Alkylation of the Tumor Suppressor PTEN Activates Akt and β-Catenin Signaling: A Mechanism Linking Inflammation and Oxidative Stress with Cancer

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Abstract

PTEN, a phosphoinositide-3-phosphatase, serves dual roles as a tumor suppressor and regulator of cellular anabolic/catabolic metabolism. Adaptation of a redox-sensitive cysteinyi thiol in PTEN for signal transduction by hydrogen peroxide may have superimposed a vulnerability to other mediators of oxidative stress and inflammation, especially reactive carbonyl species, which are commonly occurring by-products of arachidonic acid peroxidation. Using MCF7 and HEK-293 cells, we report that several reactive aldehydes and ketones, e.g. electrophilic α,β-enals (acrolein, 4-hydroxy-2-nonenal) and α,β- enones (prostaglandin A2, 12-14-prostaglandin J2, and 15-deoxy-Δ12,14-prostaglandin J2) covalently modify and inactivate cellular PTEN, with ensuing activation of PKB/Akt kinase; phosphorylation of Akt substrates; increased cell proliferation; and increased nuclear β-catenin signaling. Alkylation of PTEN by α,β-enals/enones and interference with its restraint of cellular PKB/Akt signaling may signal hyperplastic and neoplastic disorders associated with chronic inflammation, oxidative stress, or aging.

Introduction

Inflammation and cancer are intricately linked [1,2]. ‘Smoldering’ inflammation [3], also called para-inflammation [4], occurs in many types of pre-malignant and malignant tumors, e.g. colorectal adenoma and adenocarcinoma where the content of inflammatory leukocytes and the inflammatory enzyme cyclooxygenase-2 (COX-2) influence progression, prognosis and survival [5,6]. Non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit COX-2 may prevent certain, but not all, cancers [7]; and some NSAIDs, such as sulindac sulfone, act independently of COX and prostaglandin E2 (PGE2) inhibition [8]. Other NSAIDs, e.g. celecoxib, can paradoxically enhance tumor progression in APCMin/+ mice, which model intestinal tumorigenesis [9]. While COX-2 and its metabolite PGE2 are undoubtedly important, para-inflammation may enhance tumorigenesis by mechanisms that are incompletely understood. Innate immune mechanisms are prime candidates for investigation.

Usually, innate immune inflammation consists of a “wounding” phase to annihilate pathogens, and a “healing” phase to repair and regenerate damaged host tissue [10]. The transition between phases depends on gradual exhaustion of inflammatory mediators and conversion of certain pro-inflammatory mediators, e.g. PGD2, into anti-inflammatory metabolites, Δ12-PGJ2 [11,12,13]. Elements of the inflamed site itself, e.g. reactive oxygen species (ROS), albumin, fibroblasts and neutrophils, orchestrate this conversion [14,15,16,17]. For example, reactive oxygen species (ROS) cause non-enzymatic peroxidation of essential fatty acids, like arachidonic acid (AA) [18]. AA hydroperoxides transform readily into reactive products containing an α,β-unsaturated carbonyl [19,20] that include acrolein (2-propenal) [21,22], 4-hydroxy-2-nonenal (4-HNE) [23], and cyclopentenone prostaglandins (cyPGs), PGE2 and Δ12-PGJ2 [24]. Covalent modification of NFκB and IKKα/β proteins by these α, β-unsaturated carbonyl metabolites (i.e. protein alkylation) seems to be a “switch” to terminate inflammation [25]. Following this precedent, we hypothesized that alkylation may also act as a “switch” to initiate repair and regeneration of tissue damaged by inflammation.

PTEN (phosphatase tensin homolog on chromosome 10) is a phosphoinositide-3-phosphatase with two physiological roles: tumor suppressor and regulator of anabolic/catabolic cell signaling. The PTEN gene is frequently mutated or inactivated in advanced cancers [26]. Using MCF7 and HEK-293 cells, we report that reactive α, β-unsaturated carbonyls (acrolein, 4-HNE, and Δ12-PGJ2) inactivate the PTEN protein – not the gene - by alkylation. Inactivation of PTEN by α, β-unsaturated carbonyls leads to increased Akt signaling, enhanced nuclear β-catenin signaling, and augmented cellular proliferation. Redox signaling by PTEN may have evolved to enable cells (tissues) to stratify their response to oxidative stress. For example, transient inhibition of...
PTEN by reactive oxygen or carbonyl species, and the corresponding signaling through Akt/GSK3β/β-catenin/TCF4/Lef1 might benefit the host via increasing proliferation and regeneration of tissue damaged by acute inflammation or oxidative stress. Errant and persistent PTEN inactivation by the same molecular mechanism might favor tumor progression and provide an etiological link between ‘smouldering’ inflammation and certain cancers, especially colorectal cancer, where both the PTEN and the APC tumor suppressors restrict nuclear β-catenin signaling [27].

**Results**

The α, β–unsaturated carbonyls acrolein, 4-HNE and Δ12-PGJ2 covalently modify cellular PTEN

We exposed MCF-7 cells to representative α, β–unsaturated carbonyl (Figure 1C) or H₂O₂, then selectively tagged any proteins that had oxidized or carbonylated thiols using NEM-biotin (Figure 1A). We then sequestered proteins with a biotin epitope onto NeutrAvidin (NA) beads and identified carbonylated PTEN by SDS-PAGE and immunoblotting. MCF-7 cells treated with 10 μM Δ12-PGJ₂, 4-HNE, or acrolein contained carbonylated PTEN in amounts comparable to cells treated with 100 μM H₂O₂ (Figure 1A, NA pulldown). This method does not distinguish between oxidized and carbonylated thiols on PTEN. However, electrophoresis under non-reducing conditions, followed by western blotting, showed that PTEN migrated as a discrete isoform due to an oxidized disulfide [28,29], which occurred only in cells treated with 100 μM H₂O₂, but not in cells treated with α, β–unsaturated carbonyl (Δ12-PGJ₂, 4-HNE, acrolein) or 15-HpETE, a lipid hydroperoxide (Figure 1B).

Cyclopenteneone PG-biotin analogs are model α, β–enones that alkylate PTEN

The cysteinyl thiolate in the PTEN active site (–HC(X5)RT–) is prone to oxidation because it is a strong nucleophile, pKa ~5.

**Figure 1.** α, β–unsaturated carbonyls covalently modify cellular PTEN. (A) Diagram of the procedure to identify PTEN with an oxidized or alkylated thiol in cells exposed to ROS or α, β–unsaturated carbonyls. The anti-PTEN immunoblot shows oxidized or carbonylated PTEN (NA Pulldown) relative to total PTEN (Cell Lysate) isolated from MCF-7 cells treated 30 min with vehicle (DMSO), 10 μM Δ12-PGJ₂ or 4-HNE versus 10 min with 100 μM H₂O₂; an immunoblot from a separate experiment shows oxidized, carbonylated and total PTEN in cells treated for 30 min with vehicle or 20 μM acrolein versus 10 min with 100 μM H₂O₂. (B) Anti-PTEN immunoblot of MCF-7 cell lysates fractionated by SDS-PAGE under non-reducing conditions. PTEN oxidized to a Cys124-Cys71 disulfide appears as a faster migrating species (PTEN oxidized disulfide) only in cells treated with H₂O₂. This species was undetectable in MCF-7 cells treated 30 min with DMSO vehicle or 20 μM each Δ12-PGJ₂, 4-HNE, acrolein or 15-HpETE. (C) Chemical structures of typical α, β–unsaturated carbonyls. Acrolein and 4-HNE are α, β enals; Δ12PGJ₂ is an α,β enone. Electrophilic β carbons are denoted with δ−. Blots are representative of results obtained in at least three independent experiments.

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This trait should also facilitate alkylation of PTEN by α, β-unsaturated carbonyls. We used CyPG-biotin analogs, which have an electrophilic β-carbon capable of nucleophilic addition (Michael reaction), as chemical models to test this hypothesis [30]. Alkylation of any cellular proteins by these analogs would introduce a biotin epitope de novo (Figure 2A). PGA₁-biotin and Δ12 PGJ₂-biotin were both taken up into MCF-7 cells and formed covalent adducts with ~20 proteins [Figure 2B]. Δ12 PGJ₂-biotin (a bi-functional dienone) was more reactive than PGA₁-biotin (mono-functional enone), agreeing with others who reported ~20-30 protein targets modified by cyPG-biotin in 3T3 cells or mitochondria [31,32]. Sequestration of de novo biotinylated cellular proteins on NA beads, followed by immunoblot with anti-PTEN antibodies, showed that PTEN formed a covalent adduct ~10-fold more readily with Δ12-PGJ₂-biotin than with PGA₁-biotin (Figure 2C, lane 4 vs lane 3).

The α, β–enone, Δ12-PGJ₂, interferes with PTEN suppression of Akt kinase

Growth factors, insulin, and other stimuli prompt PI3-K to make PIP₃, which recruits PKB/Akt kinase to the cell membrane where PDK1/2 phosphorylates Akt Thr³⁰⁸ and Akt Ser⁴⁷³ residues, respectively [33,34,35]. PTEN down-regulates PKB/Akt activation by metabolizing PIP₃ to PIP₂ [36]. α, β–unsaturated carbonyls that alkylate cellular PTEN may interfere with its suppression of Akt kinase. A representative α, β–enone, Δ12-PGJ₂, caused a concentration and time dependent increase in phospho-(T³⁰⁸)Akt in MCF-7 cells. As little as ~2 µM Δ12-PGJ₂ caused a half-maximal response (Figure 3A). Increases in cellular phospho-(T³⁰⁸)Akt were detectable at 10 min, maximal at 30 min, and durable for >120 min (Figure 3B). Δ12-PGJ₂ increased formation of phospho-(T³⁰⁸)Akt without altering formation of phospho-(S²⁴¹)PDK1 (the kinase that phosphorylates T³⁰⁸ of Akt), and without altering PTEN protein

Figure 2. CyPG biotin analogs: model α, β-unsaturated carbonyls alkylate cellular PTEN. (A) Michael addition reaction between PTEN and a Δ12-PGJ₂-biotin analog. Following treatment of cells with cyPG-biotin, proteins with a de novo biotin epitope were sequestered onto neutravidin beads (NA Pulldown), then fractionated by SDS-PAGE for immunoblotting. (B) Anti-biotin immunoblot of proteins from lysates of MCF-7 cells treated with DMSO (lane 1), 10 µM PGA₁-biotin (lane 2), and 10 µM Δ12PGJ₂-biotin (lane 3). Δ12-PGJ₂ biotin formed a covalent adduct with proteins more readily than PGA₁-biotin (arrowheads). (C) Anti-PTEN immunoblot of cellular PTEN that formed a covalent adduct with cyPG-biotin (NA Pulldown) relative to total PTEN (Cell Lysate) from MCF-7 cells treated with DMSO (lane 1), 1 or 10 µM PGA₁-biotin (lanes 2, 3), and 1 or 10 µM Δ12PGJ₂-biotin (lanes 4, 5). Blots are representative of results obtained in at least three independent experiments.
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expression (Figure 3C). These data suggest that Δ12-PGJ2 interfered with PTEN’s capacity to restrain activation of Akt kinase. Consistent with this interpretation, co-treatment of cells with cyPGs plus 50 μM LY294002, lowered levels of phospho-(T308)Akt relative to total Akt (mean ± s.e.m) from n=3 separate experiments. (B) Immunoblot of phospho-(T308)Akt relative to total Akt in lysates from MCF-7 cells treated with 20 μM Δ12-PGJ2 for 0–120 min. The bar graph shows the increase in phospho-(T308)Akt/total Akt (mean ± s.e.m) from n=4 separate experiments. (C) Immunoblot of phospho-(T308)Akt, PTEN, phospho-(S241)PDK1 from lysates of MCF-7 cells treated 30 min with 20 μM Δ12-PGJ2 (lane 2, 3) and 20 μM PGA1 (lane 5, 6) in the presence of the PI3-K inhibitor 50 μM Ly294002 (lane 3, 6) or DMSO vehicle (lane 2, 5). (D) Immunoblot of phospho-(T308)Akt relative to total Akt in lysates from MCF-7 cells treated 4 hrs with vehicle, the cyclopentenones - PGJ2, Δ12-PGJ2, and 15-deoxy-Δ12-PGJ2, or their precursor PGD2. For (C) and (D), blots are representative of results obtained in three independent experiments. doi:10.1371/journal.pone.0013545.g003

Table 1.

| Test Compound | PTEN Activity % Inhibition |
|---------------|---------------------------|
| Vehicle       | 0                         |
| 10 μM PGB1    | 5 ± 2                     |
| 1 μM PGA1     | 37 ± 5                    |
| 1 μM PGA2     | 40 ± 3                    |
| 1 μM PGJ1     | 48 ± 5                    |
| 1 μM Δ12-PGJ2 | 56 ± 4                    |
| 1 μM 15-deoxy-Δ12, Δ12-PGJ2 | 73 ± 3          |
| 10 μM Acrolein | 40 ± 6                  |
| 10 μM 4-hydroxy-2-nonenal | 57 ± 2             |

Mean ± sem, n = 3. doi:10.1371/journal.pone.0013545.t001
Phosphorylation of Akt substrates with an RxRxx-phospho-S/T epitope, coincided with increased phospho-(T308)Akt in MCF-7 cells treated with 10 μM Δ12-PGJ2 (Figure 4A). Akt kinase activation also coincided with Akt-dependent proliferation in MCF-7 cells treated with 1–10 μM Δ12-PGJ2 (Figure 4B). This finding is consistent with reported bi-phasic actions of cyPGs, whereby they increase proliferation of cultured cells at ~1 μM [37,38], while they decrease proliferation or cause apoptosis by modifying other protein targets at ~50 μM [39].

α, β–unsaturated carbonyls cause a time-dependent accumulation of cellular phospho-(S473)Akt kinase (active) → phospho-(S9)GSK3β (inactive) → β-catenin and a rise in nuclear β-catenin signaling

GSK3β converts β-catenin to phospho-(S33/37/T41) β-catenin, which is rapidly eliminated by the 26S proteasome [40]. GSK3β acts in concert with the tumor suppressor APC. In cells with mutant APC, or when WNT ligands stimulate cells with wild type APC, GSK3β fails to phosphorylate β-catenin, which allows it to accumulate, associate with other nuclear transcription factors and express its target genes (e.g. c-myc, cyclin D1) [41]. PTEN can block β-catenin accumulation/signaling by favoring retention of active GSK3β and inactive PKB/Akt kinase in some [42,43], but not all experimental systems [44,45]. Accordingly, inactivated PTEN should augment β-catenin signaling by favoring retention of inactive phospho-(S9)GSK3β and active phospho-(S473) Akt kinase. We thus hypothesized that these electrophilic mediators may affect β-Catenin signaling through this mechanism. To investigate this further, we used HEK 293 cells which have an intact β-Catenin signaling pathway. We found that the different α, β–unsaturated carbonyls that alkylated PTEN (6 μM Δ12 PGJ2, 6 μM 4-HNE and 20 μM acrolein) all caused a time-dependent rise in phospho-(S473)Akt (i.e. active Akt kinase), with a

[Figure 4. α, β–unsaturated carbonyls interfere with Akt kinase and downstream signaling in MCF7 and HEK 293 cells. (A) Immunoblots of phospho-(T308)Akt, total Akt, and several phosphoproteins with (K/R)-x-(K/R)-xx-(S/T), a motif recognized and phosphorylated by active phospho-(T308)Akt, in lysates from MCF-7 cells treated 0 and 10 μM Δ12-PGJ2. (B) Relative proliferation of MCF-7 cells (mean ± s.e.m, n = 4) treated with 0, 1, and 10 μM Δ12-PGJ2 alone (■), or in the presence of 10 μM of inhibitor IV (□), an Akt kinase inhibitor. (C) Immunoblots of phospho-(S473)Akt, total Akt; phospho(S9)GSK3β; GSK3β; and β-catenin and tubulin in lysates from HEK 293 cells after treatment for 0, 1, 3, 6 and 16 hrs with 6 μM Δ12PGJ2, 6 μM 4-HNE or 20 μM acrolein. For (A) and (C), blots are representative of results obtained in three independent experiments.

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corresponding rise in phospho-(S9)GSK3b (i.e. inactive GSK3b) and b-catenin (Figure 4C). To determine if \(\alpha,\) \(\beta\)-unsaturated carbonyls enhanced nuclear b-catenin signaling, we used HEK 293 cells engineered to stably express WNT3A and Super-TopFlash (STF) reporter gene [46]. These cells, called STF3A cells, thus secret WNT3A, an autocrine/paracrine stimulus for FZD receptors that slows APC-dependent degradation of b-catenin (Figure 5A). Nuclear b-catenin signaling in STF3A cells is

**Figure 5.** \(\alpha,\) \(\beta\)-unsaturated carbonyls enhance nuclear b-catenin signaling in STF3A cells: structure activity relationships. (A) STF3A cells harbor a stably integrated transgene which expresses WNT3A (1). When secreted, WNT3A elicits autocrine/paracrine stimulation of Fz receptors (2) which inhibits APC-dependent turnover of b-catenin (3). If b-catenin is not phosphorylated by GSK3b, it can accumulate as a b-catenin:LEF dimer (4) and bind to promoters on stably integrated b-catenin:luciferase reporter gene (SuperTop Flash) (5). Luciferase activity in cell lysates is proportional to nuclear b-catenin signaling. (B) Nuclear b-catenin signaling (luciferase luminescence) in STF3A cells (2 \times 10^4 cells/well) grown for 24 hrs in medium containing 0, 10, or 30 ng/ml of DKK1 (mean + s.d., n = 3). DKK1, a wnt antagonist, inhibited b-catenin signaling (C) Nuclear b-catenin signaling in STF3A cells (2 \times 10^4 cells/well) grown for 24 hrs in medium containing 20 \(\mu\)M of cyPGs; \(\alpha,\beta\)-enals or primary PGs. Several reactive electrophiles enhanced b-catenin signaling by \(-2\sim4\) fold. Histogram represents the mean + s.e.m., n = 4. doi:10.1371/journal.pone.0013545.g005
proportional to their luciferase expression (activity), and they are responsive to DKK1, a WNT antagonist that inhibited β-catenin signaling in STF3A cells in a concentration-dependent manner (Figure 5B).

Several reactive carbonyl metabolites, each with an electrophilic α,β enone or enal substituent, enhanced expression of the β-catenin:luciferase reporter gene in STF3A cells (Figure 5C). Luciferase reporter activity rose by ~4-fold over baseline (p<0.01) in STF3A cells incubated with Δ12PGJ2 or 4-HNE; by ~3-fold (p<0.01) in cells with other PGJ analogs, acrolein or 4-ONE; and by ~1.5-fold (p<0.05) in cells with PGA2. Consistent with our mechanistic hypothesis, neither PGB2 nor MDA had a detectable effect. PGB2 is a cyPG, but tautomerism prevents the charge delocalization required to create an electrophilic β carbon, which is required for protein alkylation. MDA (β-hydroxy-acrolein) penetrates cell membranes poorly because it is >99% ionized at physiological pH ~7.4 used in our experiments. Neither PGE2 nor other primary PG metabolites of COX-1 or -2 had any effect on nuclear β-catenin signaling in STF3A cells. We draw attention to the fact that ectopic over-expression of EP receptors in HEK 293 cells was required to elicit any PGE2 mediated β-catenin signaling [47]. The weak response to PGE2 and other PG’s in Figure 5C may reflect the constitutive levels of EP, FP, DP or IP receptors in STF3A cells or rapid metabolism of PGs, or both.

Enhanced β-catenin signaling in STF3A cells was concentration dependent between 2–20 μM for acrolein, 4-HNE and Δ12 PGJ2 (Figure 6A). Depletion of cellular GSH to ~10% of baseline by treatment with 100 μM BSO potentiated β-catenin signaling, e.g. in STF3A cells treated with 2 and 6 μM Δ12 PGJ2 (Figure 6B). This is consistent with the role of reduced glutathione in the conjugation of reactive metabolites, and protection of redox sensitive proteins from alkylation [20].

Discussion

The PTEN tumor suppressor gene is frequently mutated or inactivated in advanced cancers [26,48]. PTEN is a phosphoinositide-3-phosphatase that metabolizes PIP3 to PIP2 [36], thereby
counter-regulating PKB/Akt, a serine/threonine kinase proto-oncogene that controls anabolic growth and specification of cell fate [33,34,35]. PTEN, itself, is regulated post-translationally by phosphorylation [49], acetylation [50], and reversible oxidation of its catalytic cysteine124 residue [28,29]. Oxidation of cellular PTEN can involve H$_2$O$_2$ derived from NADPH oxidase [51], superoxide dismutase [52], or enzymatic peroxidation of arachidonic acid (AA) by COX-1, COX-2 or 5-LOX [53]. All of these enzymes are commonly over-expressed and activated by inflammation or neoplastic transformation. PTEN oxidation, and any attendant pathophysiology, varies with the degree of cellular exposure to reactive oxygen species (ROS). In these studies, we demonstrate that the chemistry which facilitates oxidation of PTEN can also facilitate its alkylation by electrophilic α,β-unsaturated and α,β-ethylenes [19,20].

PTEN epitomizes the adaptation of redox-responsive thiols for cell signaling, as well as their potential vulnerability to by-products of oxidative stress and inflammation. PTEN is inactivated by two distinct redox-mediated processes: 1) intra- or inter-molecular disulfide formation by ROS and 2) thiolate carbonylation (Michael addition) by electrophilic α,β-unsaturated carbonyls (Figure 1–3). Hydrogen peroxide (H$_2$O$_2$), a prototypical ROS, inhibits cellular PTEN by directly oxidizing its catalytic Cys124 to a sulfenic acid intermediate, which then forms an inactive, intra-molecular Cys$_{124-71}$ disulfide [28,29]. Our data show that several representative, electrophilic carbonyl species (α,β-ethenes and α,β-enamides), which can occur endogenously as byproducts of lipid peroxidation during inflammation or oxidative stress, alkylate and inactivate PTEN. Inactivation of PTEN by redox-mediated processes causes an increase in activity of the proto-oncogene Akt. Hyperactivation of Akt increases proliferation and survival of many different cancers.

Signaling by H$_2$O$_2$ spans a wide pathophysiological continuum [54] and a comparable role for reactive electrophiles seems plausible. Reactive carbonyl species such as acrolein, 4-HNE and Δ12PGJ$_2$ represent a sub-set of electrophiles commonly produced during oxidative stress and inflammation. These findings might extrapolate to electrophilic agents lacking a carbonyl but containing another electron withdrawing groups, and we refer to them generally as “reactive electrophiles”. First, like H$_2$O$_2$, reactive electrophiles occur in vivo during inflammation and oxidative stress [16,18,19,22]. Second, reactive electrophiles covalently modulate other proteins that regulate important signaling processes; i.e. LKB1/STK11 [55], NFκB [56], and IKKβ. Third, H$_2$O$_2$ and reactive electrophiles both originate from a combination of spontaneous and enzymatic processes, which often coincide in inflamed tissues [57]. H$_2$O$_2$ derives from superoxide anion, O$_2^-$, the primary metabolite of NADPH oxidases. Spontaneous and enzymatic dismutation converts O$_2^-$ into H$_2$O$_2$. Likewise, spontaneous and enzymatic lipid peroxidation generates acrolein and 4-HNE [18,37]. cyPGs originate from the lipid endoperoxide PGH$_2$, the primary metabolite of COX-1 and -2. Enzymatic and spontaneous scission of endoperoxide bonds converts PGH$_2$ into PGE$_2$ and PGD$_2$, albumin/serum then causes their dehydration into PGE$_2$ and PGD$_2$, and their isomers [14,16,24].

While speculative, it appears that ROS and reactive electrophiles (H$_2$O$_2$, acrolein, 4-HNE, Δ12 PGJ$_2$) may have both evolved to play disparate roles in innate immunity: 1) annihilating pathogens and 2) resolving inflammation. Analogue to inactivation of NFκB and IKKζβ, temporary inactivation of the PTEN tumor suppressor protein by its alkylation, and attendant activation of PKB/Akt kinase proto-oncogenes, might help normalize morphology and histology at acutely inflamed tissues by releasing their restriction on cell proliferation, anabolic growth and fate specification [34,35]. In ordinary situations repair and resolution should help terminate innate immune inflammation (Figure 7,Q). However, this mechanism might also confer inescapable risks if PTEN were inactivated errantly or persistently. Furthermore, reactive electrophiles also inactivate other notable tumor suppressors, including p53 [30] and LKB1/STK11 [53]. This combined and sustained inactivation of tumor suppressors could contribute significantly to inflammation-associated tumorigenesis and subsequently prolong the cycle of tumor-associated para-inflammation (Figure 7,Ψ). Overall, our data and model align with the observation that tumors are wounds that fail to heal [30]. In this situation, tumor progression may derive partly from mal-adaptation of a molecular mechanism that evolved to terminate and resolve innate immune inflammation.

Inflammation is a critical component of tumor progression. Many cancers arise from sites of infection, chronic irritation and inflammation. The tumor microenvironment, which is comprised largely of inflammatory cells, plays a major role in the neoplastic process, fostering proliferation, survival, and migration. We show herein that reactive carbonyl species that are commonly produced during inflammation covalently modify and inactivate PTEN tumor suppressor. Importantly, the mechanism we describe might also extrapolate to: 1) other electrophilic species generated by inflammation, oxidative or xenobiotic stress (i.e. other α,β-unsaturated aldehydes and ketones; aliphatic or vinyl epoxides; quinones, chlorohydrazins, chloramines, vinyl sulfones; and 2) other members of the PTP superfamily that are redox sensitive. These studies extend our understanding of the mechanisms by which inflammation contributes to the initiation and progression of cancer.

Materials and Methods

Materials

We used minimum essential medium (MEM), supplements, bovine insulin, gentamicin, human embryonic kidney HEK-293 cells, and HEK-293 cells containing the Epstein Barr virus nuclear antigen 1 gene (HEK-EBNA1) (Invitrogen; Carlsbad, CA); MCF-7 cells (HTB-22, American Type Culture Collection; Manassas, VA); PGs, cyPG-biotin analogs, and WST proliferation assay kits (#10008883) (Cayman Chemical; Ann Arbor, MI); Complete™ protease inhibitor mixture (Roche Molecular Biochemicals; Indianapolis, IN); lysis buffer 0.6% Igepal CA-630 in PBS (Promega; Madison, WI); NeutrAvidin conjugated beads, NEM-biotin and goat anti-biotin polyclonal antibodies (#31052) ( Pierce Chemical; Rockford, IL); a PI3-K inhibitor, LY294002 (#9001), polyclonal antibodies against PTEN (#9352), Akt (#9271), phospho-(Thr308)Akt (#9375), phospho-(Ser473)Akt (#9271), phospho-(Ser473)GSK3β (#9336), GSK-3β (#9332), phospho-(Ser380/Thr380)β catenin (#9561S) K/R-x-K/R-x-x-S/T[PO4] epitopes (#9614) and phospho-(Ser34)PDK1 (#3061) (Cell Signaling Technologies; Danvers, MA); β-catenin (#C19220) (BD Transduction Laboratories; Franklin Lakes, NJ); HRP (horseradish peroxidase) conjugated secondary antibodies (Santa Cruz Biotechnology; Santa Cruz, CA; polyclonal IgG, biotinylated IgG, goat anti-mouse antibody, and DKK-1 (#63268), L-Buthionine-sulfoximine (BSO) (B2515), H$_2$O$_2$ 30% solution (H-1009), malondialdehyde (Malondialdehyde; Sigma-Aldrich; St Louis, MO; DKK-1 (#1096-
DK-010 (R & D Systems; Minneapolis, MN) and Luciferin (Cat. # 10101-2) (Biotium Inc., Hayward, CA).

Cell culture
MCF-7 breast cancer cells (ATCC) were grown in MEM with 10% v/v FBS, 2 mM L-glutamine, 1.5 g/l NaHCO3, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.01 mg/ml bovine insulin, and 0.01 mg/ml gentamicin. HEK-293 cells (ATCC) were grown in MEM with 10% fetal calf serum, 100 units of penicillin/streptomycin, 2 mM L-glutamine, and 1 mM pyruvate.

Identification of modified PTEN by tagging with biotin-conjugated maleimide
MCF-7 cells (MEM 1% v/v FBS) were treated for 30 min with vehicle, or reactive electrophiles (10 μM Δ12-PGJ2, 4-HNE, acrolein), or 100 μM H2O2. Media was removed; cells were frozen at -280°C for 15 min; transferred to vacuum and incubated 1 h, 25°C with 1 ml of O2-free extraction buffer (50 mM NaHPO4, pH 7.0, 1 mM EDTA, 10 mM NEM [N-ethyl maleimide], 10 mM IAA [iodoacetic acid], 1% Triton X-100, 5 mM NaF, 50 μg/ml leupeptin and 50 μg/ml aprotinin). This treatment selectively alkylates all reduced thiols in PTEN, but not oxidized thiols or thiols modified by Michael addition with reactive electrophiles. Samples were washed in 1 ml of O2-free extraction buffer then transferred to a 15-ml conical tube. After adding SDS to a final concentration of 1% v/v, the mixture was held 2 h at 25°C in the dark, and proteins were precipitated with TCA [trichloroacetic acid], 10% v/v for 1 h. The precipitate was washed twice with acetone to remove traces of TCA, NEM, and IAA. Precipitated proteins were solubilized and oxidized or modified cys residues were reduced in 0.1 ml of O2-free reducing buffer (50 mM Hepes-NaOH, pH 7.7, 1 mM EDTA, 2% SDS and 4 mM DTT) for 30 min at 50°C. Reduced proteins were subsequently biotinylated with 0.9 ml of a solution containing 50 mM NaHPO4 pH 7.0, 1 mM EDTA, and 1 mM biotin conjugated to polyethylene oxide-maleimide for 30 min at 50°C. Proteins were precipitated in 10% v/v TCA for 1 h. The precipitate was isolated by centrifugation, washed with dry ice-chilled acetone, and solubilized in 0.3 ml of the same solution without SDS. 15 μg protein was assayed by immunoblot for PTEN. A separate sample (200 μg protein) was added to 100 μL immobilized NA beads in 1 ml PBS, 0.4% v/v Tween 20. This suspension was rotated 16 hr at 4°C, centrifuged, and beads were washed twice with PBS/0.4% v/v Tween 20. Loading buffer (50 μL) with 5% BME was added directly to beads, boiled for 10 min to release maleimido-biotinylated proteins, and 20 μL was assayed by immunoblot for total and oxidized PTEN.

Identification of PTEN occurring as an intra-molecular disulfide
MCF-7 cells (MEM 1% v/v FBS) treated for 30 min with 20 μM Δ12-PGJ2, 4-HNE, acrolein, 15-HpETE or for 10 min

Figure 7. Model Depicting Hypothetical Roles of PTEN Alkylation in Inflammation and Cancer.
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with 100 μM H2O2 were lysed and proteins (15 μg) in the lysates were fractionated by non-reducing SDS-10% PAGE, followed by anti-PTEN immunoblot to distinguish native PTEN from oxidized PTEN occurring as an intra-molecular Cys125-Sys145 disulfide [20].

Identification of PTEN covalently modified by cyclopentenone PG-biotin analogs

MCF-7 cells (MEM 1% v/v FBS) treated 1 hr with 1–10 μM of the aminopentylbiotinamide analogs of PGA1 or Δ12 PGJ2, were lysed, sonicated 10× for 1 s at 4°C, then centrifuged 10,000× g for 10 min. Supernatant with 100 μg of protein was incubated with 100 μl of NA beads in 1 ml PBS with 0.4% Tween 20 for 16 h at 4°C to sequester proteins containing a biotin epitope introduced by reaction of cyPG-biotin analogs. The beads were then centrifuged at 500× g for 5 min to isolate neutravidin-biotin complexes (NA pulldown). The beads were washed 3× with 1 ml of PBS/0.4% Tween 20 then boiled 5 min in Laemmli loading buffer with 5% BME to release bound proteins. These samples were analyzed by immunoblotting for PTEN or proteins with a biotin epitope.

Western immunoblotting

Following treatment with reactive electrophiles, H2O2, or enzyme inhibitors, MCF-7 or HEK-EBNA cells were lysed in 250 mM sucrose, 50 mM Tris pH 7.4, 5 mM MgCl2, 1 mM EGTA, 1× Complete™ protease inhibitor, 2 mM NaF and 2 mM sodium orthovanadate. Samples were dissolved in 50 μl of Laemmli loading buffer, 0.5% BME and heated at 95°C for 10 min. Samples (15–30 μg protein) were fractionated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% w/v nonfat dry milk in TBS-T, then incubated for 16 h at 4°C with primary antibodies directed against PTEN (1:1000), Akt (1:1000), phospho-(T308)Akt (1:1000), phospho-(S473)Akt (1:1000), phospho-(S241)PDK1 (1:1000), GSK3b (1:1000), phospho-(S9)-GSK3b (1:1000), PDK1 (1:1000), Akt substrate proteins containing the K/R-x-K/R-x-x-S/T[P] epitope, phospho-(S216)PDK1, and PTEN.

Akt phosphorylation and signaling

~1×106 MCF-7 cells (MEM 1% v/v FBS) were treated with 0–20 μM Δ12-PGJ2, 0 min at 37°C, or with 20 μM Δ12-PGJ2 for 0–120 min to determine concentration and time dependence. ~1×106 MCF-7 cells were also treated 30 min at 37°C with 10 μM of various PGs, including PGD2 and its cyPG dehydration products PGJ2, Δ12-PGJ2, and 15-deoxy-Δ12, Δ14-PGJ2; PGE2 and its cyPG dehydration products PGA2, its epimer 8-iso-PGA2, and its isomer PGB2, to determine structure-activity relationships. In certain experiments cells were also treated with 50 μM LYS294002, 5 μM troglitazone, or 10 μM Akt inhibitor IV. Lysates from treated cells (15 μg protein) were fractionated on SDS-10% PAGE and proteins were transferred to PVDF membranes for immunoblot analysis of Akt, phospho-(T308)Akt, phospho-(S473)Akt, Akt substrate proteins containing the K/R-x-K/R-x-x-S/T[P] epitope, phospho-(S216)PDK1, and PTEN.

Wnt/β-catenin signaling in STF3A cells

STF cells are HEK-293 cells containing a stably integrated SuperTopFlash (STF) transgene with TCF binding sites upstream of a luciferase reporter gene [59]. STF cells have negligible β-catenin/TEF transactivation and luciferase expression unless they are exposed to a Wnt ligand, e.g. WNT3A. We derived a subsidiary cell line, designated STF3A, by transfecting parental STF cells with a linearized pPGK + Wnt3A plasmid and a linearized blasticidin resistance plasmid for cell selection. STF cells grown on 1-cm plates were transfected using LipofectAMINE 2000 (Invitrogen). At 24 h after transfection, cells were serially diluted, re-plated and grown in medium with blasticidin (10 μg/ml) for 14 d. Forty colonies were screened for β-catenin/TEF luciferase activity by measuring luciferase activity normalized to total protein concentration. The selected STF3A clones, which stably expressed and secreted WNT3A, were maintained at 37°C in DMEM with 10% FBS, penicillin/streptomycin, and 1% non-fetal bovine serum. STF3A cells were incubated for 24 h at 37°C, washed with 200 μl fresh medium containing 0–300 ng/ml DKK1; 2–20 μM of α,β-ene-containing cyPGs; 2–20 μM of α,β-ene metabolites derived from lipid peroxidation (acrolein, MDA, crotonaldehyde, 4-HNE, 4-ONE); 20 μM of primary PGs, PGH2, PGE2, PGF2α, PGD2, PGL2; 20 μM BSO, a glutathione synthesis inhibitor; other inhibitors, or DMSO vehicle. STF3A cells were incubated for 24 h at 37°C, washed with 200 μl PBS at 4°C, and lysed with 20 μl lysis buffer. Luciferin (60 μl/well) was added and luciferase activity in the lysates was quantified by fluorimetry. LDH activity in the lysate, an index of cell count, was quantified by spectroscopy. The ratio of luciferase/LDH activity is proportional to nuclear β-catenin signaling in STF3A cells.

Inhibition of PTEN Enzymatic Activity

Inhibition of PTEN with 0–30 μM reactive electrophiles was quantified by using a PTEN enzyme assay kit (# 17–351, Upstate Biotechnology).

Statistical analysis

Statistical significance at p<0.05 was assessed by analysis of variance (ANOVA) with Bonferroni’s post-hoc test for comparisons among groups.

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Author Contributions

Conceived and designed the experiments: TMC. Performed the experiments: TMC KE. Analyzed the data: FAF. Contributed reagents/materials/analysis tools: GSC DV. Wrote the paper: FAF.

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Electrophiles Inactivate PTEN

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