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Sex-specific perinatal expression of glutathione peroxidases during mouse lung development

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ABSTRACT

Reports indicate that antioxidant enzymes like the glutathione peroxidases (GPx) can be regulated by sex steroids. The GPx, a major class of antioxidants involved in H_2O_2 and lipid hydroperoxides neutralization, showed an age- and sex-specific expression in many adult organs including the lung. High levels of androgens in the male lung are known to delay the surge of surfactant synthesis during gestation in several species. However, the impact of male androgens on antioxidant GPx early in life remains to be determined. The objective was to study the lung sex-specific expression of GPx during BALB/c mouse perinatal development. The mRNA expression of four seleno-dependent *Gpx* (*Gpx1* to *4*) in the lung of both sexes was characterized by real-time PCR from gestational day 15 to postnatal day 30, covering the entire canalicular, saccular and alveolar stages. Immunohistochemistry of GPx-1, -3 and -4, and seleno-dependent GPx enzymatic assays were also performed in the lung. We found a transient lower *Gpx1* mRNA level in male than in female lungs during the first 5 days after birth, corresponding to the saccular phase. This dimorphic expression was concomitant to a sex difference in GPx enzymatic activity corrected for blood. It is, to our knowledge, the first report of a sex dimorphism for murine lung enzymatic antioxidant defenses during the perinatal period.

1. Introduction

The abrupt transition from a relatively low oxygen intrauterine environment to the higher ambient oxygen tension at birth is associated with increased oxidative stress markers in preterm neonates (Buonocore et al., 2002). To counteract the oxidative stress at birth, an upregulation of lung antioxidants occurs in the last stage of gestation. The latter is concomitant to a surge of surfactant synthesis in many mammalian species (Frank, 1991). Despite that androgens lead to a delayed surfactant surge in males of several species, little is known about the existence of sex differences in lung enzymatic antioxidant defenses during the perinatal period (Torday and Nielsen, 1987). Adult sex dimorphisms for endogenous enzymatic antioxidants have been documented for superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases (GPx) in multiple tissues of rodents including the lung (Hackenhaar et al., 2009), liver (Pinto and Bartley, 1968), kidney (Riese et al., 2006; Schomburg et al., 2007), brain (Ali et al., 2006), heart (Barp et al., 2002), blood (Riese et al., 2006; Schomburg et al., 2007) and skeletal muscles (Yeh et al., 1998).

The GPx constitute a major family of enzymatic antioxidants involved in the detoxification of hydrogen peroxide and lipid hydroperoxides. At least four members of this family are found in the lung. GPx-1 to -4 are all dependent on selenium (SeGPx) for their synthesis (Toppo et al., 2009). GPx-1 is a ubiquitous intracellular enzyme accounting for most of the mouse pulmonary GPx activity (Cheng et al., 1998; Ho et al., 1997). GPx-2 is also cytosolic, but mostly found in gastrointestinal tissues (Toppo et al., 2009). Moreover, GPx-2 has been reported to be highly inducible by hyperoxia in the mouse lung (McGrath-Morrow et al., 2009). As the only pulmonary extracellular GPx, GPx-3 is highly expressed by the mouse lung and found in bronchoalveolar fluids (Kim et al., 1999). In contrast to the previously described GPx, GPx-4 an intracellular enzyme found also in lung, has the unique ability to directly neutralize oxidized membrane lipids without prior phospholipase A_2 activity (Knopp et al., 1999).

Abbreviations: BPD, bronchopulmonary dysplasia; CAT, catalase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; GD, gestational day; GPx, glutathione peroxidases; GSH, reduced glutathione; GSSG, oxidized glutathione; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase 1; PN, postnatal day; *Sdha*, succinate dehydrogenase complex subunit A; SeGPx, seleno-dependent glutathione peroxidase; SOD, superoxide dismutase; TBH, tert-butyl hydroperoxide.

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Since the first report of a sexual GPx activity dimorphism in the rat liver attributed to the ovarian cycle (Pinto and Bartley, 1968), the modulation of GPx activity *in vivo* by estradiol treatment has been observed in few other studies involving the rat liver (Pinto and Bartley, 1969) and the bovine reproductive tract (Lapointe et al., 2005). Furthermore, the effects of hormones on GPx are not limited to the estradiol. Indeed, experiments involving either, castration of male rats or testosterone treatments in female rats suggested that androgens may decrease GPx activity (Burk et al., 1980; Capel and Smallwood, 1983).

Elevated steroid levels are not unique to the postpubertal period. Indeed, in both human and mice fetuses, high levels of androgens were reported in males (Boucher et al., 2010; Stahl et al., 1991). To our knowledge, no studies specifically addressed the existence of sex differences in antioxidant defenses like GPx during the murine perinatal period in lungs. We hypothesized that lung GPx expression and activity are detrimentally affected in male during the perinatal period in BALB/c mice. BALB/c mice were selected because of their known lung sexual hormonal profile during the perinatal period (Boucher et al., 2010). We investigated whether sexual differences existed in the mRNA expression for *Gpx1* to *4* during lung development from the 15th day of gestation to the 30th postnatal day, therefore covering the entire canalicular, saccular and alveolar stages (Cardoso, 2000). We also performed GPx enzymatic assays and immunohistochemistry of three of the four SeGPx.

2. Materials and methods

2.1. Animals

All protocols and procedures were approved by the Animal Care and Use Committee and the Institutional Review Board of the Centre de Recherche du Centre Hospitalier Universitaire de Québec. BALB/c mice (Charles River Laboratories, St-Constant, QC, Canada) were kept under 12 h light/dark cycles and were allowed free access to tap water and to standard mouse chow (Harlan Teklad Global Diet, Madison, WI, USA; selenium content of 0.23 mg/kg). Females at the estrus stage were mated overnight and the morning on which the copulatory plug was observed was termed gestational day (GD) 0.5, whereas parturition marked the beginning of postnatal day 0 (PN0). Pregnant females were kept into individual cages until sacrifice. Gravid females were euthanized by CO_2 inhalation; pups were either killed by decapitation following hypothermia-induced anesthesia (PN0 to PN5) or by intra-peritoneal injection of pentobarbital sodium (PN5 to PN30). For the postnatal enzymatic analysis, BALB/c mice were put in harems and pregnant females were separated when the pregnancy was visibly established. Pups from these females were anesthetized using isoflurane (Baxter Corporation, Toronto, ON, Canada); pups PN5 and younger were killed by decapitation whereas PN20 mice were killed by CO_2 exposure and then decapitated. Using heparin-coated capillaries, blood samples were then collected at the neck region, frozen immediately on dry ice and kept at −80 °C until use. Sex was determined by examination of the anogenital distance and gonadal morphology. Sex was further confirmed by PCR amplification of the *Sry* gene (Bresson et al., 2010).

2.2. RNA extraction and cDNA synthesis

Lungs were collected and pooled by sex and litter. Three to 5 litters were pooled to create each replicate (Table 1). The RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and was followed by RNA purification on a CsCl gradient as reported before (Boucher et al., 2010). Reverse transcription of 4 μg of RNA was conducted using Superscript II (Invitrogen, Burlington, ON, Canada) in accordance with the manufacturer's instructions. The cDNA was diluted 10 times in sterile water and used as the template for quantitative PCR.

Table 1
Number of foetuses/pups used for mRNA expression analysis according to litter, stage of development and sex.

Litter[a]	1		2		3		4		5	
Sex	M	F	M	F	M	F	M	F	M	F
GD15.5	3	6	5	4	3	4				
GD17.5	4	4	3	4	2	1				
GD19.5	4	4	3	2	2	3	2	3		
PN0	6	2	3	4	2	4	3	6		
PN1	2	5	2	3	2	6	2	6		
PN2	3	4	2	3	5	4				
PN3	4	3	5	3	3	4	3	2	2	4
PN5	3	5	3	3	5	2				
PN7	3	2	3	2	5	2				
PN15	2	2	2	3	3	3				
PN20	4	3	2	4	5	2				
PN30	3	4	2	2	2	2				

[a] Male (M) and female (F) foetuses or pups from the same litter (replicate) were separately pooled for mRNA analysis of Figs. 1 and 2. GD, gestational day; PN, postnatal day.

2.3. Quantitative PCR (qPCR)

Specific primers for mouse *Gpx1*, *2*, *3* and *4* were designed based on known sequences using Primer3 software (Rozen and Skaletsky, 2000) as follow: *Gpx1* (forward: 5′-gtgcgaagtgaatggtgaga-3′; reverse: 5′-ctgggacagcagggtttcta-3′) based on GenBank Accession NM_008160 for the amplification of a fragment of 252 bp; *Gpx2* (forward: 5′-accagttcggacatcaggag-3′; reverse: 5′-aactttgaggagccgtttga-3′) based on NM_030677 for a fragment of 358 bp; *Gpx3* (forward: 5′-ctcctgagaccagccaagac-3′; reverse: 5′-atgggggtgttgagatacca-3′) based on NM_008161 for a 234 bp fragment and *Gpx4* (forward: 5′-atgcccgatatgctgagtgt-3′; reverse: 5′-gctagagatagcacggcagg-3′) based on NM_001037741 for a 335 bp fragment.

Since the lung structure evolves considerably during its growth and development, three housekeeping genes known to be stable throughout mouse lung development were used (Boucher et al., 2009). The glyceraldehyde-3-phosphate dehydrogenase (*Gapdh)* (forward: 5′-gtcggtgtgaacggatttg-3′; reverse: 5′-aagatggtgatgggcttcc-3′, based on NM_008084 for a 215 bp fragment amplified; the hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1)* (forward: 5′-agtcccagcgtcgtgattag-3′; reverse: 5′-aatccagcaggtcagcaaag-3′) based on NM_013556 for a fragment of 228 bp, and the succinate dehydrogenase complex, subunit A (*Sdha*) (forward: 5′-acacagacctggtggagacc-3′ and reverse: 5′-caaagggcttcttctgctgt-3′) based on NM_023281 for a 179 bp fragment.

After establishing the standard curves, the qPCR were carried out with a LightCycler (Roche Diagnostics, Laval, Quebec) as previously described (Boutet et al., 2009). The annealing temperature was set up for each gene based on the selected primers (*Gpx1*: 64 °C; *Gpx2*: 56 °C; *Gpx3*: 63 °C; *Gpx4*: 57 °C; *Gapdh*: 61 °C; *Hprt1*: 63 °C; *Sdha*: 59 °C). Standard curves were obtained from serially diluted amplicons in PCR-grade water. The mixtures were prepared as follows: 5 μL of cDNA were added to 15 μL of mixture containing 0.25 μM of each primer, 3 μM $MgCl_2$ and 2 μL of FastStart Master SYBRGreen I mix (Roche Diagnostics, Laval, QC, Canada) completed with PCR-grade water. The reactions were performed in triplicates on a LightCycler instrument (Roche Diagnostics). Briefly, the steps consisted of a denaturation at 95 °C for 10 min followed by cycles of amplification (95 °C for 0 s, annealing temperature for 5 s and 72 °C for 20 s) with a single acquisition of fluo-