The Constitutively Active Orphan G-protein-coupled Receptor GPR39 Protects from Cell Death by Increasing Secretion of Pigment Epithelium-derived Growth Factor*

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GPR39 is a constitutively active orphan G-protein-coupled receptor capable of increasing serum response element-mediated transcription. We found GPR39 to be up-regulated in a hippocampal cell line resistant against diverse stimulators of cell death and show that its overexpression protects against oxidative and endoplasmic reticulum stress, as well as against direct activation of the caspase cascade by Bax overexpression. In contrast, silencing GPR39 rendered cells more susceptible to cell death. An array analysis of transcripts induced by GPR39 revealed up-regulation of RGS16 (inhibitor of G-protein signalling 16), which suggested coupling to Go13 and induction of serum response element-mediated transcription by the small GTPase RhoA. In line with this, co-expression of GPR39 with RGS16, dominant-negative RhoA, or serum response factor abolished cell protection, whereas overexpression of the serum response factor protected from cell death. Further downstream the signaling cascade, GPR39 overexpression leads to increased secretion of the cytoprotective pigment epithelium-derived growth factor (PEDF). Medium conditioned by cells overexpressing GPR39 contained 4-fold more PEDF, and when stripped off it lost most but not all of its protective properties. We conclude that GPR39 is a novel inhibitor of cell death, which might represent a therapeutic target with implications for processes involving apoptosis and endoplasmic reticulum stress like cancer, ischemia/reperfusion injury, and neurodegenerative disease.

G-protein-coupled receptors (GPCRs) 2 constitute the largest family of cell surface transmembrane proteins (1); they are activated by a wide variety of natural ligands, and pharmacological alteration of their signaling constitutes one of the most successful approaches to the treatment of human disease, which makes GPCRs the most targeted protein superfamily in pharmaceutical research (2). We recently presented a screening system able to discriminate protective and detrimental receptors involved in oxidative stress (3) to identify targets for the multitude of human diseases caused or aggravated by oxidative stress. In this proof-of-concept study, we noticed that the relative mRNA content of cytoprotective GPCRs (the most prominent being VPAC2, a receptor for the neuroprotective vasoactive intestinal peptide VIP) was increased in glutamate-resistant (HT22R) cells generated by repeated exposure of the parental cell line HT22 to high concentrations of glutamate and further propagation of the few surviving cells. HT22 cells are derived from embryonal mouse hippocampal cells and are considered to be a model system of cell death by oxidative stress. In this model system, increased extracellular glutamate blocks the gradient-driven glutamate/cysteine antiporter Xc−, depleting the cells of cysteine. Cysteine is required for the synthesis of the important antioxidant glutathione, and the sequence of events after depletion of intracellular glutathione involves the activation of 12-lipoxygenase, the accumulation of intracellular peroxides, activation of a cyclic GMP-dependent calcium channel, and eventually a caspase-independent cell death with features of necrosis and apoptosis (reviewed in Ref. 4). Cell death mediated by the proapoptotic protein Bax, in contrast, leads to cytochrome c release from the mitochondria and formation of a complex between Apaf-1 and pro-caspase-9, leading to caspase-9 activation. Caspase 9 in turn then activates the executioner caspases 3 and 7, which results in classical apoptosis. A third pathway leading to cell death is death by endoplasmic reticulum (ER) stress, where the accumulation of excess unfolded proteins in the ER activates, although not exclusively, the ER stress-specific caspase 12, which then activates caspase-9 independently of cytochrome c (reviewed in Ref. 5).

In this contribution, we investigated the expression of 82 orphan GPCR in HT22R and wild type cells, which identified GPR39 as a candidate regulator of cell death. GPR39 indeed protected against glutamate toxicity, ER stress, and Bax-mediated cell death by coupling to Go13, leading to activation of the
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RhoA pathway and secretion of the cytoprotective pigment epithelium-derived growth factor (PEDF) and other factors.

EXPERIMENTAL PROCEDURES

Cells, Proteins, siRNA, and Plasmids—HT22 and HT22R cells were cultured and generated as described (6). Immortalized mouse embryonic fibroblasts (a kind gift from Christoph Borner, University of Freiburg, Freiburg, Germany) and human HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium (PAA laboratories) supplemented with 10% fetal calf serum and 100 IU/ml penicillin, 100 μg/ml streptomycin. For cell death analysis by flow cytometry, the cells were plated in 24-well plates and transfected with 0.4 μg of Bax-EGFP and the indicated constructs. 24 h later, the cells were resuspended in 100 μl of medium and analyzed by flow cytometry.

For toxicity experiments, 5000 HT22 or 8000 HEK-293T cells were seeded in 100 μl of medium into 96-well plates in the presence of the indicated amounts of PEDF (BioProducts MD) or ROCK1 inhibitor Y-27632 (Sigma). Transfections were carried out 24 h before seeding. Tunicamycin, glutamate, or vehicle was added 24 h after seeding in 50 μl of a 1:1 dilution in complete medium to 50 μl of medium. Cell viability was measured 24 h later by the amount of blue formazan produced by viable cells from the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma), which is proportional to the number of viable cells as previously described (3, 6).

For cell death analysis by flow cytometry, the cells were plated in 24-well plates and transfected with 0.4 μg of Bax-EGFP and the indicated constructs. 24 h later, the cells were resuspended in 100 μl of annexin V binding buffer (BD-Pharmingen) and stained with 5 μl of annexin V-PE (BD-Pharmingen) and 5 μl of 7-AAD. Single EGFP-positive cells were gated at 488 nm and analyzed for annexin V and 7-AAD staining. The data were acquired on a FACSCalibur flow cytometer and quantified using CellStar™ software (Becton Dickinson).

To remove PEDF from GPR39-conditioned medium, 3000 wild type HT22 cells/well were seeded into a 96-well plate, and the medium was replaced 24 h later by serum-free medium conditioned by HT22 wt cells or cells overexpressing GPR39 containing 2 μg/ml α-PEDF (rabbit polyclonal antiserum; Bio-Products MD) or control antibody (rabbit polyclonal antichicken antiserum; Davids Biotechnology). Tunicamycin in the indicated amounts was added 24 h later, and cell death was measured as described above.

Microarray Analysis—Total RNA from HEK-293T cell clones 1 and 17 was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and quality was assessed by a 2100 Bioanalyzer (Agilent Technologies). Synthesis of double-stranded cDNA and in vitro transcription were carried out according to Affymetrix protocols. In brief, 3 μg of total RNA from cultured cells were reverse transcribed with SuperScriptII and T7-oligo(dt) promoter primer for 1 h at 42°C, followed by 2 h of incubation at 16°C for second strand cDNA synthesis. After sample clean-up, the cDNA served as a template for subsequent in vitro transcription carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary (cRNA) amplification and biotin labeling for 16 h at 37°C. The quality of cRNA was assessed in a 2100 Bioanalyzer. Biotinylated cRNA was cleaned up, and 15 μg were fragmented by metal/heat-
induced fragmentation. The fragmented cRNA was hybridized against GeneChip Human genome U133 Plus 2.0 in an Affymetrix GeneChip hybridization oven for 16 h at 45 °C and 60 rpm. Each microarray was washed and stained with streptavidin–phycoerythrin using Affymetrix GeneChip Fluidics Station 450 and scanned with Affymetrix Scanner 3000. Raw data were analyzed with Affymetrix MAS5 algorithm, and probe sets with a detection p value of p < 0.01 were used for further analysis.

**PEDF Quantification**—PEDF mRNA was quantitated in HEK293 cells by real time PCR 48 h after transfection using the SYBRgreen core kit (Eurogentech) and QuantiTect primer assays (QT00100730, Qiagen) according to the manufacturer’s protocol using a 7500 real time PCR system (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as endogenous control, and the results are given as cycles over endogenous control. PEDF concentrations were determined by using a sandwich enzyme-linked immunosorbent assay for PEDF (microwells coated with specific polyclonal antibody for full-length recombinant human PEDF antigen and detection with high titer polyclonal antibody for biotin (BioProducts MD). Briefly, the assays were performed incubating the samples (100 µl each, diluted 1:50) for 1 h at 37 °C into duplicate wells. The samples were aspirated, and the wells were washed. Afterward 100 µl of reconstituted PEDF detector antibody was added to each well and incubated 1 h at 37 °C. After washing five times, 100 µl of streptavidin–horseradish peroxidase working solution was added to each well and incubated for 30 min at 37 °C. After another five washing cycles, 100 µl of tetramethylbenzidine substrate was added to each well and incubated for 20 min at room temperature. The concentration of PEDF in each specimen was calculated by interpolation from the standard curve and given as ng/ml.

**Immunocytochemistry**—For immunocytochemistry, HEK293T cells or mouse embryonic fibroblasts were transfected in 6-well plates as described above and seeded into four-well chamber slides (Labtek) 24 h later. Again 24 h later, the cells were fixed for 20 min in 4% paraformaldehyde, preincubated in phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 20 for 30 min. Rhodamine-phalloidin was incubated for 1 h and 4′,6′-diamino-2-phenylindole for 15 min at room temperature. The images were captured with an Olympus IX-81 inverse fluorescence microscope and deconvoluted by CellR software (Olympus).

**SRE Reporter Assay**—HEK 293T cells were transiently transfected in a 48-well plate with the serum response element luciferase reporter plasmid (pSRE-Luc, Stratagene) and the indicated expression constructs. 48 h after transfection cells were washed and lysed in 200 µl cell lysis buffer (Promega), lysate was centrifuged at 12,000 × g for 1 min, and 20 µl of supernatant were transferred to a white 96-well microtiter plate. 100 µl of luciferase assay buffer (Promega) was injected to each well directly before measurement. Luminescence was measured by a Genios Pro microplate reader (Tecan) and integrated for 10,000 ms.

**Statistical Analysis**—The data were summarized as the means ± S.D., and the statistical significance was assessed using two-tailed t tests or analysis of variance with Tukey’s multiple comparison test as indicated.

**RESULTS**

**HT22 Cells Are Resistant against Cell Death Caused by Diverse Cytotoxic Pathways**—HT22R cells have been described before; they are resistant against oxidative stress elicited by glutamate and hydrogen peroxide by up-regulation of the glutamate/cysteine exchanger xCT (6) and the cytoprotective G-protein–coupled receptor VPAC2 (3). We now extended the description of their protective phenotype to cell death elicited by ER stress and overexpression of Bax. Treating HT22 wild-type cells with tunicamycin, a compound that inhibits protein glycosylation in the ER thus generating an unfolded protein response, led to a prominent induction of cell death even more pronounced than the cell death caused by glutamate toxicity. HT22R cells, in contrast, were protected (~18.7% difference at 5 µg/ml; Fig. 1A). Transfection of a Bax–EGFP fusion construct and quantification of early and late apoptotic cells by measuring...
phosphatidylserine translocation with annexin V and 7-AAD staining of dead cells in EGFP-positive transfected cells also demonstrated the protective phenotype of HT22R cells (~15.8% difference after 24 h; Fig. 1B). In summary, we conclude that HT22R cells are not only protected against oxidative glutamate toxicity but also against cell death caused by ER stress and Bax overexpression.

**HT22R Cells Differ in the Expression of Orphan GPCRs**—To identify novel drug targets implicated in protection against oxidative stress and cell death in general, we investigated the expression of 82 orphan GPCRs in wild type and HT22R cells by quantitative PCR. VPAC_{2} served as positive control. Half of all investigated transcripts did not yield reliable amplification curves, probably because of lack of expression. Another 36 transcripts were up-regulated less than 4-fold, and only five, including the positive control, were up-regulated more than 4-fold in HT22R cells (Fig. 1C). The expression of GPR37 and GPR101 differed significantly in the three different mRNA preparations used in the analysis and were not analyzed further. GPR15 and GPR39, however, were reproducibly regulated more than 4-fold, with GPR39 being more abundant. Both receptors were initially identified for their homology to known receptors (7, 8) with GPR39 being closely related to the neurotensin receptors and GPR15 to the angiotensin and bradykinin receptors (9). Analysis of membrane localization of GPR39 using flow cytometry and a specific antibody directed against the third extracellular loop revealed an ~2-fold increase corroborating the observed regulation at the mRNA level (Fig. 1D). Western blotting resulted in no visible bands, probably because of the still low expression level and the inherent difficulties of detecting GPCRs in Western blots.

**GPR39, but Not GPR15, Protects against Diverse Stimuli of Cell Death**—Both candidate receptors GPR15 and GPR39 were cloned from HT22R cDNA and overexpressed in wild type HT22 cells. GPR39, but not GPR15, protected against glutamate toxicity (~12% increase in viability at 40 mM glutamate), against direct oxidative stress caused by hydrogen peroxide (~19.5% at 1.25 mM H_{2}O_{2}), and tunicamycin (~19.6% at 5 mg/ml) (Fig. 2A). GPR39 also protected against direct activation of the caspase cascade by overexpression of Bax (~31.4%) in favor of a more general protective mechanism (Fig. 2B). In these experiments, only EGFP-positive transfected cells were counted, whereas in the experiments investigating cell death caused with treatment with glutamate, H_{2}O_{2}, or tunicamycin viability was quantitated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assays in a mixed population with an app. transfection efficiency of 50%, thus explaining the apparently more prominent protection against Bax-induced cell death. The reverse experiment, transfecting HT22R cells with a pool of four different siRNAs in contrast increased susceptibility to glutamate toxicity (~19.8% decrease in viability at 40 mM glutamate; Fig. 2C). Knockdown efficiency was measured by quantitative PCR and constituted 1.79 \Delta C_{T} values over GAPDH (\Delta \Delta C_{T}), corresponding to a ~3.5-fold reduction in GPR39 mRNA abundance. To exclude the possibility that the effect of the pooled siRNA corresponded to off target effects, we repeated this experiment with vector-expressed microRNA, which yielded very similar results. Overexpression of GPR39 microRNA driven by RNA polymerase II promoter increased cell death of HT22R cells caused by 40 mM glutamate by ~16.5% (Fig. 2C). In this approach, GPR39 mRNA was reduced ~3-fold. The differences in glutamate susceptibility between the siRNA and the vector-driven microRNA experiments are most likely caused by the different transfection agents used. These data strongly suggest that GPR39 has a protective phenotype, because overexpression and silencing (using two different approaches) resulted in opposite effects on cell viability. The protective phenotype was not due to mutations in the GPR39 coding region because the cDNA cloned from HT22R cells corresponded to the sequence deposited with NCBI.

**GPR39 Overexpression Induces Its Inhibitor RGS16**—We next investigated the effect of stable overexpression of GPR39 tagged with an N-terminal hemagglutinin on cell death in human HEK293 cells. We determined the surface expression of 36 monoclonal colonies by flow cytometry using a fluorescein isothiocyanate-labeled monoclonal hemagglutinin antibody and chose six clones exhibiting low, middle, and top surface expression for glutamate toxicity studies, which yielded a pos-
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PRSS23, proteins of the extracellular matrix like the integrin-binding protein (EDIL3) or collagen II alpha 1 (COL2A1), the major histocompatibility complex-binding nuclear protein MAGEB2, and zinc finger protein TRIM38. Furthermore, proteins involved in transcription and signal transduction like PEDF, the putative high mobility group transcription factor TNRC9, and the transcriptional activator Cited1 were identified (Fig. 3B). To verify the array results, we confirmed the up-regulation of PEDF by quantitative PCR, which yielded similar results (Fig. 3C). Of utmost interest was, however, the increased expression of the regulator of G-protein signaling RGS16, because some RGS proteins attenuate signals that induce their expression in a feedback desensitization loop, as shown for RGS16 in lysophatidic acid signaling (10) or for RGS1 in the stimulation of B-lymphocytes by platelet-activating factor (11). The fold change of RGS16 almost matched the fold change of GPR39, which is what is expected.

GPR39 Protects in Gα13/RhoA/SRE-dependent Manner and Is Inhibited by RGS16—Indeed, co-expression of GPR39 with RGS16 completely abolished the cytoprotective effect of GPR39 overexpression (viability at 10 μg/ml Tn: vector < 8.2% versus GPR39 ~ 29.7%, p < 0.05, versus GPR39 + RGS16 ~ 5.3% (not significant) as determined by analysis of variance with Tukey’s Multiple Comparison Test; Fig. 4A). RGS16 directly binds to Gα13 and translocates the complex to detergent-resistant membranes (10). This reduces p115Rho-GEF binding and leads to a reduced capacity of p115Rho-GEF to catalyze the nucleotide exchange on the small GTPase RhoA. In line with this, dominant-negative RhoA T19N (vector ~ 14.65% versus GPR39 ~ 26.2%, p < 0.05, versus GPR39 + RhoA T19N ~ 19.1% versus vector (not significant) analysis of variance; Fig. 4B) as well completely abolished the cytoprotective effect similar to pharmacological inhibition of the Rho-associated coiled-coil forming protein serine/threonine kinase p160ROCK, which lies downstream of RhoA, with the specific inhibitor Y-27632 (12) (vector ~ 2.3% versus GPR39 ~ 15.8%, p < 0.05, versus GPR39 + Y-27632 ~ 4.9% (not significant) analysis of variance; Fig. 4C).

Also, overexpression of GPR39 in serum-starved mouse embryonal fibroblasts resulted in prominent actin stress fiber polymerization, which was inhibited by co-expression of RGS16, further proving the activation of the Gα13/RhoA axis by GPR39 and its inhibition by RGS16 (Fig. 4D). RhoA regulates SRE-mediated gene expression; accordingly, GPR39 increased SRE but not cAMP response element induction 4-fold using a luciferase reporter read-out. We even observed a decrease in cAMP response element-mediated transcription, which was enhanced by RGS16 (Fig. 4E, upper panel). We used exactly the same conditions used for cell death assays, meaning in the presence of serum, which might explain the more pronounced induction of SRE described by Holst et al. (13). The induction of SRE-Luc was completely abolished by co-expression of RGS16 or a dominant-negative RhoA (T19N) (Fig. 4E, lower panel), suggesting that the protective effect of GPR39 overexpression is mediated by SRE induction. This view is strengthened by the fact that overexpression of a constitutively active serum response factor, but not of a construct lacking the DNA-binding domain (SRFΔM-VP16), similarly protected against ER...
stress (SRF, ~40.9%; vector, ~7.6%; SRFΔM-VP16, ~20.6%; viability at 10 mg/ml Tn; Fig. 4F), as shown previously for apoptosis elicited with the natural toxin jasplakinolide (14). Moreover, co-expression of a dominant-negative mutant of SRF (15) completely abolished the protective effect of GPR39 and was even detrimental on its own (Fig. 4F, right panel).

We infer that the protective action of GPR39 is mediated by a Ga13/RhoA/p160ROCK/SRF-mediated increase in SRE-dependent transcription.

PEDF Mediates Part of the Cytotoxic Action of GPR39—PEDF is a soluble cytoprotective factor (reviewed in Ref. 16) found increased in clone 17, which could mediate the protective action of GPR39. PEDF was also up-regulated about 4-fold after transient overexpression of GPR39, which was abolished and even reduced by co-expression of dominant-negative SRF. Overexpression of SRF-VP16 but not SRF lacking the DNA-binding domain (ΔM) resulted in a similar up-regulation (Fig. 5A). At the protein level, cells overexpressing GPR39 secreted 4.5-fold more PEDF than control cells (Fig. 5B). PEDF dose-dependently protected against ER stress although not as potently as GPR39 overexpression (Fig. 5C). In these experiments, we used only 2% serum to avoid degradation of PEDF and limit the effect of PEDF contained in the serum, which makes a side-by-side comparison of these experiments difficult.

The reverse experiment of removing PEDF decreased the protective effect of medium conditioned by GPR39-overexpressing cells, suggesting a prominent role for PEDF in GPR39-mediated...
cell protection. On the other hand, even conditioned medium stripped of PEDF retained a clear-cut protective effect, suggesting additional factors (Fig. 5D). In summary, we believe that PEDF is at least one and may be the major downstream effector of GPR39-induced cell protection.

**DISCUSSION**

We conclude that GPR39 protects against oxidative, ER, and mitochondrial stress by inducing PEDF and probably other protective transcripts in a Gα13/RhoA/SRE-dependent manner (summarized in Fig. 6). This pathway is similar to that induced by other GPCRs like those activated by lysophosphatidic acid or the protease-activated receptors that bind thrombin. For both ligands conflicting results regarding their role in cell death have been described. Lysophosphatidic acid activates a large variety of GPCRs that can couple to distinct G-proteins, which renders the elucidation of a particular pathway difficult (reviewed in Ref. 17). In the case of thrombin, dose seems to play the major role. In low concentrations thrombin protects astrocytes and other cell types against cell death, whereas high concentrations are detrimental (reviewed in Ref. 18). Both of these responses are, however, dependent on RhoA. In apoptotic concentrations (1 μM thrombin), RhoA activity rapidly increases within minutes, whereas low concentrations (10 nM) lead to a small but consistent increase over hours (19). In the case of GPR39, the ligand is not known and can therefore not be added in higher concentrations to examine a similar dichotomy. We think that the almost linear correlation of GPR39 expression, and its ability to protect against cell death observed by us suggests that GPR39 is constitutively active and leads to low level and thus protective activation of the RhoA pathway. The constitutive activation of GPR39 was already suggested by Holst et al. (13), who also observed that GPR39 dose-dependently activates SRE-dependent transcription. It can, however, not be excluded that the unknown ligand of GPR39 is present either in the serum or produced by the cells in an auto- or paracrine manner. Alternatively, GPR39 might be reversely regulated, e.g. by a natural inverse agonist.

Zhang et al. (20) reported that GPR39 binds obestatin, a peptide derived by posttranslational cleavage from the grehlin prepropeptide, and stimulates in Chinese hamster ovary and HEK293 cells cAMP production and only to a lower degree SRE activation. Obestatin is supposed to suppress food intake, inhibit jejunal contraction, and decrease body weight gain (20). The activation of GPR39 by obestatin has been challenged recently, and it is now generally accepted that obestatin is not the ligand of GPR39. Holst et al. (21) found no reproducible effect of obestatin in GPR39-expressing cells, whereas zinc ions stimulated inositol phosphate turnover, cAMP production, arrestin mobilization, as well as cAMP response element dependent, and SRE-dependent transcriptional activity in GPR39 expressing cells. Also, no specific binding of obestatin could be detected in two different types of GPR39-expressing cells using three different radio-iodinated forms of obestatin. In our hands, obestatin also had no effect on the viability of wild type HEK-293T cells or GPR39-overexpressing cells, whereas zinc killed dose-dependently (data not shown) in the time frame of our experiments as generally accepted (reviewed in Ref. 22). When treated for hours as in our experiments, the detrimental properties of zinc probably override its modulatory effect on GPR39 signaling, which was previously studied in experiments of much shorter duration.

GPR39 consists of two exons separated by a large intron, which contains, according to a recent report, an alternative C terminus resulting in a truncated 5TM receptor (23). The second exon also corresponds to the 3′-untranslated region of LYPD1, a putative signaling protein probably expressed both as a membrane-associated and as a secreted protein. It was suggested by the authors that the GPR39 mRNA previously reported to be expressed in nervous tissue mainly corresponds to LYPD1, whereas GPR39 is predominantly expressed in the gastrointestinal system. Our screening primers were indeed designed on the part of GPR39 shared with LYPD1, because this fact was not known at the time we began our study. Control exon-spanning primers resulted in a slightly decreased expression corresponding to the lower expression found by flow cytometry. However, all of the subsequent experiments were done with full-length 7TM GPR39, and the silencing constructs are based solely on the first exon unique for GPR39, thereby excluding any effect of the splice isoform or LYPD1. Egerod et al. (23) suggested that the truncated form of GPR39 might modulate the function of the full-length protein similar to the 5TM form of the closely related ghrelin receptor, which seems to be capable to suppress the function of the full-length recep-
tor (24). A similar modulation of the effect of GPR39 on cell death remains to be investigated.

In summary, we conclude that constitutive activation of the SRE pathway and not activation by obestatin mediates the protective action of GPR39 overexpression against endogenous and exogenous oxidative stress, ER stress, and activation of the caspase cascade by Bax overexpression. GPR39 is a novel inhibitor of cell death, which might represent a therapeutic target with implications for processes involving apoptosis and ER stress like cancer, ischemia/reperfusion injury, and neurodegenerative disease.

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