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Original Contribution

Reactive oxygen species induced by p66Shc longevity protein mediate nongenomic androgen action via tyrosine phosphorylation signaling to enhance tumorigenicity of prostate cancer cells

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ABSTRACT

Steroid hormones exhibit diverse biological activities. Despite intensive studies on steroid function at the genomic level, their nongenomic actions remain an enigma. In this study, we investigated the role of reactive oxygen species (ROS) in androgen-stimulated prostate cancer (PCa) cell proliferation. In androgen-treated PCa cells, increased cell growth and ROS production correlated with elevated p66Shc protein, an authentic oxidase. This growth stimulation was blocked by antioxidants. Further, elevated expression of p66Shc protein by cDNA transfection encoding wild-type protein, but not a redox-deficient (W134F) mutant, was associated with increased PCa cell proliferation. Conversely, knockdown of p66Shc expression by shRNA resulted in diminished cell growth. Increased p66Shc expression in PCa cells enhanced their tumorigenicity in xenograft animals. Importantly, p66Shc protein level is higher in clinical prostate adenocarcinomas than in adjacent noncancerous cells. Expression of redox-deficient p66Shc mutant protein abolished androgen-stimulated cell growth. In androgen-treated, H_2O_2-treated, and p66Shc cDNA-transfected PCa cells, cellular prostatic acid phosphatase, an authentic tyrosine phosphatase, was inactivated by reversible oxidation; subsequently, ErbB-2 was activated by phosphorylation at tyrosine-1221/1222. These results together support the notion that androgens induce ROS production through the elevation of p66Shc protein, which inactivates tyrosine phosphatase activity for the activation of interacting tyrosine kinase, leading to increased cell proliferation and enhanced tumorigenicity. Our results thus suggest that p66Shc protein functions at the critical junction point between androgens and tyrosine phosphorylation signaling in human PCa cells.

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Introduction

Steroid hormones including the androgen DHT are involved in regulating diverse physiological functions. Whereas the pro-growth role of steroids via gene regulation in cells has been intensively studied, their nongenomic actions have received much less attention and require further investigation.

ROS, depending on their intracellular level, are proposed to mediate diverse cellular activities [1,2]. For example, ROS-induced protein and DNA adducts are proposed to be involved in carcinogenesis [1,3]. Higher intracellular ROS levels are found in various carcinomas than in corresponding noncancerous tissues and are proposed to be involved in various stages of cancer progression, characterized by oxidative nuclear damage [2,4–6]. Evidently, elevated levels of intracellular H_2O_2 and oxidant

Abbreviations: Ab, antibody; AcP, acid phosphatase; AI, androgen independent; AR, androgen receptor; AS, androgen sensitive; cPAcP, cellular prostatic acid phosphatase; Cyt *c*, cytochrome *c*; DCF-DA, dichlorofluorescein diacetate; DHT, 5α-dihydrotestosterone; EGF, epidermal growth factor; ErbB-2, human epidermal growth factor receptor-2; FBS, fetal bovine serum; NAC, *N*-acetylcysteine; PCa, prostate cancer; pNPP, *para*-nitrophenyl phosphate; PTP, protein tyrosine phosphatase; pTyr, phosphotyrosine; ROS, reactive oxygen species; Shc, Src homolog and collagen homolog protein; SR, steroid-reduced; Tyr-P, tyrosine phosphorylation; TSP, l-(+)-tartrate-sensitive acid phosphatase; TRP, l-(+)-tartrate-resistant acid phosphatase; VES, vitamin E succinate; WT, wild type

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enzymes, such as NAD(P)H oxidase 1, correlate with cancerous and metastatic properties of PCa cells [4]. However, the molecular mechanism by which ROS increase tumorigenicity is yet to be elucidated.

ROS also play a role in growth regulation. Growth factors stimulate cell growth at least in part by increasing ROS levels that inhibit growth-inhibitory PTPs through oxidation of their active-site cysteine residues, leading to activation of corresponding receptor tyrosine kinases [7–10]. Recent advances have revealed the existence of histidine-dependent PTPs, a subgroup of the PTP superfamily [11]. In parallel, histidine can also be oxidized in cells, forming oxo-histidine [12]. However, the biological significance of histidine oxidation in proteins is much less understood. Whereas androgenic stimulation of AS PCa cells also increases ROS production [13]; the molecular mechanism by which ROS mediate androgen action remains an enigma.

Human prostatic acid phosphatase (PAcP) exists as cellular (cPAcP) and secretory forms in differentiated prostate epithelia [14,15]. Various posttranslational modifications contribute to the differences in their biochemical properties [15]. A transmembrane form of PAcP was recently reported and could function as an analgesic in mice [16]. Nevertheless, the role of this transmembrane protein in humans remains unknown. Several lines of evidence support the notion that cPAcP functions as a tumor suppressor [14–17]. Evidently, PAcP-knockout mice develop prostate carcinomas spontaneously [16]. Intratumoral injection of a plasmid encoding WT PAcP protein, but not an inactive mutant, into xenograft prostate tumors led to tumor suppression [17], supporting the notion that tumor suppression is dependent on its phosphatase activity. In PCa cells, cPAcP functions as a neutral, histidine-dependent PTP with ErbB-2 as its primary substrate [14,15,17–19]. Interestingly, the PTP and tumor suppressor activities of PAcP are dependent on its active site His12, but not cysteine residues near the active domain [17–19]. In these cells, cPAcP dephosphorylates pTyr of ErbB-2 and attenuates its downstream signaling and cell growth. Upon growth stimulation, e.g., DHT, cPAcP activity is inhibited by a yet unknown mechanism, which leads to ErbB-2 activation by Tyr-P and cell proliferation [14,15,17–19]. The mechanism of androgen-induced cPAcP inactivation remains to be investigated.

p66Shc is predominantly expressed in epithelia and can mediate apoptotic stress signals [20–25]. p66Shc, an oxidase, increases ROS levels by oxidizing Cyt *c* in mitochondria or through SOS-mediated Rac1 activation at the cell membrane [22,26,27]. Despite p66Shc being a life-span determinant in mice, its association with human longevity remains under investigation [20,28]. Rather, data from human thyroid, prostate, ovarian, and colon cancer tissues and cell lines show elevated p66Shc protein levels, implying its tumorigenic role in humans [29–32]. In parallel, p66Shc protein level is increased in breast cancer cell lines with high metastatic ability [32], although inconsistent results were observed in primary breast tumors [33,34]. Collectively, p66Shc plays a critical role in steroid hormone-related carcinogenesis, in part by promoting cell proliferation [13,30,35,36]. In sex steroid-treated prostate, testicular, and breast cancer cells, p66Shc protein levels are elevated and cell growth is increased [30]. Accelerated PCa cell proliferation by p66Shc cDNA transfection is preceded by increased mitochondrial ROS production [13]. However, it is not known how steroid and p66Shc-ROS signaling promotes PCa cell proliferation.

In this study, we report a novel molecular mechanism of nongenomic androgen action on PCa cell growth stimulation. Our data, to the best of our knowledge, for the first time clearly reveal a novel cross talk between androgen and Tyr-P signaling connected by p66Shc via ROS production that results in PTP inhibition and subsequently ErbB-2 activation, leading to the enhanced tumorigenicity of PCa cells.

Materials and methods

Materials

RPMI 1640 medium, gentamicin, and L-glutamine were obtained from Invitrogen (Carlsbad, CA, USA). FBS and charcoal/dextran-treated FBS were from Atlanta Biologicals (Lawrenceville, GA, USA). Polyclonal Abs recognizing ErbB-2 and glutathione peroxidase 1 (GPx1), and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG Abs were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-pTyr (clone 4G10) was obtained from Millipore (USA). Anti-pY1221/2 of ErbB-2 Ab was obtained from Cell Signaling (USA). Polyclonal Abs recognizing all three isoforms of Shc protein, the Ab specifically recognizing p66Shc protein, and anti-pY 1248 of ErbB-2 Ab were obtained from Upstate (Lake Placid, NY, USA). Anti-β-actin Ab, mouse anti-human PAcP Ab, DHT, NAC, and vitamin E succinate (VES) were from Sigma (St. Louis, MO, USA). Rabbit anti-human PAcP Ab (ATM-3) has been described previously [14,37–39]. Plasmids encoding Myc-tagged wild type and W134F mutant and S36A mutant of p66Shc cDNA are described in previous publications [13]. The p66Shc cDNA plasmid was originally provided by Dr. Pier Giuseppe Pelicci at the European Institute of Oncology (Milan, Italy) and Dr. A. Raymond Frackelton Jr. at Brown University (Providence, RI, USA) [36]. Full-length GPx1 cDNA was from OriGene Technologies (Rockville, MD, USA). All other reagents were as described previously [13,36–38].

Cell lines, hormone treatment, and plasmid transfection

Source and DHT treatment of AS LNCaP-FGC, MDA PCa2b, and VCaP cells and AI PC-3 human PCa cell lines were described previously [14,17,37]. Briefly, LNCaP-FGC, MDA PCa2b, VCaP, and PC-3 were originally purchased from the American Type Culture Collection (Rockville, MD, USA) and routinely maintained in their respective regular medium [18,40]. LNCaP C-33 cells are androgen sensitive, whereas C-81 cells exhibit the androgen-independent phenotype [14,17,18].

For DHT treatment, cells were seeded in regular medium for 3 days and then steroid-starved for 48 h in an SR medium, i.e., phenol red-free RPMI 1640 medium containing 5% charcoal/dextran-treated FBS (v/v), 2 mM glutamine, and 50 μg/ml gentamicin. After being fed with fresh SR medium, experimental PCa cells were exposed to 10 nM DHT, and control cells received the solvent ethanol alone for various periods of time as indicated for each experiment.

For cell growth experiments, cells were counted in a Cellometer (Nexcelom, MA, USA). cDNA and short hairpin RNA (shRNA) transfections were done as described previously [13,14,36]. The stable subclones S-31, S-32, and S-36 of LNCaP C-33 cells overexpressing p66Shc were established after p66Shc cDNA transfection [13,36].

AcP assay

AcP assay was performed as described previously with pNPP as the substrate in citrate buffer, pH 5.5 [37,38]. In LNCaP C-33 cells, L-(+)-tartrate-sensitive AcP (TSP) activity is routinely used to represent PAcP activity because over 90% of TSP activity in these cells is represented by cPAcP [37,38]. PAcP-specific AcP activity was analyzed in the immunocomplex by anti-PAcP Ab [37,38]. Reversible oxidation of PAcP was analyzed by incubating the total lysate or immunoprecipitated PAcP with or without 10 mM dithiothreitol (DTT) for 10 min at room temperature and then analyzing for AcP activity.

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