Increased expression of Leydig cell haem oxygenase-1 preserves spermatogenesis in varicocele

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BACKGROUND: Oxidative stress is involved in the pathogenesis of testicular disorders. Haem oxygenase-1 (HO-1) plays an important cytoprotective role against oxidative stress. We investigated the presence of oxidative stress, represented by generation of 4-hydroxy-2-nonenal (4-HNE)-modified proteins, and expression of HO-1 in varicocele testes of human. METHODS: Thirty testicular biopsies from patients with left varicocele and 10 from patients with normal spermatogenesis were included. Generation of 4-HNE-modified proteins was examined as a marker of oxidative stress. Expression of HO-1 was assessed by western blotting and immunohistochemistry. The expression was compared with clinico-pathological parameters. RESULTS: Increased generation of 4-HNE-modified proteins was observed in varicocele testes. HO-1 expression was significantly correlated with varicocele grade (P < 0.01 in grade 2 and 3 compared to control) and expression of 4-HNE-modified proteins (r = 0.508, P < 0.01). The immunoreactivity was increased in Leydig cells in varicocele testes. There were significant correlations between age, total motile sperm count, Johnsen’s mean score and HO-1:4-HNE-modified protein ratio (r = 0.206, 0.405 and 0.408, P = 0.027, 0.027 and 0.025 respectively). CONCLUSIONS: In testes with varicocele, there are increases in 4-HNE-modified proteins, suggesting that oxidative stress is present. Increased HO-1 expression, mainly in Leydig cells, is considered to protect the cells against oxidative stresses in varicocele testes.

Key words: haem oxygenase-1/human/oxidative stress/varicocele

Introduction

Varicocele is characterized by the stasis of the internal spermatic vein, leading to elevated scrotal temperature, testicular hypoxia and retrograde blood flow of adrenal and renal metabolites. However, the exact aetiology of this condition remains unknown. Several studies have suggested that oxidative stress, which is mainly caused by reactive oxygen species (ROS), is involved in the pathogenesis of varicocele in human and rat (Kökşal et al., 2000, 2002; Romeo et al., 2003). Under normal conditions, antioxidants maintain ROS at low levels. Varicocele reduces the antioxidant defences not only in seminal plasma but also in blood plasma (Barbieri et al., 1999). In testes with varicocele, an imbalance between oxidants and antioxidants has a significant effect upon spermatogenesis.

In response to oxidative stress, haem oxygenase (HO) system, which contains 32 kDa inducible form (HO-1) and two constitutive forms (HO-2 and -3), plays an important role in cell protection (Maines, 1997). Carbon monoxide (CO), biliverdin and Fe, which are haem degradation products generated by HO, have antioxidant activities (Vogt et al., 1995). Besides oxidative stress, HO-1 is induced by heavy metals, inflammatory mediators and oxidized low density lipoproteins (Otterbein and Choi, 2000). To date, there have been few reports focused on the expression and the role of HO-1 in testis. Using rat, HO-1 activity in testis has been shown to increase after heat shock (Maines and Ewing, 1996). Ozawa et al. (2002) reported that CO generated by Leydig cell HO-1 triggered apoptosis of germ cells and modulated spermatogenesis under exposure of cadmium chloride (Ozawa et al., 2002). In humans, Middendorff et al. (2000) observed the expression of HO-1 in Sertoli cells and suggested a functional role of CO on soluble guanyl cyclase-dependent cGMP production in seminiferous tubule. The functional significance of HO-1 in testis is not fully understood, because HO-1 activity can result in either cell protection or injury depending on the experimental setting.

A consequence of oxidative stress is membrane peroxidation, primarily involving polyunsaturated fatty acids. The levels of lipid peroxidation products are increased in testis and semen in patients with varicocele (Sharma and Agarwal, 1996; Kökşal et al., 2002). A specific and stable end-product of lipid peroxidation, the aldehyde 4-hydroxy-2-nonenal (4-HNE), can diffuse within, or even escape from, the cell and attack targets far from the site of the original free radical event (Esterbauer et al., 1991). 4-HNE is a potent alkylating agent that reacts with DNA and proteins, generating various forms of adducts (cysteine, lysine and histidine residues).
(Esterbauer et al., 1991) that are capable of inducing specific cellular stress responses such as cell signalling and apoptosis (Uchida, 2003).

Using testicular biopsy specimens from the patients with or without varicocele, expression of 4-HNE-modified proteins was analysed as a parameter of oxidative stress. We further examined the effects of varicocele on the expression and localization of HO-1. Then, we specifically studied the effects of the expressions of 4-HNE-modified proteins and HO-1 on the clinical parameters of spermatogenesis to elucidate the role of HO-1 in varicocele testes.

Materials and methods

Subjects

Varicocele was diagnosed after a physical examination, and duplex and colour Doppler ultrasonography. Testicular volume was determined using punched-out orchidometer. Semen examinations were performed at least twice before varicocelectomy. Total motile sperm count (TM) was calculated by multiplying sperm concentration ($\times 10^8$/ml) and semen volume (ml). At the end of microsurgical inguinal or sub-inguinal varicocelectomy, biopsy samples were obtained from left testes via scrotal incision. The biopsy specimens were divided into two pieces. A part of the specimen was quickly frozen and kept at $-80^\circ$C for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Another one was fixed in Bouin’s solution for histological assays. All the physical examinations, operation and biopsy were performed by one of the authors (K.S.). Thirty testicular biopsies from left testes of the patients with left varicocele (subclinical: 4; grade 1: 9; grade 2: 12; grade 3: 5) were included in this study (mean age 34.1 ± 0.8 years; range 23–51). As controls, we used 10 testicular biopsy specimens. Eight samples were obtained from obstructive azoospermia patients with normal spermatogenesis and without apparent interstitial fibrosis. Two samples were obtained from the patients with retrograde ejaculation (mean ± SD: 35.8 ± 0.9 years; range: 27–43). After the explanation of the purpose of this study, informed consents from all patients and ethical approval were obtained.

SDS–PAGE and western blotting

The frozen testes were homogenized in 10 volumes of sucrose-Tris-EGTA buffer (pH 7.4) with protease inhibitors (β-mercaptoethanol, phenylmethylsulphonyl fluoride, pepstatin A, leupeptin) with a homogenizer three times each for 30s at the maximum speed. Equal amounts of protein (20 μg) were electrophoresed on 12% gels for HO-1 or 7.5% for HNE-modified protein and transferred to polyvinylidene difluoride membranes. These membranes were blocked with 5% non-fat dried milk in Tris-buffered saline for one hour. The membranes were incubated with rabbit polyclonal anti-HO-1 antibody (at a dilution of 1:800; StressGen Biotechnologies Corp., Victoria, BC, Canada) or mouse monoclonal anti-HO-1 antibody (at a dilution of 1:1000; Japan Institute for the Control for Aging, Shizuoka, Japan) in 1% bovine serum albumin overnight at room temperature. Then the membranes were reacted with secondary antibody at room temperature for one hour. The antigens were visualized with an ECL western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and quantified using an image analyzer (Densitograph AE-6900M; Atto Co., Tokyo, Japan). The data were calculated as percentage of control and expressed as mean ± SE. For the evaluation of HNE-modified protein, the sum of total bands per lane between 247 and 43 kDa were calculated.

Immunohistochemistry

The testicular specimens were fixed in Bouin’s solution for 2 h and embedded in paraffin. Two serial 4 μm thick sections were cut and stained with haematoxylin–eosin staining for Johnsen’s score count or immunostaining for HO-1. The sections for immunohistochemistry were mounted on silan-coated glass slides (Dako Japan, Kyoto, Japan), deparaffinized in xylene and rehydrated in graded ethanol. After inhibition of endogenous peroxidases in hydrogen peroxide solution, antigen retrieval was performed by heating in citrate buffer (pH 6) at 98°C for 30 min. Overnight incubation was carried out using the same rabbit anti-HO-1 polyclonal antibody (at a dilution of 1:100) at 4°C. Goat anti-rabbit immunoglobulin was used as a secondary antibody (1:100; Dako, Kyoto, Japan) for one hour. Sections were counterstained with hematoxylin. Omitting the first antibody served as a negative control and revealed no signal.

Statistical analysis

The Statview J 4.02 program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analysis. Data are expressed as the mean ± SE. The significance of differences was evaluated by the unpaired t-test to compare with the control group. To examine the association between expression of HO-1, generation of 4-HNE-modified proteins, HO-1/4-HNE-modified protein ratio and clinicohistological parameters, Spearman rank correlation coefficients were carried out. $P < 0.05$ is considered statistically significant.

Results

Representative immunoblot for 4-hydroxy-HNE-modified protein and its quantification are shown in Figure 1. Several bands of 4-HNE-modified proteins were detected in control testes, suggesting that these proteins were basally modified by 4-HNE. In testes with varicocele, a large number of proteins were detected, suggesting that increased lipid peroxidation occurred (Figure 1A). The increase in 4-HNE modification showed up to 2–3-fold higher in testes with grade 1–3 varicocele compared to control ($P < 0.01$) (Figure 1B).

![Figure 1](https://academic.oup.com/humrep/article-abstract/20/9/2608/2356717/566x264)
In control testis, HO-1 expression showed a faint 32 kDa band in western blots (Figure 2A), indicating low constitutive expression of HO-1 in normal testis. In the patients with grade 2 or 3 varicocele, the expressions were significantly higher, up to 7.5-fold, than those in the control ($P < 0.01$) (Figure 2B). There was a significant correlation in the expressions between 4-HNE-modified protein and HO-1 ($r = 0.508$, $P = 0.0042$) (Figure 3). The patterns of distribution in testis are shown in Figure 4 using the same antibody. In control testis, HO-1 immunoreactivity was faintly observed in the cytosol of Leydig cells (Figure 4A). 

Figure 2. (A) Representative haem oxygenase-1 (HO-1) immunoblots from control and varicocele patients (labelled as in B). (B) Quantification of HO-1 protein expressed as percentage of control. Data are expressed as mean ± SE. *$P < 0.01$. C = control; sub = subclinical; G1 = grade 1; G2 = grade 2; G3 = grade 3.

Figure 3. Correlation between expression of haem oxygenase-1 (HO-1) and generation of 4-hydroxy-2-nonenal (4-HNE)-modified protein.

In control testis, HO-1 expression showed a faint 32 kDa band in western blots (Figure 2A), indicating low constitutive expression of HO-1 in normal testis. In the patients with grade 2 or 3 varicocele, the expressions were significantly higher, up to 7.5-fold, than those in the control ($P < 0.01$) (Figure 2B). There was a significant correlation in the expressions between 4-HNE-modified protein and HO-1 ($r = 0.508$, $P = 0.0042$) (Figure 3). The patterns of distribution in testis are shown in Figure 4 using the same antibody. In control testis, HO-1 immunoreactivity was faintly observed in the cytosol of Leydig cells (Figure 4A).

The nuclei of various cell types in the seminiferous tubules, including spermatogonia and primary spermatocytes, showed immunoreactivity to anti-HO-1 antibody; however, they are considered as non-specific staining because of the weak band in control testes (Figure 2). In the varicocele testis, HO-1 was localized predominantly in the cytoplasm of Leydig cells throughout the specimen (Figure 4B, arrowhead). Some, but not all, Sertoli cells exhibited HO-1 immunoreactivity in normal testis (Figure 4C). There were no striking changes in Sertoli cells with or without varicocele (data not shown).

The correlations between expression of HO-1, generation of 4-HNE-modified proteins and several clinico-histological
parameters are shown in Table I. The increased levels of HO-1 and 4-HNE-modified proteins were significantly correlated with the patient age at operation (r = 0.400, 0.178, P = 0.016, 0.035 respectively). There was a significant correlation between the age and varicocele grade (r = 0.575, P = 0.0009). No significant correlation with clinical parameters about spermatogenesis was observed about left testicular volume, total motile sperm count, Johnsen’s mean score count. There was no significant correlation between expression of HO-1 or generation of 4-HNE-modified proteins and serum testosterone concentration (Table I). Figure 5 shows correlations between HO-1:4-HNE-modified protein ratio and the clinico-histological parameters. There are significant correlations between age, total motile sperm count, Johnsen’s mean score and HO-1:4-HNE-modified protein ratio (r = 0.206, 0.405 and 0.408, P = 0.027, 0.027 and 0.025 respectively). No significant correlation was observed about left testicular volume and serum testosterone concentration (r = 0.408 and 0.138, P = 0.466 and 0.131 respectively).

**Table I.** Correlations between haem oxygenase-1 (HO-1) or 4-hydroxy-2-nonenal (4-HNE)-modified protein and clinico-pathological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HO-1</th>
<th>4-HNE-modified proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.400</td>
<td>0.016</td>
</tr>
<tr>
<td>Left testicular volume (ml)</td>
<td>0.221</td>
<td>0.178</td>
</tr>
<tr>
<td>Total motile sperm count (x10⁶)</td>
<td>0.139</td>
<td>0.089</td>
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<td>Johnsen mean score count</td>
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<tr>
<td>Serum testosterone concentration (ng/dl)</td>
<td>0.027</td>
<td>0.032</td>
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</tbody>
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r: Spearman’s rank correlation coefficient; P < 0.05 was considered significant.

**Figure 5.** Correlations between haem oxygenase-1 (HO-1):4-hydroxy-2-nonenal (4-HNE)-modified protein ratio and patient age (A), left testicular volume (B), total motile sperm count (C), Johnsen’s mean score count (D).

**Discussion**

Using semen or blood from the left spermatic vein, there are several evidences, which indirectly show the presence of oxidative stress in testes with varicocele (Mitropoulos et al., 1996; Sharma and Agarwal, 1996; Barbieri et al., 1999). Using testicular biopsy specimen, we showed increased levels of 4-HNE adducts, which is a highly specific diffusible end-product of lipid peroxidation (Figure 1). Increased expression of HO-1, which was predominantly observed in Leydig cells, was also shown along with the progression of varicocele grade (Figure 2 and 4). HO-1:4-HNE-modified protein ratio positively correlated with total motile sperm and Johnsen’s mean score (Figure 5). These data are compatible with a cytoprotective role of HO-1 against oxidative stress in testes with varicocele.

ROS act as central mediators of pathologies including inflammation, infection, alcohol toxicity, neurodegenerative disease, ischaemia–reperfusion injury, cryptorchidism, endocrine disruption by environmental stress, damage from UV radiation and male infertility (Sharma and Agarwal, 1996). Spermatogenesis per se requires extensive restructuring in the seminiferous epithelium. Stage-specific expression of superoxide dismutase and glutathione peroxidase play important antioxidant roles (Mrkuk et al., 2002). On the other hand, association between HO-1 activity in Sertoli cells and CO was suggested to act on the cyclic guanosine 3',5'-monophosphate signalling system in human testis (Middendorff et al., 2000). Ample evidence supports the notion that HO-1 serves to provide potent cytoprotective effects in many in vitro and in vivo models of oxidant-induced cellular and tissue injury (Otterbein et al., 2000). Up-regulation of HO-1 in varicocele testes (Figure 2) may have a role in conferring cytoprotection to vulnerable testicular cells in the face of sustained oxidative stress by producing the three major catalytic by-products; CO, ferritin, and bilirubin. Different from HO-2

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or -3, HO-1 is an inducible type of HO and a small amount of HO-1 was detected in normal condition (Figure 2 and 4A). As shown by Middendorff et al. (2000), HO-1 is considered to play a role in normal spermatogenesis.

Concerning the observation that thermal stress per se can induce HO-1 expression (Maines and Ewing, 1996), it is unclear whether increased oxidative stress results in the expression of HO-1. Testicular macrophages are known to produce ROS during inflammation or infection (Wei et al., 1988). We observed that inflammatory cells produced massive nitric oxide (NO), one of the ROS, in an inducible NO synthase (iNOS)-dependent manner (Shiraishi et al., 2001). In addition, endothelial NADPH oxidase contributes as a source of increased ROS (Shiraishi et al., 2003b). Inflammatory cells and endothelium are considered as sources of ROS; however, these observations do not characterize the pathophysiology of varicocele. It is therefore necessary to investigate the mechanisms of ROS on the expression of HO-1.

Due to the limitation of small samples obtained from testicular biopsy, it is hard to apply the methods widely used for the evaluation of oxidative stress in animals. 4-HNE, the most abundant product of lipid peroxidation that induces ROS production by disrupting the mitochondrial membrane potential, is a highly reactive molecule and can pass among subcellular compartments; thereby it has the potential to interact with many cell proteins (Uchida, 2003). The stability and abundance of 4-HNE make it a commonly used parameter of oxidative stress. Using anti-4-HNE-modified protein antibody, we detected many proteins modified by 4-HNE, which suggests an increased oxidative stress in varicocele testes (Figure 1). The pattern of 4-HNE-modified proteins on our western blots differed from those seen in the experimental rat vasectomy (Shiraishi et al., 2003a). This could reflect species differences or could be a defect of the rat model. We were unable to obtain accurate and conclusive results about localization of 4-HNE-modified protein by immunohistochemistry because of high cross-reactivity of the antibody to other molecules (Uchida, 2003). We have therefore tried to elucidate molecules that are vulnerable to the modification by 4-HNE and which will ultimately provide a therapeutic target. By N-terminal amino acid sequence and immunoprecipitation, we identified a 53 kDa protein as albumin (unpublished data). Several molecules have been found to be modified by 4-HNE (Uchida, 2003).

Intense expression of HO-1 in Leydig cells (Figure 4B) is in agreement with the function of the cells as a major source of HO-1 in the testis; however, there are several controversies in the distribution of HO-1 between rat and human in normal condition. Maines and Ewing (1996) observed the detectable levels of HO-1 protein only in the Sertoli cells in rats and its localization suggests a functional role of CO as a part of the normal testicular cGMP signalling system (Middendorff et al., 2000). Ozawa et al. (2002) reported that HO-1 was expressed mostly in the interstitial macrophages, but not in Leydig cells. We observed the increased expression of HO-1 in Leydig cells (Figure 4B), in agreement with Maines and Ewing (1996). After treatment with CdCl₂, increased HO-1 immunoreactivity was seen localized to Leydig cells, while there was no change in Sertoli cells. Diemer et al. (2003), using Leydig tumour cells, reported that oxidative stress resulted in perturbed function of steroidogenic acute regulatory protein through a loss of mitochondrial membrane potential. There is no correlation between expression of HO-1 and serum testosterone concentration (Table I), nevertheless intratesticular testosterone, dihydrotestosterone or estradiol should be determined to elucidate the effect of HO-1 expression on steroidogenic function of Leydig cells.

We should be aware of the many aspects of HO-1 to understand its role in physiology and pathophysiology of testes. Schipper et al. (1999) reported that free ion and CO generated from HO-1-mediated haem catabolism caused the abnormal patterns of iron deposition in brain and mitochondrial insufficiency in various human neurodegenerative disorders. It is of interest to note that germ cell apoptosis is paradoxically promoted after induction of HO-1 (Ozawa et al., 2002). In this study, HO-1 is considered to act as cytoprotection, contributing to the improvement of spermatogenesis in varicocele testes (Figure 5). Usefulness of antioxidants in treating men subject to oxidative stress has been reported (Mruk et al., 2002). As well as varicocelectomy, antioxidant therapy may open a new therapeutic strategy against varicocele.

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References


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