear whether GADD45 plays a direct role in apoptosis. Recent studies suggest that GADD45 may mediate genotoxic stress-induced apoptosis via activation of c-Jun N-terminal kinase (JNK) and/or p38 MAPK (37, 38). This data, including the present information, suggests that enhanced expression of GADD45 followed by MAPK activation may be involved in the apoptosis of RGM1 cells treated with indomethacin.

Effects of Rebamipide on Apoptosis-Related Gene Expression. To clarify the cytoprotective mechanism of rebamipide against indomethacin-induced cell death, we compared the levels of mRNA expression in RGM1 cells pre-treated with and without rebamipide. We succeeded in finding four genes based on the following criteria: genes that are up-regulated at least 2.0-fold after 6-hr exposure to indomethacin in comparison with the vehicle-treated cells, and also genes that are down-regulated at least 2.0-fold after 6-hr pretreatment with rebamipide (10 μM) in comparison with 6-hr vehicle treatment prior to the 6-h exposure to indomethacin. As shown in Table 3, we could list four genes. Among them, GADD45α gene probe (rc_AI070295_at) is the most up-regulated gene after the indomethacin treatment and reduced to 0.33-fold by the pretreatment with rebamipide. In the Rat Genome U34A array (Affymetrix), four probe sets were included for the GADD45α gene: rc_AI070295_at, rc_AI070295_g_at, L32591mRNA_at, and L32591mRNA_g_at. The expression of all four probes was up-regulated at least 4.5-fold after indomethacin exposure and down-regulated by the pretreatment with rebamipide (Table 4). In addition, we further validated the expression of GADD45α mRNA by real-time quantitative PCR (Fig. 4). As shown in Fig. 4, we found GADD45α and GADD153 mRNA expression before stimulation to be faint. In contrast, these levels were significantly increased 6 hr after stimulation with indomethacin. The increase in GADD45α mRNA expression was significantly inhibited by the pretreatment with rebamipide at a concentration of 10 μM; however, the increase in GADDα153 mRNA expression was not affected by rebamipide. The expression patterns of GADD45α and GADD153 mRNA agreed with those observed in the GeneChip experiments.

The enhanced expression of GADD45α mRNA after indomethacin exposure may be induced by direct or indirect mechanism. Recent studies demonstrated that

TABLE 4. LEVELS OF mRNA EXPRESSION FOR GROWTH ARREST AND DNA DAMAGE-INDUCIBLE GENE (GADD45α)

	*Average difference**			*Indomethacin/vehicle*		*Indomethacin + Rabamipide/ indometahcin*	
Probe Set ID	*Vehicle*	*Indomethacin*	*Indomethacin + Rebamipide*	*Fold Change†*	*Change‡*	*Fold Change†*	*Change‡*
rc_AI070295_at	18.9	323.7	82.0	10.56	I	0.33	D
rc_AI070295_g_at	151.4	921.7	490.8	5.66	I	0.5	D
L32591mRNA_at	537.3	2750.4	2460.2	5.28	I	0.87	NC
L32591mRNA_g_at	968.5	4411.5	3913.0	4.59	I	0.87	NC

*Average difference indicates expression level of the gene.
†Fold chages in average difference values were calculated using Affymetrix software algolithm (GCOS ver. 1.0).
‡I, D, and NC indicates increase, decrease, no change in the average difference.

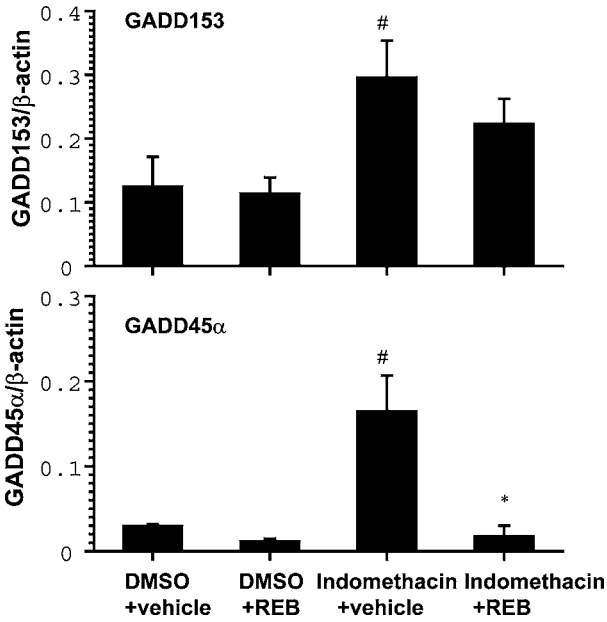

Fig 4. Real time-PCR showing the effect of rebamipide on GADD45α and GADD153 mRNA expression in RGM1 cells exposed to indomethacin. RGM1 cells were pretreated for 6 hr with rebamipide (REB, 10 μM) followed by indomethacin (500 μM for 6 hr). Real time-PCR analysis of total RNA extracts was performed as described in Materials and Methods. The relative expression was calculated as the density of the product of the respective target gene divided by that for β-actin from the same cDNA. $^{\#}p < 0.05$ compared with dimethylsulfoxide (DMSO)-treated group, and $^{*}p < 0.05$ compared with indomethacin–treated group.

the peroxisome proliferators-activated receptor (PPAR)-γ ligand-responsive element in the GADD45α promoter was mapped by promoter-deletion studies to a 153-bp region between -243 and -81 bp relative to the transcription start site that harbors an Oct-1 motif (39). Since indomethacin is known to act as a PPAR-γ ligand, indomethacin could induce GADD45α expression directly. In addition to the direct effect on GADD45α promoter, indomethacin may enhance the levels of its expression through the pathway activated by indomethacin-induced DNA damage or cellular toxicity. Further studies will be required to clarify the mechanism by which indomethacin could induce GADD45α expression, and the involvement of GADD45α in the inhibition of indomethacin-induced gastric mucosal cell apoptosis by rebamipide.

In conclusion, the present study using GeneChip analysis first demonstrated the enhanced expression of GADD45α and the activation of MAPK-associated apoptotic signaling in RGM1 cells treated with indomethacin. It also showed that rebamipide, a gastroprotective drug, inhibits the apoptosis associated with the inhibition of GADD45α expression.

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