Regulation of BiP Gene Expression by Cyclopentenone Prostaglandins through Unfolded Protein Response Element*

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Δ12-Prostaglandin (PG) J₂, a cyclopentenone prostaglandin, plays a role in various stress responses. BiP, a stress-inducible chaperone protein, is implicated in protein folding and translocation in endoplasmic reticulum and induced in the condition of accumulation of unfolded proteins. Here, we examined the effect of Δ12-PGJ₂ on the expression of the BiP gene. Δ12-PGJ₂ markedly stimulated the expression of the BiP gene in a time- and concentration-dependent manner in HeLa cells. This stimulation was specific for cyclopentenone PGs among various PGs. Cycloheximide pretreatment completely inhibited the Δ12-PGJ₂-induced expression of the BiP gene, suggesting that the effects on nascent protein synthesis are involved in the signaling mechanism. Δ12-PGJ₂ markedly stimulated the promoter activity of the 5'-flanking region of the BiP gene through the unfolded protein response element. Furthermore, Δ12-PGJ₂ stimulated the enhancer activity of the 3'-half of the unfolded protein response element, and this stimulation required three nucleotides within this region. Gel mobility shift assay demonstrated that this region was occupied with two specific nuclear protein factors with different mobilities in the control cells, and Δ12-PGJ₂ induced the dissociation of the protein-DNA complex with lower mobility. These findings indicate that Δ12-PGJ₂ stimulates the expression of BiP gene through the 3'-half of the unfolded protein response element.

Eicosanoids are oxygenated metabolites of arachidonic acid and are regarded as modulators of cellular functions in various physiological and pathological processes (1). Eicosanoids are divided into two groups, conventional eicosanoids and cyclopentenone-type prostaglandins (PGs), according to their mechanisms of action. Cyclopentenone PGs, such as Δ12-PGJ₂ and PGA₁₂, exert a variety of biological actions, including cessation of cell growth, cell differentiation, and development (2, 3). Among them, the prominent action of cyclopentenone PGs is a stress response. Cyclopentenone PGs are produced in response to various stress stimuli and then are actively transported into cells and induce the expression of various stress-related protein genes, including those of a family of cytosolic heat shock proteins (HSPs) (4, 5), ribosome-inactivating protein (6), and heme oxygenase (7). The PG-induced stress protein gene expression has been shown to require de novo protein synthesis (5, 8). The requirement of de novo protein synthesis for the cyclopentenone PG-induced gene expression suggests that the site of stress actions of the PGs may be on the regulation of nascent protein processing. However, the molecular characterization of stress actions of cyclopentenone PGs has been hardly carried out.

BiP is a member of the HSP family of molecular chaperones and plays an important role in the translocation of nascent proteins across the endoplasmic reticulum (ER) membrane and in their subsequent folding and assembly (9). Although BiP mRNA is synthesized constitutively, transcription of the BiP gene is induced by the accumulation of unfolded proteins in the ER, which is experimentally induced by various stress treatments, including depletion of ER Ca²⁺ stores, reducing environment, and block of protein glycosylation (10). The promoter region of the yeast BiP gene has been identified to contain a regulatory domain, unfolded protein response element (UPR) that responds to the accumulation of unfolded proteins in the ER (11), which is highly conserved among various species (12). Thus, BiP is a key regulator for newly synthesized protein homeostasis, and the gene expression is critically controlled by an unfolded protein response pathway. From these findings, we postulated that the stress actions of cyclopentenone PGs may be correlated with an unfolded protein response linked to BiP gene expression. Therefore, we examined the effects of the PGs on the BiP gene expression. We report here that cyclopentenone PGs induce the expression of the BiP gene through UPR.

EXPERIMENTAL PROCEDURES

Materials—Δ12-PGJ₂ was a generous gift from Teijin Ltd. (Tokyo), Δ12-PGA₁₂, and tk-CAT vector were kindly supplied by Drs. M. Suzuki (Gifu University) and M. Imagawa (Osaka University), respectively. Other agents were obtained from commercial sources as follows: [α-32P]dCTP (3,000 Ci/mmol) from DuPont NEN; 1M Cl⁻ deoxycholormethylphenoil (54 mCi/mmol) from Amersham Corp.; PGA₁₂, PGA₂₅, PGD₂₅, PGE₁, PGE₆, PGE₂, PGF₂a, and PGJ₂ from Cayman Chemical (Ann Arbor, MI); AZ3139 from Calbiochem; and arsenite, diethyleneetriamine, and tunicamycin from Sigma. The sources of other materials are given in the text.

HeLa cells were grown in high glucose Eagle’s minimal essential medium supplemented with 10% calf serum, 4 mg/ml glutamine, and 0.06 mg/ml kanamycin under humidified air containing 5% CO₂ at 37 °C. Northern Blots—Total RNA from HeLa cells was isolated using an I so gen RNA isolation kit (Nippon, Tokyo), and 5 μg of each RNA was separated by electrophoresis on a 1.5% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Corp.), and hybridized with a 32P-labeled BamHI/Sall fragment of pSV-BiP (13). The same filter was rehybridized with 32P-labeled glycerylaldehyde 3-phosphate dehydrogenase (G3PDH) cDNA probe (Clontech). Hybridization was carried out at 65 °C in 6 × SSC, and the filter was washed at 65 °C in 2 × SSC. The filter was autoradiographed with x-ray film (Fuji RX). The radio-
activity was determined with a Fuji BAS 2000 imaging analyzer (Fuji, Tokyo).

Construction of Plasmid DNA—Two different sizes of the 5'-flanking region of the human BIP gene (nucleotide residues –126 to +90 and –92 to +90, relative to the transcription start site) were obtained by means of the polymerase chain reaction from genomic DNA prepared from HeLa cells, as described previously (14). The polymerase chain reaction product was first constructed into pCR 2.1–TOPO vector (Invitrogen), and then the HindIII/XbaI insert was constructed into pCAT-126CAT and –92CAT, containing each 5'-flanking region of the BIP gene, upstream of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. The sequence of the cloned 5'-flanking region was confirmed by sequence analysis by the dideoxynucleotide chain-termination method. For the functional analysis of enhancer activity of UPR using a heterologous promoter, synthesized DNA fragments, encoding various mutated UPR, were inserted into tk-CAT vector, which carries the thymidine kinase (tk) promoter upstream from the CAT gene (15), and the DNA fragments were located upstream from the tk promoter in the forward and reverse directions.

Transfection and CAT Assay—HeLa cells were transiently transfected with the plasmid DNA constructs by the DEAE-dextran method (16). After the cells (3 × 10⁶ cells/assay) had been incubated for 30 min at 37°C in 0.5 ml of serum-free high glucose Eagle's minimal essential medium containing 5 μg of plasmid DNA and 10 μg of DEAE-dextran (Pharmacia Biotech Inc.), they were incubated for 10 min at 37°C with a dimethyl sulfoxide-hypertonic solution (28 μl Tris-HCl, pH 7.5, containing 0.4 M sucrose, 8% polyethylene glycol 4000, 84 mM NaCl, and 10% dimethyl sulfoxide). They were then cultured in high glucose Eagle's minimal essential medium containing 10% calf serum. Reactions were started by the addition of the test agents. After incubation for the indicated times, cellular extracts were prepared by four cycles of freezing and thawing. The CAT assay was carried out as described previously (16). Cellular extracts containing equal amounts of protein were treated at 65°C for 10 min to inactivate deacetylase and then incubated for 4 h at 37°C with 1-β-[32P]deoxychlamphenicol (50 nCi) and 0.1 mg/ml acetyl CoA. The reaction mixtures were extracted with ethyl acetate and then separated on a silica TLC plate (F-1500, Schleicher & Schuell). After development of the TLC plate, the radioactive [32P]-labeled product was visualized by autoradiography with x-ray film (Fuji, Tokyo). CAT activity was normalized as to the ratio of the specific radioactivity of the [32P]-labeled product in the CAT assay and radioactivity of the internal standard [32P]-labeled 9.5-kb HindIII/XbaI fragment for each sample.

RESULTS

Induction of BIP mRNA by Δ¹²-PGJ₂—We examined the effect of Δ¹²-PGJ₂ on the expression of the BIP gene in HeLa cells by Northern blot analysis. As shown in Fig. 1A, Δ¹²-PGJ₂ progressively increased the mRNA level of BIP, the level reaching 6-fold of the basal level by 24 h. Δ¹²-PGJ₂ caused marked accumulation of the mRNA in a concentration-dependent manner, the maximum being reached at 10 μM (Fig. 1B). On the other hand, the mRNA level of G3PDH did not change.

We next examined the specificity of stimulation of the BIP gene expression for various PGs. As shown in Fig. 2A, Δ¹²-PGJ₂ and Δ¹²-PGA₁ markedly increased the mRNA level of BIP, PGA₁ also increased it, but the level was very low. However, the other PGs had no ability to accumulate the mRNA. We further examined the effects of various stress inducers on the expression of the BIP gene. As shown in Fig. 2B, tunicamycin and A23187 strongly increased the mRNA level of BIP. Arsenite also elevated the mRNA level, but diethylmalate showed very low activity. Δ¹²-PGJ₂ was the most potent inducer of the BIP mRNA when it was compared with these inducers.

The BIP gene has been shown to be induced by the accumulation of improperly folded proteins newly synthesized in the ER (10). Then, we examined whether the Δ¹²-PGJ₂-induced expression was dependent on de novo protein synthesis. As shown in Fig. 3, cycloheximide pretreatment strongly abolished the Δ¹²-PGJ₂- and tunicamycin-induced accumulation of BIP mRNA, suggesting that de novo protein synthesis is necessary for Δ¹²-PGJ₂-induced expression of the BIP gene.

Stimulation of Promoter Activity of the 5'-Flanking Region of the BIP Gene by Δ¹²-PGJ₂—To determine whether UPR is the cis-regulatory element for Δ¹²-PGJ₂-induced expression of the BIP gene, we examined the effect of Δ¹²-PGJ₂ on the transient
The CAT reporter gene harboring the 5’-flanking region of the BiP gene in HeLa cells. As shown in Fig. 4, \( \Delta^{12}\)-PGJ \(_2\) markedly stimulated the promoter activity of -126CAT, containing UPR, by about 10-fold, but -92CAT without UPR had lost the \( \Delta^{12}\)-PGJ \(_2\) responsiveness, indicating that the stimulation of promoter activity by \( \Delta^{12}\)-PGJ \(_2\) is mediated by UPR. We further examined the effects of several inducers of BiP on the promoter activities of two plasmids. As shown in Fig. 5, \( \Delta^{12}\)-PGJ \(_2\), arsenite, and tunicamycin selectively stimulated the promoter activity of -126CAT, while A23187 slightly stimulated the activities of both plasmids. Thus, UPR is involved in the induction of BiP gene expression by \( \Delta^{12}\)-PGJ \(_2\), arsenite, and tunicamycin but not by A23187. Among these inducers, \( \Delta^{12}\)-PGJ \(_2\) caused the most potent stimulation of the promoter activity.

To dissect the \( \Delta^{12}\)-PGJ \(_2\) responsive domain in the UPR sequence, we constructed the UPR sequence mutated at either the 5’- or the 3’-half fused to another CAT gene with a heterologous promoter, tk promoter, and then examined the effects of \( \Delta^{12}\)-PGJ \(_2\) on the enhancer activities of the mutant genes. As shown in Fig. 6, \( \Delta^{12}\)-PGJ \(_2\) stimulated the enhancer activity of the 5’-half-mutated UPR in the forward or reverse orientation but not of the 3’-half-mutated UPR, indicating that the \( \Delta^{12}\)-PGJ \(_2\) responsive element is located in the 3’-half of UPR. To identify more precise nucleotides responsible for the \( \Delta^{12}\)-PGJ \(_2\) response within the 3’-half UPR, we mutated three nucleotides in the 3’-half UPR, which are highly conserved among various UPRs (11). This point mutation completely abolished the \( \Delta^{12}\)-PGJ \(_2\)-induced stimulation of the enhancer activity of the 3’-half of UPR. These results indicate that these three nucleotides in the 3’-half of UPR are essential for the stimulation of the enhancer activity.

Identification of Associated Transcription Factor on UPR—To identify a nuclear protein that specifically recognizes this region of UPR, we carried out a gel mobility shift assay using the DNA fragment of the 3’-half UPR sequence. As shown in Fig. 7, three retarded bands were detected, and the formation of two protein-DNA complexes with the intermediate and low mobilities were competed for by an excess of unlabeled 3’-half of UPR fragment but not by that of the three nucleotide-mutated 3’-half of UPR fragment, indicating that these two nuclear protein factors specifically recognize the three nucleotides of the sequence of 3’-half of UPR. Two specific protein-DNA complexes were observed in the control cells, but \( \Delta^{12}\)-PGJ \(_2\)-induced dissociation of the protein-DNA complex with the lower mobility but not the major complex with the intermediate mobility.

**DISCUSSION**

\( \Delta^{12}\)-PGJ \(_2\) exerts stress responses through the synthesis of a variety of stress proteins (2, 3). Among the \( \Delta^{12}\)-PGJ \(_2\)-induced protein syntheses, the mechanism for the synthesis of cytosolic HSPs has been well characterized (4, 5). \( \Delta^{12}\)-PGJ \(_2\) induces the marked expression of the HSP genes through activation of heat shock factors, which bind to the heat shock element located in the 5’-flanking region of the HSP gene (8). On the other hand, the 5’-flanking region of mammalian BiP gene does not contain a heat shock element, and thus the heat shock factor is not a regulatory transcription factor for expression of the BiP gene (14). Here, we demonstrated that \( \Delta^{12}\)-PGJ \(_2\) stimulated the promoter activity of the 5’-flanking region of the BiP gene and then induced the BiP mRNA in HeLa cells, which was sensitive to cycloheximide (Figs. 1, 3, and 4). This action was mediated by UPR, which is a cis-acting element for accumulation of unfolded proteins. This is a new example of cyclopentenone PG-induced gene expression through a cis-regulatory element other than a heat shock element.

Among various PGs, the induction of BiP mRNA is specific for cyclopentenone PGs (Fig. 2). Furthermore, we showed that
of thiol-containing proteins is reversible, but that of \( \Delta^{12}\text{-PGJ}_2 \) or \( \Delta^{7}\text{-PGA}_1 \) is irreversible (23). The stronger induction of the BiP mRNA by \( \Delta^{12}\text{-PGJ}_2 \) and \( \Delta^{7}\text{-PGA}_1 \) may be ascribed to their ability to form stable bis-conjugates with thiol groups. Arsenite is also a thiol-binding molecule and forms a metal-protein complex. Arsenite markedly stimulated promoter activity of the 5'-flanking region of the BiP gene through UPR and then induced the BiP mRNA (Figs. 2 and 5), suggesting that its binding to the thiol groups of proteins is involved in the activation of promoter activity through UPR. Thiol reactive agents, including cyclopentenone PGs and arsenite, may induce the BiP gene expression by interaction with the thiol groups of proteins, and this interaction may induce unfolded protein response.

We identified three nucleotides within the 3'-half of the UPR sequence that are essential to the \( \Delta^{12}\text{-PGJ}_2 \)-stimulated enhancer activity (Fig. 6). These nucleotides are highly conserved in UPR sequences of BiP genes of various species from yeast to human and in UPR sequences of GRP94, another ER-resident chaperone (11). Thus, the 3'-half of the UPR sequence, including these three nucleotides, is a crucial element for enhancer activity of UPR. We further identified two specific nuclear proteins, which bind to the 3'-half of UPR, and these putative transcription factors specifically recognized the three nucleotides in the 3'-half of UPR sequence, which are essential for the \( \Delta^{12}\text{-PGJ}_2 \)-induced stimulation of enhancer activity of UPR, indicating that these factors regulate the \( \Delta^{12}\text{-PGJ}_2 \)-induced BiP gene expression (Fig. 7). The homologous UPR binding activity was also reported to be constitutive, and no differences between control and stressed cells were found in both yeast and mammalian cells (24, 25). On the other hand, we revealed here that the two nuclear factors stably bound to the 3'-half of UPR in the basal condition and that \( \Delta^{12}\text{-PGJ}_2 \) induced dissociation of the factor with the lower mobility from the DNA (Fig. 7). Thus, there are two types of nuclear factors for UPR: one nuclear factor constitutively binds to UPR, and the binding of the other factor is regulated by \( \Delta^{12}\text{-PGJ}_2 \), indicating that the two factors are functionally different. The \( \Delta^{12}\text{-PGJ}_2 \)-induced dissociation of the factor may lead to the \( \Delta^{12}\text{-PGJ}_2 \)-induced stimulation of enhancer activity of UPR. The dissociated factor may play an important role in suppression of the enhancer activity of UPR.

We demonstrated here that \( \Delta^{12}\text{-PGJ}_2 \)-induced expression of the BiP gene through UPR. This suggests that the stress actions of cyclopentenone PGs are mediated by unfolded protein response. Expression of the BiP gene is induced following exposure of eukaryotic cells to diverse conditions, which are related to pathophysiological stress (10). This response has been suggested to be important for protein homeostasis, from the folding and assembly of newly synthesized proteins to their repair or degradation, during the pathogenesis of disease states, including tissue injury and inflammation (26). Cyclopentenone PGs are final products of the arachidonate cascade, and the cascade serves endogenous stress molecules, cyclopentenone PGs, in such stress conditions. The induction of BiP by the PGs through UPR may play an important role in cytoprotective regulation of protein folding in the stress conditions. This study will contribute not only to our understanding of the BiP gene expression mechanism but will also facilitate elucidation of the molecular mechanisms of cyclopentenone PG actions.

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**Fig. 6.** Enhancer activity of UPR using tk promoter. A, sequences of the mutant DNA fragments used for enhancer activity are shown. Wild-type sequence of the UPR is shown in capital letters. Bold letters represent the mutated sequences of the 3'- or 5'-half of the UPR region (3'mCAT and 5'mCAT), and underlined bold letters indicate further point-mutated nucleotides of 5'-half-mutated UPR (pm5'mCAT). B, indicated DNA fragments were inserted into tk-CAT vector in the forward (F) or reverse (R) direction. After cells (3 x 10^6 cells) had been transiently transfected with the constructed vectors, they were treated for 12 h with either or without PGA1 or PGA2 forms a monoconjugated chloramphenicol of the untreated cells transfected with tk-CAT vector, respectively.

**Fig. 7.** Nuclear protein binding to 3'-half of UPR. After cells (3 x 10^6 cells) had been treated with PGA1 or PGA2, the nuclear extracts were prepared. The gel mobility shift assay was performed using the 32P-labeled DNA fragment in the absence (lanes 1 and 4) or presence of a 50-fold excess of the unlabeled 3'-half of UPR (lanes 2 and 5) or the three nucleotide-mutated 3'-half of UPR (lanes 3 and 6), as described under "Experimental Procedures." I and II represent specific nuclear protein-DNA complexes, and NS shows nonspecific binding. The results are representative of three independent experiments that yielded similar results.

\( \Delta^{12}\text{-PGJ}_2 \) and \( \Delta^{7}\text{-PGA}_1 \) induced expression of BiP among the cyclopentenone PGs. Generally, the biological actions of PGA1 and PGA2 are much weaker than those of \( \Delta^{12}\text{-PGJ}_2 \) and \( \Delta^{7}\text{-PGA}_1 \) (2, 3). Cyclopentenone PGs are thiol-binding molecules. We recently reported that the binding of \( \Delta^{12}\text{-PGJ}_2 \) to the intracellular particulate fractions is N-ethylmaleimide sensitive, indicating that the binding sites of \( \Delta^{12}\text{-PGJ}_2 \) are thiol groups (18). PGA1 or PGA2 forms a monoconjugate with a thiol (19, 20), but \( \Delta^{12}\text{-PGJ}_2 \) or \( \Delta^{7}\text{-PGA}_1 \) can form a bis-conjugate with two thiols (21, 22). Furthermore, the binding of PGA1 to synthetic polymer-supported thiols as the model
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