Angiotensin II Induces Oxidative Stress in Prostate Cancer

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Abstract

Angiotensin II has been shown to be a cytokine especially acting as a growth factor. A local renin-angiotensin system has been identified in the prostate gland, and the physiologic function of angiotensin II seems to be similar in prostate cancer, as we previously reported. In the present study, we explored the biological role of angiotensin II in oxidative stress of prostate cancer cells. Activated Akt was determined, and the expression of oxidative stress-related proteins (p47phox, manganese superoxide dismutase 2, glutathione peroxidase) was examined by Western blotting in LNCaP cells, which were stimulated with angiotensin II and/or an angiotensin II receptor type 1 blocker, candesartan. To examine DNA damage induced by angiotensin II, 8-hydroxy-2-deoxyguanosine was determined, and Western blots were analyzed to detect checkpoint proteins including p53, Chk2, and cdc2. Immunocytochemical studies of inducible nitric oxide synthase and superoxide anion radical (O2-) were done in LNCaP cells stimulated with angiotensin II. The phosphorylation of Akt was induced by angiotensin II treatment and inhibited by candesartan, as well as byLY294002, an inhibitor of phosphoinositide 3-kinase. Oxidative stress-related proteins were up-regulated by angiotensin II and inhibited by pretreatment with candesartan or catalase. The level of 8-hydroxy-2-deoxyguanosine was increased by angiotensin II and conversely decreased by catalase. Immunocytochemical studies showed that angiotensin II enhanced an inflammatory marker, inducible nitric oxide synthase, and the production of O2 radical. The hypothesis that angiotensin II has the potential to induce oxidative stress, which may be implicated in carcinogenesis of the prostate gland through long-term exposure to chronic inflammation is proposed. (Mol Cancer Res 2008;6(2):250–8)

Introduction

Prostate cancer is a major public health problem in men and the second leading cause of cancer death in the United States and Western developed countries (1). Recently, the prevalence of prostate cancer has also been increasing in Japan. Although the etiology has not been clarified, a possible cause is considered to be the westernization of the diet and lifestyle in Japan. At present, prostate cancer is thought to have a multifactorial etiology, with both environmental and genetic factors. We previously reported some genetic changes in prostate cancer cells and tissue detected using differential display method or GeneChip analysis. For example, the liplin-a2 and nmt55 genes have been identified using differential display method. The liplin-a2 gene was down-regulated by dihydrotestosterone in prostate cancer cells, and the nmt55 gene was up-regulated in prostate cancer tissue (2, 3). Also, we found that neuroserpin (protease inhibitor 12) expression was higher in prostate cancer than in normal prostate tissue by GeneChip analysis (4). Although numerous investigators have examined and identified genes related to prostate cancer, the mechanisms of prostate cancer development and progression remain obscure.

Because Virchow discovered leukocytes in neoplastic tissues and made the first connection between inflammation and cancer, the association of chronic inflammation with the development of cancer has been well recognized (5, 6). Under chronic inflammation, the release of reactive oxygen species (ROS) and reactive nitrogen species influences DNA damage in proliferating epithelium, leading to permanent genomic alterations, such as point mutations, deletions, or rearrangements (7). Oxidative or nitrosative stress occurs when excessive ROS and/or reactive nitrogen species are produced, overcoming cellular antioxidant defenses. Recently, a large number of investigations have consolidated the evidence that oxidative stress is implicated in carcinogenesis of the prostate gland. There have been accumulated reports indicating a reduction of antioxidant defense (8-10), elevated levels of ROS-related and reactive nitrogen species–related enzymes (11, 12), and
increased levels of hydrogen peroxide (11) in prostatic intraepithelial neoplasia (PIN) and cancer. Nevertheless, the cause of oxidative stress linked to prostatic carcinogenesis has not been clarified.

Angiotensin II, which is a major effector peptide of the renin-angiotensin system (RAS), is well known to be an important factor in hypertension. The classic RAS has been identified in the kidney, heart, and vessel walls and characterized in terms of blood pressure and electrolyte/Fluid homeostasis. On the other hand, involvement of a local RAS, with autocrine/paracrine roles rather than endocrine roles, has recently been documented in relation to growth/differentiation in specific organs. Interestingly, these phenomena also seem to occur in several cancer cells in which a local RAS is involved in the progression of cancer (13, 14). Recently, we have reported that angiotensin II affects the signal pathways in prostate cancer cells. Angiotensin II activates the signal transduction of mitogen-activated protein kinase and signal transducers and activators of transcription 3 and angiogenesis in prostate cancer cells (15). Also, angiotensin II facilitates the secretion of some growth factors and cytokines from prostate stromal cells, resulting in cell proliferation of prostate cancer (16). Of interest, many investigators have clarified that angiotensin II induces oxidative stress in vascular cells. Angiotensin II stimulates the production of ROS in endothelial cells by up-regulating the subunits of NADP+/NADPH (NADPH) oxidases (17, 18).

Apart from the attention RAS has attracted in relation to its roles in the circulation, another function of local RAS related to cell proliferation and angiogenesis has recently been focused on, especially in carcinogenesis. To confirm the postulate that angiotensin II would induce ROS in prostate cancer cells, we examined the expression of oxidative stress-related proteins and DNA damage induced by angiotensin II and/or an angiotensin II receptor type 1 blocker (ARB). Our results support the hypothesis that angiotensin II generated in the prostate gland may be a cause of oxidative stress linked to prostatic carcinogenesis.

Results

Time Courses of Akt Phosphorylation

In the male reproductive system, the angiotensin II concentration in semen is higher than that in blood (19), which is important for enhancement of sperm motility and perforation of the oocyte. Earlier investigation of semen extender showed that 10 μmol/L angiotensin II increased retention of spermatid stems (20), and another report indicated that 100 nmol/L angiotensin II stimulated the acrosome reaction in spermatozoa (19). Therefore, 10 μmol/L angiotensin II may not be a high level in a physiologic condition. As we previously reported, 10 μmol/L angiotensin II stimulated the cell growth of prostate cancer cells in LNCaP and DU145 cells (14). Akt has been implicated in the regulation of a variety of signal transduction pathways, including those involved in cell proliferation (9, 21-23). To confirm the activation of Akt in LNCaP cells treated with angiotensin II, we did a time-course Western blot analysis. As shown in Fig. 1, phosphorylated Akt was time-dependently enhanced by 10 μmol/L angiotensin II treatment. Activation of Akt was shown from 1 h after the start of angiotensin II stimulation and gradually increased until 24 h. This phenomenon is different from the patterns of phosphorylations of mitogen-activated protein kinase and signal transducers and activators of transcription 3 shown in our previous report (14).

We then did Western blot to confirm the inhibition of Akt phosphorylation induced by angiotensin II. Figure 2A shows that Akt phosphorylation was induced in a dose-dependent manner by angiotensin II treatment at 1 and 10 μmol/L for 3 h. The Akt phosphorylation induced by 10 μmol/L angiotensin II was inhibited by pretreatment with 1 and 10 μmol/L candesartan, an ARB, for 30 min (Fig. 2B). Also, the Akt phosphorylation induced by 10 μmol/L angiotensin II was completely inhibited by pretreatment with 40 μmol/L LY294002, an inhibitor of phosphoinositide 3-kinase for 30 min (Fig. 2C). Also, pretreatment with 1,000 units/mL catalase, an antioxidant enzyme, for 30 min inhibited the Akt phosphorylation induced by 10 μmol/L angiotensin II for 3 h in LNCaP cells (Fig. 2D).

Expression of AT1 Receptor in LNCaP Cells

To confirm expression of the angiotensin II receptor type 1 receptor AT1 receptor, LNCaP cells were treated with 10 μmol/L angiotensin II for 24 h. As shown in Fig. 3, expression of the AT1 receptor was enhanced by angiotensin II treatment. Unlike Akt phosphorylation shown in Fig. 2, expression of the AT1 receptor was not inhibited by pretreatment with 10 μmol/L candesartan (CV11974) or 1,000 units/mL catalase for 30 min. Therefore, this result indicates that candesartan and catalase do not influence the expression of the AT1 receptor in LNCaP cells.

Expression of Oxidative Stress-Related Proteins Induced by Angiotensin II and ARB Treatment

We then did Western blot to examine whether oxidative stress-related proteins were affected when prostate cancer cells (LNCaP) were stimulated by angiotensin II treatment. Besides the major generation of ROS by mitochondrial respiration, superoxide is also generated by a family of enzymes known as NADPH oxidases, and p47phox is a crucial cytosolic regulatory subunit of NADPH oxidases. The expression of p47phox protein was enhanced by 10 μmol/L angiotensin II treatment for 24 h, as shown in Fig. 4 (top). As anticipated, this augmentation of p47phox expression was inhibited by pretreatment with 10 μmol/L candesartan or 1,000 units/mL catalase for 30 min.

To defend cells against oxidative stress, cells have multiple antioxidant enzymes. Mitochondrial superoxide dismutase 2

![FIGURE 1](image-url) Western blots of phosphorylated Akt, total Akt, and actin. LNCaP cells cultured in steroid-free medium were harvested at the indicated times after angiotensin II (Ang-II; 10 μmol/L) treatment. The cells were lysed, and detergent extracts were immunoblotted with each antibody. Total protein (20 μg) was run and transferred to Immobilon-P transfer membrane and probed with phosphorylated Akt and total Akt antibody.
(SOD2) is one of the first lines of defense. Because accumulated H₂O₂ leads to the production of hydroxyl radicals, cells are equipped with multiple enzymatic pathways for their removal. Glutathione peroxidase is one of three major pathways in the second line of defense against oxidative stress. We examined the expression of antioxidant enzymes SOD2 and glutathione peroxidase in LNCaP cells treated with 10 μmol/L angiotensin II for 24 h. As a result, the patterns of their protein expression were similar to that of p47phox expression. In brief, the expression of both antioxidant enzymes was enhanced by angiotensin II treatment for 24 h, and pretreatment with candesartan or catalase inhibited their expressions. The results of inhibition of both antioxidant enzymes by pretreatment with candesartan or catalase are convincing, because the pretreatment reduced superoxide generation associated with inhibition of p47phox.

**Superoxide Anion (O₂⁻) Induction by Angiotensin II Treatment**

We determined the generation of the O₂⁻ radical, another important member of the ROS family, using fluorescent dye staining. Dihydroethidium (HE), which is a specific fluorescent dye for O₂⁻, exhibits a red color after reaction with O₂⁻.

After treatment with candesartan, catalase, or LY294002 for 30 min, LNCaP cells were treated with 10 μmol/L angiotensin II for 5 h. As shown in Fig. 5, angiotensin II enhanced the formation of the O₂⁻ radical (Fig. 5B) compared with control (Fig. 5A), whereas candesartan decreased O₂⁻ radical formation inside cells (Fig. 5C). Also, catalase decreased formation of the O₂⁻ radical induced by angiotensin II (Fig. 5D), because catalase is an antioxidant enzyme. Figure 5E shows that LY294002, an inhibitor of phosphoinositide 3-kinase, decreased the formation of the O₂⁻ radical induced by angiotensin II. We did semiquantitative digital image analysis of the O₂⁻ radical formation. Figure 5F indicates that angiotensin II enhanced the formation of the O₂⁻ radical ~28-fold higher than control and candesartan, catalase, and LY294002 decreased it, with which relative ratios compared with control were 0.6, 2.7, and 1.9, respectively.

To confirm whether angiotensin II enhances the formation of the O₂⁻ radical in prostate cancer cells, polyethylene glycol (PEG)–SOD, which is a representative antioxidant enzyme, was used as a positive control to show superoxide was being produced. Figure 6 shows that PEG-SOD clearly decreased formation of the O₂⁻ radical induced by angiotensin II (Fig. 6C). Semiquantitative image analysis indicates that PEG-SOD decreased O₂⁻ radical formation enhanced by angiotensin II at 14.2-fold down to 1.6-fold compared with control (Fig. 6D). These results provide direct evidence that angiotensin II can induce the generation of ROS in LNCaP cells and candesartan, as well as antioxidant enzymes, and an inhibitor of phosphoinositide 3-kinase can inhibit it.

**Expression of Checkpoints Proteins and Measurement of 8-Hydroxy-2’-Deoxyguanosine Level**

To confirm whether angiotensin II can provoke DNA damage in LNCaP cells, Western blot analyses to detect checkpoints proteins of DNA damage were done. After treatment with candesartan, catalase, or LY294002 for 30 min, LNCaP cells were treated with 10 μmol/L angiotensin II for 24 h. Figure 7A shows that angiotensin II induced the phosphorylation of p53 (Ser¹⁵), Chk2 (Thr⁶⁸), and cd2 (Tyr¹³), and candesartan or catalase inhibited them. 4-Hydroxynonenal, a product of cell membrane lipid peroxidation, has been suggested to be a key mediator of oxidative stress–induced cell death (24, 25). Like the phosphorylation of checkpoint proteins, modification of proteins with 4-hydroxynonenal was enhanced by angiotensin II treatment and inhibited by candesartan or catalase (Fig. 7A). We also investigated whether androgen (dihydrotestosterone) influenced oxidative stress in LNCaP cells, which resulted in the induction of 4-hydroxynonenal by dihydrotestosterone in a dose-dependent manner (data not shown).

**FIGURE 2.** Phosphorylation of Akt induced by angiotensin II treatment and diminished by candesartan, LY294002, and catalase. A, LNCaP cells were stimulated with 1 or 10 μmol/L angiotensin II for 3 h. B, Cells were pretreated with 1 or 10 μmol/L candesartan (CV) for 30 min and harvested after 3 h of 10 μmol/L angiotensin II exposure. C, Cells were pretreated with 40 μmol/L LY294002, an inhibitor of phosphoinositide 3-kinase, for 30 min and harvested after 3 h of 10 μmol/L angiotensin II exposure. D, Cells were pretreated with 1,000 units/mL catalase, an antioxidant enzyme, for 30 min and harvested after 3 h of 10 μmol/L angiotensin II exposure. All Western blots of protein lysates were probed with antibodies to phosphorylated Akt (p-Akt) and total Akt.

**FIGURE 3.** AT1 receptor expression induced by angiotensin II with/without candesartan and catalase. Western blots of AT1 receptor and actin. LNCaP cells were pretreated with 10 μmol/L candesartan or 1,000 units/mL catalase for 30 min and harvested after 24 h of 10 μmol/L angiotensin II exposure. Western blots of protein lysates were probed with anti-AT1 receptor and anti-actin antibodies.
As an indicator of DNA damage induced by oxidative stress, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is routinely used. We examined the 8-OHdG level in the media of LNCaP cells treated with angiotensin II and/or candesartan (CV11974). The formation of 8-OHdG in LNCaP cells was significantly increased by treatment with 10 μmol/L angiotensin II (Fig. 7B) and significantly suppressed by 1 or 10 μmol/L CV11974 treatment (P < 0.02). These results were consistent with those of Western blots, which showed the pattern of checkpoint proteins and 4-hydroxynonenal expression induced by angiotensin II or candesartan treatment.

Inducible Nitric Oxide Synthase Expression in Prostate Cancer Cells with Angiotensin II and ARB Treatment

To confirm whether angiotensin II induces inflammation in prostate cancer cells, we did immunocytochemical staining of an enzyme dominantly expressed during inflammatory reactions. Inducible nitric oxide synthase (iNOS) is expressed in a variety of acute or chronic inflammatory human diseases, as well as in various types of cancer, including prostate cancer (26). iNOS antibody was used to detect the expression in LNCaP cells. The secondary antibody for iNOS was Cy3, labeled and raised against rabbit IgG. LNCaP cells were stimulated with 10 μmol/L angiotensin II after treatment with two different ARBs, candesartan and telmisartan (Tel), for 30 min. After 8 h stimulation, staining of iNOS and secondary antibodies was done. Figure 8 shows that intracellular expression of iNOS was induced by stimulation with angiotensin II (Fig. 8B) compared with the control (Fig. 8A), whereas candesartan and telmisartan diminished

[Image of figures and tables]

FIGURE 4. Expression of oxidative stress-related proteins induced by angiotensin II with/without candesartan and catalase. LNCaP cells were pretreated with 10 μmol/L candesartan or 1,000 units/mL catalase for 30 min and harvested after 24 h of 10 μmol/L angiotensin II exposure. Western blots of protein lysates (20 μg) were probed with antibodies to p47phox, glutathione peroxidase (GPx), SOD2, and actin.

FIGURE 5. Determination of O₂⁻ production by H&E staining using confocal fluorescence microscope. Cells were plated onto a glass slip in a two-well plate for 24 h, followed by pretreatment with candesartan (10 μmol/L), catalase (1,000 units/mL), or LY294002 (40 μmol/L). Cells were then treated with angiotensin II (10 μmol/L) for 5 h. H&E was applied to the cells 30 min before treatment was completed. After being stained, the cells were washed twice with PBS and fixed with 10% buffered formalin. The images were captured with a confocal fluorescence microscope. A, Control. B, Angiotensin II. C, Angiotensin II and candesartan. D, Angiotensin II and catalase. E, Angiotensin II and LY294002. F, Semiquantitative digital image analysis of O₂⁻ production by H&E staining in each panel.
iNOS expression induced by angiotensin II (Fig. 8C and D). Like in LNCaP cells, similar phenomena were shown in DU145 cells (data not shown).

Discussion

Although angiotensin II is well known to be an important factor in hypertension, it has also been reported to play a central role in proliferation and/or differentiation in specific organs, linking it to the progression of cancer (13, 14). Previously, we have reported that angiotensin II facilitates the secretion of some growth factors and cytokines from prostate stromal cells, resulting in cell proliferation of prostate cancer (16). Given that angiotensin II induces oxidative stress in vascular cells, it is very interesting to speculate that angiotensin II may function as an inducer of oxidative stress implicated in carcinogenesis. In the present study, we examined whether angiotensin II has the potential to evoke oxidative stress in prostate cancer cells. The expression of SOD2, one of the mitochondrial antioxidant enzymes, was time-dependently enhanced by angiotensin II treatment in prostate cancer cells (data not shown). Also, angiotensin II induced the expression of p47phox, one of cytosolic NADPH oxidases, whereas the angiotensin receptor blocker candesartan (CV11974) inhibited its expression in prostate cancer cells. Furthermore, angiotensin II induced the expression of antioxidant enzymes, glutathione peroxidase, and SOD2, and candesartan inhibited their expression. It is well known that oxidative stress induces DNA and protein adducts, and similarly, angiotensin II induced the expression of 8-OHdG and modification of protein with 4-hydroxynonenal expression in prostate cancer cells.

Early reports revealed the existence of RAS in the prostate; all components of RAS, including angiotensinogen, renin, ACE, and angiotensin receptors (27-31), have been identified in the prostate. Intriguingly, a 3-fold to 5-fold higher concentration of angiotensin II was confirmed in seminal fluid in comparison with that in blood (32), which strongly supports the existence of RAS in the prostate gland. Likewise, it was reported that there was a high concentration of angiotensin II in pancreatic cancer tissue compared with that in normal tissue (33), and RAS components, including angiotensinogen, angiotensin II receptor type 1, and renin, have been identified in the islets of Langerhans (34). These findings provide supportive evidence that the local RAS in these organs, especially angiotensin II, may exert paracrine effects in the development of prostate and pancreatic cancer. The possible mechanism of carcinogenesis by angiotensin II is expected to be induction of oxidative stress in these organs.

We previously clarified that angiotensin II acted as a growth factor in prostate cancer and stromal cells (14, 16). Interestingly, earlier investigators have reported that angiotensin II induced oxidative stress in vascular cells, for example, demonstrating that angiotensin II stimulated the production of ROS in endothelial cells by up-regulating the subunits of NADPH oxidases (17, 18). In the present study, we confirmed that angiotensin II–induced production of the O$_2^-$ radical in prostate cancer cells, as shown in Fig. 5. Higashi et al. confirmed that NADPH oxidase is the most important source of ROS in the vasculature (35). It is well known that NADPH oxidase is composed of cytosolic components, such as p47phox, p67phox, and RC1, and membrane-spanning components, such as p22phox and gp91phox. Angiotensin II–induced NADPH oxidase activation is one of the major sources of ROS in the pathophysiologic mechanism of atherosclerosis (35, 36). These observations are consistent with the findings of the present study, in which angiotensin II induced the expression of p47phox and production of the O$_2^-$ radical.
It is likely that induction of ROS subjects the cell to a state of oxidative stress, leading to damage of cellular DNA and proteins (37). A growing body of evidence has shown that excessive lipid peroxidation generated by oxidative stress may be involved in carcinogenesis. Especially, 4-hydroxynonenal is a major product of lipid peroxidation, and its level becomes relatively high in cells under oxidative stress (38). An augmented level of 4-hydroxynonenal was observed in oxidative stress-related degenerative diseases (39, 40). Furthermore, accumulation of 8-OHdG has been shown to lead to G:C-to-T:A transversion mutations that are prevalent in mutated oncogenes and tumor suppressor genes (41, 42). As shown in the present data, angiotensin II stimulation augmented DNA and protein markers, such as 8-OHdG, in prostate cancer cells. In general, DNA damage triggers the cell cycle checkpoints G2-M and G1-S through activation of kinases, e.g., phosphorylation of Chk2 at Thr68 and multiple sites in p53 (43, 44). Our data also showed that angiotensin II induced the phosphorylation of Chk2 (Thr68) and p53 (Ser15). Chronic oxidative stress has been implicated in neoplastic transformation (45) and promotion of tumorigenesis (46). The present study provides evidence that ROS generated by angiotensin II may contribute to DNA damage and be involved in carcinogenesis of the prostate gland.

The present data showing that angiotensin II enhanced iNOS expression and ARBs inhibited it in prostate cancer cells support the evidence that angiotensin II is a peptide involved in inflammation. In vascular smooth muscle cells, iNOS may be induced by various cytokines, including interleukin 1β, tumor necrosis factor α, IFN-γ, and interleukin 6 (47). Also, iNOS was strongly observed in inflamed epithelium. Earlier studies have shown that iNOS is expressed in epithelial cells in patients with colitis who are prone to colon cancer (48-50). Regarding the link between iNOS and cancer development, the expression of iNOS was higher in cancer specimens than in normal tissue in esophageal, colon, and thyroid carcinomas (51, 52). Baltaci et al. reported that the expression of iNOS was higher in high-grade PIN and prostatic carcinoma samples than in benign prostatic hypertrophy and low-grade PIN samples. These findings suggest that nitric oxide produced by iNOS may be involved in prostate tumorigenesis (53). In addition, Calvisi et al. revealed that iNOS and NADPH oxidase were involved in ROS generation during c-Myc/transforming growth factor α hepatocarcinogenesis and were inhibited by antioxidant agent treatment (54).

There have recently been several reports suggesting a relationship between chronic inflammation and prostate cancer. It is plausible that ROS released by inflammatory cells during cycles of cellular damage and regeneration in the organset result in permanent DNA damage (55). The prostate gland is a common site of chronic inflammation. Although most focal lesions of prostatic atrophy are considered quiescent, prostate epithelial cell proliferation is increased in some lesions, and thus, focal prostatic atrophy, which is associated with chronic inflammation, is considered to be proliferative (56).

**FIGURE 7.** DNA damage in LNCaP cells by angiotensin II treatment. A, LNCaP cells were pretreated with 10 µmol/L candesartan or 1,000 units/mL catalase for 30 min and harvested after 24 h of 10 µmol/L angiotensin II exposure. Western blots of protein lysates (20 µg) were probed with antibodies to checkpoint proteins, including phosphorylated p53 (Ser15), phosphorylated Chk2 (Thr68), phosphorylated cdc2 (Tyr15), and actin. To examine the DNA damage by angiotensin II treatment, Western blot was also done using anti--4-hydroxynonenal (4HNE) antibody. B, 8-OHdG level was measured after treatment with angiotensin II at the indicated concentrations or simultaneously with 10 µmol/L CV11974 and 1 µmol/L telmisartan, an ARB, diminished iNOS expression induced by angiotensin II treatment (C and D).

**FIGURE 8.** Immunocytochemical staining of iNOS in LNCaP cells. Intracellular expression of iNOS was induced by stimulation with 10 µmol/L angiotensin II (B) compared with the control (A), whereas 10 µmol/L CV11974 and 1 µmol/L telmisartan, an ARB, diminished iNOS expression induced by angiotensin II treatment (G and D).
Pathologic findings support the hypothesis that chronic inflammation may be involved in prostate cancer development. Novel observations have been reported regarding the pathologic entity of focal prostatic epithelial atrophy (57), which is associated with chronic inflammation with proliferative change, designated “proliferative inflammatory atrophy.” More interestingly, these areas are commonly adjacent to high-grade PIN or local foci of cancer in the prostate of older men (58). Proliferative inflammatory atrophy lesions have been regarded as a precursor lesion of prostate cancer (59). In an experimental study, prostatic tissue with inflammation caused by bacterial infection showed atypical hyperplasia and areas of dysplasia showed stronger staining for oxidative DNA damage and greater epithelial cell proliferation than normal prostatic gland (60). The pathogenesis of the development of prostate cancer possibly involves ROS generated through various actions of androgen, infection, or angiotensin II, as mentioned above. Theoretically, it is proposed that long-term exposure to ROS may cause DNA and protein damage, cell proliferation, and enhancement of oncogenes, linking it to the development of proliferative inflammatory atrophy, PIN, and, finally, prostate cancer (Fig. 9).

Androgen is also likely to play a central role in prostate carcinogenesis. There has been increasing evidence to support the hypothesis that oxidative stress induced by androgen, at least in part, contributes to carcinogenesis. Androgen-induced ROS may directly or indirectly result from its influence on mitochondria (61). An in vitro study using androgen-sensitive human prostate cancer cells, LNCaP, showed that stimulation with a physiologic level of androgen resulted in an increased level of ROS (62). Supporting this hypothesis, Sun et al. suggested that prostate-specific antigen, a representative androgen-dependent protein, markedly stimulates the generation of ROS in LNCaP cells. They also showed that the effect of testosterone on ROS was suppressed by flutamide and by anti–prostate-specific antigen antibody (63).

In conclusion, epidemiologic, experimental, and clinical studies have implicated oxidative stress in the development and progression of prostate cancer. The present study indicated that oxidative stress caused by the local generation of angiotensin II may be involved in the development of prostate cancer. A greater understanding of the molecular events associated with oxidative stress will contribute to better strategies for the chemoprevention of prostate cancer.

Materials and Methods

Cell Lines

LNCaP cells, a human prostate cancer cell line, were obtained from the American Type Culture Collection. LNCaP cells were cultured in F-12 medium supplemented with 10% FCS under 5% CO2 before the experiments. In the experiments, LNCaP cells were cultured in phenol red–free RPMI plus 0.1% bovine serum albumin and stimulated with reagents. Cells were used for each experiment within 10 to 12 passages.

Reagents

Angiotensin II was purchased from Auspep Pty. Anti-SOD2 antibody was purchased from Millipore. Anti-p47phox, anti-AT1 receptor, and anti-iNOS antibodies were purchased from Santa Cruz Biotechnology. Anti–4-hydroxynonenal antibody was purchased from NOF Co. Anti-Akt, phosphorylated Akt, phosphorylated p53 (Ser15), phosphorylated Chk2 (Thr68), and phosphorylated cdc2 (Tyr15) antibodies were purchased from Cell Signaling Technology. Catalase, PEG-SOD, and LY294002 were purchased from Sigma. The ARBs candesartan (CV11974) and telmisartan were provided by Takeda Pharmaceutical Co. and Boehringer Ingelheim, respectively.

Immunocytochemical Staining

Cells plated on culture slides, Lab-Tek Chamber Slide (Nalge Nunc International), were rinsed with PBS twice before they were fixed in 2% paraformaldehyde/PBS at room temperature for 15 min. Fixed cells were washed with 100 mmol/L ammonium chloride for 10 min and permeabilized with 0.1% Triton X-100/PBS for 10 min. These cells were blocked with 10% normal goat serum/PBS for 1 h. Primary antibody against iNOS was diluted 1:100 in 10% normal goat serum/TBST [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.05% Tween 20] and applied for 1 h at a 37°C chamber. After an additional three washes with TBST for 5 min, cells were incubated with Cy3-labeled secondary antibody for 1 h in a 37°C chamber. Before cells were mounted in antifade reagent, they were washed thrice in TBST for 5 min. As a control, sections were incubated without treatment with angiotensin II or nonprimary antibody. Images were captured using a fluorescence microscope (BZ-8000; Keyence).

Superoxide Anion (O$_2^-$) Assay

Dihydroethidium (HE), which is a specific dye for O$_2^-$, is oxidized by O$_2$ to ethidium, which stains the nucleus a bright

FIGURE 9. Pathogenesis of prostate cancer possibly involves ROS generated by various actions of androgen, infection, or angiotensin II. Theoretically, it is proposed that long-term exposure to ROS causes DNA and protein damage, cell proliferation, and enhancement of oncogenes, linking it to the formation of proliferative inflammatory atrophy (PIA), PIN, and, finally, prostate cancer.
fluorescent red. The cells were plated onto a glass slip in a two-well plate at 2 × 10^4 cells per well and incubated for 24 h. Cells were then treated with 10 μmol/L angiotensin II for 5 h after pre-treatment with candesartan, catalase, or LY294002 for 30 min. HE was added into the cell culture 30 min before treatment was completed. After being stained, the cells were washed in cold 1 × PBS and fixed with 10% buffered formalin. The slip was mounted on a glass slide and observed using a fluorescence microscope (BZ-8000, Keyence) fitted with an argon-ion laser.

Semiquantitative Digital Image Analysis

To quantify the levels of O_{2} production, we used Image J (NIH) to perform semiquantitative digital image analysis of H&E staining. The panels of Figs. 5 and 6 were split into red and blue images, and the mean level values of red and blue images were measured. The levels of O_{2} production were determined by dividing the mean level of red by that of blue in each panel.

Determination of 8-OHdG Level

LNCaP and DU145 cells were cultured on culture slides in phenol red–free RPMI plus 0.1% bovine serum albumin for 24 h, and the medium was collected immediately at 24 h after 10 μmol/L angiotensin II exposure with/without 10 μmol/L CV11974 or 1 μmol/L telmisartan as indicated in the figures. 8-OHdG level was measured as previously reported, according to the manufacturer’s instructions (64). In brief, culture supernatants after these treatments as described above were centrifuged at 10,000 × g for 10 min, and the supernatants were used for the determination of 8-OHdG level using a quantitative sandwich ELISA kit (NOF Co.) with a determination range of 0.125 to 10 ng/mL. Absorbance was determined with a microplate reader (Bio-Rad) at 450 nm. All analyses and calibrations were done in triplicate. A standard curve was created using Excel (Microsoft 2003 version) by plotting the logaritum of the mean absorbance of each sample versus the sample concentration.

Western Blot Analysis

LNCaP cells were cultured in phenol red–free RPMI plus 0.1% bovine serum albumin for 24 h. Then cells were pretreated with 10 μmol/L CV11974, 1,000 units/mL catalase, or 40 μmol/L LY294002 for 30 min and harvested at several points as indicated in the figures after 10 μmol/L angiotensin II exposure. Cells in the appropriate conditions were washed twice with ice-cold PBS, lysed in ice-cold buffer consisting of 20 mmol/L Tris (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 0.1% SDS, 0.5% Nonidet P-40, 100 mmol/L sodium fluoride, 200 mmol/L sodium orthovanadate, 1 mmol/L EGTA, 2 mmol/L phenylmethylsulfonfluryl fluoride, 1 mg/mL leupeptin, and 3 mg/mL aprotinin, and centrifuged (30 min, 4°C, 14,500 × g). After quantitation, 20 μg of each cell lysate were added to SDS gel loading buffer (containing a reducing agent) and boiled for 5 min. The samples were subjected to SDS-PAGE on 10% gel and electrotransferred to Immobilon-P (Millipore). After blocking the membrane with 5% albumin, Western blotting was done using the antibody of interest, and the product was detected with an enhanced chemiluminescence detection system (Amersham).


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