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RESEARCH ARTICLE

A specific fluorescence probe for hydrogen peroxide detection in peroxisomes

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Abstracts
Hydrogen peroxide is an important mediator in cell signalling and cell death. Apart from the mitochondrion the peroxisome is the most important cellular site for the generation and scavenging of hydrogen peroxide. Peroxisomes contain various oxidases, e.g. for the metabolism of long-chain fatty acids, polyamines, and for the oxidation of urate, which form hydrogen peroxide. Widely-used chemical probes for the detection of hydrogen peroxide like dichlorofluorescein diacetate (DCFDA) often lack in specificity and the possibility of compartment-specific measurement. To overcome these disadvantages, Belousov et al. developed the novel hydrogen peroxide sensitive fluorescent protein HyPer. In the present study the HyPer protein was fused with the PTS1 tag for a specific hydrogen peroxide detection in peroxisomes. The localization of the HyPer protein in the peroxisomes was confirmed by immunofluorescence and the functionality by fluorescence microscopy and flow cytometry analyses. The presented HyPer-Peroxi fluorescent protein is a valuable tool for studying hydrogen peroxide generation within the peroxisomes.

Keywords: *HyPer, beta-oxidation, peroxisome*

Introduction

With the rapid growth of interest in free radicals in biology there is a need for identification of specific reactive oxygen species at distinct subcellular locations. Among biologically relevant reactive oxygen species (ROS) hydrogen peroxide has the greatest stability and intracellular concentration. The cellular effects of hydrogen peroxide depend on the concentration, in lower concentrations around 10^{-8} M hydrogen peroxide can induce proliferation, when the concentration rises it causes growth arrest and finally induces apoptosis (10^{-4} M) [1]. Cellular damage is associated with initiation and progression of many diseases, including neurodegenerative disorders, diabetes, atherosclerosis and cancer [2]. As hydrogen peroxide is generated by many compartmentalized enzymes there is a need for tools, which recognize local variations in the hydrogen peroxide concentration. The chemical probe dichlorodihydrofluorescein ($DCFH_2$) is the commonest probe used to detect hydrogen peroxide [3], but this fluorescent dye lacks the possibility to discriminate between different reactive oxygen species and their intracellular site of formation.

To overcome these disadvantages, Belousov et al. [4] developed a genetically encoded biosensor for hydrogen peroxide by inserting the circularly permuted yellow fluorescent protein (cpYFP) into the regulatory domain of *E.coli* OxyR, which is specifically sensitive to hydrogen peroxide [5]. This newly designed fluorescent probe called HyPer exhibits two excitation peaks at 420 and 500 nm and one emission peak at 516 nm. Upon exposure to hydrogen peroxide the excitation peak at 420 nm decreased in proportion to the increase in the peak at 500 nm [4].

Besides the mitochondrion the peroxisome is the main intracellular site where hydrogen peroxide is both generated and scavenged. Peroxisomes contain various acyl-CoA oxidases for the metabolism of long-chain fatty acids, polyamine oxidase for the metabolism of *N*-acetyl spermine/spermidine or urate oxidase for the oxidation of urate [6]. In rat liver, for example, peroxisomal oxidase activity accounts for

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~ 20% of total oxygen consumption [7]. To prevent oxidative damage by free radicals derived from hydrogen peroxide the peroxisome contains catalase, which is a marker enzyme for the peroxisome [8]. To analyze the generation and scavenging of hydrogen peroxide it is important to have a compartment-specific probe for hydrogen peroxide detection. In the present study we therefore fused the cDNA coding for HyPer with the peroxisomal targeting sequence 1 (PTS1) to target this protein to the peroxisome and measured hydrogen peroxide in living cells.

Methods

Tissue culture of insulin-producing and hepatoma cells

RINm5F insulin-producing cells were cultured in RPMI 1640 medium, supplemented with 10 mM glucose, 10% (v/v) foetal calf serum (Biowest, Nuaillé, France), penicillin and streptomycin in a humidified atmosphere at 37°C and 5% CO_2 as described previously [9]. The RINm5F cell clone over-expressing catalase (RINm5F-Cat) was generated as described previously and the expression was analyzed by enzyme activity measurement [10].

MH-7777A hepatoma cells were cultured in DMEM medium, supplemented with 25 mM glucose, 10% (v/v) foetal calf serum, 4 mM L-glutamine, 10 nM insulin (Sigma, St. Louis, MO), 10 µM dexamethasone (Sigma), penicillin and streptomycin in a humidified atmosphere at 37°C and 5% CO_2.

Cloning of HyPer vectors

For *in vivo* analyzes of cytosolic hydrogen peroxide generation, the cDNA of the hydrogen peroxide-sensitive fluorescent HyPer protein [4] was sub-cloned from the pHyPer-dCyto plasmid (Evrogen, Moscow, Russia) into the lentiviral transfer plasmid pLenti6/V5-MCS (Invitrogen, Karlsruhe, Germany). Therefore, HyPer-dCyto cDNA was amplified using Phusion proofreading polymerase (NEB, Frankfurt, Germany) and the composite primers (Hyper-Cyto-XbaI-fw (5′ TATCTAGACGCCACCATGGAGATGGCAA 3′) and Hyper-Cyto-Bsp119I-rv (5′ GCTTCGAATTAAACCGCCTGTTTTAAAACTT 3′)) to introduce restriction sites for XbaI and Bsp119I. After digestion the HyPer-dCyto cDNA was ligated to the corresponding restriction sites of the pLenti6/V5-MCS plasmid. To construct the expression vector for the HyPer-Peroxi protein that allowed detection of peroxisome-generated hydrogen peroxide, the PTS1 sequence [11,12] was fused to the 3' end of the HyPer cDNA by PCR using composite primers (HyPer-PTS1-XbaI-fw (5' TATCTAGACGCCAC-CATG-GAGATGGCAA 3′) and HyPer-PTS1-Bsp119I-rv (5′ GCTTCGAATTACAGCTTG-GAAACCGCCT-GTTTTAAAAC 3′)) and the pHyper-dCyto plasmid as template. Then, the HyPer-Peroxi cDNA was sub-cloned into the XbaI/Bsp119I site of the pLenti6/V5-MCS plasmid.

Preparation of lentiviruses

To express the HyPer-Cyto and HyPer-Peroxi protein in the insulin producing RINm5F cell line, lentivirus was prepared according to Zufferey et al. [13]; 5×10^6 293FT cells were transfected with the packaging plasmid pPAX2 (37.5 µg), the envelope plasmid pcDNA-MDG (7.5 µg) and the transfer plasmids pLenti6/V5-MCS-HyPer-Cyto or pLenti6/V5-MCS-HyPer-Peroxi (25 µg) by calcium phosphate precipitation. The virus particles were harvested from the culture medium 48 h later and purified by ultracentrifugation (70 000 g, 2 h). The virus titers (3–5 $\times$ 10^7 infectious particles) were quantified by a Taqman qPCR assay as described elsewhere [14].

Lentiviral transduction

The RINm5F, RINm5F-Cat and the MH-7777A cell lines were infected with HyPer-Cyto or HyPer-Peroxi lentivirus with a MOI of 10. The cells were selected for HyPer expression using blasticidin (1 µmol/L).

Analyses of hydrogen peroxide generation by HyPer proteins

RINm5F cells (2×10^5) that over-expressed HyPer-Cyto or HyPer-Peroxi were seeded onto black 24-well glass-bottom plates (Zellkontakt, Nörten-Hardenberg, Germany) and cultured for 24 h. Cells were treated with hydrogen peroxide (100 µM) and immediately analyzed via fluorescence microscopy. Live cell imaging was performed using a CFP-YFP dual filter (excitation 427 nm and 504 nm/emission 520 nm) with a cell^R/Olympus IX 81 inverted microscope system (Olympus, Hamburg, Germany). CellR software (Olympus) was used for imaging and analysis.

The changes of the fluorescence ratio (488/530 nm and 404/520 nm) of RINm5F, RINm5F-Cat and MH-7777A cells over-expressing HyPer-Cyto or HyPer-Peroxi were quantified by flow cytometry (LSRII, BD, San Jose, CA). For this purpose, the cells were trypsinized and treated with different concentrations of hydrogen peroxide and the fluorescence ratio was measured immediately by flow cytometry. For data analysis, *FlowJo* software (Tree Star, Ashland, OR) was used.

Sensitivity of HyPer against nitric oxide or peroxynitrite donors

RINm5F cells over-expressing HyPer-Cyto or HyPer-Peroxi were treated for 2 h with SIN-1 (3-morpholino-sydnonimine, Cayman, Ann Arbor, MI), which

generates peroxynitrite, or for 18 h with SNAP (S-nitroso-N-acetyl-penicillamine, Cayman), which generates nitric oxide. Afterwards the cells were trypsinized and the fluorescence ratio was measured by flow cytometry. For data analysis, *FlowJo* software (Tree Star) was used.

Immunocytochemical staining

For immunocytochemical staining of RINm5F cells over-expressing HyPer-Peroxi, 1×10^5 cells were seeded overnight on collagen-coated glass slides and subsequently fixed with 4% paraformaldehyde. After a washing step the cells were permeabilized and blocked with PBS plus 0.2% Triton X-100 and 1% BSA. The slides were incubated with primary antibodies (anti-peroxisomal membrane protein 70, 1:1000, Invitrogen, Karlsruhe, Germany; anti-cytochrome c oxidase IV 1:50, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS with 0.1% Triton X-100 and 0.1% BSA at room temperature for 60 min. Following this step the cells were washed with PBS and incubated with secondary antibodies for 60 min (1:200). Secondary antibodies from donkey were conjugated with Cy5 obtained from Dianova (Hamburg, Germany). For nuclear counterstaining, 300 nM DAPI was used for 5 min at room temperature. Finally, the cells were washed and mounted with Mowiol/DABCO (Sigma) anti-photobleaching mounting media. Stained cells were examined on a cell^R/Olympus IX81 inversed microscope (Olympus) and microscopical images were post-processed with *AutoDeblur* and *AutoVisualize* (Autoquant Imaging, New York, NY).

Results

Intracellular localization of the HyPer protein in RINm5F cells

To proof the intracellular localization of the HyPer protein targeted to the peroxisomes in RINm5F cells, fixed cells were stained for the peroxisomal membrane protein 70 (PMP-70) or the mitochondrial protein cytochrome oxidase 4 (COX-4). The fluorescence (504/520 nm) of the HyPer protein was clearly detectable as green spots after fixation with paraformaldehyde. Staining of the peroxisomes (PMP-70) showed a distinct co-localization with the HyPer-Peroxi protein (Figure 1A). In contrast the HyPer-Peroxi protein showed no co-localization with the mitochondria (Figure 1B).

Fluorescence ratio changes of insulin-producing and hepatoma cells expressing the hydrogen peroxide sensitive protein HyPer either in the cytosol or in the peroxisomes

To analyzed if the novel constructed HyPer protein targeted to the peroxisomes (HyPer-Peroxi) is able to detect hydrogen peroxide, the changes of the fluorescence ratio were analyzed via live cell fluorescence microscopy dependent on hydrogen peroxide treatment and compared to the cytosolically located HyPer protein (HyPer-Cyto). Untreated RINm5F-HyPer-Cyto and RINm5F-HyPer-Peroxi cells showed a strong yellow fluorescence resulting from an overlay of the fluorescence at 504/520 nm (red) and the fluorescence at 427/520 nm (green). When the HyPer protein was over-expressed in the cytosol, the fluorescence signal was homogenously distributed over the cell, whereas the fluorescence signal of the HyPer-Peroxi protein appeared dotted (Figure 2). After short treatment with hydrogen peroxide (100 μM) the fluorescence at 427/520 nm decreased and the fluorescence at 504/520 nm increased, which results in a strong fluorescence shift towards red. The fluorescence shift was comparable in the cytosol and in the peroxisomes, which indicates that the new HyPer-Peroxi protein was able to detect hydrogen peroxide.

Relative quantification of hydrogen peroxide with the HyPer protein through measurement of the fluorescence ratio via flow cytometry

To analyze if the sensitivity of the HyPer-Peroxi protein is comparable to that of the cytosolically located

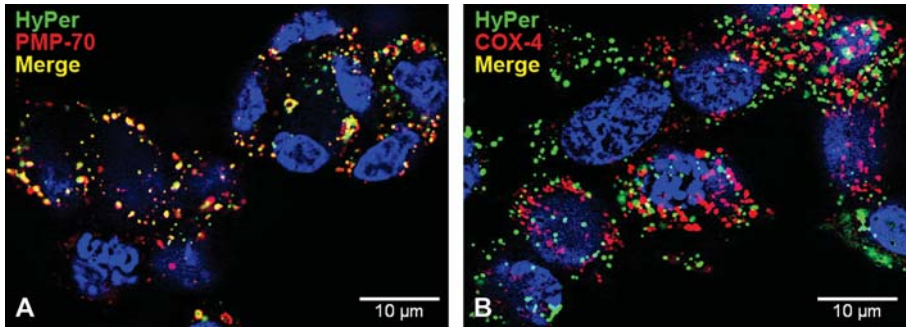

Figure 1. Immunocytochemical staining for peroxisomes and mitochondria in HyPer-Peroxi over-expressing RINm5F cells. RINm5F insulin-producing cells over-expressing the hydrogen peroxide-sensitive protein HyPer in the peroxisomes were seeded overnight on collagen-coated coverslips. After fixation with 4% paraformaldehyde the cells were stained for the peroxisomal membrane protein 70 (PMP-70 red, A) or the mitochondrial respiratory chain enzyme cytochrome c-oxidase IV (COX-4 red, B) followed by counterstaining with DAPI (blue). The HyPer protein was detected through excitation at 504 nm and emission at 520 nm (HyPer green).

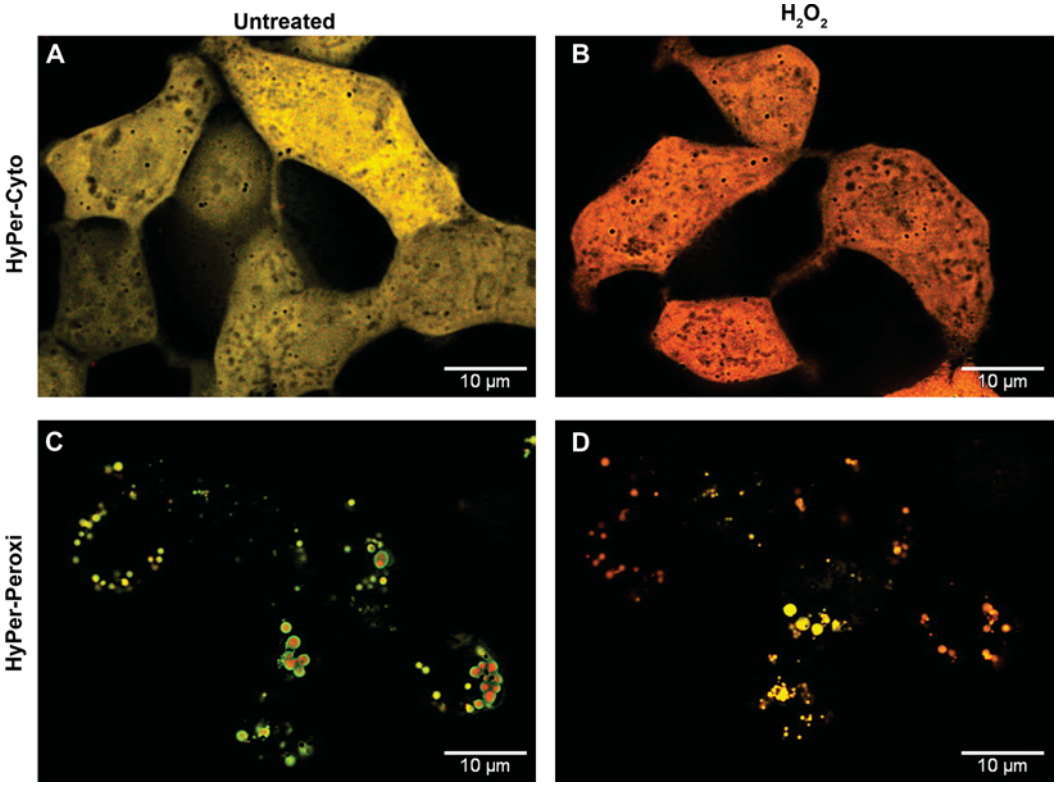

Figure 2. Hydrogen peroxide detection via live cell fluorescence microscopy of RINm5F insulin-producing cells over-expressing HyPer-Cyto or HyPer-Peroxi. Cells that stably expressed the hydrogen peroxide sensor protein HyPer in the cytosol (HyPer-Cyto) or peroxisomes (HyPer-Peroxi) were treated with 100 µM hydrogen peroxide and images were taken immediately. Shown are representative images of untreated cells and after exposure to hydrogen peroxide. In the images, the fluorescence at 504/520 nm is depicted in red and the fluorescence at 427/520 nm in green. Increased hydrogen peroxide concentration goes along with a colour change from green via yellow towards red.

protein, the cells were treated with different concentrations of hydrogen peroxide and the fluorescence ratio changes were measured immediately by flow cytometry. Both proteins showed a comparable increase of the fluorescence ratio with increasing hydrogen peroxide concentrations (Figure 3). The fluorescence ratio of the HyPer-Cyto and the HyPer-Peroxi protein increased strongly in a hydrogen peroxide concentration range from 10–75 µM. When the cells were treated with 100 µM hydrogen peroxide a plateau was reached independent of the subcellular localization (Figure 3A). Additional over-expression of catalase, which converts hydrogen peroxide to molecular oxygen and water, in the RINm5F-HyPer-Cyto or the RINm5F-HyPer-Peroxi cells resulted in a weaker increase of the fluorescence ratio both in the cytosol and in the peroxisomes (Figure 3A). The suitability of the HyPer-Peroxi protein as a hydrogen peroxide indicator was analyzed also in the hepatoma cell line MH-7777A. Comparable to RINm5F cells the fluorescence ratio of the HyPer-Peroxi protein in MH-7777A cells correlated with hydrogen peroxide concentrations similar to the HyPer-Cyto protein (Figure 3B). However, in the MH-7777A cells the fluorescence ratio increase was weaker than in RINm5F cells because of the higher endogenous level of catalase expression.

Insensitivity of the HyPer protein against reactive nitrogen species

To assess a potential effect of reactive nitrogen species on the HyPer protein RINm5F-HyPer-Cyto or RINm5F-HyPer-Peroxi cells were treated with different concentrations of either SIN-1 (a peroxynitrite donor) or SNAP (a nitric oxide donor). Both chemical compounds led to no significant changes (Anova/Dunnett's) of the fluorescence ratio of HyPer-Cyto or HyPer-Peroxi (Figure 4).

Discussion

Hydrogen peroxide plays important roles in many biological processes in many organisms, from relatively simple bacteria to complex multicellular plants and animals [2,15]. Clearly a major obstacle in studying the roles of hydrogen peroxide in biology has been the lack of widely available specific tools and methodologies. Fluorescence chemical dyes like DCF allow sensitive measurement of hydrogen peroxide formation in cells, but often lack specificity [3,16]. Recently, Belousov et al. [4] developed the hydrogen peroxide-specific fluorescent sensor protein HyPer. This specific probe is a fusion protein of OxyR, a reversible oxidizable *E. coli* protein [5], and cpYFP

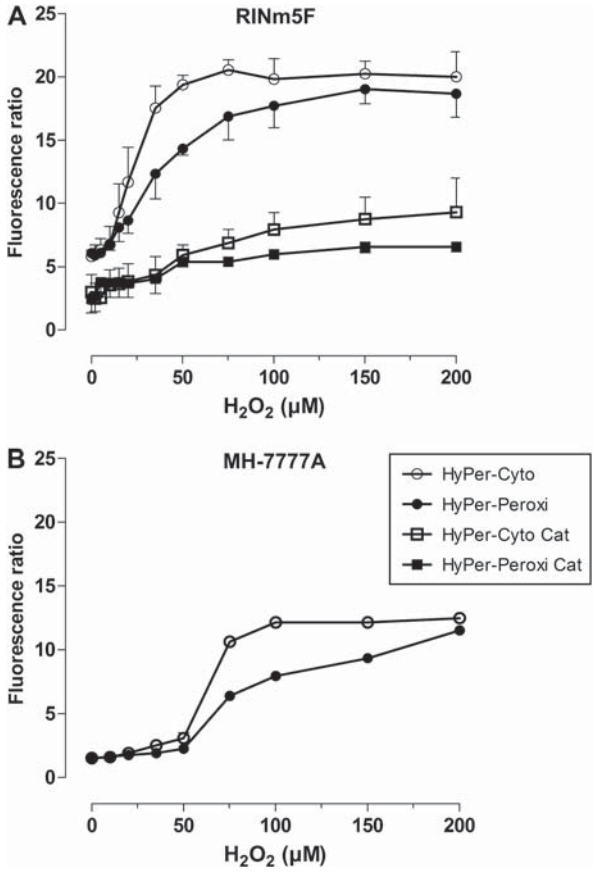

Figure 3. Hydrogen peroxide detection via flow cytometry in HyPer-Cyto and HyPer-Peroxi over-expressing RINm5F, RINm5F-Cat and MH-7777A cells. RINm5F insulin-producing cells (A, circles), RINm5F cells over-expressing catalase (Cat, A, squares) and MH-7777A cells (B, circles) over-expressing the hydrogen peroxide sensor protein HyPer either in the cytosol (HyPer-Cyto, open) or peroxisomes (HyPer-Peroxi, filled) were treated with different concentrations of hydrogen peroxide immediately before measurement. The fluorescence ratio (488/530 nm/404/520 nm), which is an indicator for the hydrogen peroxide concentration, was measured by flow cytometry. Shown is the fluorescence ratio change dependent on the hydrogen peroxide concentration. Data are means ± SEM from two or three individual experiments.

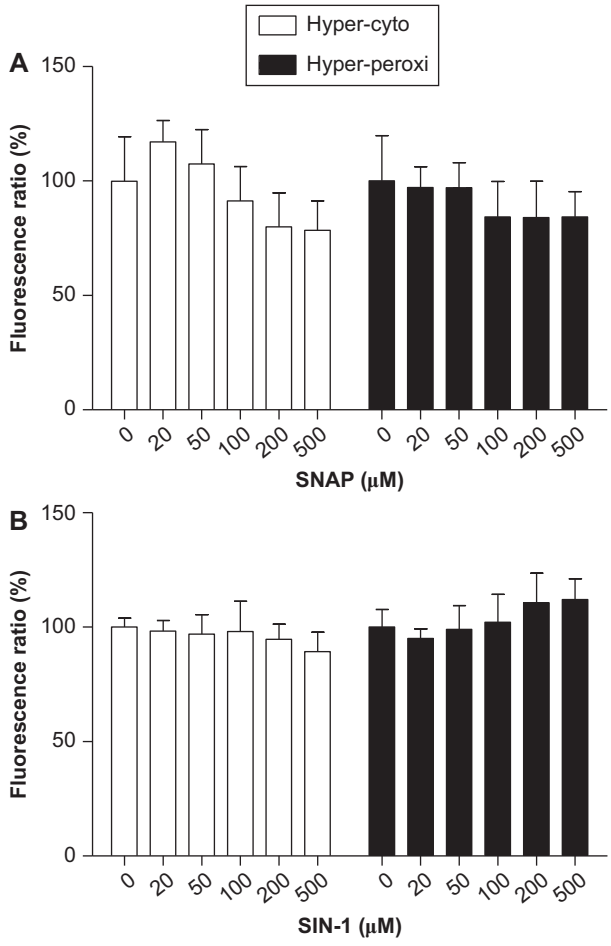

Figure 4. Fluorescence ratio changes of HyPer-Cyto and HyPer-Peroxi detected by flow cytometry. RINm5F insulin-producing cells over-expressing HyPer-Cyto (open bars) or HyPer-Peroxi (black bars) were treated for 18 h with SNAP (A) or for 2 h with SIN-1 (B). Afterwards the cells were trypsinized and the fluorescence ratio (488/530 nm/404/520 nm) was measured by flow cytometry. Shown are the relative changes in the fluorescence ratio in comparison to untreated cells (0 μM). Data are means ± SD from four or five individual experiments.

that transfers the conformational changes of the OxyR after oxidation by hydrogen peroxide into a fluorescence shift. The ratiometric measurement of the fluorescence signals enables the readout independent of the amount of protein expressed. The HyPer protein demonstrates sub-micromolar affinity to hydrogen peroxide and at the same time it is insensitive to other oxidants like superoxide radicals, nitric oxide, peroxynitrite or oxidized glutathione [4]. Although it was described for OxyR in bacteria that the conformation is dependent upon the redox state (GSH/GSSG ratio) of the cell [5], the HyPer protein showed no fluorescence changes upon exposure to oxidized glutathione up to 1 mM [4]. From ours as well as experiments from other groups [4] it cannot be excluded that the extremely reactive hydroxyl radical is also able to oxidize the HyPer protein. However, due to the fact that hydroxyl radicals are typically generated from hydrogen peroxide in the Fenton reaction [17], the potentially unspecific detection of hydroxyl radicals with the HyPer probe is an indirect confirmation of the presence of H_2O_2.

Another disadvantage of common chemical dyes is the lack of intracellular compartment-specific ROS detection. However, HyPer gives as a protein the opportunity of specific targeting and expression in distinct compartments.

In the present study the HyPer protein was fused to the PTS1 tag (HyPer-Peroxi) for specific peroxisomal expression confirmed by immunostaining.

The functionality of the HyPer-Peroxi protein was analyzed by fluorescence microscopy and flow cytometry after hydrogen peroxide treatment. The sensitivity of HyPer-Peroxi was comparable to that of the cytosolically located HyPer protein, indicating that hydrogen peroxide could be detected in the same concentration

range in the peroxisomes as in the cytosol. Overexpression of catalase in the RINm5F-HyPer-Peroxi cells reduced the hydrogen peroxide dependent changes of the fluorescence ratio, which indicates that the HyPer-Peroxi protein specifically detects hydrogen peroxide. Reactive nitrogen species like nitric oxide and peroxynitrite have no significant effect on the fluorescence ratio of the HyPer protein, which gives further evidence for the specifity of the HyPer-Protein. This is in accordance with the findings of Belousov et al. [4], that reactive nitrogen species appeared to be unable to induce changes in the HyPer fluorescence.

Peroxisomes are involved in a variety of important cellular functions, e.g. detoxification or metabolism, in eukaryotic cells [18]. The oxidases, which participate in these processes, transfer hydrogen from their respective substrates to molecular oxygen, yielding hydrogen peroxide [19]. An organelle-specific quantitative measurement of hydrogen peroxide could be of importance for the analysis of the oxidase-activities and their involved pathways. For example, the breakdown of fatty acids does not only take place in the mitochondria, another important organelle for the metabolism of fatty acids is also the peroxisomes [18]. During the beta-oxidation in the peroxisomes potentially cytotoxic hydrogen peroxide is generated by the acyl-CoA oxidases, whereas during mitochondrial beta-oxidation the hydrogen is transferred by the acyl-CoA dehydrogenases to NAD^+ or FAD. Therefore, the measurement of peroxisomally generated hydrogen peroxide is of interest for studying the mechanisms of ROS-mediated lipotoxity [20,21].

Oxidative stress in general plays an important role in many diseases like cancer, diabetes, athereosclerosis and neurodegenerative diseases [22]. Although the peroxisome is a major organelle for oxidative metabolism it is typically not in the scientific focus. With the HyPer-Peroxi fluorescent protein presented herein we offer a valuable tool for studying hydrogen peroxide generation within the peroxisomes in cellular disease model systems.

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Declaration of interest

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