Olfactory epithelium destruction by ZnSO4 modified sulfhydryl oxidase expression in mice

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Experimental destruction of olfactory neurons stimulates proliferation and differentiation of local neural precursors and is used as a model to study *in vivo* mechanisms for degeneration and regeneration of the nervous system. Quiescin-sulfhydryl oxidases (QSOX) have a potential role in the control of the cell cycle or growth regulation and have recently been described in the central nervous system. In mice, we show an expression of QSOX in olfactory mucosa. Northern- and western-blot analysis show that the destruction of olfactory epithelium is associated with a reversible reduction in QSOX expression. Interestingly, QSOX is not localized in olfactory neurons (ON) but in cells of the lamina propria, suggesting that olfactory epithelium destruction may act as a signal of down-regulation of QSOX expression. *NeuroReport* 16:179–182 © 2005 Lippincott Williams & Wilkins.

Key words: Degeneration; Mice; Olfactory epithelium; QSOX; Regeneration; Sulfhydryl oxidase; $ZnSO_4$

INTRODUCTION

The olfactory epithelium of adult mammals is a pertinent model to study regeneration of the nervous system. Olfactory neurons (ON) are known to have a limited lifespan and to be renewed by permanent neurogenesis from local neuronal progenitors, called globose basal cells [1–4]. Several models have been developed (axotomy, bulbectomy or intranasal perfusion of a chemical) to damage the whole population of mature ON. Following these experimental lesions, basal cells are able to increase their proliferative activity and result in an almost complete regeneration of the tissue a few weeks following the lesion [5,6]. One of the most commonly used methods is intranasal perfusion of $ZnSO_4$ [7]. In our laboratory, this method has been adapted to mice with the aim of controlling the extent of neuronal loss and giving supporting tissue the capacity to restore the epithelium [8]. In these conditions, a complete neuronal cell loss and hyposmia has been observed in a large proportion of mice 4–7 days after intranasal perfusion, and recovery was noticed a few weeks after the lesion. At the same time, a stimulation of basal cells division has been observed 4–7 days following neuronal destruction [8]. Mechanisms involved in the control of neural cell proliferation remain a central question and factors that could play a role still need to be elucidated.

One important factor influencing neuronal cell proliferation could be a member of the quiescin-sulfhydryl oxidase (QSOX) family. These enzymes catalyse the formation of disulfide bonds in different proteins and are present in a wide range of multicellular organisms [9]. QSOX enzymes have been described in a large variety of tissues [9–11] but they are highly expressed in secretory epithelial cells [9]. They have been found extracellularly and since they contain a signal peptide are presumed to be secreted. Although we do not yet know their exact biological function, they seem to play diverse roles, some of which could be related to the cell cycle or regulation of growth.

QSOX enzymes have been recently described in the nervous system [12–14] and to have a high expression in the olfactory bulb, but no study has yet investigated the olfactory epithelium. The relationship of this olfactory tissue with its natural neuronal regeneration and these QSOX enzymes with their hypothetical physiological role in growth regulation are intriguing. Thus, we have investigated how the mouse olfactory mucosa expresses QSOX in normal conditions, and how this expression is modified during degeneration and recovery of the olfactory epithelium.

MATERIALS AND METHODS

Subjects: Naïve, adult (4–6 weeks old) male outbred CD-1 mice (IFFA Credo, France) were used for experiments in accordance with the principles of laboratory animal care (NIH publication 86–23, revised 1985). Animals were housed up to four per cage, with free access to food and water and maintained in a light and temperature-controlled environment. Olfactory epithelium damage was performed as previously described [8]. A bilateral 16 µl $ZnSO_4$ 10% intranasal (i.n.) perfusion was administered to lightly anaesthetised mice (5 mg/kg 0.3 M chloralhydrate solution) and animals were sacrificed in function of three

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post-perfusion (PP) time points: 4 days PP, when animals are hyposmic and when there was a massive destruction of mature ON by $ZnSO_4$ and a peak of ON precursor proliferation; at 14 days PP, when mice are recovering progressively their olfactory function and ON precursor proliferative activity has dropped to control level; and at 25 days PP, when the olfactory epithelium is partially regenerated and mice have recovered their olfactory function. Results from $ZnSO_4$ treated animals were compared to those obtained with non-treated mice considered as controls. For Northern blotting and Western blotting investigations, mice were decapitated under deep anaesthesia. For immunohistochemistry, mice were deeply anaesthetized and then perfused through the ascending aorta with 50 ml 0.9% NaCl followed by 100 ml ice-cold 1% paraformaldehyde fixative in 0.1 M PB. The bulk of olfactory turbinates were then quickly removed, frozen and stored at −45°C until use for biochemical studies or postfixed in the same fixative for 4 h at 4°C, immersed overnight at 4°C in a 15% cryoprotective sucrose solution, and quickly frozen over liquid nitrogen for immunohistochemical studies.

Behavioural study: For each mouse the level of ON destruction was assessed by measuring their ability to discriminate between an odourless solution (distilled water) and a repulsive one (15% butanol) within a T-maze test as described previously [8]. Animals showing a significant disturbance in discrimination ability were chosen for experiments.

Protein extraction and Western blotting: Olfactory mucosa were homogenized using a potter in lysis buffer, (i.e., 0.5% NP40 in 50 mM Tris–HCl pH 8) containing 120 mM NaCl, 0.1 mM Na_3VO_4, 1 mM EDTA and a protease inhibitor cocktail used according to the manufacturer's directions (Sigma, P8340, Saint-Quentin Fallavier, France). After 1 h incubation at 4°C under agitation, lysates were cleared by centrifugation at $10\,000 \times g$ for 10 min. After protein quantification by Bradford assay on each sample, they were diluted in NP40 buffer to a final concentration of 8 mg/ml and mixed to equal volume of 2 × protein sample buffer and kept frozen until used. Electrophoresis was performed by SDS-PAGE by loading 20–40 μg proteins in each lane, according to the standard technique of Laemmli [15]. After electrotransfer onto PVDF membrane (Millipore, Immobilon-P, Guyancourt, France) and blocking in PBS/5% milk/0.1% Tween 20 solution, blots were incubated for 2 h at room temperature with the primary antibodies. These antibodies were rabbit polyclonal anti-QSOX (rat, 1/500) and rabbit anti actin (1/1000). After incubation for 1 h with the appropriate peroxidase-linked secondary antibody (Dako, 1/500 and 1/1000, DakoCytomation, Trappes, France), immunoreactivity was detected by chemiluminescence using a commercial kit (Pierce, Biotechnology, Rockford, Illinois).

RNA extraction and Northern-blotting: Total RNAs were isolated according to the procedure of Chomczynski and Sacchi [16]. RNA samples (20 μg) were denatured, electrophoresed in 1.2% agarose gels and blotted onto positively charged nylon filters according to the vaccugene method (Pharmacia, Amersham Bioscience, Orsay, France). The filters were baked (80°C, 1 h), prehybridized, hybridized with ^{32}P-labelled *QXOX* cDNA probe in 50% formamide, 0.12 M sodium phosphate pH 7.2, 0.25 M NaCl, 7% SDS for 24 h at 42°C. The 1035 bp guinea pig *QSOX* specific probe (nucleotides 885–1919 of the cDNA) was generated by PCR and radiolabelled by nick translation. The identity between the guinea pig sequence and the mouse sequence was about 80% over the whole sequence.

Immunohistochemistry: Olfactory mucosa were serially cut into 20 μm coronal sections on a cryostat-microtome, mounted on gelatinized slides and stored at –45°C until treatment. Sections were incubated for 5 min in PBS-0.3% Triton X-100 and then for 30 min in PBS containing 10% fetal calf serum. Sections were then incubated with diluted primary antibody (anti-rat QSOX: 1/500 in PBS, 1% fetal calf serum) overnight at room temperature, in a humid chamber. After washing, tissue sections were incubated for 1 h at room temperature in 1/100 diluted solution of appropriate peroxidase-linked secondary antibody. The peroxidase activity was revealed in a solution containing diaminobenzidine tetrahydrochloride (0.5 M) and 0.01% hydrogen peroxide. Sections were observed with a Nikon microscope (Eclipse E600) and photographed with a Nikon digital camera (Coolpix 990).

RESULTS

QSOX expression is modulated in the olfactory epithelium in function of ON degeneration and regeneration after $ZnSO_4$ intranasal perfusion: In the control condition, one transcript of 2.4 kb (Fig. 1a), and a single protein with a mol. wt of 66 kDa (Fig. 1b) were detected in the olfactory mucosa. This mRNA and protein expression followed the same pattern during ON degeneration and regeneration. Both were dramatically reduced 4 days PP, when ON were damaged as determined by the loss of odour discrimination by the T-maze test (not shown). This reduction of expression

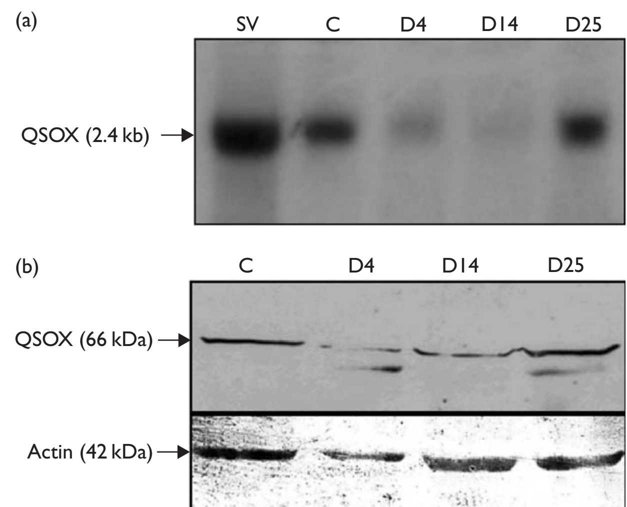

Fig. 1. (**a**) Northern-blot analysis of *QSOX* expression in seminal vesicles (SV), an organ known for having a high QSOX expression; and in olfactory mucosa in control conditions (C) (non-treated animals) and at 4 days (D4), 14 days (D14) and 25 days (D25) after $ZnSO_4$ i.n. perfusion. (**b**) Western-blot analysis of QSOX and actin expression in olfactory mucosa in control conditions (C) and at 4 days (D4), 14 days (D14) and 25 days (D25) after $ZnSO_4$ i.n. perfusion.

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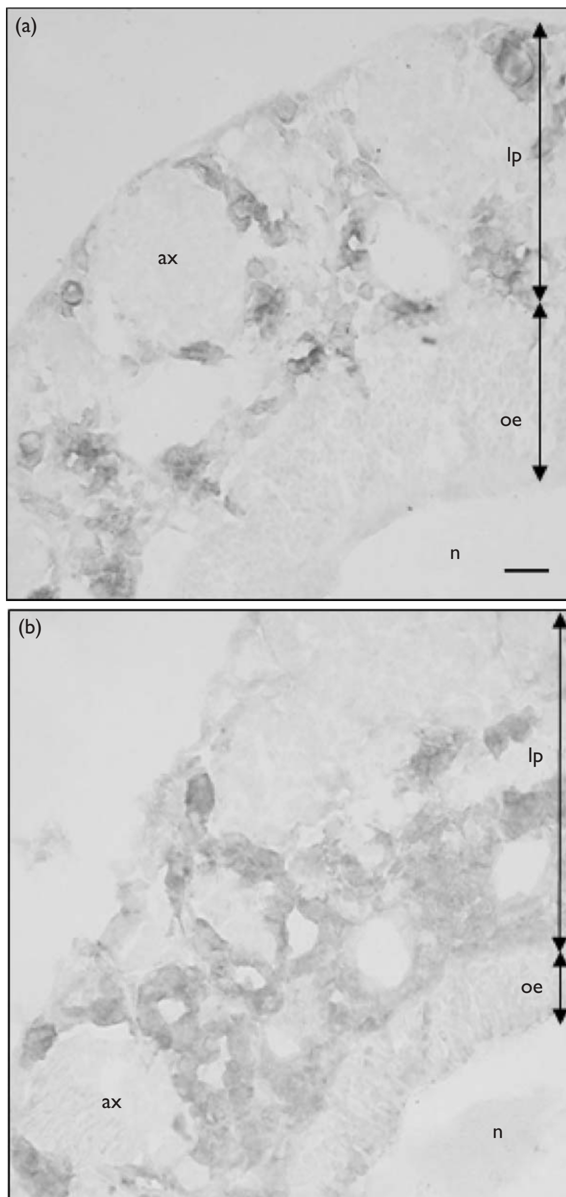

Fig. 2. QSOX immunohistochemistry on control (**a**) and 4 days after $ZnSO_4$ i.n. perfusion (**b**). lp: lamina propria, oe: olfactory epithelium, n: nasal cavity, ax: axon bundles (olfactory nerve). Bar=50 μm.

was still observed 14 days PP while mice were gradually recovering their discrimination ability. At 25 days PP, while olfactory performance was fully restored, mRNA and protein expressions were back to a control level. QSOX protein variations were observed while the amounts of actin in these same extracts were quite unchanged. By western blot analysis, a second reactive band with a slightly smaller size (which could correspond to a proteolytic form of QSOX), was also detected at 4 and 25 days PP.

QSOX expression occurs in cells of the lamina propria: No specific labelling of QSOX protein was detected in olfactory neurons. The expression was strictly restricted to cells located in the underlying lamina propria, around the axons bundles of olfactory nerve and resembled the staining of ensheathing cells (Fig. 2a). This immunohistochemistry method did not possess the ability to show a reduction of staining in the olfactory epithelium of mice treated with $ZnSO_4$ (Fig. 2b) compared to observations in saline treated mice.

DISCUSSION

The present work shows, in control conditions, a QSOX expression in the olfactory mucosa. It has already been reported that there is a high expression in the olfactory cortex, where a strong immunostaining was observed in the mitral and in the periglomerular cells [12,13]. Olfactory neurons present in the olfactory epithelium directly project to the glomerules where they synapse with mitral cells. This work is the first to show that the receptive part of the olfactory system also expresses QSOX, but interestingly, this expression is not detected in olfactory neurons but in cells of the lamina propria.

Only one transcript was detected in the olfactory mucosa while two transcripts were expressed in the complete brain [12]. Until now the CNS has been the only tissue in mice and rat where two transcripts were found to be expressed [12]. We could expect to also observe two different mRNAs in the olfactory epithelium as it is a nervous tissue in which neurons have a unique projection to the olfactory bulb. A distinction appears to be well established between the CNS and peripheral organs concerning QSOX gene expression.

Here, the biochemical analysis in function of the post-perfusion time shows that the temporary destruction of olfactory epithelium is associated with a QSOX reversible reduced expression. In control conditions, this expression being observed only in the lamina propria, the major destruction of the mucosa after the lesion does not account for the reduced amount of QSOX detected. Thus, we can conclude that the destruction of the olfactory epithelium induce a down regulation of QSOX expression in the lamina propria.

Exact biological functions of sulfhydryl oxidases are not known, but they are likely to play important roles in oxidative protein folding in a wide range of tissues [9]. The reduction of their expression as observed in our model might then induce an important modulation of the functionality of their protein targets. They could also play a role in the control of the cell cycle and they have been shown to be upregulated in quiescent cellular conditions [11,17,18]. In our model, the destruction of OE is followed by a regeneration of the tissue, based on the proliferation of basal cells [1,4]. This proliferative activity with a peak between 4 and 7 days PP [8] follows the same time course than the reduction of QSOX expression. Based on our findings and on what we already know about QSOX functions, the reduction of QSOX expression could find its origin in the need for basal cells to increase their proliferative activity. This is not so surprising as it is more and more considered that cellular redox state play an important role in the regulation of cellular growth and proliferation [19].

Interestingly, QSOX expression was monitored in the lamina propria. Immunostaining resembled the staining of ensheathing cells [20,21], and particularly the glial sheath of olfactory nerve bundles. These cells are expected to play an important role for olfactory neuronal turnover [21–23]. This

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might mean that QSOX is more or less directly implicated in neurogenesis.

This study permitted us to observe how the level of QSOX expression can be modified in physiological conditions of degeneration and regeneration of a nervous tissue. More investigations now need to be performed to determine its role in neurodegenerative conditions.

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