Testosterone and prostate specific antigen stimulate generation of reactive oxygen species in prostate cancer cells

Xiao-Ya Sun1, Steven P. Donald and James M. Phang2

Metabolism and Cancer Susceptibility Section, Basic Research Laboratory, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD 21702, USA

1Present address: Richmond, CA 94806, USA
2To whom correspondence should be addressed
Email: phang@mail.ncifcrf.gov

Prostate specific antigen, the clinical marker for prostate cancer, is a neutral serine protease whose function is to lyse seminal proteins. Recent work by our laboratory has suggested that prostate specific antigen stimulates the generation of reactive oxygen species in prostate cancer cells. Using 2′,7′-dichlorofluorescin diacetate, a dye that fluoresces in the presence of hydrogen peroxide or hydroxyl radicals, we found that prostate specific antigen markedly stimulated reactive oxygen species generation in LNCaP cells. The effect was concentration dependent and its specificity was supported by the fact that anti-prostate specific antigen antibodies abolished the response. Since testosterone stimulates the production of prostate specific antigen, we considered that the reactive oxygen species response to testosterone may be linked to prostate specific antigen. We found that the testosterone effect on reactive oxygen species was blocked by flutamide and by anti-prostate specific antigen antibody. Additionally, though PC3 and DU145 could not respond to testosterone, they readily increased reactive oxygen species in response to prostate specific antigen. Focusing on the mechanism of the prostate specific antigen effect, we tested two other serine proteases, trypsin and chymotrypsin, but found no effect on reactive oxygen species in LNCaP cells. Nevertheless, serine protease inhibitors, α1-antichymotrypsin, α2-macroglobulin and Bowman–Birk inhibitor, blocked reactive oxygen species generation stimulated by prostate specific antigen. This apparent paradox was investigated with the use of a specific anti-‘prostate specific antigen’ antibody which recognizes an epitope away from the catalytic site and which does not inhibit protease activity. Despite the lack of inhibition of proteolytic activity, this antibody blocked the effect of prostate specific antigen on reactive oxygen species generation. These findings suggest that although the integrity of the prostate specific antigen molecule is necessary for stimulating reactive oxygen species generation, its proteolytic activity is not. The underlying mechanism is currently under investigation.

Introduction

Prostate specific antigen (PSA), the clinical marker for prostate cancer, is a neutral serine protease whose recognized biologic function is to lyse proteins in the seminal coagulum (1). The production of PSA by cultured prostate cancer cells is androgen responsive through the nuclear androgen receptor (2). More recently, studies have shown that PSA is not restricted to prostate tissue (3). Albeit at low concentrations, PSA has been identified in female breast tumors and normal endometrial tissue. It has also been found in tumors from a variety of tissues. Not only has the prostate specificity of PSA undergone revision, but also its function has extended beyond those for seminal liquefication. Investigators have shown that PSA has mitogenic activity through release of IGF-I from its binding protein (4), activation of transforming growth factor-β and modulation of cell adhesion (5). Of special interest is the report that PSA may have anti-angiogenic activity leading to the proposal that it may function in tumors as an endogenous anti-angiogenic protein (6).

The effect of androgens on prostate cancer cells, including the stimulation of PSA production, has been long established. Recently, a number of workers have shown that treatment of prostate cancer cells with androgens produces redox changes which depending on concentration of androgens used may be mediators of either mitogenic or apoptotic responses (7). This is of interest because reactive oxygen species (ROS) has been linked to activation of transcriptional factors as well as apoptotic signaling. In our previous work using prostate cancer LNCaP cells, we found that PSA is readily released into the incubation medium with androgen stimulation (8). We therefore examined the coupling of androgen treatment and PSA in the stimulation of ROS accumulation. These studies showed that the androgen effect on ROS is mediated, at least in part, by PSA.

Materials and methods

Materials

Testosterone, flutamide and Bowman–Birk Inhibitor were from Sigma (St Louis, MO). Prostate specific antigen (PSA), purified from human seminal fluid, α1-antichymotrypsin (ACT), α2-macroglobulin (α2M) and anti-PSA monoclonal antibodies were obtained from Scripps Laboratories (San Diego, CA). The antibody inhibiting PSA proteolytic activity designated Ab1 was from clone BP005S, lot BB285004. The antibody without inhibition of proteolytic activity designated Ab2 was from clone AP002S, lot BC193001. 2′,7′-Dichlorofluorescin diacetate (DCF) was purchased from Molecular Probes (Eugene, OR). RPMI 1640 was obtained from Gibco-BRL Life Technologies (Rockville, MD). Fetal bovine serum (FBS) and phosphate-buffered saline were purchased from Biofluids (Rockville, MD). Four-well tissue culture chambers were obtained from Nalge Nunc (Naperville, IL). Reagents for performing chymotrypsin assays were obtained from Sigma (St Louis, MO). Chromagenix S-2586 was from DiaPharma (West Chester, OH).

Prostate cancer cells

LNCaP, PC-3 and DU-145 human prostate cancer cells were obtained from American Type Culture Collection (Rockville, MD) and were maintained in RPMI 1640 with 10% FBS. Upon achieving 75–80% confluence, cells were incubated for 1 day in serum-free medium before the experiment.

Measurement of reactive oxygen species

On the day of the experiment, cells were treated with PSA or other reagents for 2 h in serum-free medium. The treatment medium was removed and the monolayer was exposed to PBS containing 50 µM DCF, a dye which fluoresces.
when exposed to hydrogen peroxide or hydroxyl radicals (9). Cells were exposed to DCF for 20 min, then immediately scanned in DCF-free PBS using a laser-activated adherent cell cytometer (ACAS; Meridian Instruments, Okemos, MI) using 488 nm for excitation and 560 nm for fluorescence emission (10,11). A typical scan is shown with a pseudocolor scale for fluorescence intensity. Quantitation was based on analysis of fluorescence per cell or per area from at least three separate experiments and was expressed relative to untreated control preparations.

Measurement of PSA released from LNCaP cells

Cells were incubated in RPMI 1640 with 10% FBS with or without 100 nM testosterone and the media were collected at 48 h. Prostate specific antigen in the medium was determined using an immunoenzymometric method according to standards and instructions supplied by the source (Hybritech, San Diego, CA). Accumulation of PSA in the medium was linear with duration of incubation.

Measurement of PSA proteolytic activity

The serine protease activity of PSA was measured using the chromogenic substrate S-2586 (DiaPharma, West Chester, OH) (12). The reaction mixture containing 50 mM Tris–HCl, 150 mM NaCl, pH 8.7, 150 µM substrate and 5 µg PSA was analyzed on a Beckman DU-65 spectrophotometer at 405 nm for 60 min at 37°C. Two monoclonal antibodies were used to assess their effect on the proteolytic activity of PSA. Either Ab1 or Ab2 (clones BP005S or AP002S, respectively; Scripps Laboratories, San Diego, CA) were included in the incubation mixture at a concentration of 30–40 µg/ml before the addition of substrate.

Western immunoblots for PSA

Monoclonal antibodies used in the proteolytic assay were assessed for binding activity against PSA. Purified PSA (5.0 µg) in 100 µl buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.8) was added to an equal volume of Laemmlli’s buffer with 5% 2-mercaptoethanol. After boiling for 5 min, the total sample was applied and electrophoresed on a 12% preparative acrylamide gel (Bio-Rad, Hercules, CA). Transfer to nitrocellulose membrane was done using a semi-dry blotter (Bio-Rad) at 15 V for 30 min. The blot was then sectioned and probed with various dilutions of primary antibody, either Ab1 or Ab2, followed by a secondary anti-mouse IgG antibody conjugated to horseradish peroxidase (1:2000). Blocking and washing steps were done with Tris-buffered saline with 3% non-fat milk, pH 8.0 and Tris-buffered saline with Tween 20, pH 8.0, respectively (Sigma). Detection was performed using ECL plus (Amersham, Arlington Heights, IL) with exposures between 10 and 60 s.

Results

Testosterone stimulates ROS generation in LNCaP cells, but not in PC3 and DU145 cells

It is well established that androgens (testosterone and its metabolites) play an important role in the normal growth and function of the prostate (13). Changes in androgen metabolism or responsiveness to androgens have been implicated in the formation of benign prostatic hypertrophy and prostate cancer (13). A recent study has shown that the synthetic, non-metabolizable androgen, R1881, generates ROS in prostate cancer LNCaP cells (7). Using DCF, a dye that fluoresces in the presence of hydrogen peroxide or hydroxyl radicals, we confirmed that testosterone markedly stimulated ROS generation in LNCaP cells (Figure 1A). Actual scans of fluorescence with pseudocolors representing intensities are shown with control cells in the left panel and cells treated with 80 nM testosterone for 48 h in the right panel. When LNCaP cells were exposed to testosterone, they generated ROS levels markedly higher than vehicle-treated controls. The effect of testosterone on ROS levels was concentration dependent (data not shown) and was blocked by flutamide, an inhibitor of androgen-receptor dependent effects (Figure 1B). Consistent with this finding, androgen non-responsive cells, PC-3 and DU145, do not increase their ROS levels with testosterone treatment (Figure 1C). This result is similar to that described by others (7) and supports the interpretation that the testosterone effect on ROS is mediated through the androgen receptor.
PSA stimulates ROS generation in LNCaP cells

Previous studies have shown that LNCaP prostate cancer cells, but not other prostate cancer cells i.e. PC-3 and DU145, produce PSA when treated with testosterone (8). To determine if PSA plays a role in ROS generation in prostate cancer cells, LNCaP cells were treated with or without 100 ng/ml PSA for 2 h. Surprisingly, we found that PSA markedly stimulates ROS generation as shown in Figure 2A. Actual scans of fluorescence with pseudocolors representing intensities are shown. This effect of PSA on ROS generation is concentration dependent and abolished by antioxidant N-acetylcysteine (data not shown). To determine if ROS generation is a specific biological effect of PSA, we conducted experiments under the following conditions: cells exposed to 100 ng/ml PSA, denatured PSA (preheated to 60°C for 1 h), PSA plus 2 µg/ml anti-PSA antibody (IgG) or non-immune 1 µg/ml IgG. Figure 2B shows that denatured PSA has no effect on ROS generation, whereas anti-PSA blocked PSA stimulated ROS generation. These results suggest ROS generation by PSA is a specific biological effect in LNCaP cells.

ROS generation by PSA in androgen receptor-negative cells

To further characterize the PSA effect on ROS generation in different cell types, we compared the ROS response to testosterone and PSA in androgen-responsive and non-responsive cell lines. As shown in Figure 1C, only LNCaP responded to testosterone whereas PC-3 and DU145 were without effect. However, when exposed to 100 ng/ml PSA, both PC3 and DU145, as well as LNCaP, responded with increased ROS generation. This finding clearly indicates that PSA is downstream to androgens in inducing ROS generation in prostate cancer cells. The effect of testosterone on ROS is mediated through the androgen receptor, whereas the effect of PSA on ROS is not.

Abrogation of testosterone-stimulated ROS generation in LNCaP cells

Since testosterone stimulated the production of PSA, which in turn stimulates ROS generation, we considered that PSA may be the mediator of the ROS response to testosterone. To test this hypothesis, we added monoclonal anti-PSA antibodies to LNCaP cells undergoing treatment by testosterone. When
LNCaP cells were exposed to 100 nM testosterone for 48 h, they generated ROS levels markedly higher than vehicle-treated controls. As shown in Figure 3B, the presence of anti-PSA antibodies (10 µg/ml) completely blocked the ROS response to testosterone in LNCaP cells, whereas IgG used as a control was without effect (data not shown).

PSA-stimulated ROS generation is distinct from its protease activity

Prostate specific antigen is a member of the serine protease family. We asked whether it was possible to stimulate ROS generation mediated by the proteolytic activity of PSA. Focusing on this effect of PSA, we tested two other serine proteases, trypsin and chymotrypsin, but they had no effect on ROS in LNCaP cells (Figure 4A). Thus, this effect of PSA could not be generalized to other serine proteases. Interestingly, several serine protease inhibitors, α1-antichymotrypsin, α2-macroglobulin and Bowman–Birk Inhibitor, blocked ROS generation stimulated by PSA (Figure 4B). These protease inhibitors by themselves were without effect (data not shown). Their effect on PSA proteolytic activity was directly measured in a spectrophotometric assay designed to measure chymotrypsin-like activity by cleavage of a chromogenic substrate. At a 3:1 molar ratio (inhibitor to PSA), α1-antichymotrypsin, Bowman–Birk Inhibitor and α2-macroglobulin inhibited proteolytic activity by 80, 69 and 26%, respectively. Presumably, the inhibition of PSA proteolytic activity is mediated by direct protein–protein interaction with PSA. Thus they may interfere with the ability of PSA to interact with proteins other than its proteolytic substrates. To distinguish between these two possibilities, we took advantage of two commercially available monoclonal anti-PSA antibodies, one inhibits proteolytic activity (Ab1) and one does not (Ab2), presumably because the latter binds to a PSA domain away from its catalytic site. We confirmed the properties of these two antibodies by monitoring their respective inhibitory effect on PSA proteolytic activity (Figure 5A). Confirming their respective properties, we found that Ab1 was markedly inhibitory in the assay whereas Ab2 was not. Binding properties were confirmed by western immunoblotting (Figure 5B). Despite the lack of inhibition of proteolytic activity, Ab2 blocked the effect of PSA on ROS generation to a level indistinguishable from Ab1, which, in contrast, inhibited proteolytic activity (Figure 5C). Thus, the ability to inhibit the PSA stimulation of ROS generation could be separated from its proteolytic activity. Presumably, the integrity of the PSA molecule is necessary for stimulating ROS generation, but this stimulatory effect could be blocked even when proteolytic activity remained intact.

Discussion

Our current studies corroborated published reports by others that androgens stimulated ROS generation in LNCaP cells (7). That this effect required androgen receptors was supported by the failure to elicit a response in cells without functional receptors and by the blockade of the effect by flutamide. Since androgens stimulate the production and release of PSA into the medium, it was of great interest that PSA, itself, could stimulate ROS generation. The previously described effect on ROS was observed at concentrations of androgen suggesting an effect separate from its effect on cell proliferation (7). In fact, the effect on ROS was observed at concentrations of androgens, which retarded cell proliferation. In keeping with this distinction, we observed that the effect of PSA on ROS was demonstrable in cells without androgen receptors. These findings not only distinguish ROS generation from the proliferative response to androgens but also suggest that PSA is the necessary intermediate in the androgen-dependent stimulation of ROS. Additional supporting evidence comes from studies in which anti-PSA antibodies abrogated the testosterone effect on ROS generation.

Previously considered only for its proteolytic liquefaction of the seminal coagulum, PSA now has been associated with a number of biologic effects. Mitogenic activities have been identified and related to the finding that PSA treatment results in the release of insulin-like growth factor-I (IGF-I) from insulin-like growth factor binding protein-3 (IGFBP-3), which binds and inactivates IGF-I (4). Furthermore, PSA may activate latent transforming growth factor-β (5). Recently, PSA was found to have anti-angiogenic activity in that the activities...
PSA stimulates ROS generation of both fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) are inhibited (6). In general, investigators have reasoned that the mechanism is related to the fact that PSA is a serine protease and a member of the human kallikrein multigene family of enzymes for which various growth factor precursors are substrate (14). Evidence supporting the biologic effect of PSA being mediated by its proteolytic activity is usually presented as blockade of the biologic effect by α1-antichymotrypsin (ACT) (15).

An alternative explanation is based on protein–protein interaction mediated by PSA at the cell surface. That anti-PSA antibodies abrogated the PSA effect on ROS suggests that PSA produced and secreted by the cells is acting at an extracellular site. Prostate specific antigen has extensive homology with γ-nerve growth factor (56%), epidermal growth factor-binding protein (53%) and α-nerve growth factor (51%) (16). This homology with known ligands suggests that PSA may interact with proteins other than as a protease. Molecules, which interact with PSA, i.e. Bowman–Birk inhibitor, α2-macroglobulin and α1-antichymotrypsin, inhibit a number of PSA biologic effects (17). On first consideration, their ability to inhibit PSA-catalyzed proteolytic activity leads to the hypothesis that PSA stimulation of ROS generation is mediated by proteolysis. Certainly, these inhibitors block the stimulation of ROS generation by PSA. However, their ability to bind a variety of proteins is well-known (17,18). We were fortunate that two monoclonal anti-PSA antibodies were available with differential effects on the proteolytic activity of PSA. Taking advantage of this distinction between the antibodies, we compared their respective abilities to bind PSA, inhibit proteolytic activity and inhibit ROS generation. We found that Ab2 bound PSA and inhibited ROS generation, but had no effect on its proteolytic activity, whereas Ab1 bound PSA and inhibited both proteolytic activity and ROS generation. Presumably, Ab2 recognizes an epitope on the molecule away from the active proteolytic site. Thus, the inhibition of proteolytic activity was not a necessary condition for Ab2 to inhibit ROS generation. To our knowledge, this is the first demonstration that an inhibitor of a biologic effect of PSA did not also inhibit its proteolytic activity.

Although proteolysis appears unnecessary for the observed PSA effect, the possibility that PSA is stimulating ROS generation by protein–protein interaction is speculative. This putative effect could be due to non-specific interaction with cell surface membranes and interference with transporter or other membrane functions, or PSA could be specifically interacting with a cell surface receptor. We find the latter hypothesis attractive, but a more direct interaction, e.g. binding to a receptor site, is required to make this plausible (18). Either of these protein–protein interactions could be blocked by Ab2 without inhibiting the proteolytic activity of PSA. The caveat, which cannot be ruled out, is that certain substrate proteins in vivo are blocked from PSA-mediated degradation.

Fig. 5. (A) Proteolytic activity of PSA and differential inhibition by antibodies. Proteolytic activity was measured as described under Materials and methods. In a volume of 1 ml, the proteolytic cleavage of 150 µM chromogenic substrate was catalyzed by 5 µg PSA at 37°C with no other addends (○); in the presence of 30 µg Ab1 (clone BP005S; □) or 40 µg Ab2 (clone AP002S, ■). The blank reaction was monitored in the absence of PSA (△). (B) Western immunoblots for PSA using Ab1 and Ab2. The procedure is described under Materials and methods, but briefly, 5.0 µg purified PSA was electrophoresed on 12% preparative acrylamide gel, transferred to nitrocellulose membrane using a semi-dry blotter and the blot was sectioned and probed with primary antibody, monoclonal anti-PSA Ab1 or Ab2 (1 µg/ml each). Detection using a secondary antibody by the ECL method is described under Materials and methods. The duration of exposure was 10 and 60 s for Ab1 and Ab2, respectively. (C) The effect of anti-PSA antibodies on stimulation of ROS generation by PSA. LNCaP cells were treated for 2 h with 100 ng/ml PSA or with PSA together with 2 µg/ml Ab1 or Ab2, respectively. ROS was monitored as described in the legend for Figure 1A. Data represent mean ± SD of at least three determinations.
by Ab2 even though the degradation of the test substrate is not, but this possibility seems unlikely.

Nevertheless, our studies suggest that PSA can activate ROS production in cells with or without an androgen-response mechanism and that the effect of testosterone on ROS generation may, indeed, be mediated by the secretion of PSA. The generation of ROS appears independent of the well-known proteolytic activity of PSA and may be activated by protein–protein interaction between PSA and a putative membrane receptor or by binding to an intracellular protein or organelle. These intriguing possibilities are under investigation in our laboratory.

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References


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