Evidence for an adaptation in ROS scavenging systems in human testicular peritubular cells from infertility patients

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Introduction

Most, but not all reactive oxygen species (ROS) molecules belong to the class of free radicals (Warren et al., 1988) and are highly reactive (Papa & Skulachev, 1997; Valko et al., 2007). Oxidative stress results from an imbalance between the production of potentially damaging ROS molecules and their removal by ROS-scavenging systems. It is assumed that this plays a role in human male reproductive health (Turner & Lysiak, 2008; Makker et al., 2009). Spermatozoa, in particular, are highly susceptible to ROS-induced damage and therefore oxidative stress in semen has been a major topic of many studies (Aitken et al., 1989; Agarwal et al., 2005; Bennetts & Aitken, 2005; Makker et al., 2009; Gharagozloo & Aitken, 2011).

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Summary

Fibrosis, increased amounts of immune cells and expression of COX-2 in the testes of infertility patients provide circumstantial evidence for a specific testicular milieu, in which reactive oxygen species (ROS) could be increased. If ROS level increase and/or ROS scavengers decrease, the resulting testicular oxidative stress may contribute to human male infertility. Primary peritubular cells of the human testis, from men with normal spermatogenesis (HTPCs) and infertile patients (HTPC-Fs), previously allowed us to identify an end product of COX-2 action, a prostaglandin derivative (15dPGJ2), which acts via ROS to alter the phenotype of peritubular cells, at least in vitro. Using testicular biopsies we now found 15dPGJ2 in patients and hence we started exploring the ROS scavenger systems of the human testis. This system includes catalase, DJ-1, peroxiredoxin 1, SOD 1 and 2, glutathione-S-transferase and HMOX-1, which were identified by RT-PCR/sequencing in HTPCs and HTPC-Fs and whole testes. Catalase, DJ-1, peroxiredoxin 1 and SOD 2 were also detected by Western blots and in part by immunohistochemistry in testicular samples. Western blots of cultured cells further revealed that catalase levels, but not peroxiredoxin 1, SOD 2 or DJ-1 levels, are significantly higher in HTPC-Fs than in HTPCs. This particular difference is correlated with the improved ability of HTPC-Fs to handle ROS, which became evident when cells were exposed to 100 μM H₂O₂. H₂O₂ induced stronger responses in HTPCs than in HTPC-Fs, which correlates with the lower level of the H₂O₂-degrading defence enzyme catalase in HTPCs. The results provide evidence for an adaptation to elevated ROS levels, which must have occurred in vivo and which persist in vitro in HTPC-Fs. Thus, in infertile men with impaired spermatogenesis elevated ROS levels likely exist, at least in the tubular wall.
Adaptation in ROS scavenging systems in human testis

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Reactive oxygen species and oxidative stress in the testis, in contrast, are not well explored (Turner & Lysiak, 2008). Although not known for spermatogonial stem cells, ROS can lead to damage or influence the physiological signalling in other stem cells (Kanda et al., 2011; Ko et al., 2011; Park et al., 2011; Shao et al., 2011). Because Leydig cell steroidogenesis implicates constant generation of ROS species (Lee et al., 2009), the male gonad is endowed with effective antioxidant defence mechanisms (Zini & Schlegel, 1997; Aitken & Roman, 2008). A number of conditions, namely, varicocele, cryptorchidism, testicular torsion, inflammation, toxins and testicular ageing (Aitken & Roman, 2008; Turner & Lysiak, 2008), may lead to an increase in ROS production thereby causing testicular oxidative stress. In a rat varicocele model furthermore a reduced testicular antioxidant capacity was reported (Özdamar et al., 2004) and in Leydig cells from old rats the likewise reduced levels of antioxidants are linked with the decline in testosterone production (Zirkin & Chen, 2000; Cao et al., 2004; Luo et al., 2006).

Recent studies indicated that in human infertility patients testicular inflammation is a common underlying factor. This is reflected by immune cell infiltration of the testis. Macrophages, for example, accumulate in the interstitial areas and the walls of seminiferous tubules (Frungieri et al., 2002b). Furthermore, significantly elevated numbers of mast cells (Meineke et al., 2000; Welter et al., 2011) are part of an unexplored immune response (Spies et al., 2007). Importantly, ROS are produced by immune cells (Valko et al., 2007) and hence it is conceivable that an inflammatory milieu may be created, which includes locally elevated levels of ROS. These immune cells are able to influence cells of testicular peritubular wall. This is of interest because a recent study reported that the prostaglandin (PG) PGD2 and its metabolite, 15-deoxy-delta-(12–14)-prostaglandin-J2 (15dPGJ2), induced the generation of ROS in human testicular peritubular cells (HTPC) isolated from testicular biopsies of men with normal spermatogenesis (Schell et al., 2010). As a result, a phenotype switch of these cells occurred, namely a larger cell size, a reduction of smooth muscle properties and a diminished ability to contract. Antioxidant treatment (N-acetylcysteine; NAC) at least in cultured peritubular cells, blocked these changes. Evidence for a loss of contractility markers is observed in human testes of infertile men (Schell et al., 2010) and animal studies recently suggested that it may be a factor involved in the etiology of male infertility (Chang et al., 2004; Welsh et al., 2009). Yet, it remains to be shown, whether 15dPGJ2 is indeed present in infertility patients or not.

Assuming increased oxidative stress in the peritubular wall of infertility patients (Schell et al., 2010), this study focuses on a comparison of primary testicular peritubular cells from patients with impaired spermatogenesis and testicular fibrosis (HTPC-Fs) with cells from men with normal spermatogenesis (HTPCs) in regard of their equipment of ROS-scavenging enzyme systems and their reaction to exogenous oxidative stress.

Materials and methods

Isolation of cells, cell culture and human testicular samples

Primary cell culture of human testicular peritubular cells (HTPC) was isolated of testicular biopsies of men with normal spermatogenesis or impaired spermatogenesis and testicular fibrosis (HTPC-F) based on histological analysis, as previously described (Albrecht et al., 2006; Schell et al., 2007, 2008, 2010). The local ethics committee has approved the study and participants granted written informed consent.

Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) + high glucose (4 g/l) + 10% fetal calf serum (both from PAA GmbH, Colbe, Germany) to 90–100% confluence, unless indicated otherwise and were used from passage 5 to 13. Treatment with 100 μM H2O2 (Fluka, Hamburg, Germany), 1 mM NAC (N-acetylcysteine; Sigma Aldrich, Deisenhofen, Germany) and 10 μM 15dPGJ2 (15-deoxy-delta-(12–14)-prostaglandin-J2, Cayman Chemicals, IBL International, Hamburg, Germany) were performed for different periods of time as described. Cells were pre-incubated with NAC (1 mM) for 1 h when used in combination with 10 μM 15dPGJ2.

For immunohistochemistry studies paraffin-embedded human testicular biopsies with normal and impaired spermatogenesis were used as described previously (Frungieri et al., 2002b; Albrecht et al., 2006). For additional studies, diagnostic testicular biopsies from adult men (age range: 27–40 years old) with fertility disorders were used. The Ethics Committees, the Durand Hospital (Buenos Aires, Argentina) and the Instituto de Biología y Medicina Experimental, CONICET (Buenos Aires, Argentina) approved the study. Biopsies were assigned to the following groups: Sertoli cell only (SCO) syndrome (n = 6) and germ cell arrest (GA) (n = 3). The aetiology of testicular failure was heterogeneous, most patients presented idio-pathic infertility or non-obstructive azoospermia without infectious processes. Biopsies were cut in small pieces: one was used for histological studies, another one for immunoblotting and immunoassay analyses. For immunoblotting human testicular biopsy samples were homogenized in 20 mM Tris-HCl (pH 8), 137 mM NaCl buffer containing 10% glycerol, 1% lysis buffer (NP40; Sigma, Hamburg, Germany) and 1% of a protease inhibitor mixture (P8340; Sigma) (Frungieri et al., 2002b).
Cell counting

With an automated cell counting device (Casy System, Casy; Schäfer Systems, Reutlingen, Germany) the total number of cells was calculated. Measurements were performed as described for cell volume measurement (Schell et al., 2010).

Isolation of RNA and RT-PCR

Cells were cultured to sub-confluence, washed twice with phosphate-buffered saline (PBS) and solubilized in RLT buffer (Qiagen GmbH, Hilden, Germany) containing 1% mercaptoethanol. With the Qiagen RNeasy minikit RNA was isolated and reverse transcription was performed using random 15mer primers. For RT-PCR experiments total RNA (400 ng) was used. Used primers spanned at least one intron. PCR products were visualized by ethidium bromide staining and verified by sequencing (Frungieri et al., 2002a,b). Details for primers and PCR are shown in Table 1.

Western blot

Western blotting was performed as described previously (Frungieri et al., 2002a,b). Monoclonal rabbit anti-human catalase (1 : 1000) and anti-human DJ-1 (Epitomics, Hamburg, Germany; 1 : 10 000) and polyclonal rabbit anti-human peroxiredoxin 1 (Abcam, Cambridge, UK; 1 : 10 000) and polyclonal rabbit anti-human SOD 2 (Sigma-Prestige, St. Louis, MO, USA; 1 : 1000) were used. Resulting bands were compared by using densitometric measurements (NIH-Image J 1.42 q; National Institutes of Health, Bethesda, MD, USA) and normalization to β-actin. Graphs show the means and SEM of 3–6 independent experiments.

Immunohistochemistry

Immunohistochemistry was performed using slices of samples from different patients with diagnosis from beginning fibrosis, over germ cell arrest to Sertoli cell only and healthy patients. The protocol was described elsewhere (Albrecht et al., 2006; Schell et al., 2008). Catalase antibody dilution was 1 : 100 and the SOD 2 antibody dilution was 1 : 1500.

ATP assay

Viability of cells was determined by measuring the cellular amounts of ATP by the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany). Measured ATP is directly proportional to metabolically active, viable cells in culture. Cells were seeded on 96 well plates in sextuples and incubated overnight in DMEM without phenol red and FCS. Stimulation was performed with 100 μM H$_2$O$_2$ for 2 h, the same incubation time than in ROS measurements. ATP assays were repeated at least three times.

ROS measurements

Generation of ROS was evaluated with dichloro-dihydro-fluorescein diacetate (Carboxy-H$_2$DCFDA, Molecular Probes; Invitrogen, Darmstadt, Germany), which diffuses into the cells and is enzymatically converted by intracellular esterases and oxidized into the highly fluorescent compound DCF. DCF allows the detection of ROS, namely of H$_2$O$_2$, peroxyl radicals, peroxynitrite anions. Cells were loaded with 10 μM H$_2$DCFDA for 30 min at 37 °C and 5% CO$_2$ in extracellular fluid (ECM; containing 140 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, and 25 mM glucose; pH 7.4). For fluorescence microscope, cells were seeded on cover slips to sub-confluence and were used for experiments after 24–48 h. Cells were transferred to ECM and were stimulated with 100 μM H$_2$O$_2$ for 2 h and with 10 μM 15dPGJ2 for 4 h. After treatment cells were loaded with the dye diluted in ECM and observed every 5 min in a fluorescence microscope. For fluorometer use (Fluostar BMG Labtech, 2012 The Authors International Journal of Andrology © 2012 European Academy of Andrology)
Offenburg, Germany) 500 000 cells were split and seeded on 96 well dishes and were allowed to adhere for measurements performed 48–72 h later. First, cells were transferred to ECM for 2 h, loaded with H$_2$DCFDA as described above and treated for 2 h during measurement (37 °C, excitation 490 nm, emission 520 nm).

15dPGJ2 assay

Human testicular biopsies (n = 9) were homogenized in 20 mM Tris-HCl (pH 8), 137 mM NaCl buffer containing 10% glycerol, 1% lysis buffer (NP40, Sigma) and 1% of a protease inhibitor mixture (P8340, Sigma). The same volume of perchloric acid 0.4 N was added, samples kept 40 min on ice and centrifuged 30 min at 15 000 × g. Supernatants were collected and NaOH 0.5 N was added to reach a final pH 3.5. Subsequently, 90 μg protein was used to determine 15dPGJ2 concentration employing a commercially available kit (Assay Designs, Ann Arbor, MI, USA), as previously described (Frungieri et al., 2002a,b; Schell et al., 2007).

Statistical analyses

Data analysis was performed using GraphPad Prism® (Prism 4.0a; GraphPad Software, Inc., La Jolla, CA, USA). Curves are compared by nonlinear regression with a polynomial function second order and maximal ROS values after 2 h and Western blotting results were compared by t-test. All values shown are the mean ± SEM.

Results

Antioxidant systems in HTPCs and HTPC-Fs: catalase, peroxiredoxin 1, DJ-1 and SOD 2

To explore the repertoire of ROS-scavenging enzymes of human testicular peritubular cells, we performed a RT-PCR screening in HTPCs/Fs and human testes samples, followed by sequencing. Results showed expression of catalase, peroxiredoxin 1, DJ-1 (PARK7), SOD 1 and 2 (superoxide dismutase 1 and 2), HMOX-1 (heme oxygenase 1) and glutathione-S-transferase in both HTPCs and HTPC-Fs (Fig. 1a). Fig. 1b depicts the involvement of some of these in the generation of H$_2$O$_2$ (SODs) and its degradation (catalase and peroxiredoxin 1).

Using biopsies the expression of four representative antioxidant factors, catalase, peroxiredoxin 1, DJ-1 and SOD 2 was corroborated by Western blotting. In HTPCs and HTPC-Fs from different patients catalase (n = 4), peroxiredoxin 1 (n = 5), DJ-1 (n = 6) and SOD 2 (n = 6) were also readily found (Figs 2 & 3). The densitometric evaluation of the Western blot results showed significantly lower expression levels of catalase in HTPCs than in HTPC-Fs (Fig. 2a,b). Levels of peroxiredoxin 1, DJ-1 and SOD 2, although variable between individuals, were not different between the groups HTPCs and
HTPC-Fs (Fig. 3a–f). Immunohistochemistry confirmed that catalase and SOD 2 protein are present in peritubular cells of the human testes (Fig. 4a,b).

HTPC-Fs and HTPCs respond differently to H$_2$O$_2$

When HTPCs were compared with HTPC-Fs, differences in the H$_2$O$_2$-degrading system, namely catalase levels became obvious. Thus, we employed H$_2$O$_2$ (100 μM) to reliably induce, in a standardized fashion, oxidative stress in HTPCs and HTPC-Fs (Fig. 5a), which was identified using a fluorescence microscope and quantified by fluorometric analyses (Fig. 5b,c). The results of these experiments performed with cells from four different individuals per group showed that the kinetics of ROS generation strikingly differed and that the levels of ROS were statistically significantly higher in HTPCs than in HTPC-Fs after 2 h H$_2$O$_2$ stimulation (100 μM; n = 4; Fig. 5c). The results highlight that cellular ROS-scavenging systems, especially ones related to H$_2$O$_2$ degradation differ between HTPCs and HTPC-Fs.

The influence of H$_2$O$_2$, at the high concentration used in the ROS experiments, on cell viability was monitored by cellular ATP levels (results not shown). ATP levels were reduced to 51.5 ± 10.1% of untreated controls after 2 h in HTPCs (n = 3) and to 50.1% ± 2.9% in HTPC-Fs (n = 3). The difference was not statistically significant.

Evidence for the ROS-inducing factor, 15dPGJ2, in human testes

A previous study found that a highly reactive prostaglandin derivative 15dPGJ2 induced the generation of ROS in HTPCs (Schell et al., 2010). Similar actions were found when HTPC-Fs were studied (see supporting information, Figure S1). Results of 15dPGJ2 assay revealed that biopsies of testes of infertility patients actually contain 15dPGJ2 in vivo (1.207 ± 0.067 pmol/mg protein;
mean ± SEM; n = 9). Levels in SCO-syndrome samples (n = 6; 1.187 ± 0.091 pmol/mg protein) did not differ from levels in GA syndrome biopsies (n = 3; 1.247 ± 0.109 pmol/mg protein) and COX-2 was found in immunoblots in these biopsies (data not shown).

Discussion

By comparing HTPC-Fs, which are derived from men with existing impairment of spermatogenesis and fibrosis, with HTPCs from men with normal spermatogenesis, we found evidence for higher levels of the H₂O₂ metabolizing enzyme, catalase. H₂O₂ is a prominent member of the family of ROS molecules and the increased levels in HTPC-Fs imply that already in vivo, in the tubular wall of patients, an adaptation to higher ROS levels must have occurred, which persisted in vitro.

In HTPCs and HTPC-Fs we detected a wide spectrum of ROS scavenging factors. We were able to study four of them further, namely catalase, peroxiredoxin 1, DJ-1 and SOD 2. These are involved in the generation or degradation of H₂O₂. H₂O₂ was in the focus of our study. H₂O₂ like O₂⁻ and OH⁻ is a common ROS species, but because of a longer half-life it is able to act in a wide radius (Turner & Lysiak, 2008). It can have not only deleterious consequences, but also can serve as both an intracellular, as well as a cell-to-cell extracellular signalling molecule (Finkel, 2011).

To date only limited reports about antioxidant factors in testes are published. Yet, these four factors are mentioned directly or indirectly in human or rat testis in the literature (Bauche et al., 1994; Zini & Schlegel, 1996, 1997; Yoshida et al., 2003; Luo et al., 2006; O’Flaherty & de Souza, 2011). They were verified in vivo by Western

Figure 5 Results of ROS measurements.
(a) ROS generation increased over time in HTPCs (left) and HTPC-Fs (right) after the addition of 1 mM H₂O₂ as monitored by fluorescence microscopy (bar represents 10 μm). (b) Increase of ROS levels over time: Stimulation was performed with 100 μM H₂O₂ (n = 4) and measurements were terminated after 120 min. The kinetics of ROS production differed significantly between HTPCs and HTPC-Fs (p < 0.0001; n = 4). (c) The comparison of the endpoint fluorescence values after 120 min exposure to H₂O₂ showed that ROS generation in HTPCs is significantly higher than in HTPC-Fs (t-test; p < 0.05; n = 4; ±SEM).
unknown ROS species are responsible for phenotypic adaptations. Catalase is also the main degrading enzyme of $H_2O_2$, located mainly in peroxisomes (Chance et al., 1979; Lardinois, 1995), yet a low amount exists also in cytosol or in mitochondria and in endoplasmic reticulum (Antunes et al., 2002). Catalase activity increases concomitantly to prevalent $H_2O_2$ levels and the initial rate of $H_2O_2$ removal is proportional to the concentration of catalase (Chance et al., 1979; Lardinois, 1995). Hence, higher catalase amounts indicate higher levels of $H_2O_2$. Higher catalase levels observed in HTPC-Fs should be associated with better degradation of $H_2O_2$. The $H_2O_2$-induced generation of ROS indeed was significantly lower in HTPC-Fs compared to HTPCs. This result allows us to conclude to increased antioxidant ability, because of higher catalase, rendering HTPC-Fs less susceptible to $H_2O_2$-related actions.

What the specific physiological and a pathological role of $H_2O_2$ in peritubular cells may be, remains to be shown. It is however a noteworthy fact that it is involved in myocyte hypertrophy, apoptosis and myocardial fibrotic remodelling. In an animal model of cardiomyopathy higher catalase antagonized this action of $H_2O_2$ (Qin et al., 2010). Enlarged peritubular cells and fibrosis are also typical in human testes from infertility patients and unknown ROS species are responsible for phenotypic changes of peritubular cells in vitro (Schell et al., 2010). $H_2O_2$ may be one among them and be a player in human testicular fibrosis, typical for infertility patients.

Peroxiredoxin is a second molecule involved in removal of $H_2O_2$, which is assumed to be more important in the peroxide-removing system in animals than catalase and GPx1 (O’Flaherty & de Souza, 2011). Peroxiredoxin was described in human testis, especially in spermatozoa and seminal plasma (O’Flaherty & de Souza, 2011). It is also known as a modulator of the androgen receptor (Park et al., 2007), which is expressed by peritubular cells. However, our results did not provide evidence for an alteration in the levels of this scavenger protein.

DJ-1 appears to interact with SODs, probably also with catalase, to influence activity and action of this antioxidant defence enzymes (Xu & Möller, 2010). DJ-1 is known from its role in the pathogenesis of Parkinson disease and its essential role in the cellular defence to oxidative stress (Dekker et al., 2003) in brain. DJ-1 was specifically selected because immuno-reactive human peritubular cells were shown, but not commented, in a figure of a previous study on this protein (Yoshida et al., 2003). Results of our study support the site of expression, but they did not indicate alteration in the levels of this scavenger protein.

The SODs are described in different publications to be part of the antioxidant defence system of the testis (Bauche et al., 1994; Zini & Schlegel, 1997; Luo et al., 2006). SOD 2, as an example for several SODs, was examined because of its major role in producing intracellular $H_2O_2$, namely by degrading superoxide to $H_2O_2$ (Fig. 1b).

Thus, two of the four ROS scavengers, studied in HTPCs and HTPC-Fs, did not differ between HTPCs and HTPC-Fs, while a tendency to a higher expression of the third, namely SOD 2, and the higher catalase level point to a selective adaptation that must have occurred in vivo and persist in vitro. This is assumed because the isolation and culture conditions for HTPCs and HTPC-Fs are identical.

The increased catalase levels and the other variable results are reflected to some degree by the results of the ATP assay, which was used to explore the toxic actions of $H_2O_2$ used at a very high concentration, however, solely for the experimental purpose of robust ROS measurements. The results obtained were not statistically significant, yet results in HTPCs were much more variable and they may reflect the overall changes in antioxidant enzymes that became obvious in course of this study.

How changes in antioxidant enzymes are brought about in vivo and then are maintained under culture conditions is not known. It is, however, possible that an adaptation to higher levels of ROS, including higher levels of $H_2O_2$, in the tubular walls of infertility patients initiated the observed change of catalase protein level. Potential sources for ROS, including $H_2O_2$ in vivo could be macrophages, which are increased in infertility patients. In addition, cells, which in infertility patients express COX-2, namely Leydig cells, macrophages and mast cells, must be considered (Valko et al., 2007; Matzkin et al., 2010; Welter et al., 2011). COX-2 activity and actions of down-stream products, namely 15dPGJ2, which is present in biopsies of infertile testes, also lead to ROS generation (Schell et al., 2010; and this study). Thus, the inflammatory environment of the tubular wall in patients suffering from infertility could be a crucial factor driving expression of catalase.

However, the results do not allow us to unambiguously conclude as to whether assumed high levels of ROS may have resulted in oxidative stress in the human testis, i.e. a true imbalance between the production of ROS and their removal by their scavenging systems, which would result in damage to testicular cells. The answer to this question awaits a further elucidation of the full spectrum of antioxidant defence mechanisms used by human peritubular and other testicular cells.
Given that H$_2$O$_2$ can act as a diffusible ROS and that stem cells may be regulated by ROS, future studies should be specifically aimed to explore the direct role of ROS for the neighbouring spermatogonial stem cell (Kanda et al., 2011; Ko et al., 2011; Park et al., 2011; Shao et al., 2011). Changes in intracellular ROS levels may likewise affect cellular signalling pathways in peritubular cells (Finkel, 2011) and thus modulate functions of these cells. It is likely that peritubular cells contribute in several ways to the stem cell niche and to spermatogenesis (Spinnler et al., 2010) as well as sperm transport (Schell et al., 2010).

Additional insights into this complex system are necessary to provide a basis for evidence-based novel treatment strategies of male sub- or infertility (Atitken & Roman, 2008; Haidl et al., 2008; Turner & Lysiak, 2008; Makker et al., 2009; Gharagozloo & Aitken, 2011).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. ROS measurements using HTPC-Fs and 15dPGJ2. ROS generation increased over time in HTPC-Fs after the addition of 10 µm 15dPGJ2 (stimulation time 4 h) and is inhibited by 1 mM NAC (pre-incubation 1 h, followed by co-stimulation with 15dPGJ2) as monitored by fluorescence microscopy (bar represents 10 µm).

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