Endocr (2010) 38:167–173
DOI 10.1007/s12020-010-9362-0

ORIGINAL ARTICLE

Atypical induction of the unfolded protein response by mifepristone

N. Dioufa · E. Kassi · A. G. Papavassiliou · H. Kiaris

Received: 11 January 2010 / Accepted: 22 June 2010 / Published online: 11 July 2010

Abstract Mifepristone is a synthetic progesterone antagonist that is being used widely for the treatment of various conditions such as endometriosis, glaucoma, meningiomas, breast, ovarian and prostate cancer, as well as for research purposes, in the conditional induction of gene expression by using artificial plasmid-based systems. Here, we report that exposure of A549 human lung cancer cells to mifepristone caused an atypical induction of the cellular unfolded protein response, as evidenced by the time-dependent stimulation of RNA levels of the chaperone Grp94 and PDIa, as well as the endoplasmic reticulum stress-associated receptors ATF6, PERK and eIF2 but not of their downstream target, transcription factor ATF4. This profile was very different from that of progesterone, which at the same dose as mifepristone, failed to induce all of the ER-stress-related genes examined, apart from PERK. Furthermore, XBP1, a transcription factor that is regulated predominantly by alternative splicing by the IRE1 receptor, remains unspliced and therefore inactive either by mifepristone or progesterone treatment. Finally, the pro-apoptotic molecules CHOP and BIM are only induced in the presence of tunicamycin in the culture medium. Tunicamycin, the most commonly used pharmacologic inducer of ER stress that triggers the canonical ER stress response, was used for comparison purposes. Our results suggest that mifepristone can elicit an atypical ER stress response when used at different doses and for different time points. The subsequent induction of UPR should be taken into consideration when this agent is being used either for therapeutic or for experimental uses.

N. Dioufa · E. Kassi · A. G. Papavassiliou · H. Kiaris (✉)
Department of Biochemistry, University of Athens Medical School, M. Asias 75, 115 27 Athens, Greece
e-mail: hkiaris@med.uoa.gr

Keywords ER stress · UPR · Mifepristone · Progesterone · Lung cancer

Introduction

The endoplasmic reticulum (ER) is the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins [3, 21]. The lumen of the ER constitutes a unique cellular environment, where the highest concentrations of calcium are found, and where calcium-dependent molecular chaperones reside, such as glucose-regulated protein 78 kDa (GRP78), GRP94, and calreticulin, which help stabilize protein-folding intermediates. The ER lumen also has an oxidative environment, which is crucial for the formation of disulphide bonds, mediated by protein disulphide isomerase (PDI) and for the proper folding of many proteins destined for secretion or for display on the cell surface. Also post-translational modifications of proteins such as glycosylation and lipidation occur in the ER, and lipid membrane biosynthesis and production of cholesterol are also mediated by the ER [6, 7, 19] Protein folding is an essential process for protein function in all organisms; therefore, all cells have evolved sophisticated mechanisms to ensure proper protein folding. All proteins that transit the secretory pathway in eukaryotic cells first enter the ER lumen, where they fold and assemble into multi subunit complexes prior to transit to the Golgi compartment [11]. The efficiency of protein folding depends on appropriate environmental, genetic, and metabolic conditions. Conditions that disrupt protein folding present a threat to cell viability. Quality control is a surveillance mechanism that permits only properly folded proteins to exit the ER. Misfolded proteins initiate activation of an adaptive signaling cascade, known as the unfolded protein response (UPR)

[11, 21, 26]. Numerous pathophysiological conditions are associated with ER stress, including ischemia, neurodegenerative diseases, diabetes, and cancer [7, 12, 23]. The UPR is mediated through 3 ER transmembrane receptors: Pancreatic ER kinase (PKR, like ER kinase PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). In resting cells, all 3 ER stress receptors are maintained in an inactive state through their association with the ER chaperone GRP78/BiP. On accumulation of unfolded proteins, GRP78/BiP dissociates from the three receptors, leading to their activation and triggering the UPR [23], which is a prosurvival response, aiming to reduce the accumulation of unfolded proteins and to restore normal ER functioning [21]. However, if protein aggregation is persistent and the stress is not resolved, the cell is led to apoptotic death. Recent studies suggested that either a cytosolic pool of BiP, or a subpopulation of it, existing as an ER transmembrane protein, may form a complex with caspase 7 and caspase 12 at the ER surface, and prevent their release and activation, highlighting the importance of GRP78/BiP as an antiapoptotic protein [18].

Mifepristone is a synthetic norsteroid that binds with high affinity to the human progesterone and glucocorticoid receptors and acts as both a progesterone and glucocorticoid antagonist. In the absence of progesterone, mifepristone acts as a partial agonist. Mifepristone is known as RU486 and has been used as an abortifacient at high doses (3–10 mg/kg) [16] and is also used for the treatment of endometriosis, major depression with psychotic features, glaucoma, meningiomas, breast, ovarian and prostate cancer. In research it is used in inducible gene regulatory systems, designed to allow the control of the spatiotemporal expression of transgenes in vitro and in vivo, in a ligand-dependent manner [22]. In the inducible system, mifepristone acts as an agonist to activate gene transcription by binding to a truncated human progesterone receptor. The concentrations used in the system are non toxic and exert no known pleiotropic effects on mammalian cells that lack endogenous progesterone and glucocorticoid receptors [22].

Our original aim was the creation of a mifepristone-inducible system to express various chaperones conditionally. During the course of these experiments we noticed that some ER stress chaperones were unexpectedly overexpressed, even in control mock-transfected cells. Given recent evidence reporting the induction of ER stress by the selective progesterone receptor modulator, asoprisnil [24], this observation prompted us to explore systematically if mifepristone interferes with the regulation of ER stress and triggers the UPR. Therefore, A549 human lung cancer cells, that previously were shown to develop a canonical UPR [5] and also express progesterone receptors [4], were exposed to progesterone, mifepristone and tunicamycin, a glycosidase inhibitor and widely used inducer of ER stress, for various time points, and the levels of a panel of 11 ER stress-associated proteins were assessed. Our results show that indeed mifepristone induces ER stress, but the profile of the resulting UPR is distinct from that of tunicamycin.

Materials and methods

Cell culture

A549 cancer cells were cultured in 6-well plates at 37°C in a humidified atmosphere of 5% CO_2–95% air in DMEM supplemented 10% FBS (vol/vol, Invitrogen). Cells were treated with 50 nM Mifepristone (Invitrogen), 5 μg/ml Tunicamycin (Sigma) and 50 nM Progesterone (Sigma), for 48, 24, 12, 6, 2, and 0 h. Cells were plated at 60–70% confluency levels, and were allowed to seed for 24 h before treatment.

RNA isolation–cDNA formation

RNA was isolated using Trizol RNA isolation protocol (Invitrogen), according to the manufacturer's instructions. cDNA was obtained after a two-step reaction using AMV-RT (Promega) using 2 μl from the extracted RNA for each reaction. Cells were approximately 90% confluent when RNA was isolated.

Semi-quantitative RT-PCR

PCRs were performed using Go-Taq polymerase (Promega) according to manufacturer's instruction. The primers used are shown in Table 1. PCR products were resolved in 2% agarose, stained with ethidium bromide, and analyzed by scanning densitometry by using the ImageJ image analysis software. All PCR reactions were performed at conditions according to which preliminary experiments have determined the exponential phase of the reaction. Each experiment has been performed at least three times independently, and similar results were obtained. For each RT-PCR amplification reaction, the result of a representative experiment is shown. Statistical analysis has been performed and SEM is demonstrated in the graphs. Each gene expression, at different time points, is expressed versus control (0 h) and versus actin levels.

Western blot analysis

Proteins were extracted from cultured A549 cells as previously described [8]. Briefly, the cells were lysed at 4°C for 20 min using a lysis buffer (Ripa, Invitrogen and

Table 1 Sequence of PCR primers used in this study

Gene	Sense primer	Antisense primer
ATF4	taggggcctcctacctttgt	gtgtcatccaacgtggtcag
ATF6	Gcctttattgcttccagcag	tgagacagcaaaaccgtctg
BIM	Gagatatggatcgcccaaga	gtgctgggtcttgttggttt
BIP	Tagcgtatggtgctgctgtc	tttgtcaggggtctttcacc
CHOP	agtgccacggagaaagctaa	ccatacagcagcctgagtga
ERP29	Ccagtacccctacggtgaga	tggatggctccaaccttaac
GRP94	Tgggaagaggttccagaatg	gttgccagaccatccgtact
PERK	ccagccttagcaaaccagag	tgccctaaagggacacaaac
XBP1	ggagttaagacagcgcttgg	actgggtccaagttgtccag
eIF2	gccaaattgccctatctcaa	cagaaaaatgggcaaaggaa
PDIA2	ggagtttggtgtgacggagt	aggtcctggaagaagccaat

proteinase inhibitor). Whole cells lysates were subsequently centrifuged at 13,000 rpm for 10 min at 4°C and the supernatants were collected. Protein content in the supernatants was determined by the Bradford assay. Each 100 μg aliquot of the proteins extracted from cells was electrophoresed on a 10% SDS-PAGE under reducing conditions. The proteins were electrophoretically transferred from gels to nitrocellulose membranes. The blots were exposed overnight to primary antibodies, followed by 1 h of incubation with secondary antibodies. The antigen–antibody complexes were detected with the ECL chemiluminescence detection system (Thermo Scientific). The experiments were repeated with at least three different cultured specimens with similar results and the reported results are representative. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Results and discussion

Initially we monitored by semiquantitative RT–PCR the profile of UPR induced by tunicamycin, a widely used stimulator of ER stress. A summary of our findings is shown in Fig. 1. As expected, we found that tunicamycin induced the expression of all the genes involved in all three major branches of UPR pathways (IRE1, ATF6, PERK), even after only 2 h of treatment, and all of them showed greatest expression after 48 h (Figs. 5, 6). As previously observed, PDIa2 expression levels decrease in the presence of tunicamycin [15]. In the presence of mifepristone (Figs. 2, 5, 6), PERK, ATF6, and marginally Erp29 were induced after 2 h of treatment. Interestingly, there was significant induction of the molecular chaperone Grp94 which is a marker of the ER stress. On the other hand, progesterone only induced PERK mRNA expression. XBP1, which is mostly modulated by IRE1, remains unspliced when cells are treated either with mifepristone or

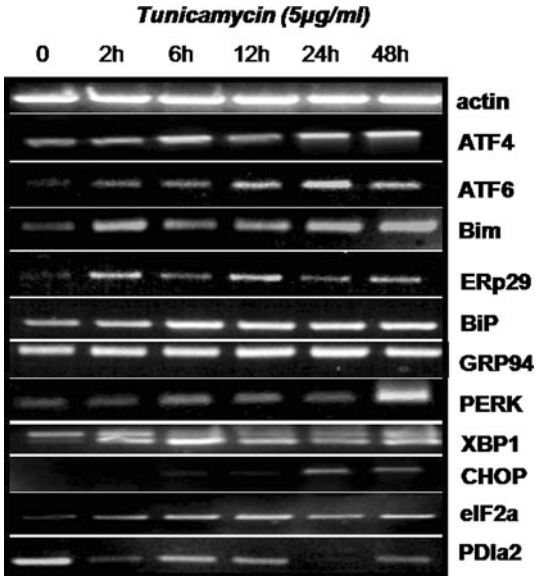

Fig. 1 RT-PCR analysis of various ER stress-associated transcripts following exposure to tunicamycin for the indicative times

progesterone (Figs. 2, 3). The proapoptotic molecules BIM and CHOP were only induced in the presence of tunicamycin. BiP, ERp29 and GRP94 chaperone induction were also assessed by western blot (Fig. 4). Tunicamycin induced Grp78/Bip and Grp94, and reduced Erp29 at a protein level. No induction has been noticed in mifepristone and progesterone-treated cells.

PERK is a Ser/Thr protein kinase. Upon dissociation from GRP78/BiP, PERK is activated by dimerization and autophosphorylation, and phosphorylates eukaryotic initiation factor 2 (eIF2) [21, 26], which leads to the inhibition of

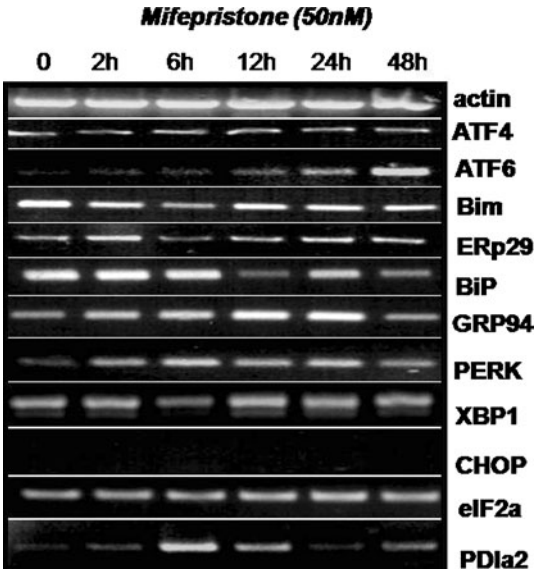

Fig. 2 RT-PCR analysis of various ER stress-associated transcripts following exposure to mifepristone for the indicative times

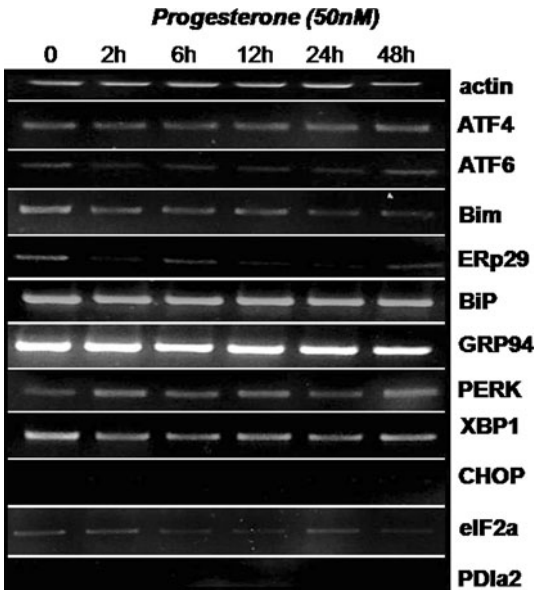

Fig. 3 RT-PCR analysis of various ER stress-associated transcripts following exposure to progesterone for the indicative times

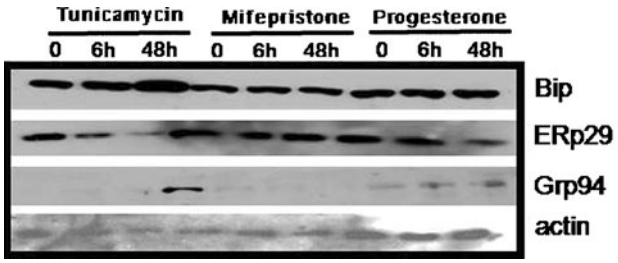

Fig. 4 Western blot analysis of BiP, Grp94, and ERp29 following exposure to tunicamycin, progesterone, and mifepristone

general protein translation, resulting in the reduction of protein load in the ER. However, some mRNAs, including the one encoding transcription factor ATF4, gain a selective advantage for translation, under conditions in which eIF2 is phosphorylated [11]. ATF4 is a member of the bZIP family of transcription factors and encodes a cAMP response element-binding transcription factor (C/EBP). Among the documented or putative targets of ATF4 are genes encoding the ER chaperones GRP78/BiP and GRP94, and the transcription factor C/EBP homologous protein (CHOP), whose induction is strongly dependent on ATF4 and promotes apoptotic cell death. Despite the fact that the PERK branch of UPR is stimulated mainly by the aforementioned mechanism that operates at the post-transcriptional level, we noted a considerable induction of the transcripts that encode for proteins such as PERK and eIF2. However, while at the RNA level PERK is induced in the presence of tunicamycin, mifepristone and progesterone also lead to a major induction of PERK, but amongst its downstream targets, only eIF2 seems affected by mifepristone, whereas ATF4 expression levels remain stable in both cases. This perhaps explains the lack induction of its downstream target, CHOP. This observation is consistent with the finding that different doses of the selective progesterone receptor modulator, asoprisnil, at different time points induced the expression of different UPR genes, showed the induction of pPERK, ATF4, GRP78, and CHOP, while the IRE1–XBP1 axis remains unaffected in the presence of 10^{-7} M asoprisnil in the culture medium [24].

ATF6 proteins are bZIP transcription factors, similar to ATF4. ER stress leads to the release of GRP78/BiP, frees ATF6 proteins to translocate to the Golgi, where resident proteases cleave at a juxtamembrane site, releasing the transcription factors into the cytosol and allowing them to migrate into the nucleus to regulate gene expression [9, 25]. Although ATF6 can induce CHOP mRNA expression, no reports have linked ATF6 to ER stress-induced apoptosis; therefore, it seems that ATF6-mediated signals are purely pro-survival and aim to counteract ER stress [11, 21]. Our experiments indicate that only mifepristone leads to a dose-dependent induction of ATF6, whereas progesterone does not seem to affect its expression. The expression profile of PDIa2, in the presence of mifepristone is more consistent with the one of ATF6, which is a transcription factor that mediates its expression, but is atypical considering that canonical ER stress reduces PDIa levels [15], (Fig. 1).

IRE1 is a type I transmembrane protein that has both a Ser/Thr kinase domain and an endoribonuclease domain [7, 21]. On activation, the endoribonuclease activity of IRE1 removes a 26 nucleotide intron from XBP1mRNA, previously induced by ATF6 [19]. The generated frameshift splice variant XBP1 (sXBP1) mRNA has diverse targets, including ER chaperones [11, 21], leading to the reduction of the protein load on the ER. Although the IRE1–XBP1 axis seems to have pro-survival effects, overexpression of IRE1 results in apoptotic cell death [7, 11]. It is thought to be the last arm of the UPR to be activated, with PERK being the first, closely followed by ATF6. Perhaps PERK and ATF6-mediated pathways attempt to resolve the stress before the activation of the IRE1 pathway. Once activated, IRE1 initially aids the UPR by splicing XBP1 but ultimately terminates it by relieving the transcriptional inhibition by inducing P58 ^{IPK} [18]. At this point, either the cell returns to normal functioning or if the stress persists, IRE1 triggers apoptosis by recruiting ASK1–JNK [6, 10, 21]. The observation that the IRE1–s XBP1 seems unaffected in the presence of progesterone and mifepristone may be due to the fact that the duration of the ER stress provoked, or the potency of the substances used was not enough to lead to apoptotic cell death through this well-established pathway of ASK1–JNK. In our experiments, we studied the ER-related apoptosis by studying the expression profiles of

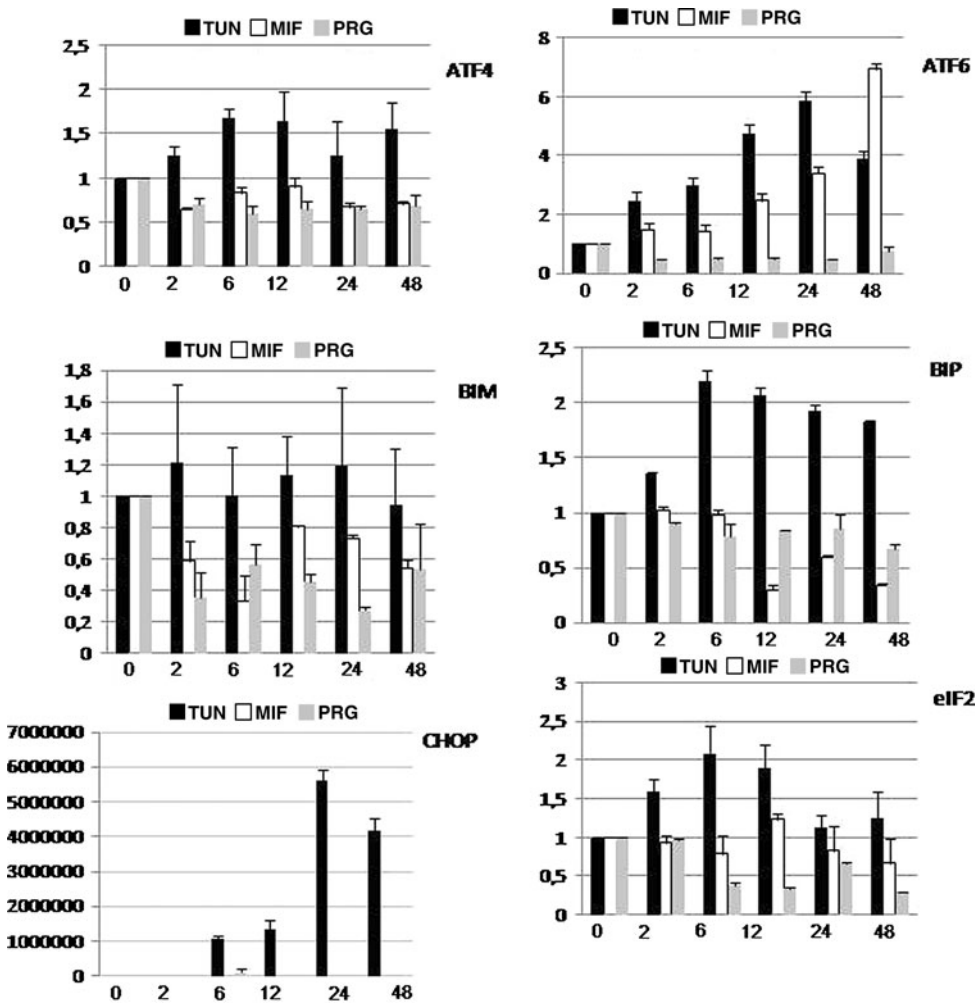

Fig. 5 Graphical presentation of RNA expression levels of ATF4, Bim, CHOP, ATF6, BiP, and eIF2 following exposure to tunicamycin, mifepristone, or progesterone for various time periods ranging from 2 to 48 h. Each *bar* indicates the density and intensity of each band, compared with the control (0 h) and the corresponding actin band

CHOP and BIM. The transcription factor C/EBP homologous protein operates as a downstream component of ER stress pathways, at the convergence of the IRE1, PERK, and ATF6 pathways [3, 17, 18]. Overexpression of the CHOP protein induces apoptosis, but this effect can be blocked by BiP overexpression [1] In addition, recent data indicate that CHOP binds an element in the promoter of the gene encoding BIM protein. It is noteworthy that neither progesterone, which is a natural hormone, and does not seem to cause ER stress, nor mifepristone, which is a synthetic compound, and exerts some kind of ER stress in cell culture, manages to lead the cell to apoptotic death by inducing the expression of CHOP or BIM, in comparison to tunicamycin which induces both.

Amongst our genes of interest was also ERp29, which is a recently characterized protein, of 29 kDa, which resides in the lumen of the ER and mediates the secretion of proteins [2]. ERp29 is widely expressed and highly conserved amongst mammalians [20]. It has a C-terminal domain, similar to the one of other proteins involved in ER stress (tetrapeptide KDEL) [13] and has a homology with PDI. Also ERp29 is shown to be implicated in the malignant conversion of mammary epithelial cells [14].

In conclusion, according to our findings, mifepristone seems to induce ER stress by inducing genes such as ATF6, PERK, and eIF2, without leading to the sufficient stimulation of the apoptosis-related BIM and CHOP. Furthermore, the profile of the genes evaluated in our study was distinct from that induced by tunicamycin, since PDIa had a reverse expression profile compared with tunicamycin and Grp94 was excessively induced by mifepristone, implying the atypical induction of ER stress by this hormone analog. This may be due either to the potency of the doses of mifepristone and progesterone used, or to the duration of the stress induced, which is not potent enough to lead the cell to apoptosis. Alternatively, differential specificity of the precise molecular mechanism associated with the progesterone- and mifepristone-induced UPR may also account for these differences. Collectively, we have shown that progesterone's specific antagonist, mifepristone induces adequately the cell's UPR in a manner that is distinct from the typical UPR induction triggered by

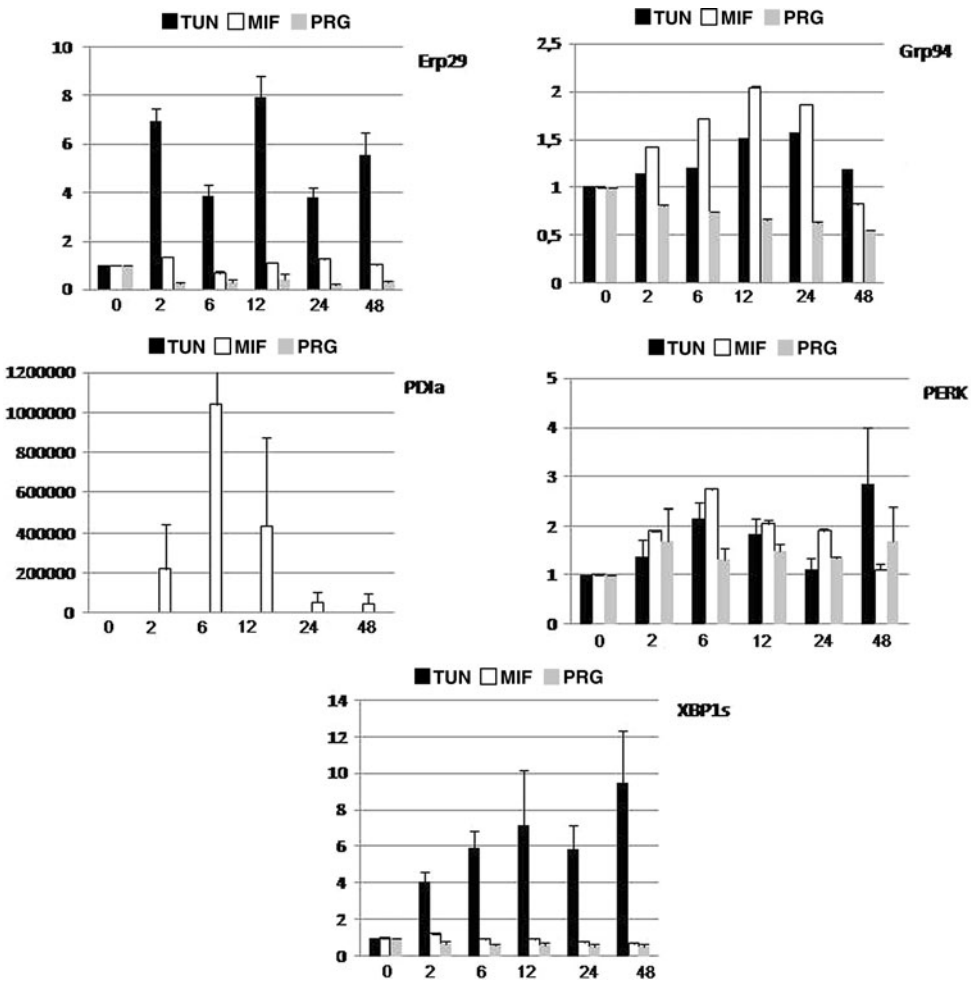

Fig. 6 Graphical presentation of RNA expression levels of ERp29, PDIa, XBP1, Grp94, and PERK following exposure to tunicamycin, mifepristone, or progesterone for various time periods ranging from 2 to 48 h. Each *bar* indicates the density and intensity of each band, compared with the control (0 h) and the corresponding actin band

tunicamycin. Besides understanding the molecular mechanism underlying the crosstalk between progesterone signaling and UPR, our findings have implications in conditions and experimental systems where these hormone analogs are being utilized.

Acknowledgments This study was supported by grants from the Greek Endocrine Society and the University of Athens (Kapodistrias).

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