Expression Pattern of Antioxidant Enzymes Genes in the Ventral Prostates of Rats Exposed to Procymidone and/or Testosterone after Castration

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ABSTRACT

Procymidone is a fungicide with anti-androgenic properties widely used to protect fruits from fungal infection, which induces an excessive reactive oxygen species production in male reproductive organs. In this study, to clarify whether procymidone affect the cellular antioxidant system of prostate at onset of puberty, gene expression patterns of the representative antioxidant enzymes such as cytoplasmic glutathione peroxidase (GPx1), phospholipid hydroperoxide GPx (PHGPx), selenoprotein P (SePP), cytoplasmic copper/zinc superoxide dismutase (SOD1), and manganese SOD (SOD2) were investigated in the rat ventral prostates exposed to procymidone using real-time RT-PCR analyses. Seven-week-old Sprague-Dawley rats castrated at 6 weeks old were treated with procymidone (25, 50, or 100 mg/kg per day) orally for 7 consecutive days after testosterone propionate (0.4 mg/kg per day) administration by subcutaneous injection. As compared to normal control animals, GPx1 mRNA expression in prostates significantly increased by the administration with TP and/or procymidone. However, PHGPx and SOD1 mRNA levels significanatly decreased by over 25 mg/kg of procymidone treatment and SePP and SOD2 mRNA levels was significanatly reduced by over 50 mg/kg of procymidone treatment. These findings indicate that procymidone may affect the antioxidant system of prostatic cells in up-regulation mode of GPx1, but in down-regulation modes of PHGPx, SePP, SOD1, and SOD2, suggesting that procymidone may affect differently the cellular antioxidant system of prostate according to the exposure doses.

(Key words : procymidone, GPx, SOD, selenoprotein P, rat prostates)

INTRODUCTION

To date, it has been reported that a variety of structurally diverse natural and synthetic chemicals, called endocrine disrupting chemicals (EDCs), can interfere with the endocrine system by mimicking or inhibiting the action of endogenous steroid hormones (Colborn *et al.*, 1993; Danzo, 1998; Baek *et al.*, 2007). EDC is a chemical with the potential to disturb hormonal homeostasis in the body. When such chemicals are within the body, they have the possibility to interact with estrogen or androgen signaling mechanisms, which is called 'endocrine disruption' (Sharpe and Irvine, 2004). The pesticide procymidone has been used as a dicarboximide fungicide for control of plant diseases and known to be present in fruit products prepared for human consumption (Ostby *et al.*, 1999). Procymidone has a binding affinity for the androgen receptor in rodents, and is considered to have anti-androgenic activity similar to flutamide because they are structurally similar (Hosokawa *et al*., 1993).

Mammalian cells have various antioxidant systems. Glutathione peroxidase (GPx) is one of the primary cellular enzymatic defense systems against damage induced by hydrogen peroxide and lipid hydroperoxide (Imai and Nakagawa, 2003). Among all known GPxs, including four selenium dependent enzymes cytoplasmic GPx (GPx1), gastrointestinal GPx (GPx2), plasma GPx (GPx3), and phospholipid hydroperoxide GPx (PHGPx), and the selenium-independent GPx involved in specific glutathione S-transferases, GPx1 is the principal enzyme that reduces H_2O_2 and other hydroperoxides in mitochondria and cytosol (Bilodeau and Mirault, 1999). PHGPx is an antioxidant that belongs to the superfamily of selenium-dependent peroxidase existing in the nucleus, mitochondria, and cytosol,

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and interacts directly with peroxidized phospholipid and cholesterol, and cholesteryl ester in biomembrane (Thomas *et al.*, 1990; Yagi *et al.*, 1996; Imai and Nakagawa, 2003). Selenoprotein P (SePP) is a major plasma and extracellular selenoprotein which is synthesized and secreted by most tissues, including testis (Burk *et al.*, 2003). It functions principally as a selenium transporter and antioxidant (Lee *et al.*, 2008). Members of the superoxide dismutase (SOD) family, a ubiquitously distributed group of enzymes that is commonly characterized by the metals that they contain and efficiently catalyze the dismutation of O^{2-}, are involved in an essential antioxidant defense system (McCord and Fridovich, 1969; Crapo *et al.*, 1992). Cytoplasmic copper/zinc SOD (SOD1) is distributed in various intracellular compartments including nucleus, cytosol, lysosome, and mitochondria (Crapo *et al.*, 1992; Okado-Matsumoto and Fridovich, 2001), while manganese SOD (SOD2) is located only in mitochondria (Weisiger and Fridovich, 1973).

The prostate is an essential male accessory sex gland that provides about 20 % of the total volume of seminal plasma. Any changes of secretions from the prostate may be reflected in reproductive disorders (Selvakumar *et al.*, 2011). Normal physiological level of androgen is important in maintaining not only a homeostasis between cellular pro-oxidants and antioxidants, but also a balance between cell death and proliferation in the prostate (Tam *et al.*, 2003a). When normal androgenic condition is disrupted, such as by anti-androgenic EDCs, oxidative stress is induced in the prostate, which may play a critical role in mediating epithelial apoptosis and involution.

In this study, to clarify whether procymidone affect the cellular antioxidant system of prostate at onset of puberty, the mRNA expressions of the representative antioxidant enzymes such as GPx1, PHGPx, SePP, SOD1, and SOD2 were investigated in the rat ventral prostates exposed to procymidone using a real-time RT-PCR analysis.

MATERIALS AND METHODS

1. Materials

Testosterone propionate (TP; purity >97%, Catalog # 205-08432) was purchased from Wako Chemical Company (Japan). Procymidone (purity >98.7%, Catalog # PS-2126) was purchased from Supelco Chemical Company (St. Louis, MO, USA). Corn oil was purchased from Aldrich (St. Louis, MO, USA). The purities of all compounds used in this study were as supplied by the chemical supplier.

2. Animals

Five-week-old male Sprague-Dawley rats were purchased from Samtaco (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, Korea) and tap water *ad libitum* throughout the experimental period. Six-week-old animals were castrated via a midline incision, 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). Procymidone (25, 50, or 100 mg/kg) were administered daily by oral route after TP (0.4 mg/kg per day) administration by subcutaneous injection within 15 min as possible for 7 consecutive days. Control animals received corn oil (the 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 at 8 weeks of age under pentobarbital anesthesia and their prostates were rapidly removed. Total RNA was extracted from the prostates using a Trizol reagent kit (Invitrogen, USA) according to the manufacturer's instructions. Two micrograms of total RNA was utilized for reverse transcription to generate cDNA, using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). The generated cDNA was employed as a template for PCR reactions. Quantitative real-time RT-PCR reactions were 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 7,500 Real-Time PCR System (Applied Biosystems, USA). The data were analyzed for the triplicates of five independent runs (mean ± SD).

5. Statistical Analysis

The data were analyzed using a one-way ANOVA followed

by Tukey's multiple comparison test. All analyses were conducted using the 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 mean ± SD.

Table 1. Primer list

Gene name	Primer
GPx1	Forward : 5'-agaaggctcacccgctct-3' Reverse : 5'-ggatcgtcactgggtgct-3'
PHGPx	Forward : 5'-cgtctgagccgcttattga-3' Reverse : 5'-atgtccttggctgcgaat-3'
SePP	Forward : 5'-gacagtggttgctcttcttcaa-3' Reverse : 5'-tcgcaggtcttccaatctg-3'
SOD1	Forward : 5'-ggtccagcggatgaagag-3' Reverse : 5'-ggacacattggccacacc-3'
SOD2	Forward : 5'-tggacaaacctgagccctaa-3' Reverse : 5'-gacccaaagtcacgcttgata-3'
β-Actin	Forward : 5'-ctaaggccaaccgtgaaaag-3' Reverse : 5'-gcctccatggctacgtaca-3'

RESULTS

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

GPx1 mRNA level in the prostates of rat administered with TP only significantly increased to 2.60-fold compared to normal control (1-fold; $p<0.05$). When administered with 25, 50, and 100 mg/kg procymidone after TP treatment, GPx1 mRNA level also significantly increased to 2.39-, 2.11- and 2.45-fold, respectively compared to normal control ($p<0.05$).

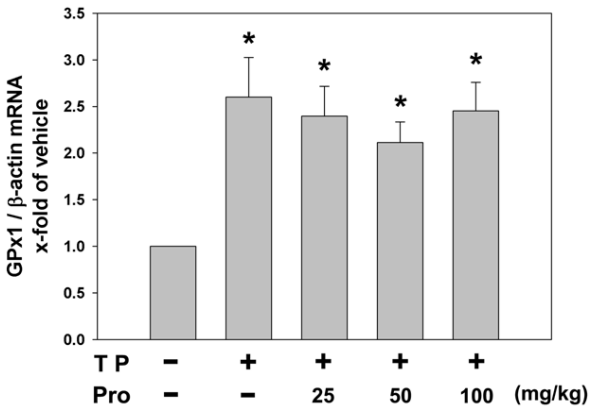

Fig. 1. Quantitative RT-PCR analysis of GPx1 mRNA in the prostates of 8-week-old Sprague-Dawley rats daily exposed to testosterone propionate (TP; subcutaneously) and/or procymidone (Pro; orally) for 7 days after castration. Data represent five independent assays (mean ± SD) performed in triplicate. * Versus control group at $p<0.05$.

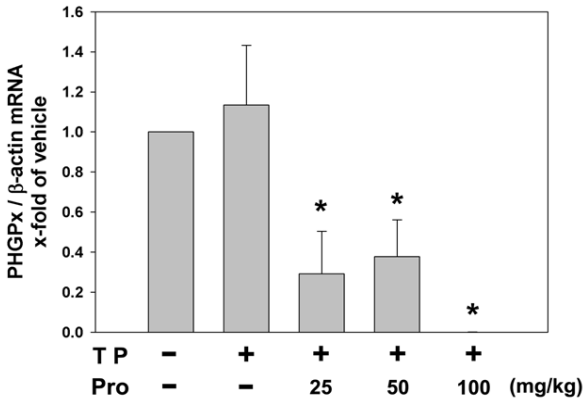

Fig. 2. Quantitative RT-PCR analysis of PHGPx mRNA in the prostates of 8-week-old Sprague-Dawley rats daily exposed to testosterone propionate (TP; subcutaneously) and/or procymidone (Pro; orally) for 7 days after castration. Data represent five independent assays (mean ± SD) performed in triplicate. * Versus control group at $p<0.05$.

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

PHGPx mRNA level in the prostates of rat administered with TP alone was higher (1.13-fold) than that of normal control (1-fold). However, when administered with 25, 50, and 100 mg/kg procymidone after TP treatment, PHGPx mRNA level significantly decreased to 0.29-, 0.37, and 0.02-fold, respectively compared to normal control ($p<0.05$).

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

SePP mRNA levels in the prostates of rat administered with TP alone, and 25, 50, and 100 mg/kg of procymidone after TP treatment decreased to 0.90-, 0.99-, 0.76, and 0.63-fold of normal control, respectively. In particular, the level significantly decreased in the groups treated with 50 and 100 mg/kg of procymidone compared to normal control ($p<0.05$).

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

SOD1 mRNA level in the prostates of rat administered with TP alone was similar to normal control (1.01-fold).

However, when administered with administered with 25, 50, and 100 mg/kg of procymidone after TP treatment, SOD1 mRNA level significantly decreased to 0.59-, 0.41-, and 0.12-

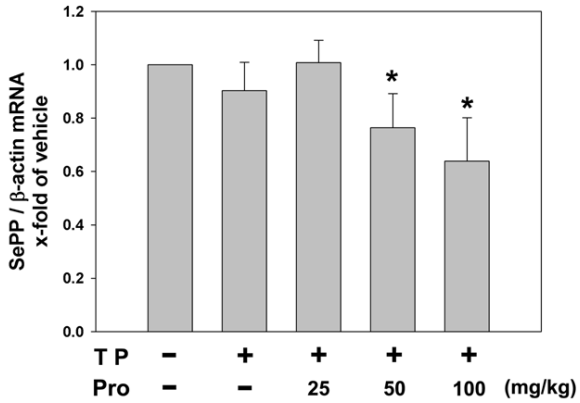

Fig. 3. Quantitative RT-PCR analysis of SePP mRNA in the prostates of 8-week-old Sprague-Dawley rats daily exposed to testosterone propionate (TP; subcutaneously) and/or procymidone (Pro; orally) for 7 days after castration. Data represent five independent assays (mean ± SD) performed in triplicate. * Versus control group at $p<0.05$.

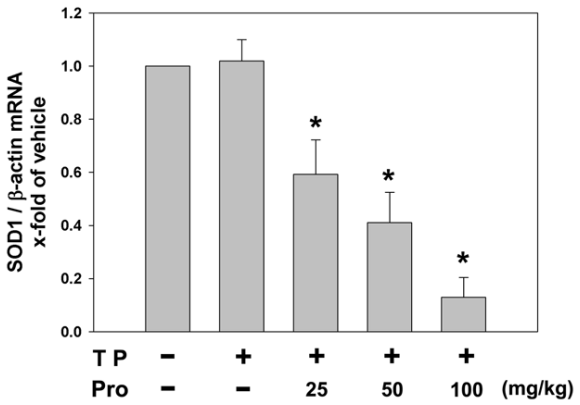

Fig. 4. Quantitative RT-PCR analysis of SOD1 mRNA in the prostates of 8-week-old Sprague-Dawley rats daily exposed to testosterone propionate (TP; subcutaneously) and/or procymidone (Pro; orally) for 7 days after castration. Data represent five independent assays (mean ± SD) performed in triplicate. * Versus control group at $p<0.05$.

fold of normal control, respectively ($p<0.05$).

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

SOD2 mRNA level in the prostates of rat administered with TP alone and 25 mg/kg procymidone plus TP increased to 1.49- and 1.35-fold of normal control. However, SOD1 mRNA level in the prostates of rat administered with 50 and 100 mg/kg of procymidone after TP treatment significantly decreased to 0.64- and 0.18-fold of normal control, respectively ($p<0.05$).

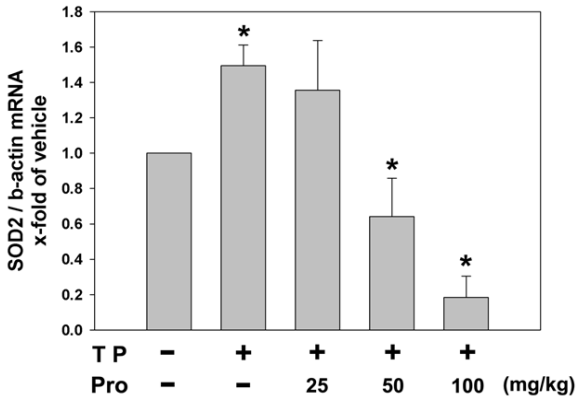

Fig. 5. Quantitative RT-PCR analysis of SOD2 mRNA in the prostates of 8-week-old Sprague-Dawley rats daily exposed to testosterone propionate (TP; subcutaneously) and/or procymidone (Pro; orally) for 7 days after castration. Data represent five independent assays (mean ± SD) performed in triplicate. * Versus control group at $p<0.05$.

DISCUSSION

EDCs can interfere with the normal embryonic development of the male and female reproductive systems of wildlife and experimental animals, ultimately disrupting normal reproductive function in adulthood (Colborn *et al.*, 1993). Estrogenic and androgenic EDCs which are structurally similar to estrogen and androgen bind to estrogen receptors (ERs) or the androgen receptor (AR), eventually altering the normal function of tissues and organs (Korach *et al.*, 1991). The expressions of ERs and AR are controlled in a cell-specific manner in reproductive organs of male and female rats (Pelletier *et al.*, 2000). Many studies have demonstrated that several chemicals, including vinclozolin, p,p'-DDE, procymidone, and linuron, have anti-androgenic activity in the male rats (Kelce *et al.*, 1997; Gray *et al.*, 1999; Ostby *et al.*, 1999; Lambright *et al.*, 2000). The administration of vinclozolin, an AR antagonist, during sexual differentiation demasculinizes and feminizes male rat offspring, such that the treated males display a female-like anogenital distance at birth, hypospadias, retained nipples, suprainguinal ectopic testes, a blind vaginal pouch, and small-to-absent accessory sex glands (Gray *et al.*, 1994). Neonatal injection of vinclozolin demasculinizes an aggressive behavior in male rats at 35 days-of-age, demonstrating that sexual differentiation is altered through anti-androgenic mechanism (Hotchkiss *et al.*, 2003). Kennel *et al.* (2003) reported that procymidone (100 mg/kg/day, orally) attenuated testosterone pro-

pionate-induced increase in the weights of sex accessory tissues in rat including seminal vesicle, levator ani and bulbocavernosus muscles, Cowper's gland and prostate, which is clearly related to the anti-androgenic effect of procymidone.

GPxs, SePP, and SODs are essential enzymes in an antioxidant defense system of mammals, and the mRNA expressions of the enzymes can be changed when exposed to estrogenic or anti-androgenic EDCs. The GPx activity in rat ventral prostate exposed to diethylstilbestrol, a xenoestrogen, significantly decreased compared to untreated control (Tam *et al.*, 2003b). The administration of cadmium, a common environmental pollutant and one of the possible EDCs, significantly down-regulates the SePP and PHGPx gene expressions in the rat testis when compared to control rats, eventually leading to testicular damage due to oxidative stress (Messaoudi *et al.*, 2010). Dhanabalan *et al.* (2010) found that administration of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), an endocrine disrupting toxicant, decreases the level of serum testosterone and the activity of SOD in epididymides of adult male rats. In the present study, GPx1 mRNA expression in prostates significantly increased by the administration with TP and/or procymidone. However, PHGPx and SOD1 mRNA levels significanatly decreased by over 25 mg/kg of procymidone treatment and SePP and SOD2 mRNA levels was significanatly reduced by over 50 mg/kg of procymidone treatment. We recently, demonstrated that the mRNA level of 3β-hydroxysteroid dehydrogenase (3β-HSD), a key enzyme in steroidogenesis of Leydig cells, significantly decreases in the rat testes treated with 0.2 or 1 mg/kg/day TP, although TP at a low dose (0.05 mg/kg/day) increases the expression of 3β-HSD mRNA (Kim et al., 2007). This phenomenon could be explained by the fact that expression of 3β-HSD is under control of negative feedback mechanism that is regulated by testosterone. Furthermore, the activities of both SOD and GPx decreases in the ventral prostates of rats exposed to polychlorinated biphenyl (PCB), whereas lipid peroxidation and H_2O_2 increases in the ventral prostate of PCB-treated rats compared to normal controls (Venkataraman *et al.*, 2004). TCDD treatment also causes the ROS level to increase and the SOD activity to decrease significantly compared to control at a dose and time dependent manner in primary Sertoli cell culture (Aly and Khafagy, 2011). These findings suggest that procymidone may affect the antioxidant system of prostatic cells in up-regulation mode of GPx1, but in down-regulation modes of PHGPx, SePP, SOD1, and SOD2, although further study will be needed in future.

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