Differential selectivity of protein modification by the cyclopentenone prostaglandins PGA₁ and 15-deoxy-Δ^{12,14}-PGJ₂: Role of glutathione

Javier Gayarre, Konstantinos Stamatakis, Marta Renedo, Dolores Pérez-Sala*
Departamento de Estructura y Función de Proteínas, Centro de Investigaciones Biológicas, C.S.I.C., Ramiro de Maeztu, 9, 28040 Madrid, Spain

Received 2 August 2005; revised 20 September 2005; accepted 29 September 2005
Available online 6 October 2005

Abstract Cyclopentenone prostaglandins (cyPG) with antiinflammatory and antiproliferative properties have been envisaged as leads for the development of therapeutic agents. Because cyPG effects are mediated in part by the formation of covalent adducts with critical signaling proteins, it is important to assess the specificity of this interaction. By using biotinylated derivatives of 15-deoxy-Δ^{12,14}-PGJ₂ (15d-PGJ₂-B) and PGA₁ (PGA₁-B) we herein provide novel evidence for the differential selectivity of protein modification by distinct cyPG. The marked quantitative and qualitative differences in the binding of 15d-PGJ₂-B and PGA₁-B to cellular proteins were related to a differential reactivity in the presence of glutathione (GSH), both in vitro and in intact cells. Therefore GSH levels may influence not only the intensity but also the specificity of cyPG action.

© 2005 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Keywords: Prostaglandin; Posttranslational modification; Michael addition; Glutathione

1. Introduction

Cyclopentenone prostaglandins (cyPG) are eicosanoids which display varied biological activities, ranging from antiinflammatory effects to inhibition of cell proliferation and viral replication. Recently, these compounds have been the subject of considerable interest due to their potential use as therapeutic agents [1,2]. In vivo studies have shown beneficial effects of cyPG in several models of inflammation and tissue injury [3,4]. These eicosanoids exert their actions through complex mechanisms not completely elucidated. Some cyPG can act as agonists of the PPAR transcription factors [5]. In addition, cyPG can form covalent adducts with cellular thiols, both in glutathione (GSH) and in proteins, due to the presence of an unsaturated carbonyl group in the cyclopentenone moiety which confers a strong electrophilicity to neighboring carbons. Several protein targets for cyPG addition have been identified. Modification of functionally important sulphydryl groups in these proteins contributes to the biological effects of these prostanoids. Binding of cyPG to transcription factors NF-κB and AP-1 [6,7] inhibits DNA binding. Modification of cysteine residues in IKK [8], thioredoxin and thioredoxin reductase [9,10] also leads to inhibitory effects. In contrast, modification of H-Ras proteins correlates with activation of Ras-dependent pathways [11] and binding of cyPG to the protein Keap-1 results in activation of the transcription factor Nrf-2 and induction of genes involved in antioxidant defense [12]. Although the structure-activity relationship of cyPG has been addressed in several studies, little is known about the specificity of protein modification by these compounds. We and others have reported that protein modification by cyPG does not occur randomly, but affects defined cysteine residues within certain proteins [12,13]. It has been proposed that a low pH in the vicinity of the adduct may contribute to stabilize the C–S bond [1,14]. The reactivity of thiols can also be affected by steric factors. Therefore, structural determinants of either the protein or the cyPG may be important for the specificity of protein modification. We hypothesized that cyPG with diverse structure could selectively modify distinct protein targets in cells and/or modify some common targets with different potency. Characterization of the selectivity of protein modification by cyPG with different structure could then provide valuable information on the mechanism of action of these compounds and potentially aid in the design of cyPG targeting specific proteins. In this work we show the existence of common and selective targets for modification by biotinylated 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂-B) and biotinylated prostaglandin A₁ (PGA₁-B) in NIH-3T3 fibroblasts and explore the factors involved in differential selectivity.

2. Materials and methods

2.1. Materials

15-Deoxy-Δ^{12,14}-prostaglandin J₂ was from Calbiochem-Novabiochem (San Diego, CA) and from Cayman Chemical (Ann Arbor, MI). Biotinylated 15d-PGJ₂ was the generous gift of Dr. F. J. Canada (San Diego, CA) and from Cayman Chemical (Ann Arbor, MI). Biotinylated PGA₁ (N-9-oxo-15S-hydroxy-prosta-10,13E-1-dien-1-oyl-N-biotinoyl-1,5- diaminohexane) was from Cayman Chemical.

2.2. Incorporation of biotinylated cyPG into NIH-3T3 proteins

NIH-3T3 cells were grown in DMEM (Invitrogen) containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For treatments, cells were incubated with biotinylated cyPG for 2 h in serum-free medium. Cell lysates were obtained
by disrupting cells in 50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM β-mercaptoethanol, 0.5% SDS, 0.2 mM sodium vanadate, 50 mM FNa containing 2 µg/ml of each of the protease inhibitors: leupeptin, pepstatin A, and aprotinin and 1.3 mM Pefablock (Boehringer), as described [13]. Protein concentration was determined by the BCA assay (Pierce). Fifteen µg of protein from each experimental condition were electrophoresed on 12.5% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). In addition, 100 µg of protein were analyzed by 2D-electrophoresis as described [13]. Incorporation of biotinylated cyPG into proteins was assessed by Western blot and detection with horseradish peroxidase-conjugated streptavidin and ECL, as described [7].

2.3. Binding of biotinylated cyPG to proteins in vitro

Formation of covalent adducts of biotinylated cyPG with proteins in vitro was explored by using total lysates from NIH-3T3. Aliquots from cell lysates containing 6 µg of protein were incubated with biotinylated cyPG for 1 h at r.t. and analyzed by Western blot.

3. Results

3.1. Modification of NIH-3T3 proteins by 15d-PGJ2-B and PGA1-B

The dienone 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) and the single enone PGA1 are the most widely used cyPG in biological studies. Both eicosanoids have been reported to exert antiinflammatory and antiproliferative effects [6,8,15]. Here we have used biotinylated derivatives of 15d-PGJ2 (15d-PGJ2-B) and PGA1 (PGA1-B) (see Fig. 1A) to explore the existence of protein targets that could be selectively modified by either cyPG. Both compounds were cell permeable and formed adducts with proteins which were resistant to electrophoresis under reducing conditions (Fig. 1B). Interestingly, we noticed important quantitative and qualitative differences in the modification of cellular polypeptides by either cyPG. Incorporation of 15d-PGJ2-B was more efficient (Fig. 1B, lower panel). In addition, the labeling patterns obtained with the two biotinylated prostanoids were not identical, as evidenced in the densitometric profiles of the corresponding gel lanes (Fig. 2A). Some of the bands showing more prominent differences are marked by arrowheads. To verify these differences we performed 2D-electrophoresis (Fig. 2B). This analysis clearly showed the presence of biotin-positive spots common to 15d-PGJ2-B and PGA1-B-treated cells (marked by arrowheads), along with spots selective of either condition. Some of the spots which consistently showed selectivity or were labeled with different intensity are highlighted in boxes. These observations indicate that the efficiency and the selectivity of protein modification by PGA1-B and 15d-PGJ2-B in intact cells display important differences.

3.2. Binding of 15d-PGJ2-B and PGA1-B to NIH-3T3 proteins in cell-free extracts

The differences shown above could be due to several factors including disparities in the reactivity of the two prostanoids towards cellular thiols or in their intracellular accumulation. To explore these possibilities we first performed a labeling assay using cell-free extracts. As shown in Fig. 3, both the quantitative and qualitative differences in protein labeling observed in intact cells were markedly reduced when cell lysates were treated with 15d-PGJ2-B or with PGA1-B. These observations suggest that factors determined by cell integrity are important for the differential modification of proteins by cyPG.

3.3. Effect of GSH on cyPG binding to proteins

The intracellular concentration of GSH has been proposed to be an important factor for the biological effects of cyPG. We therefore explored whether GSH could affect the intensity or selectivity of protein modification by biotinylated cyPG. As stated above, labeling of proteins present in lysates was similar with the two cyPG in the absence of added GSH (Fig. 4). Addition of GSH at concentrations in the range of those present in cells clearly reduced the incorporation of biotinylated cyPG into proteins in cell-free lysates. Noteworthy, this inhibition was more marked in the case of PGA1-B. In addition, GSH reduced the incorporation of biotinylated cyPG more potently in certain polypeptide bands (marked by arrowheads in...
Fig. 4), and this resulted in different labeling patterns when the biotinylated cyPG was 15d-PGJ2-B or PGA1-B. These observations suggest that GSH levels may contribute to the differential modification of cellular proteins by 15d-PGJ2-B or PGA1-B. Therefore we tested whether GSH levels could influence cyPG protein modification in intact cells. Pre-treatment of NIH-3T3 fibroblasts with the GSH synthesis inhibitor buthionine sulfoximine (BSO), increased the intensity of protein labeling by biotinylated cyPG in a dose-dependent fashion (Fig. 5A and B). Interestingly, this increase was proportionally more marked in the case of low concentrations of PGA1-B (see quantitation in Fig. 5C). In addition, inhibition of GSH biosynthesis induced a more prominent increase in the labeling of some polypeptide bands, thus altering the labeling patterns. To better observe these differences we performed 2D-electrophoresis (Fig. 6). The intensity of several spots clearly increased after BSO treatment. Some of these spots are marked by arrowheads in Fig. 6. Conversely, pre-incubation of fibroblasts with N-acetyl-L-cysteine to increase cellular thiol content, reduced the incorporation of biotinylated cyPG into cellular proteins, more intensely in the case of PGA1-B (Fig. 5D). Taken together these observations suggest that a differential reactivity in the presence of soluble thiols, such as GSH, contributes to the selectivity of protein modification by cyPG.

4. Discussion

Biological actions of cyPG are due in part to their ability to covalently bind to cellular proteins. Several recent studies have sought to modify cyPG structure in order to obtain higher antiinflammatory or antiproliferative potency [16,17]. However, the specificity of protein modification by diverse cyPG has not been previously addressed. Here we have observed that 15d-PGJ2-B is more effective than PGA1-B at modifying cellular proteins. This is in agreement with the biological potency of their parent compounds. Typically, five to ten fold higher concentrations of PGA1 are required to elicit effects comparable to those of 15d-PGJ2 on IKK activity [8], NF-κB DNA binding...
or induction of apoptosis [19]. Our results also show that incubation of intact cells with either biotinylated analog results in a different pattern of polypeptide labeling, consisting of common and selectively modified bands. Several factors could contribute to this selectivity including different permeability or intracellular distribution or alterations in protein expression induced by the various treatments. Our results identify GSH levels as an important factor influencing the potency and selec-

Fig. 4. Effect of GSH on binding of biotinylated cyPG to proteins in vitro. (A) Cell lysates were incubated with 10 μM biotinylated cyPG in the absence or presence of 2 mM GSH and biotin labeling was detected as above. Polypeptides showing intense inhibition of cyPG incorporation in the presence of GSH are indicated by arrowheads. The blot shown is representative of four assays. (B) Densitometric profiles obtained for each lane.

Fig. 5. Modulation of cellular GSH levels affects binding of biotinylated cyPG to proteins in cells. Cells were pre-incubated with BSO (at 0, 50 or 100 μM) in serum-free medium for 16 h, before treatment with 15d-PGJ$_2$-B (A) or PGA$_1$-B (B) at the indicated concentrations for 2 h. Incorporation of biotinylated cyPG into cellular proteins was assessed by Western blot. The biotin signal was quantitated by image scanning and results are shown in (C). (D) NIH-3T3 cells were incubated with 5 mM N-acetyl-L-cysteine (NAC) for 16 h. Subsequent incubation with 15d-PGJ$_2$-B (5 μM) or PGA$_1$-B (60 μM), was performed in fresh medium without NAC. Results shown are representative of three experiments with similar results.
tivity of protein modification by biotinylated cyPG, in cells and in vitro. This could be related to the formation of GSH-cyPG adducts, a process which can occur through enzymatic and non-enzymatic mechanisms and results in a reduction in the availability of free cyPG for protein modification [14]. In cells, conjugation with GSH also favors cyPG detoxification [20]. These factors can lead to an attenuation of the biological effects of cyPG [20]. Conversely, GSH depletion has been reported to enhance cyPG effects [21,22]. As it is shown here, modulation of GSH levels also leads to opposite changes in the extent of protein modification by cyPG. Interestingly, our results show that PGA\textsubscript{1-B} binding to proteins is more dependent on GSH levels, both in vitro and in cells. This may be due to the fact that single enone cyPG, like PGA\textsubscript{1}, form more stable adducts with soluble thiols than dienone cyPG, like 15d-PGJ\textsubscript{2} [14]. This would shift the equilibrium towards GSH-cyPG adduct formation in the case of PGA\textsubscript{1-B}, with the consequences outlined above. Noteworthy, early evidence supported a differential subcellular fate of single enone and dienone cyPG, with single enone being present as soluble species in a higher proportion [23]. Although the interaction of cyPG with soluble thiols is reversible, the binding to cellular proteins has been proposed to be irreversible under physiological conditions [14,24]. Several factors have been postulated to contribute to the stability of cyPG-protein interactions, including the decreased molecular motion of protein biomarkers [24], the presence of low $pK_a$ cysteine residues with a high reactivity, and the nature of the amino acids in the vicinity of the target cysteines [1]. These factors could also affect the selectivity of protein modification. In summary, our findings illustrate the differential binding to proteins of distinct cyPG and postulate a role for their unique reactivity towards GSH in this phenomenon. Identification of the proteins susceptible to be modified by various cyPG will provide a deeper insight into the factors that determine their selectivity and help to ascertain whether target-specific cyPG can be designed.

Acknowledgments: This work was supported by grants from Ministerio de Educación y Ciencia (SAF2003-05713) and, in part, by Fundación La Caixa (04/179-01). J.G. and K.S. are recipients of fellowships from C.S.I.C. We thank Dr. F.J. Can~añ~ada for helpful comments and discussion and M. Jesús Carrasco for technical assistance.

References


