Comparison of 4-hydroxynonenal-induced p53-mediated apoptosis in prostate cancer cells LNCaP and DU145

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Introduction

With the increase of detection rate of prostate cancer in recent years, an aging population and lifestyle changes have significantly increased the risk of prostate cancer, which has now become a serious threat to elderly men's health in our country [1, 2]. For advanced prostate cancer, androgen deprivation therapy (surgical castration, androgen analogues or estrogen) has been recognized as one of the effective treatments for many years [3, 8]. But after a median time of 18 to 24 months of treatment, many patients with prostate cancer gradually produce hormones of tolerance type for hormone refractory prostate cancer. The pathogenesis of type for hormone refractory prostate cancer remains unclear at present, so there are no effective treatment methods. Many animal experiments show that surgical castration can enhance the oxidative stress reaction in mice prostate tissue, and causes an increase of 4-hydroxynonenal (4-HNE) expression [9]. 4-Hydroxynonenal is a major end product from lipid peroxidation of omega-6 polyunsaturated fatty acids (PUFA) induced by oxidative stress, playing an important regulatory role in cell differentiation, proliferation and apoptosis and the expression of a multitude of genes including p53 in cells of diverse origin [9–11]. Further study showed that 4-HNE can prompt high expression of mitogen-activated protein kinase (MAPK) and protein kinase B (AKT); the expression of MAPK and AKT were significantly increased in the hormone refractory prostate cancer cells, and the expression was positively correlated with prostate cancer stage [12]. As an important cell signaling molecule, 4-HNE can induce phosphorylation of the androgen receptor for prostate cancer cells, and significantly enhance susceptibility to dihydrotestosterone (DHT), which is involved in the development of prostate cancer. This suggests that 4-HNE is probably involved in the development of prostate cancer and tolerance of androgen deprivation therapy [9, 10].

Since generation of 4-HNE has been suggested to be a common denominator in mechanisms of apoptosis caused by diverse forms of oxidative stress [13, 14], it is likely that it would also affect the expression and activation of p53. Despite the great amount of data reported in the literature defining the susceptibility of prostate cancer cells to oxidative damage, little is known about the 4-HNE-mediated activation of p53 in prostate cancer models.

In this study, the different influence of 4-HNE on prostate cancer of androgen-dependent (LNCaP cells) and androgen-independent (DU145 cells) or androgen-refractory type has been evaluated to explore its role in the pathogenesis of androgen-refractory prostate cancer. We attempted to study the effects of 4-HNE on the expression and activation of p53 in LNCaP and DU145 cells, focusing on the p53-mediated intrinsic pathway for apoptosis. Glutathione S-transferase A4-4 (GSTA4-4)-mediated metabolism of 4-HNE is one of the major determinants of the intracellular concentration of 4-HNE [15–18]. There-
fore, we have also examined the possible role of GSTA4-4 in regulation of 4-HNE-induced, p53-mediated apoptosis in prostate cancer cells.

**Material and methods**

**Materials**

DU145 (androgen-independent prostate cancer cells) and LNCaP (androgen-dependent prostate cancer cells) cells were obtained from the Shanghai Institute of Cell (Shanghai, China). 4-Hydroxynonenal was purchased from MERCK (German chemical company). The cell culture medium RPMI-1640, DMEM, CCK-8 detection kit, Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit, propidium iodide (PI), Lipofectamine 2000 transfection reagent and fetal bovine serum were from GIBCO (Shanghai, China). Bradford reagent, bis-acrylamide, and SDS for SDS-PAGE were obtained from Bio-Rad (Hercules, CA). Horseradish peroxidase labeling goat anti-rabbit IgG (H + L), Horseradish peroxidase labeling goats in mice IgG (H + L), and Western-blotting detection kit were purchased from Cell Signaling Technology Inc (Shanghai, China). 4-Hydroxynonenal was purchased from Merck (German chemical company). The cell culture medium RPMI-1640, DMEM, CCK-8 detection kit, Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit, propidium iodide (PI), Lipofectamine 2000 transfection reagent and fetal bovine serum were from Gibco (Shanghai, China). Bradford reagent, bis-acrylamide, and SDS for SDS-PAGE were obtained from Bio-Rad (Hercules, CA). Horseradish peroxidase labeling goat anti-rabbit IgG (H + L), Horseradish peroxidase labeling goats in mice IgG (H + L), and Western-blotting detection kit were purchased from Cell Signaling Technology Inc (Shanghai, China), pCNA3.1-GSTA4-4 plasmids were constructed and purified by Nanjing Jinshirui Biological Science and Technology Co., LTD (Nanjing, China). Other reagents were purchased from Nanjing Chemical Reagent Co., LTD.

**Cells and culture conditions**

Cells were cultured in DMEM (LNCaP) and RPMI 1640 (DU145) supplemented with 10% fetal calf serum in a humidified incubator at 37°C with 5% CO₂. Cell viability after recovery was observed at 2–3 days. This experiment used cells in logarithmic growth phase.

**Cell viability assay**

Cells were trypsinized, washed in 1 × PBS, and seeded (100 μl/well, approximately 3.5 × 10⁴ cells) into a 96-well plate and left overnight to attach. After 24 h, the cells were treated with concentrations of 0 (control), 20, 40, 60, 80 and 100 μM of 4-HNE and were incubated in an incubator at 37°C with 5% CO₂, Cell viability after recovery was observed at 2–3 days. This experiment used cells in logarithmic growth phase.

**Annexin V-FITC/PI analysis**

Detection of apoptosis was conducted using the Annexin V-FITC/PI apoptosis detection kit according to the manufacturer’s protocol. Approximately 1–4 × 10⁵ cells of LNCaP and DU-145 cells in 96-well plates at concentrations of 0, 20, 40 and 80 μM of 4-HNE were incubated in an incubator at 37°C with 5% CO₂ for 24 h. Cells were digested by trypsin and harvested by trypsinization, washed in 1 × PBS and subsequently incubated for 15 min at room temperature in the dark in 500 μl of 1 × binding buffer containing 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI). Afterward, apoptosis was analyzed by a FACSscan laser flow cytometer (FACSCalibur, Becton Dickinson, USA) and BD FACSDiva analysis software.

**Stable transfection with pCNA3.1 and pCNA3.1-GSTA4-4**

LNCaP and DU145 cells were plated in six-well plates at a density of 5 × 10⁵ cells per 100 mm Petri dish having 70–80% confluent cells, and used for the transfection. The cells were transfected with 3 μg of either empty vector pCNA3.1 or the target vector pCNA3.1-GSTA4 with the open reading frame (ORF) of the hGSTA4-4 sequence, using Lipofectamine 2000 reagent (Gibco, Shanghai, China) as per the manufacturer’s instructions, resulting in the GSTA4-4 high expression cell line DU145-pCNA3.1-GSTA4-4 and LNCaP-pCNA3.1-GSTA4-4, as well as control cell lines DU145-pCNA3.1 and LNCaP-pCNA3.1.

**Preparation of cell extracts and Western blot analysis**

Cells were collected, washed with cold PBS and then incubated in 100 μl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5; 1% NP-40; 150 mM NaCl; 1 mg ml⁻¹ leupeptin; 1 mg ml⁻¹ aprotinin; 1 mM Na VO₄; 1 mM NaF) at 4°C for 30 min. Cell debris was removed by centrifugation at 12,000 g for 10 min at 4°C. Protein concentrations were determined using a BCA protein content detection kit (Nanjing Keygen Biotech. Co. Ltd, China). Cell extracts were electrophoresed on a 12% SDS-polyacrylamide gel, and transferred onto nitrocellulose membrane (Roche, Shanghai, China). The blots were blocked at room temperature for 60 min in fresh blocking buffer (0.1% Tween 20 in Tris-buffered saline, pH 7.4, containing 5% nonfat dry milk), and incubated overnight at 4°C with the diluted primary antibody, which were made in 5% nonfat dry milk in Tris-buffered saline (TBS) containing 50 mM NaF and 0.05% Tween 20. After three times washing with T-TBS (Tris-buffered saline containing 0.05% Tween 20), the membrane was incubated with the diluted horseradish peroxidase-conjugated secondary antibody in TBS with 5% nonfat dry milk at room temperature for 2 h. After washing again with T-TBS, the transferred proteins were incubated with ECL solution (Nanjing Keygen Biotech. Co. Ltd, Nanjing, China) for 1 min, in accordance with the manufacturer’s instructions, and visualized on Hyperfilm ECL film (Nanjing Keygen Biotech. Co. Ltd, China) at room temperature.

**Statistical analysis**

All data of at least three independent experiments are expressed as the mean ± SD, and analyzed by Student’s t test. Values of p < 0.05 were considered to be statistically significant.

**Results**

4-Hydroxynonenal causes apoptosis in DU-145 and LNCaP cells

The efficacy of 4-HNE in inhibiting cell growth was assessed by MTT analysis. The results reported in Table 1 in-
dicated that significant differences were revealed between the two models of prostate cancer utilized (DU145 and LNCaP). In particular, HNE inhibited the growth of LNCaP cells, starting at 20 μM for 24 h, respectively, and there was dose- and time-dependent inhibition of proliferation by HNE. A significant decrease in the viable cell population, i.e. 70.45%, was observed in cells treated with 100 μM of 4-HNE on LNCaP for 48 h. In contrast, in DU145 cells, HNE caused only a slight reduction in cell proliferation starting at 40 μM for 24 h without statistical significance; even if exposed to 100 μM HNE (P > 0.05), DU145 manifested a relative resistance to supra-physiological concentrations of 4-HNE toxicity. 4-Hydroxynonenal-induced apoptosis in DU145 and LNCaP cells was further analyzed by flow cytometry. As the results in Figure 1 show, after treatment of LNCaP cells with different concentrations of 4-HNE ranging from 20 to 80 μM for 24 h, the late apoptotic or necrotic cells increased from 16.5% to 48.2% in LNCaP cells, and from 17.2% to 22.8% in DU145 cells in a dose-dependent manner, indicating that DU145 cells are more resistant to 4-HNE toxicity compared to the more susceptible LNCaP cells.

4-Hydroxynonenal activates apoptotic signaling in LNCaP cells

Numerous studies have shown that 4-HNE in many different cell types has the effect of inducing cell apoptosis [19–25]. P53 is an important gene involved in internal cell apoptosis signaling pathways. Based on these results, 4-HNE concentrations of 0–40 μM were used to examine its effect on apoptotic signaling in LNCaP and DU145 cells. Results presented in Figure 2A showed that within the 0–40 μM range, 4-HNE caused a dose-dependent increase in p-p53, p21, Bax and caspase-3 in LNCaP corresponding p53 protein which was not changed in LNCaP cells, but an effect of 4-HNE-induced activation of p53, p-p53, p21, Bax and caspase-3 was not observed in DU-145 cells treated with 4-HNE (Fig. 2B). These results showed that activation of p53-mediated intrinsic apoptotic signaling occurred in

Table 1. Inhibitory effect of 4-HNE on the cell proliferation of DU145 and LNCaP cells

| 4-HNE (μmol/l) | DU145 24 h | DU145 48 h | LNCaP 24 h | LNCaP 48 h |
|---------------|-----------|-----------|------------|------------|
| 20            | 0.32%     | 2.73%     | 10.19%     | 12.03%     |
| 40            | 2.93%     | 1.04%     | 19.46%     | 26.77%     |
| 60            | 3.08%     | 1.13%     | 22.81%     | 50.12%     |
| 80            | 2.82%     | 1.49%     | 28.07%     | 61.85%     |
| 100           | 2.15%     | 1.38%     | 34.22%     | 70.45%     |

Cell proliferation evaluated by MTT assay in DU145 and LNCaP cells untreated or treated with 20, 40, 60, 80 or 100 μM 4-HNE. The results shown were the mean of three parallel experiments (triplicate wells) for each concentration point.

Fig. 1. Effects of 4-HNE on DU145 and LNCaP cell apoptosis analyzed by flow cytometry with Annexin V-FITC, PI staining, and TUNEL assay. Annexin V-FITC in conjunction with PI staining was used to distinguish early apoptotic from late apoptotic or necrotic cells. Fluorescence intensity for Annexin V-FITC is plotted on the x-axis, and PI is plotted on the y-axis.
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LNCaP rather than DU145 cells when exposed to 4-HNE, exhibiting significant differences between DU145 and LNCaP cells. Further studies are needed to evaluate the difference between LNCaP and DU145 cells in 4-HNE-induced apoptosis in the p53-mediated pathway.

4-Hydroxynonenal activates JNK signaling pathway in LNCaP and DU-145 cells

A rise in intracellular levels of 4-HNE is a common occurrence when cells are exposed to stressors such as oxidant chemicals, UV radiation and heat shock, and sustained activation of JNK occurs during stress-induced apoptosis in many different cell types [19, 25–27]. The JNK activation is through its phosphorylation on Ser and Thr residues within its N and C-terminal regions. Therefore, we examined the phosphorylation status of JNK in 4-HNE-treated DU145 and LNCaP cells using the anti-phospho-antibody which specifically detects phosphorylated JNK. As shown in Figure 3B, 4-HNE caused a dose-dependent increase in the phosphorylation of JNK in DU145 cells, but JNK activation (phosphorylation) was not observed in LNCaP cells treated with 4-HNE (Fig. 3A). These results showed that sustained activation of JNK occurred in DU145 rather than LNCaP cells exposed to 4-HNE.

Over-expression of hGSTA4-4 inhibits 4-HNE-induced apoptosis

During stress conditions causing lipid peroxidation (LPO), the cells with induced GST isozymes have an enhanced capability to lower 4-HNE and are resistant to apoptosis caused by various oxidants [19, 28, 29]. Therefore, we studied the effect of over-expression of GSTA4 in DU145 and LNCaP cells on 4-HNE-induced apoptotic signaling. Firstly, DU145 and LNCaP cells were stably transfected with hGSTA4-4 and over expression of hGSTA4 protein was confirmed by the results of Western blot analysis (Fig. 4A). Glutathione S-transferase activity for 4-HNE was found to be enhanced in hGSTA4-4 transfected cells along with the expected decrease in the constitutive 4-HNE levels (data not shown). The extent of 4-HNE-induced apoptosis was compared in the empty vector pCNA3.1 and pCNA3.1-GSTA4-4 transfected cells by assessing the activation of caspase-3 in 4-HNE-treated LNCaP cells and JNK and p-JNK in 4-HNE-treated DU145. As shown in Figure 4B, the caspase-3 and p-JNK component activated by 4-HNE was significantly suppressed in hGSTA4-4 transfected cells as compared to that with pCNA3.1-transfected cells. These results demonstrate that the hGSTA4-4 over-expressing cells have an enhanced anti-apoptosis capability, suggesting a regulatory role of GSTA4-4 in p53-mediated, 4-HNE-induced apoptosis.

Discussion

Prostate cancer is a disease associated with aging. Also commonly associated with increasing age is a shift in the prooxidant-antioxidant balance of many tissues toward a more oxidative state, i.e., increased oxidative stress [30]. Risk factors for prostate cancer and the relationship be-

Fig. 2. Effect of 4-HNE on p53-mediated intrinsic apoptotic pathway in LNCaP (A) and DU145 (B). DU145 and LNCaP cells were treated with different concentrations of 4-HNE (0–40 µM) for 24 h at 37°C, respectively. Total protein lysates were collected as described in the Materials and methods section. The lysates were analyzed by Western blotting for p53, p-p53 (Ser15), p21, JNK, Bax and caspase-3. GAPDH was used as a loading control.
between oxidative stress have been obtained. Epidemiology, laboratory and clinical studies have clearly proved that oxidative stress has a role in the development of prostate cancer [31–33]. 4-Hydroxynonenal (HNE), as a second messenger of oxidative damage, can cause antiproliferative, prodifferentiative, and proapoptotic effects, modulate cell growth, and affect various signal transduction pathways [19–25, 34–36].

To explore the mechanism of oxidative stress in the development of prostate cancer, the differences in the antiproliferative and proapoptotic effects of 4-HNE in prostate cancer cells were examined using androgen-independent prostate cancer cells such as DU145 or androgen-sensitive prostate cancer cells such as LNCaP. Results from the present study demonstrate that a significant apoptotic effect of 4-HNE was observed on LNCaP cells but not on DU145 cells, and DU145 cells exhibited much more resistance to 4-HNE toxicity than LNCaP cells. Our results are consistent with the study of Piergiorgio et al., who found that 4-HNE induced a strong antiproliferative response in LNCaP cells and only a weak effect in DU145 cells [37]. Although both LNCaP and DU145 cell lines originated from human prostate cancer, they had significant differences in sensitivity to androgen. This indicated that prostate cancer cell apoptosis induced by 4-HNE might be related to the androgen sensitivity of prostate cancer cells.

Consequently, the expression of related molecules in cell proliferation and the apoptosis signal pathway was analyzed by Western blot in 4-HNE induced LNCaP and DU145 cells. As shown in Figure 4, hGSTA4 transfection blocked the apoptosis induced by 4-HNE, and the expression of related molecules was analyzed by Western blot in hGSTA4 transfected DU145 and LNCaP cells, or treated with different concentrations of 4-HNE (0–40 µM) for 24 h at 37°C. GAPDH was used as a loading control.
pathway, and JNK may play different roles in different tumor types. The results reported herein are similar to a previous study showing that JNK activation could be either upstream or downstream of cell death activation, depending on cell type and death-initiating agents [38, 39]. LNCaP (hormone-sensitive) and the late cell line DU145 (hormone insensitive) were prostate cancer cell lines in different development periods. Thus, anti-proliferation and pro-apoptosis of HNE-treated LNCaP cells may be increased by activating the p53 signaling pathway, and those properties of HNE-treated DU145 cells, especially a relative resistance to supra-physiological concentrations of HNE, may be restrained or reduced by activating the JNK signaling pathway.

GSTA4 is 4-HNE reduction control the level of the main enzymes in the cell. In recent years epidemiological studies on different racial types have shown that lower expression of the different categories of GST was positively correlated with prostate cancer risk [36, 40]. The present study showed that 4-HNE-induced p53 is inhibited by the excessive expression of GSTA4 through accelerating 4-HNE consumption. This indicated that the activation of p53 is 4-HNE’s direct role, or its metabolites or degradation products triggered the P53-mediated apoptosis, and 4-HNE can imitate the stress or stress-induced DNA damage in the role of apoptotic signals, suggesting that 4-HNE could cause the stress to induce the p53-mediated intrinsic apoptotic signaling pathway.

In conclusion, our tests have demonstrated that 4-HNE promotes prostate cancer cell apoptosis through the p53 signaling pathway; the differences of sensitivity to 4-HNE in LNCaP and DU145 cells may be related to the androgen sensitivity of prostate cancer cells; and the 4-HNE-induced p53-mediated apoptosis signal is regulated by GSTA-4.

Authors declare no conflict of interests.

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