Gene Expression Patterns of the Endogenous Antioxidant Enzymes in Linuron-Treated Rat Ventral Prostates after Castration

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ABSTRACT

Linuron is a pesticide with a weak anti-androgenic property, which impacts male reproductive organs. In this study, to clarify whether linuron affects the cellular antioxidant system of ventral prostate, gene expression patterns of the representative antioxidant enzymes such as glutathione peroxidase (GPx), selenoprotein P (SePP), and superoxide dismutase (SOD) were investigated in the rat ventral prostates exposed to linuron using real-time RT-PCR analyses. Sprague-Dawley rats castrated at 6 weeks old were treated with linuron (25, 50, or 100 mg/kg per oral) daily for 10 days after testosterone propionate administration (0.4 mg/kg) subcutaneously. As compared to normal control animals, mRNA levels of phospholipid hydroperoxide GPx (PHGPx), SePP, and Mn SOD significantly increased in the prostates exposed to linuron (25, 50, and 100 mg/kg). However, cytosolic GPx (100 mg/kg) and Cu/Zn SOD (25, 50, and 100 mg/kg) mRNA levels significantly decreased in the ventral prostates. These results indicate that linuron up-regulates the expressions of PHGPx, SePP, and Mn SOD mRNAs, but down-regulates the expressions of cytosolic GPx and Cu/Zn SOD in rat prostates, suggesting that linuron may have dual effects in the cellular antioxidant system of prostate.

(Key words : linuron, glutathione peroxidase, selenoprotein, superoxide dismutase, rat ventral prostate)

INTRODUCTION

Endocrine disruptors (EDs) are compounds used in various industrial products, pesticides, herbicides, and pharmaceuticals, which impact endocrine function in humans and wildlife. The characterization of chemicals with affinity for the endogenous steroid hormones causes adverse effects such as birth defects and developmental disorders (Frye *et al.*, 2012). Chemicals like flutamide, vinclozolin, p.p-DDE, procymidone, and linuron competitively inhibit the binding of androgen to androgen receptor (AR) (Waller *et al*., 1996). Androgen, the male sex steroid, plays a key role in sexual maturation at puberty and male reproductive function and behavior in adulthood (Davison and Bell, 2006). Blockade of AR signaling may induce oxidative stress in various systems.

An increase in reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, and hydroxyl radical leads to potential damage in cells. Damage induced by ROS includes alterations of cellular macromolecules such as membrane lipids, DNA, and proteins, alters cell function, and eventually can lead to cell death (Agarwal *et al*., 2003). To counteract the effects of ROS, the testes are equipped with antioxidant defense systems that prevent cellular damage. For example, superoxide dismutase (SOD) and selenoprotein (SeP) including glutathione peroxidase (GPx) are main antioxidant enzymes used to scavenge harmful ROS in most organs. SODs catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Cytolasmic copper/zinc SOD (SOD1) is distributed in nucleus, cytosol, lysosome, and mitochondrial intermembrane, and manganese SOD (SOD2) is located in mitochondrial inner membrane (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973). SePP is an abundant extracellular glycoprotein and has functions as an selenium homeostasis and oxidant defense (Burk *et al*., 2003). GPxs directly limit ROS levels such as hydrogen peroxide or reverse oxidative damage to lipids and proteins. Cytoplasmic GPx (GPx1) is the most abundant type in cytoplasm, and phospholipid hydroperoxide (PHGPx) has a high prefeence for lipid hydroperoxide (Imai and Nakagawa, 2003; Muller *et al*., 2007).

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The prostate constitutes 20～30% of semen volume. Androgen is necessary for prostate development and normal prostate function (Roy *et al*., 1999). It has also been shown that castration induces oxidative stress in the rat prostate by significantly up-regulating ROS and down-regulating antioxidant enzymes (Tam *et al*., 2003). To better understand linuron-induced antioxidant enzyme changes in ventral prostate, we first demonstrated the expression patterns of SODs, SePP, and GPxs mRNAs after linuron treatment in rat ventral prostates.

MATERIALS AND METHODS

1. Materials

Testosterone propionate (TP; purity >97%, Catalog # 208-17533) was purchased from Wako Chemical Company (Japan). Linuron (purity >98.7%, Catalog # PS372) was purchased from Supelco Chemical Company (St. Louis, MO, USA). Corn oil was purchased from Aldrich (St. Louis, MO, USA).

2. Animals

Five-week-old male Sprague-Dawley rats were purchased from Samtaco Co.(Gyeonggido, Korea) and acclimated in polycarbonate cages for 1 week. The animals were housed in an environmentally controlled room with a 12-h light/dark cycle, temperature of 21 ± 2℃, and frequent ventilation at 10 times per hour. The animals were fed a standard rat chow (Samyang Co., Gyeonggido, Korea) and tap water *ad libitum* throughout the experimental period. Six-week-old animals were castrated via a midline incision of abdomen, and test chemical treatment was not commenced until 1 week later to allow for complete recovery. All procedures were conducted in compliance with the "Guide for Care and Use of Animals" (NIH # 86-23) and approved by Chungbuk National University Animal Care Committee.

3. Experimental Design

Seven-week-old rats were weighed and randomly assigned to each groups (n = 10 per group). Linuron (25, 50, or 100 mg/kg) was administered daily by oral route after TP (0.4 mg/kg per day) administration by subcutaneous injection within 15 min as possible for 10 consecutive days. Control animals were received corn oil (a vehicle) for the same periods. The maximum limit of the volume administered per animal was 4 ml/kg per day for oral administration. The dosage level was adjusted according to body weight changes.

4. Total RNA Extraction and Real-Time RT-PCR Analysis

The rats were euthanized about 60 days of age under pentobarbital anesthesia and their prostates were rapidly removed. Total RNA was extracted from the prostates using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms of total RNA were utilized for reverse transcription (RT) to generate cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The generated cDNA was employed as a template for PCR. Quantitative real-time RT-PCR was conducted using a Power SYBR Green PCR Master Mix (Applied Biosystems, USA). The primer sets were used to amplify GPx1, PHGPx, SePP, SOD1, SOD2, and β-actin as an internal standard (Table 1). Reactions were carried out in a 7500 Real-Time PCR System (Applied Biosystems). The data were analyzed for the triplicates of five independent assays.

5. Statistical Analysis

The data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. All analyses were conducted using a Statistical Package for Social Sciences for Windows software, version 10.0 (SPSS Inc., IL, USA). Statistical significance was established at $p<0.05$. All data were expressed as the mean ± SD.

RESULTS

1. Expression Pattern of GPx1 mRNA (Fig. 1)

GPx1 mRNA level in the ventral prostate administered with

Table 1. Primer list

Gene name	Accession No.	Primer
GPx1	NM_008160	Forward : 5'-agaaggctcacccgctct-3' Reverse : 5'-ggatcgtcactgggtgct-3'
PHGPx	NM_008162	Forward : 5'-cgtctgagccgcttattga-3' Reverse : 5'-atgtccttggctgcgaat-3'
SePP	BC001991	Forward : 5'-gacagtggttgctcttcttcaa-3' Reverse : 5'-tcgcaggtcttccaatctg-3'
SOD1	NM_011434	Forward : 5'-tgcgtgctgaagggcgac-3' Reverse : 5'-gtcctgacaacacaactggttc-3'
SOD2	NM_013671	Forward : 5'-tggacaaacctgagccctaa-3' Reverse : 5'-gacccaaagtcacgcttgata-3'
β-Actin	NM_007393	Forward : 5'-ctaaggccaaccgtgaaaag-3' Reverse : 5'-gcctccatggctacgtaca-3'

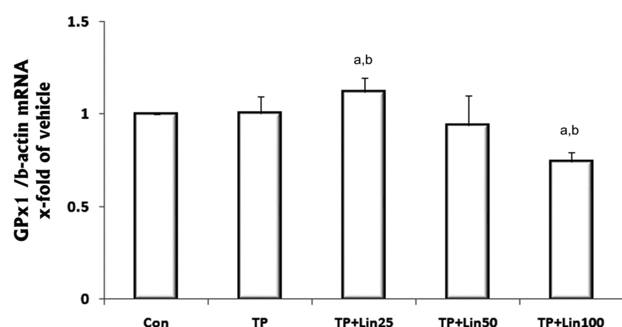

Fig. 1. Quantitative RT-PCR analysis of GPx1 mRNA in the ventral prostates of Sprague-Dawley rats (60 days old) daily exposed to testosterone propionate (TP; 0.4 mg/kg, subcutaneously) and/or linuron (Lin; 25, 50, and 100 mg/kg, orally) for 10 days after castration at 6 weeks old. Data represent five independent assays (mean ± SD) performed in triplicates. Significance vs control (a) or TP (b) group at $p<0.05$.

TP only was similar to that of normal control (1-fold). However, the level significantly increased (1.14-fold) when administered with 25 mg/kg linuron after TP treatment ($p<0.05$). 100 mg/kg of linuron administration plus TP resulted in a significant decrease in GPx1 mRNA levels compared to normal control (0.83-fold; $p<0.05$).

2. Expression Pattern of PHGPx mRNA (Fig. 2)

PHGPx mRNA level in the ventral prostate administered with TP alone was equal to that of normal control (1-fold). However, PHGPx mRNA level significantly increased (1.75-, 1.65-, and 1.84-folds, respectively) when administered with linuron (25, 50, and 100 mg/kg) plus TP compared to normal control or TP alone group ($p<0.05$).

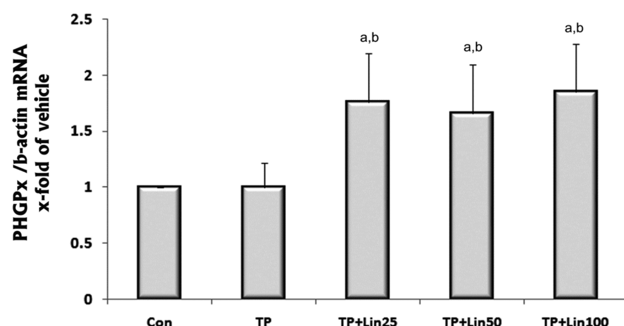

Fig. 2. Quantitative RT-PCR analysis of PHGPx mRNA in the ventral prostates of Sprague-Dawley rats (60 days old) daily exposed to testosterone propionate (TP; 0.4 mg/kg, subcutaneously) and/or linuron (Lin; 25, 50, and 100 mg/kg, orally) for 10 days after castration at 6 weeks old. Data represent five independent assays (mean ± SD) performed in triplicates. Significance vs control (a) or TP (b) group at $p<0.05$.

3. Expression Pattern of SePP mRNA (Fig. 3)

SePP mRNA level in the ventral prostate of rat administered with TP alone was lower (0.88-fold) than that of normal control (1-fold). However, the SePP level significantly increased to 1.21- and 1.30-folds of normal control when administered with linoron (25 and 100 mg/kg) plus TP ($p<0.05$).

4. Expression Pattern of SOD1 mRNA (Fig. 4)

SOD1 mRNA level in the prostate administered with TP

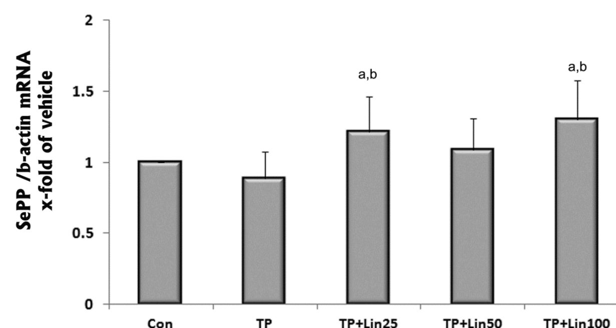

Fig. 3. Quantitative RT-PCR analysis of SepPP mRNA in the ventral prostates of Sprague-Dawley rats (60 days old) daily exposed to testosterone propionate (TP; 0.4 mg/kg, subcutaneously) and/or linuron (Lin; 25, 50, and 100 mg/kg, orally) for 10 days after castration at 6 weeks old. Data represent five independent assays (mean ± SD) performed in triplicates. Significance vs control (a) or TP (b) group at $p<0.05$.

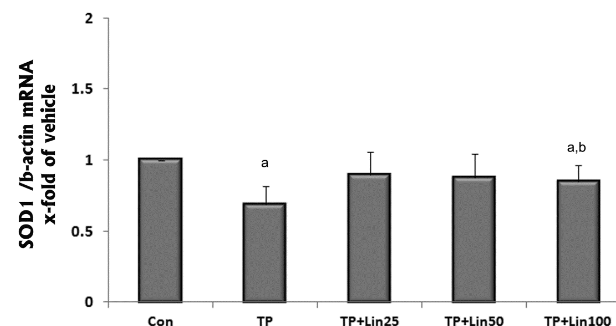

Fig. 4. Quantitative RT-PCR analysis of SOD1 mRNA in the ventral prostates of Sprague-Dawley rats (60 days old) daily exposed to testosterone propionate (TP; 0.4 mg/kg, subcutaneously) and/or linuron (Lin; 25, 50, and 100 mg/kg, orally) for 10 days after castration at 6 weeks old. Data represent five independent assays (mean ± SD) performed in triplicates. Significance vs control (a) or TP (b) group at $p<0.05$.

alone was significantly lower (0.69-fold) than that of normal control (1-fold). SOD1 mRNA level decreased to 0.89- and 0.87-folds of normal control when administered with linuron (25 and 50 mg/kg) plus TP. The linuron treatment of 100 mg/kg plus TP resulted in a significant decrease in SOD1 mRNA level compared to normal control (0.85-fold; $p<0.05$).

5. Expression Pattern of SOD2 mRNA (Fig. 5)

SOD2 mRNA level in the prostate administered with TP alone was lower (0.92-fold) than that of normal control (1-fold). However, SOD2 mRNA level significantly increased to 1.26-, 1.24-, and 1.23-folds of normal control when administered with linuron (25, 50, and 100 mg/kg, respectively) plus TP ($p<0.05$).

DISCUSSION

Various chemicals including pesticides have been identified as EDs that may bind to estrogen receptor or AR (Colborn *et al*., 1993). Linuron is a urea-based herbicide and alters differentiation of androgen-related tissues by acting AR antagonist. Linuron inhibits the TP-induced re-growth of androgen-dependent sex accessory organs. Especially, the weights of ventral prostate and seminal vesicle, the most sensitive androgen-dependant tissues, are significantly decreased, but adrenal gland weight is significantly increased by linuron treatments (Kang *et al*., 2004). However, linuron binds to AR in lower affinity than flutamide, vinclozolin, and procymidone. Linuron alters reproductive organ developments in consistent with its low affinity for AR such as reduced anogenital distance, retained nipples, and a low incidence of hypospadias and also induces an epididymal and testicular atrophy (Wolf *et al*., 1999).

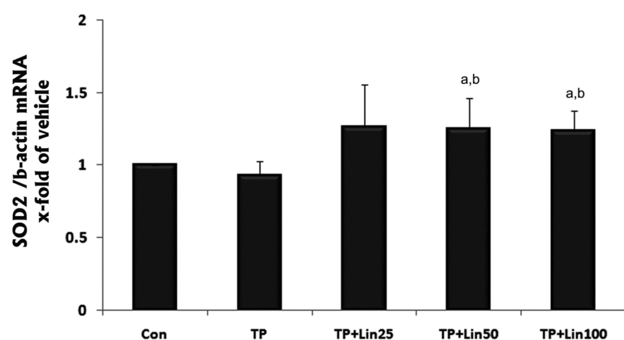

Fig. 5. Quantitative RT-PCR analysis of SOD2 mRNA in the ventral prostates of Sprague-Dawley rats (60 days old) daily exposed to testosterone propionate (TP; 0.4 mg/kg, subcutaneously) and/or linuron (Lin; 25, 50, and 100 mg/kg, orally) for 10 days after castration at 6 weeks old. Data represent five independent assays (mean ± SD) performed in triplicates. Significance vs control (a) or TP (b) group at $p<0.05$.

Castration results in a rapid and significant reduction of blood flow to mature rat ventral prostate, and induces apoptosis in the vascular cells (Shabsigh *et al*., 1998). Androgen removal may be caused by oxidative stress, which induces prostate apoptosis (Jara *et al*., 2004). Antioxidant enzymes such as SePP, GPxs, and SOD functionally are related to cellular redox regulation. Therefore, the function of antioxidant defense systems can be changed when exposed to estrogenic or anti-androgenic EDs. The GPx1 and SOD2 are down-regulated after castration in rat ventral prostate (Pang *et al*., 2002). SOD2 down-regulation directly affects AR activity in advanced prostate cancer (Sharifi *et al*., 2008). GPx is regulated in glandular epithelial cells of prostate and epididymis by testosterone (Murakoshi *et al*., 1993; Castellon *et al*., 2005). SePP mRNA expression induces the testosterone secretion in cultured Leydig cells (Nishimura *et al*., 2001).

The loss of ventral prostate weight by linuron was less apparent than that of anti-androgenic chemicals such as vinclozolin and procymidone. Administration of high doses (50 and 100 mg/kg) of linuron inhibits the TP-induced re-growth of ventral prostate (Kang *et al*., 2004). Moreover, linuron alters the expression of androgen-regulated gene (Lambright *et al*., 2000). However, linuron does not show the typical anti-androgenic effect like flutamide (Wolf *et al*., 1999). In the present study, the mRNA levels of SOD1, SOD2, and SePP decreased, but GPx1 and PHGPx mRNA levels were similar to that of normal control in rat prostates by the administration with TP alone after castration. After linuron and TP treatment, the mRNAs of PHGPx, SePP, and SOD2 increased, but SOD1 mRNA decreased. On the other hand, GPx1 mRNA significantly increased by the administration with linuron at a low dose (25 mg/kg), but decreased by administration of linuron at high dose (100 mg/kg). Taken together, linuron up-regulated the expressions of PHGPxs, SePP, and SOD2 mRNAs, but down-regulated the expressions of GPx1 and SOD1 in rat prostates. These findings suggest that linuron may have dual effects in the cellular antioxidant system of prostate, although further study will be needed in future.

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