Involvement of protein kinase in Δ^{12}-prostaglandin J_2-induced expression of rat heme oxygenase-1 gene

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Abstract **We recently identified the *cis*-regulatory element and its specific nuclear binding factors for Δ^{12}-prostaglandin (PG) J_2-induced expression of the rat heme oxygenase, HO-1 [Koizumi, T., Odani, N., Okuyama, T., Ichikawa, A. and Negishi, M. (1995) J. Biol. Chem. 270, in press]. Here we further examined the molecular mechanism underlying the Δ^{12}-PGJ_2-induced HO-1 gene expression. Protein kinase inhibitors, 2-aminopurine and staurosporine, suppressed the Δ^{12}-PGJ_2-induced HO-1 mRNA and the nuclear protein binding to the Δ^{12}-PGJ_2-responsive *cis*-regulatory element in rat basophilic leukemia cells. Furthermore, the nuclear protein binding to the element was suppressed by in vitro phosphatase treatment of the nuclear proteins from Δ^{12}-PGJ_2-treated cells. These findings suggest that Δ^{12}-PGJ_2 induces the expression of the HO-1 gene through phosphorylation of the nuclear proteins which bind to the Δ^{12}-PGJ_2-responsive element.**

Key words: Δ^{12}-Prostaglandin J_2; Heme oxygenase; Protein kinase

1. Introduction

Cyclopentenone prostaglandins (PGs), such as Δ^{12}-PGJ_2 and PGA_2, exert a variety of biological actions, including cessation of cell growth, cell differentiation and development [1]. The actions of cyclopentenone PGs are attributed to the synthesis of various proteins induced by them, such as heat shock proteins (HSPs) [2,3], γ-glutamylcysteine synthetase [4], and collagen [5], gadd 153 [6], and heme oxygenase [7].

Heme oxygenase is one of the most prominent proteins induced by Δ^{12}-PGJ_2, and it is a key enzyme in heme catabolism, oxygenizing heme to yield biliverdin, iron and carbon monooxide [8]. The biological functions of this enzyme are the production of biliverdin as a physiological antioxidant and the conservation of the iron [9]. Furthermore, carbon monooxide produced on the enzymatic degradation of heme has been suggested to function as a neural messenger [10]. Two isozymes of heme oxygenase, HO-1 and HO-2, have been identified [11]. HO-2 is constitutively expressed, while HO-1 is drastically induced in response to a variety of stresses, including heavy metals, heat shock and UV irradiation [12–14]. We previously found that Δ^{12}-PGJ_2 preferentially induced the synthesis of HO-1 in various cells involved in the reticulo-endothelial system, in which active degradation of heme by HO-1 takes place during inflammation [7]. Furthermore, we identified the Δ^{12}-PGJ_2-specific *cis*-regulatory element, the Δ^{12}-PGJ_2-responsive element, located in the 5′-flanking region of HO-1 gene, for Δ^{12}-PGJ_2-induced expression of the HO-1 gene, and two specific nuclear factors, the Δ^{12}-PGJ_2-responsive factors, which bind to the element in response to Δ^{12}-PGJ_2 [15]. However, the molecular mechanisms for the Δ^{12}-PGJ_2-induced nuclear factor binding to the element remain unclear. The DNA binding activity of nuclear transcription factors has been shown to be modulated by phosphorylation [16]. We suggest here that the Δ^{12}-PGJ_2-induced HO-1 gene expression is regulated through phosphorylation of the Δ^{12}-PGJ_2-responsive factors which bind to the Δ^{12}-PGJ_2-responsive element.

2. Materials and methods

2.1. Materials

Δ^{12}-PGJ_2 was a generous gift from Teijin Ltd. (Tokyo, Japan). [γ-^{32}P]ATP (3,000 Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA). 2-Aminopurine (2-AP) and staurosporine were from Sigma (St. Louis, MO); calf intestinal phosphatase from Boehringer-Mannheim (Mannheim, Germany); and sodium fluoride and sodium vanadate from Wako Pure Chemical Industries Ltd. (Tokyo, Japan).

Rat basophilic leukemia (RBL)-2H3 cells were obtained from the Japanese Cancer Research Bank (Tokyo, Japan). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM glutamine, 0.2 mg/ml streptomycin and 100 units/ml penicillin under humidified air containing 5% CO_2 at 37°C.

2.2. ^{35}S-Labeling conditions and SDS-PAGE

Cells cultured in 12-well plates (2×10^5 cells/well) were incubated for 3 h with 0.4 ml of methionine-free Eagle's medium containing [^{35}S]methionine (10 μCi/ml) and 10% dialyzed fetal bovine serum. After incubation, the cells were washed with ice-cold phosphate-buffered saline, lysed and subjected to SDS-10%PAGE, as described previously [17].

2.3. Northern blots

Total RNA from RBL-2H3 cells was isolated using an Isogen RNA isolation kit (Nippon-gene, 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 ^{32}P-labeled fragment, corresponding to exon 3 of the rat HO-1 gene. Hybridization was carried out at 68°C in 6 × SSC, and the filter was washed at 68°C in 2 × SSC. The filter was autoradiographed with X-ray film (Fuji RX). The radioactivity was determined with a Fuji BAS 2000 imaging analyzer (Fuji Film Co., Tokyo).

2.4. Gel mobility shift assay

A complementary pair of DNA fragments encoding region, −690 to −660, relative to the transcription start site, which contains the Δ^{12}-PGJ_2-responsive *cis*-regulatory element, was synthesized with an ABI 391 DNA synthesizer (Applied Biosystems Inc., CA), and then annealed as a probe. The fragment was radiolabeled at the 5′ ends with [γ-^{32}P]ATP using T_4 polynucleotide kinase. Nuclear extracts were prepared by detergent lysis method of Block et al. [18]. The nuclear extracts

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Abbreviations: PG, prostaglandin; HO, heme oxygenase; RBL, rat basophilic leukemia; HSP, heat shock protein.

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(2 μg) were incubated with 2 μg of poly(dI-dC) and 1 ng of a ^{32}P-labeled probe (10,000 cpm) for 30 min at 30°C in 25 mM HEPES-NaOH (pH 7.9), containing 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. The reaction mixtures were electrophoresed on native 4% polyacrylamide gels at 4°C at 150 V for 2 h in 50 mM Tris-HCl (pH 8.5), containing 380 mM glycine and 2 mM EDTA. The gels were dried on Whatman 3MM paper and then autoradiographed with X-ray film (Fuji RX).

3. Results and discussion

The purine analogue, 2-AP, a broad spectrum protein kinase inhibitor, has been shown to inhibit various gene expression [19]. We examined the effect of 2-AP on Δ^{12}-PGJ$_2$-induced synthesis of HO-1 in RBL-2H3 cells. As shown in Fig. 1, 2-AP treatment almost completely inhibited the synthesis of HO-1 induced by Δ^{12}-PGJ$_2$ without any alteration of basal protein synthesis, indicating that 2-AP specifically inhibited the Δ^{12}-PGJ$_2$-induced protein synthesis. In addition to HO-1, Δ^{12}-PGJ$_2$ induced the synthesis of HSP70, and the 2-AP treatment also completely inhibited the Δ^{12}-PGJ$_2$-induced HSP70 synthesis (Fig. 1). We further examined the effects of protein kinase inhibitors on the Δ^{12}-PGJ$_2$-stimulated HO-1 mRNA induction. In addition to 2-AP, we used other inhibitor, staurosporine, which is an inhibitor for serine/threonine protein kinases, including protein kinase C [20]. As shown in Fig. 2A, 2-AP and staurosporine suppressed the ability of Δ^{12}-PGJ$_2$ to stimulate HO-1 mRNA induction, indicating that the inhibition of the Δ^{12}-PGJ$_2$-induced HO-1 synthesis is due to suppression of the HO-1 gene expression. Recently, an element responsible for 12-*O*-tetradecanoylphorbol-13-acetate was identified in the mouse HO-1 gene, suggesting involvement of protein kinase C in induction of the mouse HO-1 gene [21]. In RBL-2H3 cells, 12-*O*-tetradecanoylphorbol-13-acetate could not induce the HO-1 gene expression (data not shown). We recently demonstrated that the Δ^{12}-PGJ$_2$-induced HO-1 gene expression was mediated by the binding of nuclear proteins, the Δ^{12}-PGJ$_2$-responsive factors, to the Δ^{12}-PGJ$_2$-responsive *cis*-regulatory element [15]. Thus, we examined the effects of these inhibitors on the Δ^{12}-PGJ$_2$-stimulated nuclear protein binding to the element. As shown in Fig. 2B, Δ^{12}-PGJ$_2$ markedly induced two nuclear protein–DNA complexes, and 2-AP and staurosporine completely abolished the Δ^{12}-PGJ$_2$-induced nuclear protein binding to the element. These results suggest that the Δ^{12}-PGJ$_2$-induced nuclear protein binding to the element is regulated by phosphorylation. To assess phosphorylation of the Δ^{12}-PGJ$_2$-responsive factors, we exposed a nuclear extract from Δ^{12}-PGJ$_2$-treated cells to calf intestinal phosphatase and examined the binding of the Δ^{12}-PGJ$_2$-responsive factors to the element. As shown in Fig. 3, phosphatase treatment completely inhibited the binding activity of the factors to the element, and this

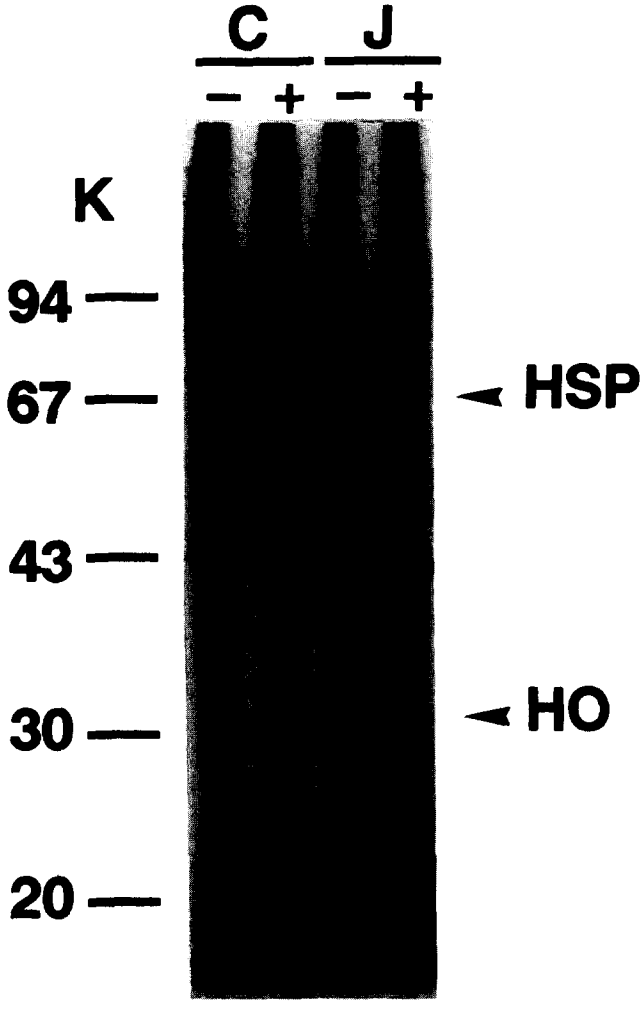

Fig. 1. Effect of 2-AP on the induction of HO-1 synthesis by Δ^{12}-PGJ$_2$ in RBL-2H3 cells. RBL-2H3 cells were incubated with the vehicle (−) or 3 mM 2-AP (+) for 3.5 h in the presence of [^{35}S]methionine, and for the last 3 h were treated with (J) or without (C) 10 μM Δ^{12}-PGJ$_2$. The cells were then lysed and subjected to SDS-10%PAGE, followed by fluorography as described in section 2. The positions of molecular weight markers are shown on the left. The arrows indicate the positions of HO-1 and HSP70.

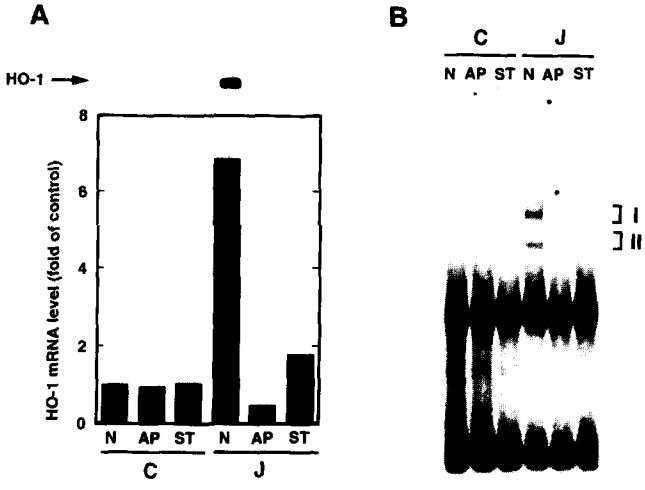

Fig. 2. Effects of 2-AP and staurosporine on the Δ^{12}-PGJ$_2$-stimulated HO-1 mRNA induction and the binding of the Δ^{12}-PGJ$_2$-responsive factors to the Δ^{12}-PGJ$_2$-responsive element. After cells had been incubated with the vehicle (N), 3 mM 2-AP (AP) or 100 nM staurosporine (ST) for 30 min, they were treated with (J) or without (C) 10 μM Δ^{12}-PGJ$_2$ for 3 h (A) or 2 h (B). (A) Total cellular RNA was extracted and subjected to Northern blot analysis as described in section 2. HO-1 mRNA values are expressed as fold of the untreated cell value. (B) Nuclear extract was prepared for the gel mobility shift assay using DNA fragment, including the Δ^{12}-PGJ$_2$-responsive element, as described in section 2. I and II represent the specific protein–DNA complexes. The results are representative of three independent experiments that yielded similar results.

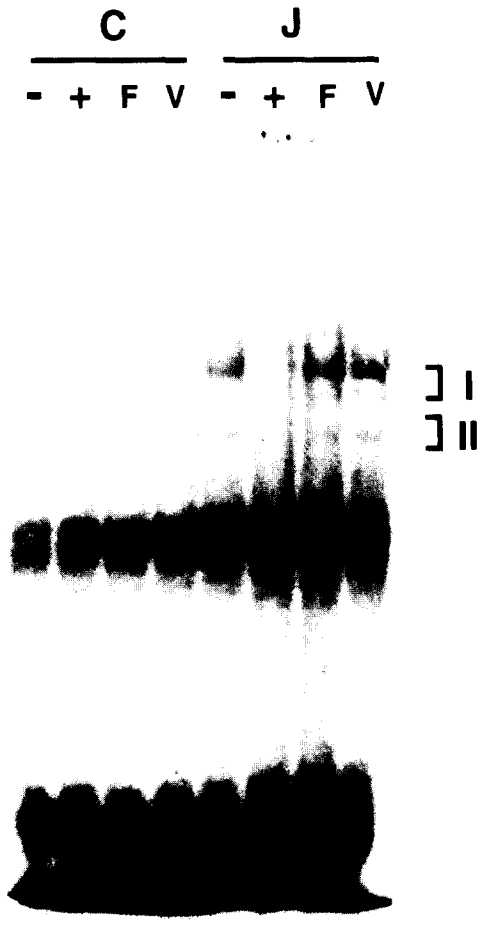

Fig. 3. Effect of phosphatase treatment on the Δ^{12}-PGJ_2-induced binding of the Δ^{12}-PGJ_2-responsive factors to the Δ^{12}-PGJ_2-responsive element. After cells had been treated with (J) or without (C) 10 μM Δ^{12}-PGJ_2 for 2 h, nuclear extracts were prepared. The nuclear extracts were incubated with the vehicle (−), 0.5 unit of calf intestinal phosphatase (+), the phosphatase and 1 mM sodium fluoride (F), or the phosphatase and 20 μM sodium vanadate (V). After incubation, DNA binding activity to the fragment, including the Δ^{12}-PGJ_2-responsive element, was measured in a gel mobility shift assay. I and II represent the specific protein–DNA complexes. The results are representative of three independent experiments that yielded similar results.

inhibition was blocked by the addition of phosphatase inhibitors, sodium fluoride and sodium vanadate, suggesting that the binding of the Δ^{12}-PGJ_2-responsive factors to the element is regulated by phosphorylation of the factors or phosphorylation of a nuclear component(s) which regulates the binding of the factors to the element.

These findings suggest that Δ^{12}-PGJ_2 stimulates phosphorylation of the Δ^{12}-PGJ_2-responsive factors, leading to the binding of the factors to the Δ^{12}-PGJ_2-responsive element and subsequent induction of HO-1 gene expression. In addition, we showed that Δ^{12}-PGJ_2-induced HSP70 synthesis was also sensitive to the protein kinase inhibitor (Fig. 1). The Δ^{12}-PGJ_2-induced HSP70 synthesis has been shown to be mediated through binding of heat shock factors to heat shock element located in the 5′-flanking region of the HSP70 gene [22,23]. The Δ^{12}-PGJ_2-induced binding of heat shock factors to heat shock element was also suppressed by 2-AP treatment in RBL-2H3 cells (data not shown). Thus, protein kinase(s) appears to be generally involved in the Δ^{12}-PGJ_2-induced gene expression through phosphorylation of the responsive transcription factors, including heat shock factors and the Δ^{12}-PGJ_2-responsive factors.

HSP70 and HO-1 belong to a stress protein family. A variety of stress proteins, including HSPs, are induced by compounds or conditions that perturb nascent protein synthesis [24]. Concerning regulatory mechanism for stress protein induction, a cDNA for a novel serine/threonine kinase, Ern 1p, has been recently isolated from yeast, and this kinase was shown to be essential for a set of specific stress gene expression induced by perturbation of nascent protein synthesis [25,26]. The Δ^{12}-PGJ_2-induced HSP70 gene expression has been shown to require de novo protein synthesis [22,23]. The Δ^{12}-PGJ_2-induced HO-1 gene expression also required de novo protein synthesis (data not shown). The requirement of de novo protein synthesis for the Δ^{12}-PGJ_2-induced gene expression suggests that Δ^{12}-PGJ_2 affects nascent protein synthesis, inducing expression of stress-related genes. Considering these findings, this type of protein kinase could be involved in the Δ^{12}-PGJ_2-induced HO-1 gene expression.

In summary, we report here that protein kinase is involved in the Δ^{12}-PGJ_2-induced HO-1 gene expression, and the site of action is on the Δ^{12}-PGJ_2-responsive factors which bind to the Δ^{12}-PGJ_2-responsive element. This study will facilitate elucidation of the molecular mechanisms underlying the actions of cyclopentenone PGs.

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