Original Paper

Aspirin Inhibits IKK- β -mediated Prostate Cancer Cell Invasion by Targeting Matrix Metalloproteinase-9 and Urokinase-Type Plasminogen Activator

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Key Words

Invasion • Matrix metalloproteinase • Nuclear factor-kappa B • Prostate cancer • Urokinase-type plasminogen activator

Abstract

Background/Aims: Aspirin has been demonstrated to possess potent chemopreventive and anticancer effects on prostate cancer. However, the more detailed molecular mechanisms of aspirin to suppress prostate cancer cell invasion have not been clearly elucidated. **Methods:** Transwell assays were performed to evaluate the effects of aspirin on cell invasion. Matrix metalloproteinases (MMPs) and serine proteinases activities in cell media were examined by gelatin zymography and ELISA. In addition, inhibitor of κ B (I κ B) kinase- β (IKK- β) phosphorylation and IKK- β kinase activity were measured to assess the effects of aspirin on IKK- β activation. **Results:** We found that aspirin suppressed the invasion and attachment in human prostate cancer cells. Aspirin treatment significantly resulted in reduction of matrix metalloproteinase-9 (MMP-9) and upregulation of tissue inhibitors of metalloproteinase-1 (TIMP-1) activity, which are the proteolytic enzymes contributing to the degradation of extracellular matrix and basement membrane in cell invasion and metastasis. Our data further showed that aspirin was able to inhibit both urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) expression in the cells. In addition, aspirin treatment caused a strong decrease in nuclear factor-kappa B (NF- κ B) activation, inhibitor of κ B (I κ B)- α phosphorylation together with translocation of NF- κ B p65 to nucleus and I κ B kinase (IKK)- β activation. Moreover, the inhibitory effects of aspirin on cell invasion were reversed by IKK- β overexpression, while the IKK inhibitor sensitizes the anti-invasive effect of aspirin in prostate cancer cells. **Conclusion:** The present research concluded that aspirin suppressed prostate cancer cell invasion by reducing MMP-9 activity and uPA expression through decreasing of IKK- β -mediated NF- κ B activation, indicating that the ability of aspirin to inhibit cell invasion might be useful in the chemoprevention of metastatic prostate cancer.

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Introduction

Prostate cancer is one of the most frequently diagnosed malignancies among men in the world [1, 2]. The initial treatment of prostate cancer is the suppression of testicular androgen production by medical or surgical castration, but many patients develop disease progression. Since there are no efficacious therapies for the advanced prostate cancer with hormone refractory or metastatic phenotypes, the need for developing new methods that could inhibit the progression or invasive abilities of prostate cancer is obvious.

An essential step in the process of cancer invasion and metastasis involves the penetration of the extracellular matrix (ECM) and basement membrane, and it requires the action of proteases [3]. A number of proteolytic enzymes, including matrix metalloproteinases (MMPs) and serine proteinases, contribute to the degradation of ECM in connection with cancer invasion. MMPs are a large family of proteolytic enzymes that can cleave the components of the extracellular matrix. The elevated activities of MMPs are associated with metastases in many types of cancers and are related to advanced cancer stage and poor patient prognosis [4]. Among the MMPs, gelatinase-A (MMP-2) and gelatinase-B (MMP-9) represent key enzymes for degrading type IV collagen, which is a major component of the basement membrane [5]. It is reported that the elevated expression of MMP-2 and MMP-9 is well known for promoting prostate cancer metastases [6], and both MMP-2 and MMP-9 are helpful in the prediction of prostate cancer progression [7]. In addition to MMPs, the plasminogen activators are also involved in cell adhesion and invasion [8]. The serine proteases urokinase-type plasminogen activator (uPA) and its highly glycosylated receptor (uPAR), together with plasminogen activator inhibitor-1 (PAI-1), are responsible for the invasive characteristics of the cancer cells [8]. uPA has the ability to degrade the components of the ECM, including laminin, fibronectin and collagen, and it is believed to be a particularly efficient proteolytic system in the prostate cancer cell invasion and metastases [9]. Moreover, high uPA expression is found in patients with prostate cancer compared with those with benign prostatic hyperplasia [10]. Thus, it appears reasonable to consider uPA as a valid therapeutic target and evaluate the benefits of blocking uPA with currently available treatment to prevent invasion and metastasis in prostate cancer.

Expression of MMPs and uPA/uPAR has been reported to be under the regulation of extracellular mediators such as growth factors and intracellular signaling by several transcriptional factors such as nuclear factor-kappaB (NF- κ B) [11, 12]. NF- κ B is a key transcriptional factor involved in regulating expression of many chemokines and cytokines, thereby playing a critical role in mediating inflammation, immunity, apoptosis, and migration in the cancer cells. It is a dimeric transcription factor consisting of homo- or heterodimer composed of p65 and p50 or closely related proteins. Generally, NF- κ B resides in the cytoplasm and forms a multiprotein complex with the inhibitor of NF- κ B (I κ B). On activation by the stimuli, the signal activates the I κ B kinase (IKK), which could phosphorylate I κ B α and lead to its ubiquitination and degradation [13]. These processes lead to the liberated NF- κ B entering the nucleus and regulation of a variety of downstream target genes [14]. Recently, it has been demonstrated that blockade of the NF- κ B activation suppresses the release of many mediators by the cells, eventually modulating the cell survival, invasion, proliferation and death [12, 15]. Therefore, modulation of NF- κ B and the related gene expression is thought to be important for cancer treatment.

Aspirin is a nonsteroidal anti-inflammatory drug which has been reported to play an important role in the chemoprevention of carcinogenesis. The anti-inflammatory activity of aspirin has been proposed through inhibiting cyclooxygenase-2, NF- κ B, iNOS and various cytokines [15, 16]. Many studies have focused on aspirin and its ability to inhibit the invasion of a number of cancer cells, including breast, colon, oesophageal and lung cancer cells [17, 18]. Moreover, there is some evidence that the use of aspirin is inversely related to the risks of developing prostate cancer [19, 20], and aspirin also influences prostate cancer growth and metastasis by down-regulation of androgen receptor and prostate-specific antigen [21,

22]. However, the potential molecular mechanisms by which aspirin inhibits prostate cancer cell invasion have not been clearly elucidated.

In the present study, we showed that aspirin suppressed prostate cancer cell invasion via modulation of proteolytic enzymes, which were dependent on the IKK- β -mediated NF- κ B activation. Our results revealed the role of aspirin as an effective agent to negatively regulate invasion and metastasis of cancer cells and suggested that the invasion prevention might be a therapeutic strategy in the management of prostate cancer.

Materials and Methods

Chemicals, reagents and cell culture

Aspirin was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 100% ethanol in the studies. The effects of aspirin in the experiments were compared with the same concentration of ethanol as vehicle. Antibodies for Histone H2B, β -actin and NF- κ B p65 were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for I κ B- α , phospho-I κ B- α , IKK- α , IKK- β and phospho-IKK- α/β were obtained from Cell Signaling Technology (Beverly, MA). The IKK- β -specific inhibitor Bay 65-1942 was from ApexBio Technology (Houston, TX). Human prostate cancer DU145, LNCaP and PC-3 cells were purchased from the American Type Cell Collection (Manassas, VA, USA) and were cultured in RPMI-1640 containing penicillin (100 IU/ml), streptomycin (100 mg/ml) with 10% fetal bovine serum (Hyclone, South Logan, UT).

Cell invasion assay

Cells were treated with vehicle or aspirin, then the transwell assays were conducted according to the previously described [23]. In brief, transwells were coated with Matrigel (BD Biosciences, San Diego, CA) for cell invasion assay. Cells were treated with vehicle or aspirin for 48 h, and then the cells were seeded at a density of 5×10^4 cells in the upper chambers of transwells. The lower chambers were filled with the media containing vehicle or aspirin. After 12 h incubation at 37 °C, the cells which had invaded through the matrigel were fixed and counted by microscope.

Cell attachment assay

Cells were treated with vehicle or aspirin for 48 h, and then the cells were seeded in 24-well tissue culture plates at a density of 10^5 cells/well in 2ml media. After incubation for 1h at 37 °C, the cells were rinsed with phosphate-buffered saline and incubated with 2ml media for another 1h at 37 °C. After washing off unattached cells, attached cells were stained with 0.5% crystal violet in methanol for 10 min and counted by microscope.

Gelatin zymography

For gelatin zymography, equal number of cells was incubated in the presence or in the absence of aspirin. Then cell media was collected and the gelatinolytic activities of MMP-2, MMP-9, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 were detected by gelatin zymography. Briefly, samples were subjected to nonreduced SDS-PAGE using a 12% gel containing 0.1% gelatin. Following electrophoresis, the gels were rinsed in 2.5% Triton X-100 solution at room temperature. The gels were then incubated overnight in reaction buffer (50 mm Tris/pH 7.4, 100 mm NaCl, 10 mm CaCl₂ and 0.02% NaN₃) at 37°C and stained with Coomassie Blue.

MMP-2, MMP-9, TIMP-1 and TIMP-2 activities assays

The activities of MMP-2, MMP-9, TIMP-1 and TIMP-2 in cell media were evaluated with MMP-2, MMP-9, TIMP-1 and TIMP-2 Amersham Biotrak™ assays (Amersham Biosciences) according to the manufacturer's instruction. These assays are based on the quantitative sandwich enzyme immunoassay technique. Before the analysis, samples were centrifuged and three assays were conducted, with each sample done in triplicate. Measurements were made using an ELISA reader in a microplate spectrophotometer and the results were calculated as the ratio of the absorbance of the aspirin-treated cells/absorbance of vehicle-treated cells.

Quantitation of uPA and PAI-1 Levels

Cells were treated with vehicle or aspirin, and then cell media was collected. The levels of secreted uPA and PAI-1 were determined using the uPA ELISA Kit (Abcam, Cambridge, UK) and PAI-1 ELISA Kit (Abcam, Cambridge, UK) according to the manufacturer's instruction. Absorbance was measured at 450 nm using a microplate spectrophotometer. Each assay was conducted in triplicate.

Luciferase assays

The NF- κ B luciferase reporter construct (NF- κ B-Luc) containing four copies of NF- κ B cis-acting elements linked to TATA box was cloned into pGL3 basic vector [24]. Cells were plated in 24-well plates, then the cells were co-transfected with NF- κ B-Luc plasmid and the internal control plasmid renilla luciferase (pRL-TK) using transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously [25]. The cells were harvested after aspirin treatment, and the luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase activity was measured and the reading was normalized to renilla luciferase activity, which served as an internal control for transfection efficiency.

IKK- β kinase activity assay

The IKK- β kinase activity was determined by HTScan IKK- β Kinase Assay Kit (Cell Signaling Technology). The kit contains GST-IKK- β kinase protein, a biotinylated peptide substrate and a phosphoserine antibody for detection of the phosphorylated form of the substrate peptide. Assay was performed according to the manufacturer's protocol.

Adenoviral infection

Cells were transfected either with adenoviruses encoding IKK- β or β -galactosidase (β -gal) as described previously [26]. Adenoviral vector transductions were carried out in the medium overnight, and then the virus-containing medium was replaced by fresh medium 24 h later. The cells were infected with IKK- β or β -gal adenovirus at multiplicity of infection (MOI) 50 for 24 h, achieving around 80% to 90% of transduction.

Nuclear fractionation

Cells were treated and then the cells were harvested with ice cold PBS. The extraction of nuclear protein was obtained by a modified protocol as previous described [13]. Briefly, the cells were collected in hypotonic lysis buffer (10 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 0.2 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride, and 1 mmol/l dithiothreitol) with protease inhibitor cocktail and incubated on ice for 5 min. The cell lysate was chilled on ice for 10 min and then vigorously shaken for 10 min in the presence of 0.6% Nonidet P-40. The nuclear fraction was precipitated by centrifugation. Nuclear fractionation was extracted by addition of high-salt buffer (20 mmol/l HEPES, pH 7.9, 400 mmol/l KCl, 0.2 mmol/l EDTA, 0.2 mmol/l phenylmethylsulfonyl fluoride, and 1 mmol/l dithiothreitol with protease inhibitor cocktail) with continuous shaking, then centrifuged and the supernatants were collected.

Western blot analysis

Cells were treated and then the cells were prepared with lysis buffer and western blot analysis was performed [25]. The proteins were separated by 10% SDS-PAGE, then transferred onto PVDF membranes and blotted with primary antibodies against Histone H2B (1:400), β -actin (1:400), NF- κ B p65 (1:400), I κ B- α (1:1000), phospho-I κ B- α (1:1000), IKK- α (1:1000), IKK- β (1:1000) and phospho-IKK- α/β (1:1000) at 4°C overnight, and then incubated with the secondary antibody (1:10000) for 2 h. The blots were normalized against β -actin or Histone H2B. The experiments were replicated at least three times.

Statistical analysis

All assays were performed in triplicate. Results were analyzed by two-sided unpaired Student's t-test using SPSS software (SPSS, Inc., Chicago, IL, USA) to assess the statistical significance of the difference between the groups. Data were presented as mean $\pm$ SD from three independent experiments. For all analyses, differences were considered significant at $P < 0.05$.

Fig. 1. Effects of aspirin on cell invasion and attachment in prostate cancer cells. (A) Cells were treated with vehicle or aspirin (0-5 mmol/l) for 48 h and followed with a 12 h invasion period, and then the penetrating cells in the lower membrane were counted. (B) Cells were treated with vehicle or aspirin (0-5 mmol/l) for 48 h before a 1h attachment assay, and the attached cells were counted. The data were expressed as the fold changes relative to the cells treated with vehicle. Results were expressed as mean $\pm$ SD of three independent experiments. * P <0.05, as compared with the vehicle group.

Results

Aspirin suppressed the invasive potential of prostate cancer cells

One of the major characteristic of metastasis is the invasive ability of cancer cells, so the effects of aspirin on prostate cancer cell invasion were examined by transwell assays. As shown in Fig. 1A, we found that the invasion of DU145, LNCaP and PC-3 cells was decreased by approximately 47%, 66% and 55% after 5 mmol/l aspirin treatment (P <0.05, P <0.05 and P <0.05, respectively). In addition, we also examined the effects of aspirin on cell attachment, and we found that 5 mmol/l aspirin reduced cell attachment by 72%, 76% and 87% in DU145, LNCaP and PC-3 cells respectively (all P <0.05; Fig. 1B).

Aspirin affected the activities of MMPs in prostate cancer cells

To examine the mechanisms underlying the anti-invasive effects of aspirin in prostate cancer cells, the levels of secreted MMP activities in cell media were examined. As shown in Fig. 2A and 2B, the activity of MMP-9 was significantly suppressed by 5 mmol/l aspirin in DU145, LNCaP and PC-3 cells. However, the activity of MMP-2 was barely affected by aspirin (Fig. 2A and 2C).

Most MMPs are inhibited by the specific inhibitors TIMPs, so we also tested whether aspirin regulated TIMP-1 and TIMP-2 activities. As shown in Fig. 3A and 3B, TIMP-1 activity was induced in DU145 and PC-3 cells after 5 mmol/l aspirin treatment, while the effect of aspirin on the activity of TIMP-1 was modest in LNCaP cells. In addition, the activity of TIMP-2 was barely affected by aspirin in all the cells (Fig. 3C

Fig. 2. Effects of aspirin on MMP-9 and MMP-2 activities. Cells were treated with vehicle or aspirin (0-5 mmol/l) for 48 h, and then the media were collected. (A) The activities of MMP-9 and MMP-2 were analyzed by gelatin zymography. The activities were expressed as the fold changes relative to the cells treated with vehicle by densitometric analysis. Additionally, MMP-9 (B) and MMP-2 (C) activities were also analyzed by Amersham Biotrak™ assay. Results were presented as the fold changes relative to the cells treated with vehicle. * P <0.05, as compared with the vehicle group.

Fig. 3. Effects of aspirin on TIMP-1 and TIMP-2 activities. Cells were treated with vehicle or aspirin (0–5 mmol/l) for 48 h, and then the media were collected. (A) The activity of TIMP-1 was analyzed by gelatin zymography. (B) TIMP-1 activity was analyzed by Amersham Biotrak™ assay. (C) The activity of TIMP-2 was analyzed by gelatin zymography. The activity was expressed as the fold changes relative to the cells treated with vehicle by densitometric analysis. (D) TIMP-2 activity was analyzed by Amersham Biotrak™ assay. Results were presented as the fold changes relative to the cells treated with vehicle. * $P < 0.05$, as compared with the vehicle group.

and 3D). The data suggested that aspirin-inhibited prostate cancer cell invasion partly via modulating both MMP-9 and TIMP-1 activities in prostate cancer cells.

Aspirin regulated the levels of uPA and PAI-1 in prostate cancer cells

It is demonstrated that uPA and PAI-1 are involved in the invasive activity of cancers and play prominent roles in cell migration [8, 27], so the effects of aspirin on the amount of uPA and PAI-1 were examined. As shown in Fig. 4A and Fig. 4B, both the levels of secreted uPA and PAI-1 were significantly decreased in prostate cancer cells after 5 mmol/l aspirin treatment.

Aspirin suppressed NF- κ B activation in prostate cancer cells

NF- κ B is constitutively activated in a variety of human malignancies including prostate cancer and it could regulate the expression of many growth genes and the cell biological behavior [15, 28]. Thus, the role of NF- κ B was determined in the cell invasion after aspirin treatment. Using a NF- κ B-luc reporter gene expression assay, we found that aspirin treatment caused strong decrease in NF- κ B activation in DU145 cells, and the similar results were found in LNCaP and PC-3 cells (Fig. 5A). Consistently, the expression of NF- κ B p65 in the nucleus, which was the indicator of NF- κ B transcription activity, was also inhibited in these cells treated with aspirin (Fig. 5B).

It is reported that PC-3 and DU145 cells have a high invasive behavior compared with LNCaP cells [29] in agreement with the present study. Therefore, we used DU145 cells, which were more aggressive and expressed high levels of MMP-9 and uPA compared with PC-3 and LNCaP cells, to perform the further experiments. We chose 5 μ mol/l aspirin, which concentration of aspirin could decrease the invasion of DU145 cells, to investigate the regulation of aspirin on the NF- κ B activation. As shown in Fig. 5C, we found that aspirin dramatically inhibited cytoplasmic level of I κ B- α degradation and phosphorylation.

Aspirin suppressed cell invasion through downregulation of IKK- β activation

The phosphorylation of I κ B by IKK is an important step in NF- κ B activation [13, 14]. To clarify the molecular mechanisms by which aspirin inhibited the activation of NF- κ B in

Fig. 4. Effects of aspirin on secreted uPA and PAI-1 levels. Cells were treated with vehicle or aspirin (0-5 mmol/l) for 48 h, and then uPA (A) and PAI-1 (B) levels were determined in the cell media. Results were presented as the fold changes relative to the cells treated with vehicle. *P<0.05, as compared with the vehicle group.

prostate cancer cells, we studied whether aspirin affected IKK activation in DU145 cells. As shown in Fig. 6A and 6B, aspirin treatment dramatically suppressed IKK- β phosphorylation and IKK- β kinase activity, but it did not affect IKK- α .

In addition, we overexpressed or downregulated IKK- β and examined NF- κ B activation and the cell invasion. As shown in Fig. 6C, the beneficial effect of aspirin on cell invasion was effectively reversed by IKK- β overexpression, and it was confirmed by the detection of MMP-9 activity (Fig. 6D), secreted uPA and PAI-1 levels (Fig. 6E). Similarly, the inhibitory effect of aspirin on NF- κ B activation was also abolished by IKK- β overexpression (Fig. 6F). Furthermore, the IKK- β -specific inhibitor Bay 65-1942 enhanced the effects of aspirin on cell invasion and NF- κ B activation (Fig. 6D, 6E, 6F and 6G). Thus, these results suggested that suppression of IKK- β -mediated NF- κ B activation might contribute to the inhibitory effects of aspirin on cell invasion.

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Fig. 5. Effects of aspirin on NF- κ B activation. (A) Cells were transfected with NF- κ B-luc reporter for 24 h, then the cells were treated with aspirin (5 mmol/l) for 48 h, and the NF- κ B transcriptional activity was measured by a luciferase activity assay. Results were presented as the fold changes relative to the cells treated with vehicle. (B) Cells were treated with aspirin (5 mmol/l) for 48 h, and the cell nuclear extracts were prepared and the extract was analyzed for NF- κ B p65 expression by western blot. Bands of interest were normalized against Histone H2B and data were provided as relative density ratios. (C) The cell cytoplasm extracts were measured for I κ B- α and p-I κ B- α expression. Bands of interest were normalized against β -actin and data were provided as relative density ratios. Data were expressed as the mean $\pm$ SD of three independent experiments. *P<0.05, as compared with the vehicle group.

Fig. 6. Role of IKK- β activation in the effects of aspirin on cell invasion. (A) DU145 cells were treated with aspirin (5 mmol/l) for 48 h. Western blot analysis was performed to detect IKK- β , IKK- α , phospho-IKK- β and phospho-IKK- α protein. Bands of interest were normalized against β -actin and data were provided as relative density ratios. (B) IKK- β kinase activity was measured by the IKK- β Kinase Assay Kit. Data were expressed as mean $\pm$ SD. * P <0.05, as compared with the vehicle group. (C) DU145 cells were infected with IKK- β adenovirus for 24 h, then the cells were treated with aspirin (5 mmol/l) for 48 h, and cell invasion was examined by transwell assays. The data were expressed as the fold changes relative to the cells treated with vehicle. Results were expressed as mean $\pm$ SD of three independent experiments. * P <0.05. (D) DU145 cells were infected with IKK- β adenovirus then treated with aspirin (5 mmol/l) for 48 h, or treated with aspirin (5 mmol/l) in the presence of IKK- β -specific inhibitor Bay 65-1942 (2 μ mol/l) for 48 h, and then the activities of MMP-9, MMP-2 and TIMP-1 were analyzed by gelatin zymography. (E) The secreted uPA and PAI-1 levels were determined in the cell media. * P <0.05, as compared with the vehicle group. (F) Expression of nuclear NF- κ B p65 and total I κ B- α was analyzed by western blot. (G) DU145 cells were treated with aspirin (5 mmol/l) in the presence of Bay 65-1942 (2 μ mol/l) for 48 h, and cell invasion was examined by transwell assays. * P <0.05.

Discussion

Suppression of cell invasion in malignant cells is a critical property of chemopreventive agents. It is reported that aspirin is effective in the inhibition of cell growth for aggressive prostate cancer [19, 20]. However, the understanding about the roles of aspirin in prostate cancer cell invasion is still lacking. In this study, our findings showed that the inhibitory effects of aspirin on prostate cancer cell invasion were attributed to the suppression of MMP-9 activity and secreted uPA expression, which were dependent on the IKK- β -mediated NF- κ B activation.

In cancer cell invasion and metastasis, the degradation of ECM has been proposed as an important process. In our study, we find that aspirin inhibits MMP-9 activity with less effect

on MMP-2 in prostate cancer cells. It is indicated that MMP-9 plays an important role in the progression of various types of cancers and the abundant expression of MMP-9 appears to be related to the invasion and aggressiveness of prostate cancer cells [30]. Many studies have shown that inhibition of the MMP-9-mediated invasion reduces the incidence of cell metastasis and cancer survival, suggesting suppression of MMP-9 is useful in the patients suffering from cancer [6, 7]. MMP-9 is inhibited by the specific inhibitor TIMP-1, which can suppress tumor angiogenesis and invasion by inhibiting the degradation of ECM [31]. Our studies also show that aspirin increases TIMP-1 activity in the cancer cells. In general, binding of TIMP-1 to the active site of the MMP-9 results in an efficient inhibition of MMP-9 activity, and overexpression of TIMP-1 reduces cancer cell growth and invasion [32]. Therefore, it implies that the balance between MMP-9 and TIMP-1 levels is a critical determinant of the prostate cancer cells metastatic activity, and it also suggests that the invasive and metastatic ability of cancer cells can be altered by changing the MMP-9: TIMP-1 ratio [33]. However, it is noteworthy that the activity of TIMP-1 in LNCaP cells is not significantly altered compared to the other two tested cells. This may contribute to the lower NF- κ B activity expressed in LNCaP cells compared to DU145 and PC-3 cells. Despite this, aspirin still has significant effects on MMP-2 and cell invasion, suggesting that TIMP-1 likely has a minor role in the anti-invasive effects of aspirin on LNCaP cells. Since the LNCaP cell line is the prostate cell line established with functional androgen receptors and prostate-specific antigen expression, we speculate that aspirin plays the unique role in the different kinds of prostate cancer cell invasion. In addition, it is demonstrated that the expression of VEGF, ICAM-1 and IL-8, which can be induced by MMP-9 expression [34, 35], is significantly higher in the more metastatic PC-3 and DU-145 cells as compared with poorly metastatic LNCaP cells [36]. Thus, these reports show the possible relationship between MMP expression and the pro-angiogenic factors, in turn, is associated with the increased invasive ability of cancers.

The up-regulation of uPA in tissues closely parallels the motility of cells, which could facilitate ECM degradation by converting the zymogen plasminogen into plasmin [37]. PAI-1 is thought to be the inhibitor of uPA [38]. In addition to binding to uPA, PAI-1 can also attach to the ECM and regulate cell adhesion and migration independent of its protease inhibitory capacity [27]. Moreover, it is shown that PAI-1 promotes, rather than inhibits, invasion and metastasis [38, 39]. It could protect cancer cells from apoptosis and enhance cancer progression [40], and the elevated expression of PAI-1 is an indicator of poor prognosis in many cancers [41]. Here, we show that aspirin treatment suppresses secreted uPA and PAI-1 levels and it indicates that aspirin may achieve the anti-invasive effects in human prostate cancer cells partly through regulation of uPA and PAI-1 levels.

Previous studies have shown that the inhibition of NF- κ B significantly decreases cell invasion and angiogenesis in the prostate cancer animal model [12]. The molecular mechanisms of the effects of aspirin on NF- κ B-mediated invasion in prostate cancers cells have not been fully defined, although some useful clues are provided by the previous data. One study reported that the inhibitory effect of aspirin on NF- κ B activation in prostate cancer cells resulting in the suppression of uPA secretion [42]. In our studies, aspirin treatment could suppress NF- κ B activation, I κ B- α phosphorylation together with translocation of NF- κ B p65 to nucleus and IKK- β activation. It is essential to emphasize that aspirin specifically inhibits IKK- β activation. IKK- β is critical for NF- κ B translocation and activation, and it could phosphorylate I κ B at N-terminal sites resulting in their ubiquitination and degradation in the cytoplasm. In our studies, we find that aspirin-mediated cell invasion inhibition is reversed by IKK- β overexpression, while the IKK inhibitor sensitizes the anti-invasive effect of aspirin in prostate cancer cells. In addition to NF- κ B, there is abundant evidence that the invasive capacity of cancer cells is related to the activation of mitogen-activated protein kinases such as ERK1/2 [43]. Recent studies have demonstrated that the phosphorylation of ERK1/2 promotes MMP secretion and increases prostate cancer cell metastasis [44]. The ERK1/2 activation can induce the expression of many transcription factors, such as AP-1. The AP-1 DNA binding element is present throughout the MMP promoters and plays a dominant role in the transcriptional activation of the MMPs [45]. The increased expression of c-Jun

and c-Fos leads to an elevated MMP expression and promotes cancer cell invasion [46, 47]. Moreover, the activation of several other pathways, including c-Jun N-terminal kinase (JNK), p38 and Akt, which could induce MMP expression and be involved in invasion of many cell types, are also known to be sufficient to induce invasive capacity of prostate cancer [48-50]. Consequently, we hypothesize that there may be some other signaling by which aspirin suppresses cell invasion, and further studies are needed to identify more evidence.

In summary, this report concluded that aspirin suppressed prostate cancer cell invasion by reducing MMP-9 activity and secreted uPA expression through decreasing of IKK- β -mediated NF- κ B activation, supporting the hypothesis that aspirin reduces the risk of invasive cancer and suggests the rationale use of aspirin in preventing metastatic prostate cancer.

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Disclosure Statement

The authors have declared no conflict of interest.

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