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Transcriptome Analysis for Cytoprotective Actions of Rebamipide against Indomethacin-Induced Gastric Mucosal Injury in Rats

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Summary **We have reported that rebamipide, a gastroprotective drug, suppresses indomethacin-induced gastric mucosal injury in humans and rats. However, the mechanisms of the cytoprotective actions of rebamipide have not been fully addressed. In the present study, we determined mRNA expression profile of the gastric mucosa treated with indomethacin in rats, and investigated the cytoprotective effects of rebamipide against indomethacin-induced injury with a high-density oligonucleotide array (Rat Toxicology U34 GeneChip array). Gastric epithelial cells were obtained by laser-assisted microdissection. Data analysis was performed with a GeneChip Operating Software, GeneSpring software 7.0, and Ingenuity Pathway Analysis. Among 1,031 probes, the expression of 160 probes (15.5%) showed at least 2.0-fold up-regulation (158 probes) and down-regulation (2 probes) 2 h after indomethacin administration in comparison with the vehicle-treated rats. The pathway analysis of the up-regulated 123 probes identified the network with a highly significant score, which consisted of known clusters of cell death, cancer, and endocrine system disorders. We succeeded in listing 10 genes that were up-regulated by the treatment with indomethacin and that were down-regulated by rebamipide, including growth arrest and DNA damage-induced 45α. In conclusion, we demonstrated that cell death, especially apoptosis, pathway is involved in the pathogenesis of indomethacin-induced gastric mucosal injury, and that inhibition of apoptosis-related genes is possibly important for the cytoprotective effect of rebamipide against this injury.**

Key Words**: cytoprotection, gastric injury, indomethacin, transcriptome, rebamipide**

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin and indomethacin have been widely used clinically as anti-inflammatory, analgestic agents, but it has been documented that NSAIDs cause gastrointestinal erosions

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and ulcers as adverse effects [*1*, *2*]. Although it has been proposed that a deficiency of endogenous prostaglandins due to inhibition of cyclooxygenase by NSAIDs is involved in these effects [*3*], the exact pathogenic mechanism remains to be elucidated. Recently, several groups including us reported that rebamipide, a gastroprotective drug, significantly reduced gastric mucosal injury induced by indomethacin in rodents [*4–7*] and humans [*8*, *9*]. We firstly reported that protective effects of rebamipide against indomethacin-induced gastric mucosal injury may result from its antioxidant effect [*4*]. In addition, we have demonstrated the gastric cytoprotection induced by rebamipide from a double blind comparative study in healthy volunteers [*8*].

More recently, we investigated the effect of rebamipide on gene expression in cultured rat gastric mucosal (RGM1) cells exposed to indomethacin [*10*]. By the analysis using DNA microarray and real-time PCR, we confirmed that the expression of growth arrest and DNA damage-induced 45α (GADD45α) in RGM1 cells is enhanced by the exposure to indomethacin and this enhancement is markedly inhibited by rebamipide. However, the effects of rebamipide on gastric mucosal gene expression *in vivo* have not been fully addressed. In order to characterize the cytoprotective effects of rebamipide on indomethacin-induced gastric mucosal injury, we developed acute gastric mucosal injury induced by indomethacin in rats and measured comprehensive changes in mRNA expression using DNA microarray in the absence and presence of rebamipide.

Materials and Methods

Reagents

All chemicals were prepared immediately before use. Rebamipide was a gift from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). RNeasy Mini kit was purchased from QIAGEN (Valencia, CA) and Rat Toxicology GeneChip U34 array and Eukaryotic Small Sample Target Labeling Assay kit were from Affymetrix (Santa Clara, CA). All other chemicals used were of reagent grade.

Preparation of rats for acute gastric mucosal injury induced by indomethacin

Male Sprague-Dawley rats weighing 190–210 g were obtained from Keari Co. Ltd. (Osaka, Japan). They were housed in stainless steel cages with wire bottoms and maintained on a 12-h light and 12-h dark cycle with the temperature and relative humidity of the animal room controlled at 21–23°C and 55–65%, respectively. They were not fed for 18 h prior to the experiments, but were allowed free access to water. Maintenance of animals and experimental procedures were carried out in accordance with the U.S. National Institutes of Health Guidelines for the Use of Experimental Animals. All experiments were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan). Gastric mucosal injury was induced by the oral administration of 25 mg/kg of indomethacin (Sigma Chemical Co., St. Louis, MO) suspended in 0.5% carboxymethyl cellulose (CMC) solution with a few drops of Tween 80 in a volume of 0.5 ml/100 g body weight [*11*]. According to our previous report [*4*], rebamipide (100 mg/kg) dissolved in 0.5% CMC solution was given to the rats by intraperitoneal injection 0.5 h before indomethacin administration. To evaluate the effect of agents on indomethacin injury, rats were divided into the following groups: 1) sham-operated rats receiving 0.5% CMC solution, 2) indomethacin-treated rats receiving 0.5% CMC solution, 3) sham-operated rats receiving rebamipide, and 4) indomethacin-treated rats receiving rebamipide. Each of the groups contained 3 rats.

Laser capture microdissection, isolation of RNA, cDNA synthesis, cRNA amplification, and GeneChip hybridization

According to our previous report [*12*], we used laser-assisted microdissection to obtain cell-specific RNA. Gastric epithelial cells, located mainly in an upper one-third of mucosa, were identified on cryostat sections (8 μm) of the specimens obtained from the stomach of the rat, and the cells were isolated by laser-assisted microdissection using an LM200 system (Olympus, Tokyo, Japan). A sample containing five hundred cells was collected from each stomach. Our experiments were performed according to the Affymetrix GeneChip Eukaryotic Small Sample Target Labeling Assay protocol (Version II). Using this protocol, we succeeded in obtaining a sufficient amount of biotinylated cRNA to perform the GeneChip analysis from the small amount of gastric epithelial cells obtained by laser-captured microdissection.

Total RNA was extracted from the mixtures of three samples using a Qiagen RNeasy kit (Qiagen, Valencia, CA) and treated with DNase to remove any residual genomic DNA. Briefly, for first strand cDNA synthesis, total RNA sample (1 μl) mixed with T7-Oligo(dT) promoter primer was incubated at 70°C in a thermal cycler for 6 min, cooled to 4°C for 2 min, and reverse transcribed for 1 h at 42°C with 3 μl of the RT_Premix_1, and cooled to 4°C. Strand cDNA synthesis was carried out by adding 32.5 μl of SS_Premix_1, and incubating for 2 h at 16°C. The resulting cDNA was cleaned up by ethanol precipitation. To perform *in vitro* transcription, the dried double-stranded cDNA pellet was mixed with the following reagents (10 μl): 4 μl DEPC-treated water, 4 μl premixed NTPs, 1 μl 10× reaction buffer, and 1 μl 10× enzyme mix, and incubated at 37°C in a water bath for 6 h. First cycle cRNA was cleaned up using the RNeasy Mini Protocol for RNA Cleanup from the handbook accompanying the RNeasy Mini Kit for cRNA purification. For the second cycle of amplification and labeling, the cRNA sample was mixed with random primers (0.2 μg/μl),

incubated at 70°C for 10 min, cooled on ice for 2 min, and incubated at 42°C for 1 h with 5 µl of the RT_Premix_2. Second strand cDNA synthesis was carried out by mixing the sample with by addition of 5 µM T7-Oligo(dT) promoter primer and incubating at 70°C for 6 min, cooling at 4°C, and incubating again with 62 µl of SS_Premix_2. The resulting cDNA was treated with 1 µl T4 DNA polymerase (5 U/µl) for 10 min at 16°C, and cleaned up by ethanol precipitation. To perform *in vitro* transcription and labeling with the ENZO BioArray High Yield RNA Transcript Labeling Kit, the dried double-stranded cDNA pellet was incubated at 37°C for 4 h with 40 µl of the following reagents: 22 µl DEPC-treated water, 4 µl 10× HY reaction buffer, 4 µl 10× biotin labeled ribonucleotides, 4 µl 10× DTT, 4 µl 10× RNase inhibition mix, and 2 µl 20× T7 RNA polymerase. Labeled cRNA target was cleaned up using RNeasy columns.

The fragmentation, hybridization, washing, and staining were carried out according to the instructions described in the GeneChip Expression Analysis Technical Manual. GeneChip arrays were hybridized with the biotinylated products (5 µg/chip) for 16h at 45°C using the manufacturer's hybridization buffer. After washing the arrays, 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 of streptavidin-phycoerythrin from Molecular Probes). The DNA chips were scanned using a specially designed confocal scanner (GeneChip Scanenr 3000, Affymetrix).

Gene expression analysis

As an initial statistical analysis, we used 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 of the difference representing perfect match - mismatch for each gene-specific probe family is calculated. GCOS showed expression changes (Increased, Decreased or Marginal) in expression levels between pairs of profiles (difference analysis). 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 score. The score is the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. A score of 3 indicates that there is a 1/1000 chance that the focus genes are in a network due to random chance. Therefore, scores of 3 or higher have a 99.9% confidence of not being generated by random chance alone.

Results and Discussion

Analysis for gene expression induced by indomethacin treatment in rats

In the present study, we used the high-density oligonucleotide microarray technique for mRNA expression profile of gastric elithelial cells in order to investigate the mechanism of mucosal injury under the conditions of indomethacin exposure *in vivo*. We used the Rat Toxicology GeneChip U34 array (Affymetrix), which contained 1,031 probes. The present study showed that the expression of 160 probes (15.5%) showed at least a 2.0-fold up-regulation (158 probes) or down-regulation (2 probes) 2 h after indomethacin administration in comparison with the vehicle-treated rats. Selective genes demonstrating alterations greater than 3.0-fold are listed in the Table 1. Genes involved in redox-related enzymes (superoxide dismutase 1, carbonyl reductase 1, glutathione peroxidase 3, glutathione S-transferase, etc.) and transcription regulators (c-fos oncogene, etc.) were included.

123 of these up-regulated 158 probes were mapped to genetic networks as defined by the IPA tool. By the pathway analysis of the up-regulated 123 probes, 5 networks were found to be significant in that they had more of the identified genes present than would be expected by chance (Table 2). The network 1 shown in Table 2 contained the majority of these genes, and had a highly significant score of 73, and consisted of known clusters of cell death, cancer, and endocrine system disorders. Figure 1 illustrated the association of the network 1 that was most significantly affected by indomethacin. These data suggest that the imbalance of gene expression between apoptotic- and anti-apoptotic genes may be involved in the pathogenesis of indomethacin-induced gastric mucosal injury, which was also supported by previous studies. It has been demonstrated that indomethacin treatment induces gastric epithelial cell apoptosis *in vivo* [*13*, *14*] and *in vitro* [*15*, *16*]. Recent our study clearly showed apoptotic cell death of gastric epithelial cells induced by indomethacin *in vitro* [*10*].

In addition, we found two major pathways by the Ingenuity pathway analysis; glutathione metabolism and inflammation (Table 3). The genes involved in glutathione metabolism included glutathione peroxidase 1 and 3, many types of glutathione S-transferases (GST), and microsomal glutathione transferase (MGST1, 3) were up-regulated after the indomethacin exposure. The glutathione metabolism, a caronical pathway recorded in this analysis, was markedly affected by indomethacin with a most highest significance of $p = 1.42 \times 10^{-7}$. This induction of these genes may result from cellular response in gastric epithelial cells against oxidative stress induced by indomethacin administration, or from the direct pharmacological effect of indomethacin. The former hypothesis is supported by several reports [*11*,

Table 1. Genes up-regulated at least 3.0-fold in gastric mucosa exposed to indomethacin

Probe Set ID	Description	Signal Intensity		fold difference
		sham	indomethacin	
M74439mRNA_i_at	—	180.1	18371.4	78.79
Y00404_s_at	superoxide dismutase 1	298.5	3426.9	10.56
D89070cds_s_at	carbonyl reductase 1	347.5	4341.7	9.85
rc_AA859372_s_at	—	195.1	1347.0	9.19
X06769cds_g_at	FBJ murine osteosarcoma viral oncogene homolog	197.7	1906.8	8.00
rc_AI228738_s_at	FK506-binding protein 1a /// FK506 binding protein 2	142.6	922.2	7.46
AFFX_rat_5S_rRNA_at	—	124.1	688.1	6.50
rc_AI172411_at	glutathione peroxidase 3	477.1	2807.2	6.06
J02844_s_at	carnitine O-octanoyltransferase	28.2	103.9	6.06
C06598_at	similar to binding protein	298.4	1828.7	4.92
U39208_at	cytochrome P450 4F6	578.8	2699.1	4.92
X53428cds_s_at	glycogen synthase kinase 3 beta	67.4	267.0	4.59
rc_AI171506_g_at	malic enzyme 1	39.9	206.2	4.00
AA848546_at	similar to programmed cell death 10	120.2	264.9	4.00
rc_AI176658_s_at	heat shock 27kDa protein 1	154.1	553.6	4.00
X07467_at	glucose-6-phosphate dehydrogenase	272.0	1103.8	3.73
rc_AI178835_at	mitogen activated protein kinase kinase 1	70.0	114.2	3.48
U95727_at	DnaJ (Hsp40) homolog, subfamily A, member 2	109.3	238.5	3.48
X04229cds_s_at	glutathione S-transferase, mu 1	159.2	578.6	3.48
D63761_g_at	ferredoxin reductase	183.9	668.8	3.48
X77117exon#1-3_at	Diaphorase 1	248.8	610.0	3.48
rc_AA945054_s_at	cytochrome b-5	271.0	1036.7	3.48
K01932_f_at	glutathione S-transferase A5	341.3	1250.4	3.48
M57428_s_at	ribosomal protein S6 kinase, polypeptide 1	76.2	215.6	3.25
rc_AA848545_at	similar to programmed cell death 10	119.0	288.9	3.25
X91988_at	signal transducer and activator of transcription 5B	163.4	454.9	3.25
L19998_g_at	sulfotransferase family 1A, phenol-preferring, member 1	209.1	605.4	3.25
X63594cds_g_at	nuclear factor of kappa light chain gene enhancer	269.1	890.6	3.25
S82820mRNA_s_at	glutathione S-transferase Yc2 subunit	282.4	928.6	3.25
U03388_s_at	prostaglandin-endoperoxide synthase 1	286.2	572.8	3.25
rc_AI176422_g_at	electron-transferring-flavoprotein dehydrogenase	287.5	842.7	3.25
M15114_g_at	stearoyl-Coenzyme A desaturase 2	102.8	468.7	3.03
U46118_at	cytochrome P450, family 3, subfamily a, polypeptide 13	138.3	355.6	3.03
J05035_at	steroid 5 alpha-reductase 1	145.2	544.8	3.03
X62952_at	vimentin	163.2	454.5	3.03
M38566mRNA_s_at	cytochrome P450, family 27, subfamily a, polypeptide 1	163.5	492.3	3.03
U03491_g_at	transforming growth factor, beta 3	175.1	604.8	3.03
M33986mRNA_at	cytochrome P450, family 19, subfamily a, polypeptide 1	186.1	427.7	3.03
rc_AA925473_at	cell division cycle 42 homolog (S. cerevisiae)	206.3	595.5	3.03
rc_AA859648_at	DnaJ (Hsp40) homolog, subfamily B, member 1 (predicted)	221.0	730.3	3.03
D00636Poly_A_Site#1_s_at	diaphorase 1	243.0	1035.5	3.03
L29232_at	insulin-like growth factor 1 receptor	244.7	319.3	3.03
X78848cds_f_at	glutathione S-transferase A5	291.6	903.7	3.03
X70369_s_at	collagen, type III, alpha 1	720.3	1642.2	3.03

Table 2. Genes in network induced by indomethacin treatment

Network ID	Genes in Network	Score	Focus Genes	Top Functions
1	APEX1, COL3A1, CRYAB, CYB5, CYCS, FASN, FOS, GADD45A, GAPD, GPX1, GSK3B, GSTA1, GSTM1, GSTM2, GSTP1, HSF1, HSPA1B, HSPB1, HSPB6, IL18, JUN, MAP2K6, MAP3K12, MAPK14, MGST3, MSH2, MYC, ODC1, PAWR, PPIA, Scd2, SOD2, TGFB3, TOP2A, TRAPPC3	73	35	Cell Death, Cancer, Endocrine System Disorders
2	ADCYAP1, APOC2, ARD1, BAG2, CANX, CCND2, CDC42, CYP19A1, FBXW11, FGF6, GEFT, GPR30, GPX3, HSPA8, HSPB8, IGF1R, KSR, LPL, MAP2K1, MAP2K1IP1, MAPK3, MOS, NAT1, NFKBIA, PBP, PCSK5, POMC, PPP1CB, PTPRR, RB1, RPS6KB1, SOD1, STIP1, UCN, Ugt2b	23	16	Cell Cycle, Cellular Growth and Proliferation, Cancer
3	ABCC1, AKR1B1, ANGPTL4, ARG1, CEBPA, CROT, CTH, CYP3A7, CYP51A1, DECR1, DIA1, EGR2, FASN, FTCD, GAL, GH1, GHRH, GHRL, HSD17B4, IFNG, Ins1, LPL, ME1, MGST1, MST1R, PPARA, PTPRN, SOCS2, SRD5A1, STAT5B, THRB, UQCRC1, UQCRC2, UQCRH, VIM	21	15	Organismal Development, Nutritional Disease, Lipid Metabolism
4	ACOX1, AGT, ANGPTL4, COX7A2, COX8A, CYP17A1, CYP27A1, Cyp2c44, CYP2E1, DAD1, EHHADH, ETFDH, FKBP1A, GAL, GPX3, GPX4, HADHA, HADHB, HSD3B1, IDH1, IL4, IL13, IL1R2, LEP, LTC4S, MAOA, PPARG, PTEN, PTGES, PTGS1, RAB10, STK11, TGFB1, Tgtp, XBP1	19	14	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
5	AHR, AIP, APC, BRF1, BUB1, CAMLG, CCS, CSNK1D, CSNK1E, CYP1A1, CYP1A2, CYR61, DHFR, DNASE1, EPHX1, FDXR, G6PD, GSK3B, HSPCB, IFI16, KLF4, MCM7, NFE2, NQO1, PHB, PSMB2, RBL2, RRM2, SHOX, SIM1, TFDP1, TP53, UBE2B, UBTF, UGT1A6	12	10	Cell Signaling, Drug Metabolism, Cell Cycle

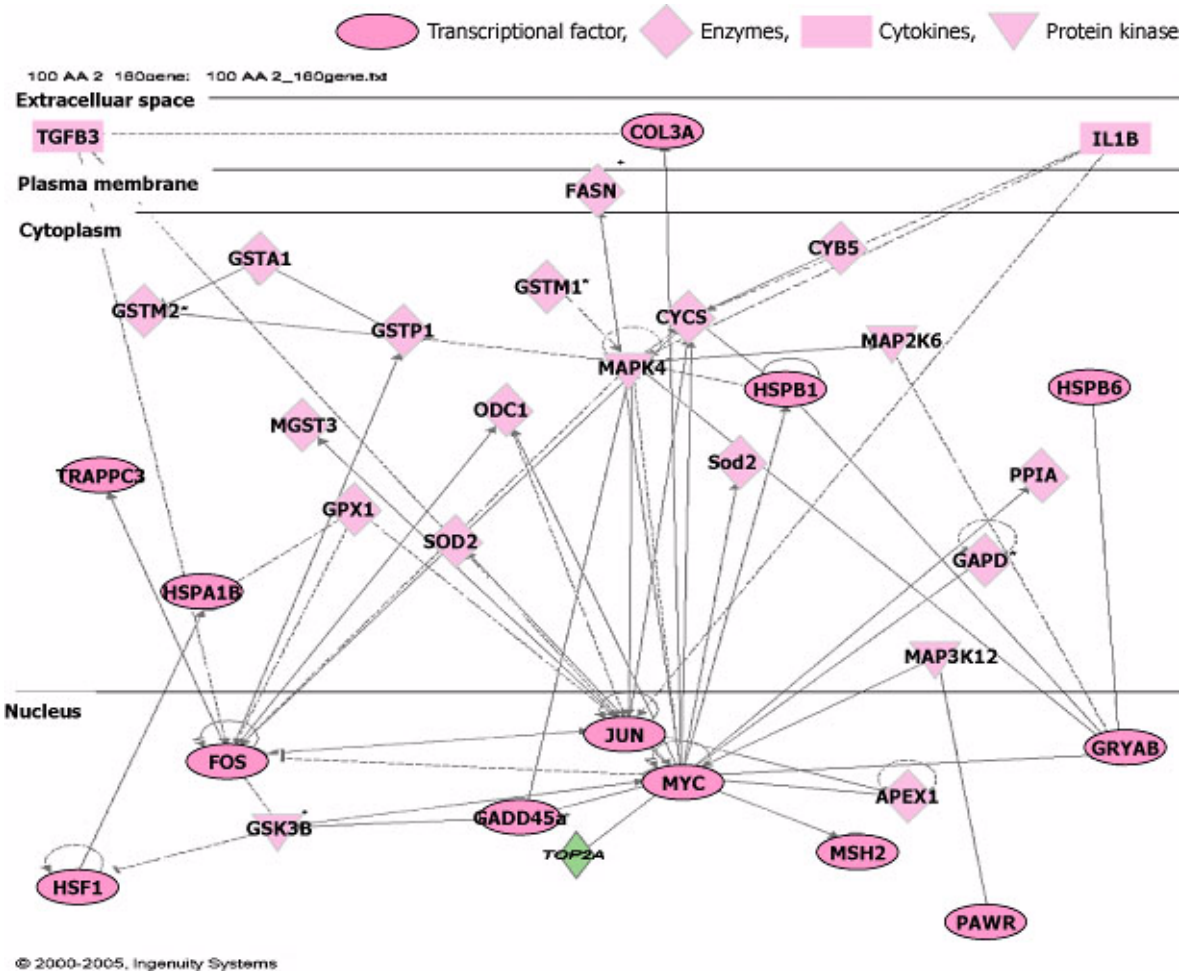

Fig. 1. Networks of genes commonly up-regulated after indomethacin administration.

Table 3. High level functions most significantly associated with indomethacin-induced gene expression profile

Canonical Pathway	Focus Gene	Significance	Genes
Glutathione Metabolism	11/61	1.42 × 10–7	G6PD, GPX1, GPX3, GSTA1, GSTK1, GSTM1, GSTM2, GSTP1, GSTT2, MGST1, MGST3
IL-6 Signaling	10/68	3.97 × 10–6	CYP19A1, FOS, HSPB1, IL1R, JUN, MAP2K1, MAP2K6, MAPK3, MAPK14, NF-KBIA
B Cell Receptor Signaling	10/114	3.78 × 10–4	CDC42, GSK3B, JUN, MAP2K1, MAK2K6, MAP3K12, MAPK3, MAPK14, NFKBIA, RPS6K131
Tryptophan Metabolism	9/98	5.42 × 10–4	CYP19A1, CYP1A1, CYP1A2, CYP3A7, CYP51A1, EHHADH, HADHA, HSD17B4, MADA

Table 4. Genes up-regulated at least 2.0-fold in indomethacin treatment and down-regulated at least 1.5-fold in rebamipide treatment

Probe Set ID	Description	Signal Intensity			Ratio	
		Vehicle	Indomethacin	Indomethacin + Rebamipide	Indomethacin/ vehicle	Indomethacin + Rebamipide /Indomethacin
rc_AI228738_s_at	FK506-binding protein 1a /// FK506 binding protein 2	142.6	922.2	547.4	7.46	0.66
rc_AI172411_at	glutathione peroxidase 3	477.1	2807.2	1892.2	6.06	0.66
rc_AA900413_at	dihydrofolate reductase	95.0	349.9	262.4	3.73	0.57
U03388_s_at	prostaglandin-endoperoxide synthase 1	286.2	572.8	673.0	3.25	0.62
X63594cds_g_at	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	269.1	890.6	526.8	3.25	0.62
U03491_g_at	transforming growth factor, beta 3	175.1	604.8	164.2	3.03	0.38
AFFX-DapX-M_at	—	20.7	70.2	42.2	2.83	0.62
rc_AI178204_at	—	55.6	86.5	78.4	2.46	0.66
D31838_at	wee 1 homolog (S. pombe) (predicted)	91.2	174.8	102.0	2.00	0.66
rc_AI070295_g_at	growth arrest and DNA-damage-inducible 45 alpha	85.4	168.6	109.5	2.00	0.62

Table 5. The levels of mRNA expression for growth arrest and DNA damage-inducible gene (GADD45α)

Probe set ID	Vehicle	Indomethacin	Indomethacin + Rebamipide	Indomethacin/Vehicle		Indomethacin + Rabamipide /Indometahcin	
				Ratio	Change	Ratio	Change
rc_AI070295_at	31.7	61.4	23.5	1.87	NC	0.44	D
rc_AI070295_g_at	85.4	168.6	109.5	2.00	I	0.62	D
L32591mRNA_at	284.3	627.3	556.1	2.64	I	0.93	NC
L32591mRNA_g_at	76.5	112.8	60.1	1.52	NC	0.87	NC

17–19], in which lipid peroxidation was enhanced by indomethacin treatment and indomethacin induced-gastric mucosal injuries were significantly inhibited by the several antioxidants *in vivo*. The induction of these genes may indicate the presence of oxidative stress in the gastric mucosa after the indomethacin administration. Recent investigation clearly demonstrated that oxidative stress was induced by the irreversible inactivation of gastric peroxidase via the direct interaction between indomethacin and gastric peroxidase [*20*]. Up-regulation of GST genes is also supported by van Lieshout *et al.* [*21*], who have demonstrated the induction of GST in the stomach by indomethacin in rats. As GST is a family of detoxifying enzymes, the enhancement of GSTs in the stomach may explain in part the anticarcinogenetic properties of nonsteroidal anti-inflammatory drugs including indomethacin.

In addition to glutathione metabolism, inflammation-associated genes were also up-regulated after the indomethacin treatment, which included fos, jun, MAP kinase, MAP kinase kinase, MAP kinase kinase kinase, NF-κB,

Table 6. Genes up-regulated at least 1.5-fold in rebamipide treatment in rats

Probe Set ID	Description	Signal intensity		Ratio
		vehicle	Rebamipide	Rebamipide /vehicle
D89070cds_s_at	carbonyl reductase 1	347.5	5844.1	4.00
rc_AA859372_s_at	—	195.1	1366.7	4.00
rc_AI171506_g_at	malic enzyme 1	39.9	360.8	4.00
X07467_at	glucose-6-phosphate dehydrogenase	272.0	1660.4	3.48
M58040_at	Transferrin receptor	40.9	208.2	3.25
D00680_at	glutathione peroxidase 3	27.2	216.5	2.83
D16308_at	cyclin D2	203.0	1238.3	2.83
M19533mRNA_i_at	peptidylprolyl isomerase A	81.9	548.2	2.83
U09793_at	Kirsten rat sarcoma viral oncogene homologue 2 (active)	29.2	184.9	2.64
rc_AI172411_at	glutathione peroxidase 3	477.1	2975.9	2.64
U03388_s_at	prostaglandin-endoperoxide synthase 1	286.2	1255.3	2.64
D88190_s_at	serine/threonine kinase 39, STE20/SPS1 homolog (yeast)	439.2	1506.9	2.30
rc_AI171506_at	malic enzyme 1	52.6	312.1	2.30
U27518_at	UDP-glucuronosyltransferase	57.8	242.2	2.30
X70369_s_at	collagen, type III, alpha 1	720.3	3777.7	2.30
rc_AA892234_at	microsomal glutathione S-transferase 3 (predicted)	480.5	1595.6	2.14
U68562mRNA#2_s_at	heat shock protein 1 (chaperonin)	108.6	466.8	2.14
X62952_at	vimentin	163.2	484.5	2.14
X53428cds_s_at	glycogen synthase kinase 3 beta	67.4	209.3	2.00
M24604_g_at	proliferating cell nuclear antigen	192.1	1020.3	2.00
rc_AA963449_s_at	cytochrome P450, subfamily 51	58.7	229.2	2.00
AB010428_s_at	cytosolic acyl-CoA thioesterase 1	118.5	519.1	1.87
AF007107_s_at	cytochrome b-5	90.6	315.6	1.87
AFFX_Rat_GAPDH_5_at	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	202.0	752.8	1.87
AFFX_Rat_GAPDH_M_at	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	493.0	1994.0	1.87
D89375_s_at	sulfotransferase family 1B, member 1	173.4	662.9	1.87
M17701_s_at	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	693.4	2398.9	1.87
rc_AA899854_at	topoisomerase (DNA) 2 alpha	224.1	816.7	1.87
S82820mRNA_s_at	glutathione S-transferase Yc2 subunit	282.4	1092.5	1.87
U95727_at	DnaJ (Hsp40) homolog, subfamily A, member 2	109.3	363.8	1.87
AJ222813_s_at	interleukin 18	82.2	374.6	1.74
D89069_f_at	carbonyl reductase 1	705.1	3095.2	1.74
U46118_at	cytochrome P450, family 3, subfamily a, polypeptide 13	138.3	386.3	1.74
AFFX_Rat_GAPDH_3_at	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	964.7	3448.5	1.62
D17310_s_at	3-alpha-hydroxysteroid dehydrogenase	129.1	484.7	1.62
rc_AA926193_at	sulfotransferase family, cytosolic, 1C, member 2	523.4	1761.8	1.62
rc_AI171243_at	replication protein A3 (predicted)	61.7	195.5	1.62
rc_AI175959_at	v-jun sarcoma virus 17 oncogene homolog (avian)	121.3	345.4	1.62
rc_AI177256_at	moderately similar to NP_795929.1 RIKEN cDNA 8030475D13	512.5	2134.4	1.62
rc_AA900199_s_at	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	269.7	775.7	1.52
rc_AA945054_s_at	cytochrome b-5	271.0	1003.5	1.52
rc_AA945867_at	v-jun sarcoma virus 17 oncogene homolog (avian)	88.3	307.0	1.52
rc_AI013834_s_at	hydroxysteroid (17-beta) dehydrogenase 4	127.8	415.9	1.52
Z78279_g_at	collagen, type 1, alpha 1	593.3	2153.6	1.52

and STAT. Ingenuity Pathway analysis showed that IL-6 signaling was associated with these genes with a significance of $p = 3.97 \times 10^{-6}$. Although it has been reported that IL-6 play a significant role in pathogenesis of gastric inflammation induced by *Helicobacter pylori* [*22*, *23*], there were no reports investigating the role of IL-6 in indomethacin-induced gastric mucosal injury. Further studies will be necessary to clarify the role of this signaling in the pathogenesis of indomethacin-induced gastric injury.

Effects of Rebamipide on Gene Expression Affected by Indomethacin in Rats

We succeeded in listing 10 genes including 2 EST based on the following criteria: genes that are up-regulated at least 2.0-fold after 2-h treatment with indomethacin in comparison with the vehicle-treated rats, and also genes that are down-regulated at least 1.5-fold after pretreatment with rebamipide in comparison with pretreatment with vehicle prior to 2-h exposure to indomethacin (Table 4). These genes were involved in regulators of NF-κB cascade (FK506-binding protein 1a, and IkBα), oxidative stress response (glutathione peroxidase 3, dihydrofolate reductase), and cell cycle regulators {transforming growth factor, wee 1, and growth arrest and DNA-damage-inducible 45α (GADD45α)}.

The down-regulation of NF-κB cascade by rebamipide is in line with the previous report, in which anti-inflammatory effect of rebamipide is derived from the inhibition of NF-κB cascade [*24*, *25*]. The inhibition of the oxidative stress-related genes by rebamipide is also consistent with the data that rebamipide is powerful scavenger of oxygen-derived free radicals [*26*, *27*]. These data suggest that cytoprotection by rebamipide against indomethacin-induced gastric injury may be related to its anti-inflammatory and anti-free radical properties.

Finally, in the Rat Toxicology U34 array, 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 1.5-fold after indomethacin exposure and down-regulated by the pretreatment with rebamipide (Table 5). The present data was in line with our previous data obtained from the *in vitro* study, showing that the expression of GADD45α was enhanced by indomethacin exposure and that this enhancement was markedly inhibited by the treatment with rebamipide [*10*]. These changes were also confirmed by real-time PCR using a gastric mucosal cell line [*10*]. These data obtained from *in vivo* and *in vitro* studies strongly suggest that GADD45α play a crucial role in indomethacin-induced cell death, and that cytoprotective action of rebamipide may, in part, be mediated by this molecule.

Effects of Rebamipide Treatment on Gene Expression in Normal Rats

Among the 1031 probes, the number of genes, the expression levels of which increased more than 1.5-fold in rebamipide-treated mucosa, was 44 including 1 EST. Many investigations have demonstrated several factors, including prostaglandins, growth factors, antioxidants, and heat shock proteins, to exert cytoprotection against gastric injuries. Among these cytoprotective factors, the expression of prostaglandin-endoperoxide synthase 1 (cyclooxygenase 1 (*cox-1*)) was up-regulated by the treatment with rebamipide *in vivo* as shown in Table 6. By an *in vitro* study using gastric epithelial cells, rebamipide treatment increased the expression of *cox-1* by 1.32-fold compared to vehicle treatment (data not shown). These results suggest that cytoprotective effects of rebamipide may be derived, in part, from Cox-1 or its generated prostaglandins. Tarnawski *et al.* [*28*] previously reported that rebamipide significantly upregulated the proangiogenic genes encoding vascular endothelial growth factor (VEGF), heparin binding epidermal growth-like factor (HB-EGF), fibroblast growth factor receptor-2 (FGFR2), and cyclooxygenase-2 (Cox2), as well as growth promoting genes, including insulin growth factor-1 (IGF-1). However, the enhanced expression of these genes was not re-confirmed in the present study. These difference may be derived the difference between experimental conditions of *in vivo* (the present study) and *in vitro* shown by Tarnawski *et al.* [*28*].

In conclusion, the present study using GeneChip analysis demonstrated the enhanced expression of apoptosis- and inflammation-related genes in the gastric epithelial cells exposed to indomethacin *in vivo*, and that inhibition of apoptosis-related genes, especially GADD45α by rebamipide is possibly important for its cytoprotective effect against this injury.

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