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# Protective effect of quercetin on ER stress caused by calcium dynamics dysregulation in intestinal epithelial cells

Yayoi Natsume*, Shinya Ito, Hideo Satsu, Makoto Shimizu

*Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo, Japan*

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ABSTRACT

Quercetin, one of the flavonoids present in plants, expresses several physiological functions including antioxidative and anti-inflammatory properties. However, its effect on intestinal epithelia remains to be elucidated. Endoplasmic reticulum (ER) stress has been attracting considerable attention since ER stress triggers such disorders as inflammation and cancer. The effect of quercetin on ER stress was investigated in this present study. Several ER stress inducers (tunicamycin, A23187, thapsigargin and brefeldin A) were added to human colonic LS180 cells or Caco-2 cells with quercetin, and the GRP78 expression as an ER stress marker was determined. The results showed that quercetin suppressed the induction of GRP78 expression by these ER stressors, excepting brefeldin A, at both the mRNA and protein levels. Additionally, XBP-1 mRNA splicing was determined to evaluate the activation of IRE1. The phosphorylation of eIF2α and shutdown of protein synthesis were determined to evaluate the activation of PERK. Although quercetin activated IRE1 and PERK when added to LS180 cells alone, it suppressed the activation of IRE1 and PERK induced by A23187 or thapsigargin. The suppressive effect of quercetin on GRP78 mRNA induction was reproduced by PI3K inihibitors (LY294002 and wortmannin), but not by vitamin C and E. LY294002 failed to suppress the GRP78 mRNA induction in combination with quercetin. In conclusion, this study indicates for the first time that quercetin suppressed the ER stress caused by calcium dynamics dysregulation by the inhibition of PI3K. This study helps to clarify the mechanism for quercetin presenting its versatility.



## 1. Introduction

Quercetin (3,3′,4′,5,7-pentahydroxylflavone) is one of the well-studied flavonoids that are ubiquitous in plants. Quercetin has been reported to show such physiological functions as anti-mutagenicity, PKC inhibition, lipoxigenase inhibition, histamine release inhibition, SOD-like activity, cell cycle regulation, anti-angiogenic activity and ACE II inhibition (Murota and Terao, 2003). However, the effect of quercetin on the small intestinal functions remains unclear, although there are many reports about the physiological properties and absorption of quercetin (Walgren et al., 2000; Sesink et al., 2005).

The endoplasmic reticulum (ER) is an organelle present in all eukaryotic cells. ER plays a leading role in lipid and protein synthesis. Membrane-spanning proteins or hydrosoluble proteins are folded by a chaperone in ER, in parallel with protein synthesis at ER-bounded ribosomes. However, this folding process is disturbed by heat shock, inhibition of sugar chain modification, dysregulation of calcium dynamics, metabolic disorder, dysregulation of cellular redox control, viral infection and so on. Under these conditions, proteins are misfolded or unfolded (ER stress) which leads to the aggregation of unusual proteins and apoptosis (Xu et al., 2005). Cells take action to recover from ER stress, such as by up-regulating the chaperone to promote protein refolding, enhancing protein degradation by the ubiquitin–proteasome system, and the temporal shutdown of protein synthesis to prevent an accumulation of misfolded or unfolded proteins.

The initial response just described is called the unfolded protein response (UPR) (Brodsky, 2007). Three sensor proteins exist on the ER membrane that sense ER stress and invoke UPR (Ron and Walter, 2007): activating transcription factor-6 (ATF6), inositol-requiring protein-1 (IRE1) and protein kinase RNA (PKR)-like ER kinase (PERK).

*Abbreviations:* ATF, activating transcription factor; CHOP, C/EBP homology protein; DME, drug-metabolizing enzyme; ER, endoplasmic reticulum; eIF, eukaryotic translation initiation factor; GRP, glucose-regulating protein; GSK-3β, glycogen synthase kinase-3β; IP3, inositol 1,4,5-trisphosphate; IRE, inositol-requiring protein; JNK, c-Jun N-terminal kinase; MRP, multidrug resistance-associated protein; PERK, protein kinase RNA (PKR)-like ER kinase; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; ROS, reactive oxygen species; SERCA, sarco(endo)plasmic reticulum $Ca^{2+}$ ATPase; SOCE, store-operated calcium entry; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase; UPR, unfolded protein response; XBP, X-box binding protein.

* Corresponding author. Tel.: +81 774 38 3038; fax: +81 774 38 3037.
*E-mail address:* xiamu4@hotmail.co.jp (Y. Natsume).

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ATF6 is a type II transmembrane protein that exists in an inactivated form. The intra-ER domain is lined with molecular chaperone GRP78. Under conditions of increased ER stress, ATF6 is dissociated from GRP78, is translocated to the Golgi apparatus, and is processed by site 1 protease (S1P) or site 2 protease (S2P). Processed ATF6 is translocated to the nucleus and up-regulates such UPR target genes as GRP78 via the ER stress response element (ERSE; CCAAT-N9-CCACG) (Yamamoto et al., 2004).

IRE1 is a type I transmembrane protein that bears kinase and endonuclease activities. Under conditions of increased ER stress, endonuclease activity is activated by oligomer formation and self-phosphorylation. After X-box binding protein-1 (XBP-1) mRNA splicing, mature XBP-1 mRNA is translated into the transcription factor that regulates the expression of such chaperones as GRP78 to protect the cell from stress. XBP-1 is the only substrate of IRE1 that has been reported, and up-regulates such target genes as ubiquitin-conjugating enzymes E2E1, EDEM, XBP-1, and GRP78.

PERK has a similar structure to that of IRE1. Upon ER stress, endonuclease activity is activated by oligomer formation and self-phosphorylation. This results in inactivation of the eukaryotic translation initiation factor-2α-subunit (eIF2α) via phosphorylation of Ser51. This regulation results in the shutdown of protein synthesis which prevents cells from exposure to the toxicity of accumulated unfolded or misfolded protein caused by ER stress. C/EBP homologous protein (CHOP) is one of the proteins highly inducible during ER stress. The PERK/eIF2α signaling pathway plays an essential role in the induction of CHOP in ER stress, and is dominant over that of the ATF6 and IRE1/XBP-1 signaling pathways (Oyadomari and Mori, 2004).

The aggregated proteins generated by ER stress show high toxicity which ameliorates the ubiquitin–proteasome pathway and promotes the dissociation of transcription factors such as CREB-binding protein and TATA-binding protein. Accumulation of unfolded proteins in the ER leads to apoptosis *via* induction of CHOP, activation of JNK and caspase-12 (Oyadomari and Mori (2004)). As a result, ER stress is responsible for causing apoptosis or "conformation disease" (Yoshida, 2007). The diseases related to ER stress include neurodegenerative disorders such as Alzheimer disease or Huntington disease, diabetes, arteriosclerosis, inflammation, and liver or kidney damage. ER stress might play a crucial role in inflammation of the intestines. ER stress activates NF-κB to induce inflammatory cytokines (Robertson et al., 2006). In addition, IRE1β (−/−) mice had higher sensitivity toward dextran sodium sulfate (DSS) that causes inflammatory bowel disease (Bertolotti et al., 2001). GRP78 was induced at the protein level in the intestinal epithelium of anti-inflammatory cytokine IL-10 (−/−) mice, and in patients of Crohn's disease or ulcerative colitis. Activation of the p38 pathway by IL-10 has suppressed GRP78 induction by ATF6 activation *via* TNF-α (Shkoda et al., 2007).

In our previous study, the administration of quercetin (5 mg/kg bw) for 2 weeks expressed the effect related to ER (xenobiotics and lipid metabolism, calcium dynamics and ribosomal protein) on mice epithelia (Natsume et al., in press). This result led to the hypothesis that multiple physiological properties of quercetin might be expressed *via* the suppression of ER stress. The effect of quercetin on the induction of ER stress markers and the activation of ER stress sensors was investigated in this present study.

## 2. Materials and methods

### 2.1. Reagents and cell culture

All chemicals were of reagent grade or better and were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. The human colon cancer cell lines, Caco-2 ($2 \times 10^5$ cells/ml) and LS180 ($1 \times 10^5$ cells/ml) (American Type Culture Collection, Rockville, MD), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS (Asahi Techno Glass, Japan), 1% NEAA (Invitrogen, San Diego, CA), 20 U/ml of penicillin (Invitrogen, San Diego, CA) and 20 μg/ml of streptomycin (Invitrogen, San Diego, CA) in 5% $CO_2$ at 37 °C. The Caco-2 cells were cultured for 2 weeks, and the experiment was conducted after their differentiation. As ER stress inducers, tunicamycin (Wako, Japan), A23187 (Calbiochem, La Jolla, CA), thapsigargin and brefeldin A were added to the cells at various concentrations and for various time periods.

### 2.2. RNA preparation

Total RNA was extracted from the Caco-2 cells or LS180 cells by the single-step acid-guanidium–phenol chloroform method using Isogen (Nippon Gene, Japan) according to the manufacturer's instructions.

### 2.3. Real time RT-PCR

First-strand cDNA synthesis was conducted by using an ExScript® RT reagent kit (Takara, Japan) according to the manufacturer's instructions. A LightCycler system (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions. The sequences of the primers and the annealing temperature were as follows: human GRP78 (NM_005347, 5′-TGA TTC CAA GGA ACA CAG T-3′ and 5′-GTC AGA TCA AAT GTA CCC A-3′, 58 °C), human CHOP (S40706, 5′-TTC TCT GGC TTG GCT GAC T-3′ and 5′-CTG GTT CTC CCT TGG TCT TC-3′, 63 °C), human GAPDH (NM_002046, 5′-TTC AAC AGC GAC ACC CAC TG-3′ and 5′-CAC CCT GTT GCT GTA GCC A-3′, 56 °C), and human β-actin (BC002409, 5′-CCA CGA AAC TAC CTT CAA C-3′ and 5′-GAT CTT CAT TGT GTG CTG GG-3′, 56 °C). An amplification program (40 cycles) for denaturation (94 °C, 15 s), annealing (15 s) and extension (72 °C, 20 s) was used. After PCR amplification, we checked that the primer dimer had not been generated by evaluating the melting curve of the PCR product. The concentration of mRNA was normalized to that of β-actin mRNA or GAPDH mRNA.

### 2.4. Western blot analysis

LS180 cells seeded on a 6-well plate (Corning, NY) were treated with quercetin and/or an ER stress inducer for a certain period of time, and total protein was recovered after washing with PBS(−) (Nissui Pharmaceuticals, Japan). The recovered sample was centrifuged at 12,000 × *g*, 4 °C for 10 min, and the resulting pellet was dissolved by 30 μl of a lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mg EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM DTT, and 1 mM $Na_3VO_4$. An inhibitor cocktail was added before use at a final concentration of 0.1%). The solution was centrifuged at 12,000 × *g* and 4 °C for 10 min, after homogenizing with a syringe fitted with a 25 G needle. The concentration of total protein in the supernatant was determined by a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). The same amount of a 2× sample buffer (125 mM Tris–HCl at pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (w/v) β-mercaptoethanol, and 0.05% (w/v) bromophenol blue) was added to the supernatant. After incubating at 95 °C for 5 min, the protein sample was subjected to an SDS–PAGE analysis (10% (w/v) gel).

Samples containing 25 μg of protein were loaded on to 10% polyacrylamide gel, and electrophoresis was conducted in an electrode buffer (25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS) with the current fixed (concentrating gel, 20 mA; resolving gel, 30 mA).

An Immobilon PVDF membrane (Millipore, Bedford, MA) was treated with methanol and then with a blotting buffer (100 mM Tris, 192 mM glycine, and 20% methanol). Bio Craft semi-dry type of blotting apparatus was used to electrophoretically transfer the gel to the PVDF membrane at 120 mA over 90 min.

After blotting, this PVDF membrane was treated with a blocking buffer (5% skim milk dissolved in PBS for GRP78, and 5% BSA dissolved in PBS for eIF2α) for 1 h at RT (GRP78) or overnight at 4 °C (eIF2α). After washing the membrane with 0.1% Tween 20 dissolved in PBS (PBS–T), this was subjected to an analysis of antibody response.

After washing the PVDF membrane three times with PBS–T for 10 min each, this membrane was treated with 1st antibodies dissolved in PBS–T (except eIF2α) or in a Can Get Signal® immunoreaction enhancer solution (Toyobo, Japan). Anti-mouse β-actin (A5441) was purchased from Sigma, anti-rabbit GRP78 (sc-13968) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-rabbit eIF2α (#9722) and anti-rabbit p-eIF2α (#9721) were purchased from Cell Signaling Technologies (Beverly, MA), anti-mouse GAPDH (AB8245) was purchased from ABCam (Cambridge, UK), and anti-rabbit HRP was purchased from ICN Biomedicals (Aurora, OH). The experimental conditions for the 1st antibody reaction were as follows: GRP78 (200× in PBS–T, 2 h, RT), eIF2α (200× in Can Get Signal, O/N, 4 °C), p-eIF2α (200× in Can Get Signal, O/N, 4 °C), and GAPDH (5000× in PBS–T, 30 min, RT). After the 1st antibody reaction, the membrane was treated with 5000× 2nd antibody dissolved in the same solution as that for the 1st antibody at RT for 30 min after washing with PBS–T. An ECL western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) was used for detecting the protein.

### 2.5. Metabolic labeling

LS180 cells seeded on a 6-well plate were treated with quercetin and/or a ER stress inducer for 90 min, and then additionally treated with 20 mCi Redivue L-