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Original Contribution

The glutathionylation of p65 modulates NF-κB activity in 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2-treated endothelial cells

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ABSTRACT

Protein glutathionylation is a posttranslational modification of cysteine residues with glutathione in response to mild oxidative stress. Because 15-deoxy-Δ12,14-prostaglandin J_2 (15 d-PGJ_2) is an electrophilic prostaglandin that can increase glutathione (GSH) levels and augment reactive oxygen species (ROS) production, we hypothesized that it induces NF-κB-p65 glutathionylation and would exert anti-inflammatory effects. Herein, we show that 15 d-PGJ_2 suppresses the expression of ICAM-1 and NF-κB-p65 nuclear translocation. 15 d-PGJ_2 upregulates the Nrf2-related glutathione synthase gene and thereby increases the GSH levels. Consistent with this, Nrf2 siRNA molecules abolish the inhibition of p65 nuclear translocation in 15 d-PGJ_2-induced endothelial cells (ECs). ECs treated with GSSG show increased thiol modifications of p65 and also a block in TNFα-induced p65 nuclear translocation and ICAM-1 expression, but not in IκBα degradation. However, the overexpression of glutaredoxin 1 was found to be accompanied by a modest increase in NF-κB activity. Furthermore, we found that multiple cysteine residues in p65 are responsible for glutathionylation. 15 d-PGJ_2 was observed to induce p65 glutathionylation and is suppressed by a GSH synthesis inhibitor, buthionine sulfoximine, by catalase, and by Nrf2 siRNA molecules. Our results thus indicate that the GSH/ROS-dependent glutathionylation of p65 is likely to be responsible for 15 d-PGJ_2-mediated NF-κB inactivation and for the enhanced inhibitory effects of 15 d-PGJ_2 on TNFα-treated ECs.

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The endogenous cyclopentenone 15-deoxy-Δ12,14-prostaglandin J_2 (15 d-PGJ_2) is the dehydration end product of prostaglandin D_2 and has been shown to display protective effects against injury and inflammation [1]. Although 15 d-PGJ_2 is a ligand for the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) [2], it has been postulated that 15 d-PGJ_2 may also modulate multiple cellular functions by mechanisms that are independent of PPARγ. These mechanisms independent of PPARγ show electrophilic activity-related biological effects, as it possesses an α,β-unsaturated carbonyl group in its cyclopentane ring. This electrophilic activity is likely to cause the activation of the transcription factor NF-E2-related factor-2 (Nrf2), a molecule that controls the expression of many thiol-regulating enzymes, including glutathione *S*-transferase and glutamylcysteine synthetase, which subsequently increases the intracellular glutathione (GSH) level [3,4]. However, 15 d-PGJ_2 also can directly form covalent adducts with the free thiols of GSH or thioredoxin to lower the intracellular GSH levels [5]. Our recent data have also indicated that these two opposing effects of electrophiles on the intracellular GSH concentration result in protein S-glutathionylation [6].

Glutathionylation is often considered to be a process that protects sensitive cysteinyl residues from irreversible oxidation. In addition to this protective effect, glutathionylation can also cause a temporary loss of protein function [1,7]. The glutathionylation of Ras activates its function, however, and leads to the downstream phosphorylation of Akt and p38, thereby increasing cell proliferation [8]. In contrast, the catalytic subunit of protein kinase A is inactivated through the glutathionylation of Cys^{199} [9]. Glutathionylation also plays a key role in the regulation of the kinase activity of MEKK1 and in the PKC-α pathway in response to oxidative stress [10,11]. Moreover, transcription factors that function in cell growth, differentiation, and apoptosis appear to be regulated by glutathionylation [12,13]. Through the introduction of a negatively charged GSH within their DNA-binding sites, glutathionylation also inhibits the DNA-binding activity of c-Jun and NF-κB [14,15]. Collectively, these data suggest that glutathionylation is a physiologically important mechanism that controls the activation of key signaling pathways.

Previous studies have found that electrophiles can give rise to thiyl radicals and, consequently, propagate protein glutathionylation [16,17]. In our previous studies, we reported that electrophiles, including chalcone, sulforaphane, carnosol, and cinnamaldehyde, show anti-inflammatory effects in endothelial cells (ECs) [18–21]. However, the mechanisms by which electrophiles modulate p65 function remain poorly defined. These electrophiles function as potent Michael reaction acceptors, which cause GSH depletion and subsequently increase mild oxidative stress [22,23]. On the other hand, electrophiles increase the GSH levels through Nrf2-related

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glutamylcysteine synthetase expression [24]. These two opposing effects of electrophiles on intracellular GSH and oxidative stress may result in the modulation of signal transduction by the sulfhydryl residues of proteins and hence exert anti-inflammatory effects that appear to be mediated through p65 glutathionylation and involve increased GSH and reactive oxygen species (ROS) interactions. In this study, we describe the phenomenon of 15 d-PGJ_2-induced p65 glutathionylation, which provides a common anti-inflammatory mechanism for electrophiles.

Materials and methods

Materials

The p65-green fluorescent protein (GFP) expression plasmid was a gift from Dr. J.A. Schmid (Department of Vascular Biology and Thrombosis Research, University of Vienna, Vienna, Austria). The plasmids expressing p65-GFP with cysteine-to-serine mutations at amino acids 38 (p65C38S), 120 (p65C120S), 160 (p65C160S), and 216 (p65C216S) were created by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA, USA). The ICAM-1/Luc [25], NF-κB/Luc [6], and pcDNA-His-GRX [6] vectors were constructed as described previously. 15-Deoxy-Δ12,14-prostaglandin J2 and CAY104105 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Bacterially derived TNFα was purchased from Calbiochem (San Diego, CA, USA). P65 antibodies were obtained from Stressgen Biotechnologies (San Diego, CA, USA). Antibodies raised against IκBα, GSH, and Nrf2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL reagents were purchased from Pierce (Rockford, IL, USA). Luciferase assay kits were purchased from Promega (Madison, WI, USA). Peroxidase-conjugated anti-rabbit and anti-mouse antibodies were obtained from Amersham (Arlington Heights, IL, USA) and nitrocellulose was obtained from Schleicher & Schuell (Dassel, Germany). Nrf2 short interfering RNAs (siRNAs) and nonsense siRNAs were purchased from Ambion (Austin, TX, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Endothelial cell cultures and transfection

The human endothelial cell line EA.hy926 (American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of air and 5% CO_2. Cells were grown in petri dishes for 3 days and allowed to reach confluence. The culture medium was then replaced with serum-free DMEM and the cells were incubated for a further 12 h before experimental treatment. For transient transfections, cells were grown to 80% confluence and transfected using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). After 24 h of recovery in 10% serum medium, the cells were then cultured in medium without serum for another 12 h before treatment.

GSH assay

GSH levels were determined using the method originally described by Kamencic et al. [26]. Briefly, cells were cultured at 37 °C in the presence or absence of specific treatment reagents, as indicated in the figures, washed twice with phosphate-buffered saline (PBS), and then incubated with monochlorobimane (2 mM) in the dark for 20 min at 37 °C. After two further washes with PBS, the cells were solubilized with 1% SDS and 5 mM Tris–HCl (pH 7.4). Fluorescence was measured by spectrofluorophotometry (Shimadzu; Rf-5301PC) with excitation and emission wavelengths of 380 and 470 nm, respectively. Samples were assayed in triplicate. The assay for detecting GSH levels in vitro was performed identically but without cell lysates. The levels of intracellular GSH were quantified using a GSH solution as a standard. Samples were assayed in triplicate.

Determination of GSSG level

The level of GSSG was determined using the method originally described by Tietze [27]. Briefly, ECs were harvested after treatment, washed twice, and resuspended in cold PBS. For GSSG analysis, the GSH scavenger 1-methyl-2-vinylpyridinium trifluoromethane sulfonate was added. GSSG levels were determined spectrophotometrically using GSH reductase-linked 5,5′-dithiobis(2-nitrobenzoic acid).

Detection of protein glutathionylation using biotin-labeled glutathione ester (BioGEE)

BioGEE (G36000; Invitrogen) is a cell-permeative biotinylated GSH that detects proteins that form adducts to reactive thiols by avidin–agarose pull down. These biotinylated proteins can be observed by SDS–PAGE [28]. The BioGEE mixture is then added to the cell culture medium at a final concentration of 100 μM. At designated time points, cell lysates are prepared, precleared with agarose beads, and then incubated with streptavidin-conjugated agarose beads (100 μl/mg protein) for 30 min at 4 °C to specifically bind protein–BioGEE complexes. After centrifugation and washing, the beads are incubated for 30 min with 10 mM dithiothreitol (DTT) in PBS/EDTA/SDS to elute proteins. Total glutathionylated proteins were detected by SDS–PAGE followed by silver staining.

RNA isolation and RT-PCR for detection of ICAM-1 and glutamate–cysteine ligase modulatory light subunit (GCLM) and heavy subunit (GCLC)

Total cellular RNA was extracted using the phenol–guanidinium isothiocyanate method [29]. Equal amounts (5 μg) of RNA from the various treatments were then reverse-transcribed for 50 min at 42 °C using 50 units of Superscript II (Invitrogen). Amplifications of the cDNA were performed in 25 μl of PCR buffer (10 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, and 0.1% Triton X-100, pH 9.0) containing 0.6 units of Taq DNA polymerase (Promega) and 30 pmol of specific primers for ICAM-1 (sense 5′-AGCAATGTGCAAGAAGATAGCCAA-3′ and antisense 5′-GGTCCCCTGCGTGTTCCACC-3′) and GAPDH (sense 5′-TATCGTGGAAGGACTCATGACC-3′ and antisense 5′-TACATGGCAACTGTGAGGGG-3′) [30]. The specific primers used for the amplification of GCLM and GCLC were as follows: GCLM, forward 5′-CAGCGAGGAGCTTCATGATTG-3′, reverse 5′-TGATCACAGAATCCAGCTGTGC-3′; GCLC, forward 5′-GTTCTTGAAACTCTGCAAGAGAAG-3′, reverse 5′-ATGGAGATGGTGTATTCTTGTCC-3′ [31]. Reaction products were separated electrophoretically on a 2.5% agarose gel and stained with ethidium bromide.

Luciferase reporter assay

For luciferase assays, the cell lysate was first mixed with luciferase substrate solution (Promega), and the resulting activity was measured using a luminometer. For each experiment, luciferase activity was determined in triplicate and normalized using β-galactosidase activity.

Transient transfection with siRNA targeting Nrf2

The siRNA sequences used in this study were as follows: human Nrf2, 5′-UCCCGUUUGUAGAUGACAA-3′ [32], and control siRNA, 5′-GCAAGCUGACCCUGAAGUUCAU-3′, purchased from Ambion. The effects of both siRNAs were demonstrated in our previous study [6].

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