Rac1 modification by an electrophilic 15-deoxy Δ^{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-Δ^{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 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.
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 interact 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 a single electron or two electron oxidation mechanisms, via either a thiol 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-Δ12,14-prostaglandin J2 (15d-PGJ2). This lipid is electrophilic and forms covalent adducts with specific cysteine residues, thereby mediating cellular signaling in a pleiotropic manner [20,21]. 15d-PGJ2 has been described to accumulate in human atherosclerotic plaques and promote anti-inflammatory pathways. A well-described target of 15d-PGJ2 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-PGJ2 has been observed to have biphasic effects in many cell types including ECs, and up-regulates reactive oxygen species [24]. In particular, 15d-PGJ2 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-PGJ2. In particular, this work explores the site specific modification of Rac1 by 15d-PGJ2 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-PGJ2

Recombinant Rac1 (rRac1) protein was prepared as previously described [27]. Biotin tagged 15d-PGJ2 (bt-15d-PGJ2) 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 thiol were added 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 MgCl2 (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-PGJ2 (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-PGJ2 in a total volume of 100 μl. Unreacted bt-15d-PGJ2 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-PGJ2 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 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-PGJ2 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-PGJ2 (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 μg/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY). Cells were used

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Methods

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Recombinant Rac1 (rRac1) protein was prepared as previously described [27]. Biotin tagge...
between passage 5 and 9 and were cultured in 6-well plates to confluence. For treatments, media was replaced with low-serum DMEM (0.5% FBS) for 16 h prior to addition of either 15d-PGJ2 or bt-15d-PGJ2, as described previously [29]. Treatment with lipid is expressed as a concentration but may be converted to amount lipid per cell, since in all experiments 2 ml of media is used in 6-well plates with confluent BAEC; we estimate that, the treatment of 10 μM bt-15d-PGJ2 is equivalent to approximately 20 fmol/cell in our conditions. Unless noted otherwise, cells were washed once prior to treatment with ice cold phosphate buffered saline (PBS) and lysed with RIPA lysis buffer [50 mM Tris–HCl, pH 7.4, 0.5% w/v sodium deoxycholate, 1% v/v SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% v/v NP-40], and protease inhibitor cocktail (PIC, Roche Diagnostics, Indianapolis, IN). Lysates were cleared by centrifugation at 16,862 g unless noted otherwise. Protein concentrations were measured by DC Lowry protein assay (BioRad, Hercules, CA) adapted for microplates. Cell viability was measured by lactate dehydrogenase release assay as previously described [30].

### Biotin affinity precipitation

Affinity precipitation of biotin was performed using Neutavidin resin. Briefly, BAEC were treated at various times and concentrations of bt-15d-PGJ2 as described in the results. After treatment, BAEC were lysed and cleared by centrifugation. Approximately, 25 μl settled resin (50 μl of 50% slurry; Thermo Scientific) was used for each experiment and was loaded into Micro Bio-Spin columns (Bio-Rad, Hercules, CA). The resin was equilibrated with RIPA buffer prior to loading protein according to the concentration of bt-15d-PGJ2 (see Table S2 for resin load in frequency). Volumes were adjusted with RIPA lysis buffer to normalize protein concentration to 1 ng/ml and incubated overnight at 4 °C on a shaker. Resin was washed six times with 200 μl volumes of RIPA lysis buffer. Resin was transferred to a fresh tube in three volumes of 200 μl RIPA and allowed to settle by gravity, and supernatant was removed. Bound proteins were eluted by heating at 80 °C for 10 min in 1.5 fold volume of 2 × sample buffer (0.1 M Tris–HCl, 4% SDS, 10% Glycerol, 0.2% Bromphenol blue, pH 6.8, containing 2% β-mercaptoethanol) on settled resin by heating at 80 °C for 10 min. Within 5 min after heating, resin was briefly centrifuged and supernatants were collected to a fresh tube for analysis by SDS-PAGE or stored at –20 °C for later analysis.

### Rac1 activity assay

A plasmid encoding a glutathione-S-transferase fusion protein with Rac1 p21 protein binding domain (GST-PBD) was a generous gift from Dr. Rakesh Patel (University of Alabama at Birmingham), and protein was expressed and purified as previously described [31]. The Rac1 activity assay was performed as previously described with minor changes [32]. Briefly, BAEC were grown to 80% confluence in 6-well plates. Media was changed to low-serum DMEM (0.5% FBS) for 16 h, then cells were treated with bt-15d-PGJ2 or EtOH vehicle for 4 h. Cells were detached by 0.05% Trypsin (Gibco, Grand Island, NY) for 3 min, followed by neutralization of trypsin with 1 ml of DMEM (10% FBS). Cells were pelleted and resuspended in DMEM (10% FBS). Cells were replated at a threefold dilution and were evaluated after both 30 min and 2 h. For each condition, 4 images were acquired and analyzed by counting the total number of cells per field (10 × magnification), and the number of cells spread/field. Data are expressed as cells spread (%) and values were calculated as (number of cells spread/total number of cells) × 100.

### Cell spreading assay

Cells were seeded at a density of 2 × 10⁵ cells/well and allowed to reach 70–80% confluence in 10% serum DMEM. Media was changed to low-serum DMEM (0.5% FBS) for 16 h, then cells were treated with bt-15d-PGJ2 or EtOH vehicle for 4 h. Cells were detached by 0.05% Trypsin (Gibco, Grand Island, NY) for 3 min, followed by neutralization of trypsin with 1 ml of DMEM (10% FBS). Cells were pelleted and resuspended in DMEM (10% FBS). Cells were replated at a threefold dilution and were evaluated after both 30 min and 2 h. For each condition, 4 images were acquired and analyzed by counting the total number of cells per field (10 × magnification), and the number of cells spread/field. Data are expressed as cells spread (%) and values were calculated as (number of cells spread/total number of cells) × 100.

### Western blot analysis

For all lysate samples, protein concentrations were measured via Lowry, DC and loaded by equal protein amounts onto 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Transferred protein and amount loaded was confirmed with Ponceau S stain. Membranes were blocked with 5% nonfat milk in TBS-T. Rac1 antibody (Millipore, Billerica, MA) or β-actin antibody (Cell Signaling) was used at a dilution of 1:3000 or 1:5000, respectively, in 0.5% milk TBS-T. Anti-mouse IgG-HRP (Amersham, Amersham, UK) secondary antibody was used at 1:10,000 in TBS-T for Rac1 and Anti-rabbit IgG-HRP (Amersham) secondary antibody was used at 1:10,000 in TBS-T for β-actin. In order to detect biotin, blots were incubated with 10 ml of Streptavidin-HRP (1:10,000 in TBS-T) for 1 h. HRP-conjugated signal was visualized using chemiluminescence using SuperSignal West Dura (Thermo Scientific, San Jose, CA), detected using a FluorChem M camera imaging system (ProteinSimple). Protein bands were quantified by analysis of pixel density using Alpha ViewSA version 3.4.0 software (ProteinSimple, San Jose, CA).
Statistical analysis

Data are reported as mean ± S.E.M. and sample sizes indicated in the legends. Statistical significance was evaluated by either standard two-tailed student t-test or via one-way or two-way ANOVA (analysis of variance) among the groups using Graph pad (version 5.0). The minimum level of significance was set at p < 0.05. The least significant difference (LSD) test was used as a post hoc test for the significant difference among the groups.

Results

Determination of Rac1 modification by 15d-PGJ2 in vitro

In order to follow modification of proteins by 15d-PGJ2, a biotin-tagged analog (bt-15d-PGJ2) was used. The chemical structure is shown in Fig. 1A. The tagged analog consists of the parent 15d-PGJ2 molecule, a linker region and a biotin tag. Bt-15d-PGJ2 forms covalent adducts to cysteine residues via the two electrophilic carbons (indicated by asterisks), and protein adducts have been detected using the biotin tag [29,34–38]. Purified recombinant Rac1 (rRac1) protein was used to assess modification by bt-15d-PGJ2 in vitro. When rRac1 was treated with equimolar amounts of bt-15d-PGJ2, a biotin signal was detected by western blot at the expected molecular weight of rRac1 (21 kDa), whereas no signal was detected in the untreated rRac1 (Fig. 1B). The treated rRac1 was also applied to nitrocellulose by dot blot in order to determine the protein modification by increasing lipid concentration. Interestingly, rRac1 modification by bt-15d-PGJ2 did not increase linearly with concentration, since maximal modification occurred at 0.5:1 (bt-15d-PGJ2: rRac1) (Fig. 1C).

Identification of sites of Rac1 modification in vitro by mass spectrometry

To determine the sites of adduct formation on rRac1, rRac1 was treated with bt-15d-PGJ2 (1:1 to 1:5 ratio, mol protein:mol lipid) and then analyzed by a series of high resolution mass spectrometry experiments to identify peptide adducts. After reacting rRac1 with lipid, the tryptic peptides were subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS; as described in the methods). Fragmentation of peptides was accomplished using collision induced dissociation (CID) or high-energy collision dissociation (HCD) from the same samples in order to maximize protein coverage. The high resolution LC–MS/MS analyses were searched by use of the SEQUEST and MASCOT search algorithms. As expected, the protein was identified as human Rac1 (UniProt entry P63000) using both search engines (Table S1). Table S1 details the peptide fragmentation method, percent coverage, and search scores (for potential bt-15d-PGJ2 peptide adducts) for each reaction condition. These results also indicated modification by bt-15d-PGJ2 occurs on two cysteine residues of Rac1.

In order to further investigate these cysteine adducts, high resolution broad band mass spectra (MS1) features were examined and manual interpretation of the MS/MS spectra was done using theoretical MS/MS peak lists with the Protein Prospector MS Product tool. Fig. 2 shows tandem mass spectra of parent ions from carbamidomethyl ([613.321]⁺, top spectrum) and bt-15d-PGJ2 adducts ([803.109]⁺, bottom spectrum) on the C157 containing peptide [148EIGAVKYLEC157SALTQR163] obtained from the 1:1 reaction by HCD. Each spectrum shows the relative abundance of the daughter ions for the respective parent ion. The parent ion, [613.321]⁺ (monoisotopic mass 1836.940 Da), was the exact mass adduction of carbamidomethyl to C157, indicating a carbamidomethyl adduct (CAM) formed by reaction of Rac1 with iodoacetamide. Daughter ions were assigned to the peptide via a sequential series of singly charged y-ions, y3–y11 (Fig. 2, top panel).

A potential adduct on the C178 peptide was identified in the MS1 high resolution spectra, but we were unable to verify the addition by MS/MS. Several proteolytic fragment ions were detected with masses consistent with addition of bt-15d-PGJ2. Manual interpretation of the MS/MS spectra could only identify the peptide sequence, but the fragmentation was not sufficient to...
unambiguously assign the bt-15d-PGJ2 adduct on the cysteine (data not shown). These results suggest that C178 is likely a target of adduct formation due to the unique mass of the adduct formed, but this site could not conclusively be confirmed. The fact that other sides of adduct formation were not detected does not rule out these sites, but if they were modified it was well below our limit of detection.

Modification of Rac1 in endothelial cells

Our group and others have previously demonstrated that 15d-PGJ2 modifies a discreet electrophile responsive proteome in multiple cell types [29,39,40]. However, whether 15d-PGJ2 modifies Rac1 in endothelial cells is not known. Primary bovine aortic endothelial cells (BAECs) were treated for 24 h with increasing concentrations of 15d-PGJ2. Cytotoxicity was not observed up to 5 μM 15d-PGJ2 (10 fmol/cell), whereas viability decreased to 78% and 24% at 10 and 20 μM 15d-PGJ2 (20 and 40 fmol/cell), respectively (Fig. S1). These results are consistent with our previous reports of 15d-PGJ2 effects on viability in endothelial cells [30].

Rac1 modification in endothelial cells was assessed by affinity precipitation using the biotin analog of 15d-PGJ2, bt-15d-PGJ2. We have previously shown that bt-15d-PGJ2 elicits similar cellular effects as 15d-PGJ2 at similar concentrations in low serum conditions [28]. Fig. 3 shows that total Rac1 levels did not change in response to the lipid over 4 h (Fig. 3A and B), and Rac1 was only detected by affinity precipitation in the presence of bt-15d-PGJ2 (Fig. 3A and C), demonstrating that Rac1 is modified by the lipid. These data represent the first evidence that the GTPase protein, Rac1, is modified by 15d-PGJ2 in endothelial cells.
modified Rac1 or β-actin was expressed as a percentage of modification from 10 μM then plotted as a function of bt-15d-PGJ2. This plot suggests that Rac1 modification increases in a linear manner up to 1 μM bt-15d-PGJ2, whereas a higher percentage of β-actin protein is modified by bt-15d-PGJ2 at lower concentrations.

Since Rac1 activity was altered in response to bt-15d-PGJ2 adduct formation, the effects of the electrophile on endothelial cell functions which are downstream of Rac1 were determined. First, endothelial cell migration was monitored in response to 15d-PGJ2 using a scratch assay. Shown in Fig. 5A are representative images of vehicle and 10 μM 15d-PGJ2-treated BAEC just after scratching and after 8 h. The migration of endothelial cells was inhibited significantly by 5–10 μM 15d-PGJ2 (Fig. 5B). Next, Rac1 is crucial for the formation of cellular protrusions (e.g. lamellipodia) such as those formed by adherent cells just after replating. Therefore, cell spreading was assessed in response to bt-15d-PGJ2. Fig. 5C shows representative images 2 h after replating. An example of a spread cell is marked a white arrow and is shown in the inset having a protrusion(s) extending from the cellular body. Cells treated with bt-15d-PGJ2 which had not spread were characterized by a round shape with a bright halo. The percent cells spread was plotted as a function of increasing concentrations of bt-15d-PGJ2 in Fig. 5D. Significant inhibition of cell spreading was observed at 10 μM bt-15d-PGJ2 compared to vehicle control.

**Discussion**

Electrophilic lipids are signaling mediators which elicit a coordinated biological response through the direct modification of protein nucleophiles. It is becoming increasingly clear that electrophilic lipids have additional advantages as signaling mediators since they form covalent adducts allowing the effect of adduction to persist and accumulate over time. In particular the electrophilic lipid, 15d-PGJ2, is a cyclooxygenase 2 (Cox2)-derived electrophilic lipid which is formed endogenously downstream of PGE2, and has been described as an anti-inflammatory mediator in macrophages and other cell types [39,41,42]. 15d-PGJ2 has also been found in atherosclerotic lesions colocalized with Cox2 [41]. Biological responses of 15d-PGJ2 have been reported to occur due to multiple mechanisms of action and via direct modification of protein thiols [40]. The formation of these protein adducts has been hypothesized to elicit specific biological responses via coordinated ad- duction to the electrophilic responsive proteome [43]. Some previously described members of 15d-PGJ2 modified proteome are β-actin, Keap1, and H-Ras. For example, Renedo et al. reported that two electrophilic lipids, PGA1 and 15d-PGJ2, modified H-Ras at two different cysteine residues [44]. Interestingly, modification of H-Ras by 15d-PGJ2 at C184 in the c-terminal hypervariable domain may alter palmitoylation of the protein and may thereby alter downstream H-Ras function via interacting with effector proteins [35]. The fact that H-Ras is modified by bt-15d-PGJ2 suggests that other GTPase proteins such as Rac1 may also be targets for the lipid. In fact, Rac1 is poised to be an important mediator of redox signaling, due to its role in a wide variety of cellular functions including reactive oxygen species formation via Nox, cellular migration, and modulation of downstream signaling pathways.

Rac1 contains seven Cys residues which are highly conserved across species and therefore, may have important physiological functions. The C189 residue on Rac1 has been well characterized, and has been demonstrated to be a site of geranylageryl addition. This modification occurs in the endoplasmic reticulum immediately following protein translation as a permanent modification, suggesting that this site not available for redox signaling. It was recently shown that Rac1 is palmitoylated at C178 [16].
PGJ2 is a relatively large hydrophobic molecule and may be sterically hindered from interaction with residues within the binding pocket, or may preferentially interact with more hydrophobic regions of the protein.

Unlike geranylgeranyl modification, palmitoylation is a reversible lipidation which allows for the transient association of a protein to detergent resistant membranes and is thereby an important regulator of localization [45,46]. Interestingly, Navarro-Lérida et al. further demonstrated that inhibiting palmitoylation by mutation C178S decreased cellular migration. Another cysteine residue of Rac1, C18, is located within the guanine nucleotide binding pocket. This residue has been shown to be modified by the nonradical oxidant glutathione, and guanine nucleotide dissociation has been shown to be increased both S-glutathionylation and by radical oxidants such as •NO2 and superoxide [18,19,47]. C18 was not found to be modified by 15d-PGJ2 in our studies. Modification of C18 by 15d-PGJ2 cannot fully be excluded, since tryptic peptides containing C18 were very large (>20 residues in length), and therefore, the addition of a lipid may have prevented the modified peptide from being detected. Although this residue has a relatively high solvent accessibility compared with other cysteines [47], 15d-PGJ2 is a relatively large hydrophobic molecule and may be sterically hindered from interaction with residues within the binding pocket, or may preferentially interact with more hydrophobic regions of the protein.

In this study, we show for the first time that C157 and C178 of Rac1 are modified by 15d-PGJ2 in vitro. C157 is located in a loop between the sixth β-strand and the α5 helix (referred to as the G-5 region) [11]. It has been recently shown that mutation of C157, a residue unique to Rho proteins, impairs the interaction of the Rho mutant protein with its guanine nucleotide exchange factor [48]. However the precise role of C157 in Rac1 function in endothelial cells is still not clear. The other modification observed in our study was at C178. This site has been shown to be palmitoylated. Interestingly, previous studies showed that mutation of this residue inhibited Rac1 localization, reduced spreading and delayed cellular migration [16]. Under our conditions (7.5 μM bt-15d-PGJ2) we found that Rac1 activity was increased at 1 h, but returns to baseline at 2 h (Fig. 4B). The fact that overall protein adducts increase over this time scale (Fig. 4A), suggests that increases in Rac1 activity are disproportionate. It is intriguing to speculate based on our recombinant studies, that there may be two distinct regulatory sites acting as allosteric mediators of Rac1 activation observed in our study.

The overall effects of 15d-PGJ2 on cells is dependent on several factors, including amount of lipid, time of exposure, and cell type and presence of effector proteins. It has been reported that sub-nanomolar concentrations of 15d-PGJ2 can stimulate cellular migration of eosinophils in response to a chemoattractant [49]. Other studies report inhibition of cellular migration in multiple cell types in response to micromolar levels of 15d-PGJ2 [33,50]. This biphasic phenomenon of 15d-PGJ2 may be explained by the accumulation...
of 15d-PGJ2 protein adducts on specific target residues over time, as described by Oh et al. [29]. In addition, it is known that adduct formation occurs on a number of target proteins simultaneously, and therefore, the observed inhibition of migration and spreading in response to 15d-PGJ2 may not be solely due to Rac1 modification. In fact, as noted in our studies, other cytoskeletal proteins such as β-actin are sensitive targets for 15d-PGJ2 modification [33,36,51]. Currently, methods of studying electrophile-proteomes are limited. Based on what we know, cytoskeletal proteins including those in the Ras superfamily may be important members of this proteome.

In conclusion, we have shown that rRac1 residues C157 and C178 are modified by bt-15d-PGJ2. Adduct formation with other cysteine residues, particularly C18 which is a likely target for redox modification, cannot be ruled out by these results. Altogether, these results suggest that modification of Rac1 by electrophilic lipids may be an additional axis of Rac1 regulation not previously described, and future studies are warranted.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2015.01.016.

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