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Research Paper

Rac1 modification by an electrophilic 15-deoxy $\Delta^{12,14}$-prostaglandin J_2 analog

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ABSTRACT

Vascular endothelial cells (ECs) are important for maintaining vascular homeostasis. Dysfunction of ECs contributes to cardiovascular diseases, including atherosclerosis, and can impair the healing process during vascular injury. An important mediator of EC response to stress is the GTPase Rac1. Rac1 responds to extracellular signals and is involved in cytoskeletal rearrangement, reactive oxygen species generation and cell cycle progression. Rac1 interacts with effector proteins to elicit EC spreading and formation of cell-to-cell junctions. Rac1 activity has recently been shown to be modulated by glutathiolation or S-nitrosation via an active site cysteine residue. However, it is not known whether other redox signaling compounds can modulate Rac1 activity. An important redox signaling mediator is the electrophilic lipid, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). This compound is a downstream product of cyclooxygenase and forms covalent adducts with specific cysteine residues, and induces cellular signaling in a pleiotropic manner. In this study, we demonstrate that a biotin-tagged analog of 15d-PGJ_2 (bt-15d-PGJ_2) forms an adduct with Rac1 in vitro at the C157 residue, and an additional adduct was detected on the tryptic peptide associated with C178. Rac1 modification in addition to modulation of Rac1 activity by bt-15d-PGJ_2 was observed in cultured ECs. In addition, decreased EC migration and cell spreading were observed in response to the electrophile. These results demonstrate for the first time that Rac1 is a target for 15d-PGJ_2 in ECs, and suggest that Rac1 modification by electrophiles such as 15d-PGJ_2 may alter redox signaling and EC function.

Introduction

Ras-Related C3 Botulinum Toxin Substrate 1 (Rac1) is a small GTP hydrolyzing (GTPase) protein in the Ras superfamily. Rac1 is ubiquitously expressed in many cell types, and regulation is both dynamic and contextual. Previous studies have described Rac1 dysregulation as a contributing factor in an array of different pathologies including cardiovascular disease and cancer [1–4]. Dysregulation of Rac1 in endothelial cells (ECs) may be important in disease initiation and progression during cardiovascular disease. Many studies have shown that Rac1 functions downstream of many cell surface receptors and is a major pathway by which ECs migrate and align in the direction of flow [5]. In addition, the modulation of EC migration is of interest in vascular restenosis, where normal EC migration and function are necessary for vascular repair after balloon angioplasty and stenting [6]. However, the mechanisms by which Rac1 can be dysregulated by vascular oxidative stress, and therefore contribute to vascular injury, are not clear.

Rac1 acts downstream of G-protein coupled receptors (such as those for sphingosine-1-phosphate and stromal cell-derived factor-1) and tyrosine kinase receptors (such as those for vascular endothelial growth factor and basic fibroblastic growth factor) and plays a major role in endothelial cell function [7–10]. Similar to other Ras GTPase proteins, Rac1 cycles between an active (GTP-bound) form and an inactive (GDP-bound) form, acting as a molecular switch dependent on the protein's GTP/GDP bound state

Abbreviations: 15d-PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; BAEC, bovine aortic endothelial cells; DMEM, Dulbecco's Modified Eagle's Medium; EC, endothelial cells; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; GST-A, glutathione agarose; GST, glutathione-S-transferase; PBS, phosphate-buffered saline; PIC, protease inhibitor cocktail; PBD, protein binding domain; SDS, sodium dodecyl sulfate; Ras-Related C3 Botulinum Toxin Substrate1 Aliases, cell migration-inducing gene 5 protein, TC-25, Ras-like protein TC25, Ras-related C3 botulinum toxin substrate 1, p21-Rac1, TC25

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[11]. In the GDP bound form, Rac1 is inactive and sequestered in the cytoplasm. Upon binding to GTP, Rac1 undergoes structural changes which allow for the interaction with cell type- specific effector proteins to elicit cellular responses. Rac1 nucleotide binding is mediated by three families of regulatory proteins including guanine exchange factors, guanine activating proteins, and guanine nucleotide dissociation inhibitors [12]. In addition to nucleotide binding, intracellular localization of Rac1 can impact the interaction of Rac1 with its effector proteins near the plasma membrane [13]. Plasma membrane localization is mediated via post-translational modifications of Rac1 including lipidation of cysteine residues. Like other members of the Rho family, Rac1 is lipidated at C189 by the 20-carbon geranylgeranyl group [14,15]. Additionally, the 16-carbon palmitoylation of Rac1 was recently described at C178; this lipidation dynamically regulates the localization of the protein to detergent resistant domains within the plasma membrane [16].

Since the discovery of Rac1, there has been growing interest in the ability of Rac1 to regulate and respond to the cellular reductive and oxidative (redox) environment. Such interest has stemmed from Rac1's direct interactions with enzymes involved in reactive species production and regulation, such as NADPH oxidase, nitric oxide synthase (NOS), and superoxide dismutase 1 (SOD1) [17]. Interestingly, a redox sensitive C18 in the GTP-binding pocket of Rac1 has been described to regulate its activity via both one electron or two electron oxidation mechanisms, via either a thiyl radical-dependent or as most recently described via S-glutathionylation-dependent mechanism [18,19].

An important redox signaling mediator produced during inflammation is the cyclopentenone prostanoid, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). This lipid is electrophilic and forms covalent adducts with specific cysteine residues, thereby mediating cellular signaling in a pleiotropic manner [20,21]. 15d-PGJ_2 has been described to accumulate in human atherosclerotic plaques and promote anti-inflammatory pathways. A well-described target of 15d-PGJ_2 is the Kelch-like ECH-associated protein 1 (Keap1) which regulates the cytoprotective transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) [22,23]. Interestingly, 15d-PGJ_2 has been observed to have biphasic effects in many cell types including ECs, and up-regulates reactive oxygen species [24]. In particular, 15d-PGJ_2 has been shown to inhibit migration in endothelial cells [25]. Since Rac1 can be redox-regulated and contributes to migration, we sought to determine whether Rac1 is a cellular target of 15d-PGJ_2. In particular, this work explores the site specific modification of Rac1 by 15d-PGJ_2 in vitro and suggests a correlation between Rac1 modification and the inhibition of migration and spreading in endothelial cells. Furthermore, this study highlights some important concepts regarding redox signaling by electrophiles and the role of the target proteins involved in electrophile dependent modulation of biological responses.

Materials

Primary bovine aortic endothelial cells (BAEC) were collected as previously described [26], or purchased (Lonza, Walkersville, MD). All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Methods

Recombinant Rac1 reactions with bt-15d-PGJ_2

Recombinant Rac1 (rRac1) protein was prepared as previously described [27]. Biotin tagged 15d-PGJ_2 (bt-15d-PGJ_2) was synthesized and purified as previously described [28]. rRac1 consisted of residues 1–192 (non-lipidated or cleaved). The protein was stored in 50% glycerol at −20 °C. rRac1 thiols were reduced with 1 mM DTT for 30 min on ice. DTT was removed by dialysis against a Chelex 100 resin (Bio-Rad)-treated buffer solution containing 50 mM Tris–HCl pH 7.5, 150 mM sodium chloride, 50 μM GDP, and 50 mM $MgCl_2$ (UltraPure) used for subsequent reactions and assays. The protein concentration of the dialyzed rRac1 was measured by BCA protein assay. Equimolar amounts of rRac1 were reacted with increasing molar ratios of bt-15d-PGJ_2 (0:1 to 5:1, lipid:protein) for 1 h at room temperature. This was 30 fmols (fmol) of Rac1 with 0.15, 30, or 150 fmol of bt-15d-PGJ_2 in a total volume of 100 μl. Unreacted bt-15d-PGJ_2 was quenched using β-mercaptoethanol (β-ME) at a final concentration of 10 μM. Biotinylated lipid adducts on rRac1 were detected either by western blot or mass spectrometry as described below.

Mass spectrometry of rRac1 adducts

Approximately 1 μg of rRac1 treated with increasing amounts of bt-15d-PGJ_2 was denatured, reduced, then digested using sequencing grade trypsin (Promega, Madison, WI). Digested peptides were loaded onto a self-prepared 11-cm,100-μm-diameter pulled tip packed with Jupiter 5-μm C18 reversed-phase beads (Phenomenex, Torrance, CA). Samples were analytically separated via nanoLC by use of an Eksigent MicroAS autosampler and 2D LC nanopump (Eksigent, Dublin, CA) via two different methods. For each method, tryptic peptides were separated by liquid chromatography using a gradient of acetonitrile containing 0.2% formic acid and eluted tryptic peptides were electrosprayed at 2 kV into a dual linear quadrupole ion trap Orbitrap Velos (Orbitrap) mass spectrometer (Thermo Fisher Scientific, San Jose, CA). In the first method samples were analyzed making use of collision-induced dissociation (CID), and in the other method, samples were analyzed using high-energy collision dissociation (HCD) for the MS/MS scans. Briefly, the mass spectrometer was set to switch between an Orbitrap full scan (m/z 300–1800) followed by successive MS/MS scans of the 10 or 15 most abundant precursor ions (parent ions). The dynamic exclusion setting was set to exclude ions for 2 min after a repeat count of three within a 45 s duration. Thermo Xcalibur RAW files were converted to mzXML files using the converter ReAdW program, then to. MGF files with the transproteomic pipeline tools software suite. For identification of rRac1 the search engines TurboSEQUEST (Thermo, Fisher Scientific) and MASCOT 2.2 (Matrix Biosciences). SEQUEST and MASCOT searches used the latest available UniRef100 database. Parent ion mass accuracy window was set to 10.0 ppm. For rRac1 modification by bt-15d-PGJ_2 the search included the mass addition of 626.387 Da on a cysteine residue. SEQUEST searches were processed and visualized using Scaffold (Proteome software Inc., Portland, OR) with the addition of searching with X!Tandem on the Scaffold software. Manual validation of parent ions was aided by the online program Protein prospector (MS-product feature) to produce lists of daughter ions for select peptides and modified peptides. Cysteine containing peptides were searched for as non-modified peptides, carboxyamidomethyl cysteine (CAM; iodoacetamide treatment results in the addition of monoisotopic mass 57.021 to Cys), and bt-15d-PGJ_2 (addition of 626.387 to Cys).

Cell culture

BAEC were cultured in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Herndon, VA) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA) and supplemented with 5.6 mM D-glucose, 4 mM glutamine and 100 μ/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY). Cells were used