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Hydrogen peroxide permeability of cellular membranes in insulin-producing cells

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Abstract

Hydrogen peroxide (H_2O_2) plays a central role in redox signalling and in oxidative stress-mediated cell death. It is generated through multiple mechanisms at various intracellular sites. Due to its chemical stability it can reach distant sites of action. However, its hydrophilicity can hamper lipid membrane passage. We therefore studied the kinetics of H_2O_2 diffusion through subcellular membranes employing the H_2O_2 biosensor HyPer in insulin-producing RINm5F cells.

Plasma- and ER-membrane-bound HyPer sensors facing the cytosolic compartment reacted twice as fast to H_2O_2 compared to sensors expressed in peroxisomes and mitochondria. Overexpression of the H_2O_2-inactivating enzyme catalase in the ER-lumen and in the peroxisomes retarded the reaction time of HyPer, both localized within the peroxisomes as well as at the cytosolic surface of the ER. The unsaturated fatty acid oleic acid did not affect the reaction of the peroxisomal HyPer sensor to H_2O_2, while the saturated fatty acid palmitic acid accelerated its reaction time to H_2O_2 in this organelle.

The results show that the plasma-, peroxisomal, and mitochondrial membrane of insulin-producing RINm5F cells are permeable for H_2O_2. Nonetheless, the organelle membranes retard H_2O_2 diffusion due to a barrier function of the lipid membrane, as documented by retarded reaction times of the intraorganellar sensors. Accelerated decomposition of H_2O_2 by catalase, expressed in the peroxisomes or the ER, further retarded the HyPer sensor reaction time. The results show that redox signalling and oxidative stress mediated toxicity are crucially dependent on physicochemical membrane properties and antioxidative defence mechanisms in health and disease.

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Keywords

hydrogen peroxide; genetically encoded sensors; beta-cells; oxidative stress; membrane permeability; free fatty acids

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1. Introduction

The biologically important reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) has been a main focus of research for many years [1-3]. Depending on its concentration it could function on the one hand as an important signalling molecule [4, 5] and on the other hand act as a mediator of oxidative stress [6-8]. One example for the toxic potential of H_2O_2 is the oxidative stress mediated pancreatic beta-cell toxicity in the development of the diabetic metabolic state [8]. H_2O_2 emerging from the peroxisomal beta-oxidation during lipotoxicity [9-12] or from the endoplasmic reticulum (ER) as a consequence of increased insulin biosynthesis [13] represents a major challenge for the pancreatic beta-cell that is badly protected against H_2O_2 toxicity due to low expression of antioxidative enzymes such as catalase (Cat) and glutathione peroxidases [14, 15]. The development of the genetically encoded biosensor HyPer for the specific detection of H_2O_2 allows the monitoring of this reactive species at distinct locations in living cells [16]. The sensor was generated by insertion of circularly permuted yellow fluorescent protein (cpYFP) into the regulatory domain of the H_2O_2-sensitive transcription factor OxyR from *E. coli* and allows the specific ratiometric measurement of H_2O_2 [16, 17].

H_2O_2 is rather stable chemically [2, 8]. Therefore, it is able to travel significant intracellular distances moving from the site of generation to the site of action and can traverse lipid membranes of subcellular organelles [2]. These membranes show limited permeability to H_2O_2 in dependence on the membrane composition and on the expression of membrane proteins facilitating H_2O_2 diffusion, known as aquaporins [18, 19]. H_2O_2 can thus pass the plasma membrane and gain access to different subcellular compartments. However, it is unknown, whether different subcellular membranes show the same permeability to H_2O_2 and to which extent they exhibit a barrier function for this ROS.

Therefore, we investigated in the present study the ability of H_2O_2 to diffuse through lipid membranes such as the plasma membrane or subcellular organelle membranes of insulin-producing RINm5F cells. With respect to the special situation of insulin-producing cells regarding low H_2O_2 decomposing capacity we additionally studied the effects of catalase overexpression either inside the peroxisomes or the ER lumen on HyPer sensor reactions to H_2O_2. Lastly, relating to the pancreatic beta-cell lipotoxicity in diabetes, we examined the effects of the saturated and unsaturated fatty acids palmitic acid (PA) and oleic acid (OA) on the membrane permeability of subcellular organelles in insulin-producing cells.

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2. Materials and Methods

2.1 Materials

RPMI 1640 tissue culture medium, penicillin and streptomycin were purchased from Biochrom (Berlin, Germany), fetal calf serum from Thermo Fisher Scientific (Waltham, MA, USA) and trypsin from PAN Biotech (Aidenbach, Germany). Bovine serum albumin (BSA) was from Serologicals Proteins Inc. (Kankakee, IL, USA), sodium bicarbonate and ethanol from ChemSolute (Renningen, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), HEPES and free fatty acid (FFA) free BSA were obtained from Serva (Heidelberg, Germany). Puromycin and blasticidin were from InvivoGen (San Diego, CA, USA). Tissue culture plates were purchased from Greiner Bio-One (Frickenhausen, Germany), Sarstedt (Nümbrecht, Germany) and Nunc/Thermo Fisher Scientific. Custom designed PCR primers were synthesized by Thermo-Fisher Scientific. Anti-peroxisomal membrane protein 70 kDa (PMP70) antibody, rabbit (polyclonal), was from Zymed (Vienna, Austria), anti-cytochrome c oxidase subunit IV (COXIV) antibody, mouse (monoclonal), from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-protein disulphide isomerase (PDI) antibody, mouse (monoclonal), from Abcam (Cambridge, UK), anti-Tag(CGY)FP, rabbit (polyclonal), from Evrogen (Moscow, Russia) and anti-mouse-Cy5 (donkey, IgG), anti-mouse-Alexa-Fluor 647 (donkey, IgG), anti-rabbit-Alexa-Fluor 647 (donkey, IgG) and anti-rabbit-Alexa-Fluor 488 (donkey, IgG) were from Dianova (Hamburg, Germany). Chamber slides (8-well) were from SPL Life Sciences Co., Ltd. (Gyeonggi-do, Korea). All other chemicals were from Merck (Darmstadt, Germany).

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2.2 Tissue culture of insulin-producing cells

RINm5F insulin-producing cells (RRID:CVCL_0501; ATCC, Manassas, VA, USA), established from rat insulinoma [20], were cultured in RPMI 1640 medium supplemented with 10 mM glucose, 10% (v/v) fetal calf serum, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2, as described previously [15]. RINm5F cells overexpressing N-glycosylation negative catalase in the ER were generated as described previously [21]. Functional expression of catalase was analysed by flow cytometry or enzyme activity measurement.

NEFAs (non-esterified fatty acids) were dissolved in 90% ethanol by heating to 60 °C and used at a final concentration of 50 µM in RPMI 1640 (PAN Biotech) with 1% fetal calf serum and a final BSA/NEFA ratio of 2%/1 mM (defined NEFA free BSA). All untreated wells were exposed to the same amount of solvent and BSA. This procedure did not significantly affect the viability in the absence of added fatty acids [22].

2.3 Subcloning of H_2O_2-sensitive sensor HyPer, pH-sensitive sensor SypHer or catalase targeted to different subcellular sites or membrane surfaces

To analyse intracellular H_2O_2 diffusion and membrane permeabilities for H_2O_2 the cDNA of the specific fluorescent H_2O_2 sensor protein HyPer [16] was subcloned into lentiviral transfer plasmids together with specific localisation signals or membrane anchor sequences.

For analysis of H_2O_2 in the cytosol (Cyto) the HyPer cDNA was amplified from pLenti6/V5-MCS-Hyper-Cyto plasmid [23] using Q5® High-Fidelity DNA polymerase (NEB, Frankfurt, Germany) and corresponding composite primers shown in Table 1. Then the HyPer-Cyto cDNA was subcloned into the EcoRI/BamHI site of the pLVX-EF1α-IRES-Puro plasmid (Clontech, Saint-Germain-en-Laye, France).

To express HyPer inside the mitochondria (Mito) HyPer cDNA with a double N-terminal mitochondrial targeting signal was amplified from the pHyPer-dMito plasmid (Evrogen) using corresponding composite primers as shown in Table 1. Thereafter HyPer-Mito cDNA was inserted into the EcoRI/XbaI site of the pLVX-EF1α-IRES-Puro plasmid (Clontech).

For peroxisomal expression (Peroxi) of the HyPer sensor protein the peroxisome target signal 1 (PTS-1) [24, 25] was fused to the 3'-end of the HyPer cDNA by PCR using composite primers as shown in Table 1 and the pLenti6/V5-MCS-Hyper-Peroxi plasmid [23] as a template. The HyPer-Peroxi cDNA was then inserted into the EcoRI/BamHI site of the pLVX-EF1α-IRES-Puro plasmid (Clontech).

To target the HyPer sensor protein to the cytoplasmic face of the plasma membrane (PM) or of the ER membrane (ERM) HyPer cDNA without stop codon was amplified from the pLenti6/V5-MCS-Hyper-Cyto plasmid [23] using corresponding composite primers shown in Table 1. The HyPer cDNA lacking the stop codon was then subcloned into the EcoRI/XbaI site of the pLVX-IRES-Neo plasmid (Clontech). The cDNAs of the membrane targeting sequences containing a short linker sequence (15 bp; ANSRV) at the 5'-end were generated by annealing of complementary phosphorylated oligonucleotides (Table 2). For localisation at the PM the tag consisted of the C-terminal CAAX motif of human K-RAS, for localisation at the cytoplasmic face of the ERM of the localisation signal of *S. cerevisiae* ubiquitin conjugase 6 [26]. PM-tag or ERM-tag cDNAs were then subcloned into the XbaI/BamHI site of the pLVX-IRES-Neo-HyPer-noStop plasmid.

For observation of cytosolic pH changes the cDNA of the pH sensor protein SypHer was amplified from the SypHer mt plasmid (Addgene, Plasmid #48251) using the primers HyPer-Cyto-EcoRI-fw and HyPer-Cyto-BamHI-rv (Table 1). SypHer-Cyto cDNA was inserted into the

EcoRI/BamHI site of the pLVX-EF1α-IRES-Puro plasmid (Clontech).

For peroxisomal targeting of the SypHer pH sensor, the PTS-1 [24, 25] was fused to the 3'-end of the SypHer cDNA by PCR using the composite primers HyPer-PTSI-EcoRI-fw and HyPer-PTSI-BamHI-rv as shown in Table 1 and the SypHer mt plasmid (addgene, Plasmid #48251). SypHer-Peroxi cDNA was inserted into the EcoRI/BamHI site of the pLVX-EF1α-IRES-Puro plasmid (Clontech).

To analyse the effects of peroxisomal catalase overexpression on the HyPer reaction to H_2O_2 the PTS-1 [24, 25] was fused to the 3'-end of the catalase cDNA by PCR using composite primers hCat-SpeI-fw and hCat-NotI-PTS1-rv and the pcDNA3-hCat plasmid [27] as a template (Table 1). The Peroxi-Cat cDNA was subsequently inserted into the SpeI/NotI site of the pLVX-EF1α-IRES-Puro plasmid and the pLVX-IRES-Neo plasmid (Clontech).

Accuracy of all DNA constructs was verified by sequencing.

Table 1

Primers for subcloning of HyPer, SypHer and catalase cDNAs into lentiviral transfer plasmids.

HyPer localisation	**Primer name**	**Sequence**
Cytosol	HyPer-Cyto-EcoRI-fw	5´-TAGAATTCATGGAGATGGCAAGCCAGC-3´
	HyPer-Cyto-BamHI-rv	5´-TAGGATCCTTAAACCGCCTGTTTTAAAACTTTATC-3´
Mitochondria	HyPer-Mito-EcoRI-fw	5´-TAGAATTCATGTCCGTCCTGACGCC-3´
	HyPer-Mito-XbaI-rv	5´-TATCTAGATTAAACCGCCTGTTTTAAAACTTTATC-3´
Peroxisomes	HyPer-PTSI-EcoRI-fw	5´-TAGAATTCATGGAGATGGCAAGCCAGCAG-3´
	HyPer-PTSI-BamHI-rv	5´-TAGGATCCTTACAGCTTGGAAACCGCCTGTTT-3´
Plasma/ER membrane	HyPer-Cyto-EcoRI-fw	5´-TAGAATTCATGGAGATGGCAAGCCAGC-3´
	HyPer-Cyto-noStop-XbaI-rv	5´-TATCTAGAAACCGCCTGTTTTAAAACTTTATCGA-3´
Catalase localisation	**Primer name**	**Sequence**
Peroxisomes	hCat-SpeI-fw	5´-TACTAGTGGGTGGAGACCCACGAGCC-3´
	hCat-NotI-PTS1-rv	5´- TATCTAGATCACAGCTTGGACTCCCTTGCCGCCAAGTGA -3´

Table 2

Sequences of complementary oligonucleotides for HyPer localisation at the plasma or the ER membrane.

HyPer localisation	**Oligonucleotide name**	**Oligonucleotide sequence**
Plasma membrane	CAAX-tag-fw	5´-CTAGAGCCAACAGCAGAGTGAAGATGTCAAAAGACGGGAAGAA AAAGAAGAAAAAATCCAAGACCAAGTGTGTCATCATGTAAG-3´
	CAAX-tag-rv	5´-GATCCTTACATGATGACACACTTGGTCTTGGATTTTTTCTTCTTTT TCTTCCCGTCTTTTGACATCTTCACTCTGCTGTTGGCT-3´
ER	Ubi-conj-6-tag-fw	5´-CTAGAGCCAACAGCAGAGTGATGGTGTACATCGGCATCGCCATC

membrane		TTCCTGTTCCTGGTGGGCCTGTTCATGAAGTAAG-3´
	Ubi-conj-6-tag-rv	5´-GATCCTTACTTCATGAACAGGCCCACCAGGAACAGGAAGATGGC GATGCCGATGTACACCATCACTCTGCTGTTGGCT-3´

2.4 Preparation of lentiviruses and stable transduction of insulin-producing cells

For stable expression of the various HyPer or SypHer sensor proteins or of peroxisomal catalase in the insulin-producing RINm5F cell line, lentiviruses were prepared as described before [28]. In brief, 5×10^6 293FT cells were transfected with the packaging plasmid pPAX2 (37.5 µg), the envelope plasmid pcDNA3-MDG (7.5 µg), and the respective transfer plasmids pLVX-EF1α-IRES-Puro-HyPer-Cyto, pLVX-EF1α-IRES-Puro-HyPer-Mito, pLVX-EF1α-IRES-Puro-HyPer-Peroxi, pLVX-IRES-Neo-HyPer-PM, pLVX-IRES-Neo-HyPer-ERM, pLVX-EF1α-IRES-Puro-SypHer-Cyto, pLVX-EF1α-IRES-Puro-SypHer-Peroxi, pLVX-EF1α-IRES-Puro-Peroxi-Cat or pLVX-IRES-Neo-Peroxi-Cat (25 µg) by calcium phosphate precipitation. The virus particles were harvested from the culture medium 48 h later and purified by ultracentrifugation (70,000 x g, 2 h). RINm5F cells were infected with a virus dilution of 1:100 in the supernatant for 6 h. After the infection the viral supernatant was replaced by fresh cell culture medium. Cells were selected for expression of HyPer, SypHer or Peroxi-Cat respectively using either puromycin (5 µg/mL) or G418 (250 µg/mL). Additionally, RINm5F cells stably overexpressing Peroxi-Cat were infected with purified HyPer-Peroxi particles, RINm5F cells stably expressing HyPer-PM were infected with purified Peroxi-Cat particles and RINm5F cells stably overexpressing ER-Cat N244 were infected with purified HyPer-ERM particles.

2.5 Immunocytochemical staining

For immunocytochemical staining of RINm5F cells expressing HyPer sensor proteins in the mitochondria, the peroxisomes and at the cytoplasmic face of the ER membrane 70,000 cells per well were seeded overnight on 8-well chamber slides and subsequently fixed with 1% paraformaldehyde. After washing the cells were permeabilized and blocked with PBS containing

0.1% Triton X-100 and 1% BSA for 20 min. After repeated washing cells were incubated with primary antibodies (Table 3) diluted in PBS containing 0.1 % Triton X-100 and 0.1% BSA at room temperature for 1 h. Thereafter, the cells were washed and incubated with secondary antibodies (Table 3) for 1 h. Cells expressing HyPer at the ER membrane were additionally stained with a second pair of primary and secondary antibodies (Table 3) following the same procedure. For nuclear counterstaining the cells were incubated with 300 nM 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Cells expressing HyPer in the cytosol or at the cytoplasmic face of the plasma membrane were handled like the other cells though without incubations with antibodies. At last, the cells were washed and mounted with Mowiol/DABCO anti-photobleaching mounting media. Stained cells were examined with an Olympus IX81 inverted microscope using a 60x super apochromatic silicon oil immersion objective (Olympus, Hamburg, Germany) and microscopic images were postprocessed using Autoquant (Media Cybernetics, Inc., Rockville, MD, USA).

Table 3
Antibodies for immunocytochemical staining.

HyPer localisation	Primary antibody	Source	Secondary antibody	Source
Peroxisomes	anti-PMP70, rabbit, 1:500	Zymed, Vienna, Austria	anti-rabbit-Alexa-Fluor 647, 1:200	Dianova, Hamburg, Germany
Mitochondria	anti-COX IV, mouse, 1:50	Santa Cruz Biotechnology, Santa Cruz, CA, USA	anti-mouse-Cy5 1:200	Dianova, Hamburg, Germany
ER membrane	anti-Tag(CGY)FP, rabbit, 1:1500	Evrogen, Moscow, Russia	anti-rabbit-Alexa-Fluor 488, 1:200	Dianova, Hamburg, Germany
	anti-PDI, mouse, 1:100	Abcam, Cambridge, UK	anti-mouse-Alexa-Fluor 647, 1:200	Dianova, Hamburg, Germany

2.6 Detection of H_2O_2 using HyPer sensors

To verify the functionality of the HyPer sensor proteins at different subcellular localisations in insulin-producing RINm5F cells and to show the antioxidative capacity of

peroxisomal and ER-luminal catalase, changes in the fluorescence ratio of the sensor upon exposure to H_2O_2 were quantified by flow cytometry. Prior to analysis, the cells were trypsinized and treated with the indicated concentrations of H_2O_2 immediately before the fluorescence ratio was determined by flow cytometry using FL-1 (488/527 nm) and FL-7 (405/527 nm) of the CyFlow ML (Sysmex, Norderstedt, Germany).

2.7 Assessment of cell viability after exposure to H_2O_2

Insulin-producing RINm5F cells expressing HyPer sensor proteins (HyPer-Peroxi, HyPer-ERM, HyPer-PM) alone or with co-expressed catalase (Peroxi-Cat, ER-Cat N244) were seeded at a density of 2×10^4 cells per well in 100 µL culture medium in 96-well plates and allowed to attach for 24 h before being incubated with different H_2O_2 concentrations ranging from 0 µM to 50 mM for 2 h in 20 mmol/L HEPES-supplemented Krebs-Ringer bicarbonate medium without glucose. H_2O_2 incubation medium was then replaced by fresh culture medium for the next 22 h. Cell viability was subsequently determined by a microplate-based MTT assay (Serva) [29].

2.8 Quantification of catalase activity

Catalase activity was quantified as described before [27]. Briefly, whole cell extracts were prepared in 50 mmol/L potassium phosphate buffer (pH 7.8) through sonication on ice with a Braun-Sonic 125 sonifier (Braun, Melsungen, Germany). The homogenates were then centrifuged at 10,000 x g and 4 °C for 10 min and the protein content of the supernatant was determined by the BCA assay (Thermo Fisher Scientific). Catalase activity was measured by ultraviolet spectroscopy, monitoring the decomposition of H_2O_2 at 240 nm.

2.9 Perifusion of insulin-producing cells with H_2O_2

RINm5F cells stably expressing HyPer sensor proteins at various subcellular localisations alone or co-expressing catalase were seeded overnight at a density of 8 x 10^6 cells on collagen-

coated glass coverslips. For analysis the glass slides were mounted in a perifusion chamber (POC Chamber System, PeCon, Erbach, Germany). Cells were first perifused with Krebs-Ringer bicarbonate medium for detection of the basal state and then with 20 µM H_2O_2 in Krebs-Ringer bicarbonate medium using a peristaltic pump at a speed of 1.1 mL/min. Live cell imaging was performed by excitation at 500/24 nm and 436/10 nm and emission at 542/27 nm using an Olympus IX81 inverted microscope system with an 40x apochromatic objective (Olympus) at a time resolution of 0.5 s – 5 s. Xcellence software (Olympus) was used for imaging and analysis. Reaction times of the H_2O_2 sensor protein HyPer were determined as starting point for HyPer ratio increase.

To analyse effects of FFA incubation on HyPer reaction times to exogenous H_2O_2 RINm5F cells stably expressing HyPer sensor proteins at various subcellular localisations alone or co-expressing catalase were seeded at a density of 4 x 10^6 cells on collagen-coated glass coverslips. Cells were cultured for 24 h and then exposed to 50 µM palmitic acid (PA) or 50 µM oleic acid (OA) for another 24 h. Controls were incubated with the same amount of solvent and BSA. Perifusion and live cell imaging were performed as described above.

2.10 Statistical Analysis

Data are expressed as means ± SEM. Statistical analyses were performed using unpaired t-test or One-sample t-test (Graphpad, San Diego, CA, USA).

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3. Results

3.1 H_2O_2 detection via flow cytometry in insulin-producing cells expressing the H_2O_2 sensor protein HyPer at different subcellular localisations

To proof the functionality of the HyPer sensor protein expressed in the cytosol (HyPer-Cyto), the peroxisomes (HyPer-Peroxi) or the mitochondria (HyPer-Mito) and on surfaces of the plasma membrane (HyPer-PM) and the ER membrane (HyPer-ERM), the cells were exposed to 100 µM H_2O_2 and the fluorescence ratio changes compared to untreated cells were subsequently detected by flow cytometry. All sensors showed a significant increase of the fluorescence ratio after treatment with H_2O_2, proving their responsivity to H_2O_2 independent of their subcellular localisation (Fig. 1).

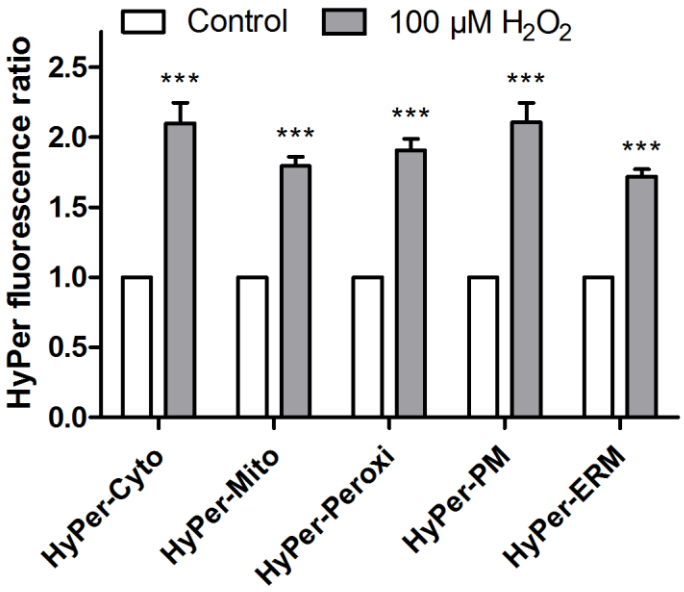

Fig. 1. Hydrogen peroxide detection via flow cytometry in insulin-producing cells expressing the H_2O_2 sensor protein HyPer at different subcellular localisations. Insulin-producing RINm5F cells stably expressing the H_2O_2 sensor protein HyPer in the cytosol (HyPer-Cyto), the mitochondria (HyPer-Mito) or the peroxisomes (HyPer-Peroxi) as well as at the cytosolic face of the plasma membrane (HyPer-PM) or the cytosolic face of the ER membrane (HyPer-ERM) were analysed by flow cytometry in basal state and immediately after treatment with 100 µM H_2O_2 (incubation time: 10 s). The HyPer fluorescence ratio, which increases with increasing H_2O_2 concentrations, was measured at 488/527 nm/405/527 nm. Data are means ± SEM from 6 to 23 independent experiments. ***$p<0.001$ (One sample t-test).

3.2 Effects of co-expression of catalase on HyPer ratio changes in insulin-producing cells

Furthermore the effects of catalase overexpressed either inside the peroxisomes or inside the ER lumen on the HyPer ratio were analysed. For cells co-expressing peroxisomal catalase and HyPer-Peroxi (Fig. 2A) or HyPer-PM (Fig. 2C) as well as cells co-expressing ER-catalase N244 and HyPer-ERM (Fig. 2B) the increase of the HyPer fluorescence ratio in response to increasing H_2O_2 concentrations was much weaker as for cells without catalase overexpression. The HyPer ratios ascended in a wider range from 0-200 µM H_2O_2 compared to 0-80 µM H_2O_2 for HyPer-Peroxi alone or 0-40 µM H_2O_2 for HyPer-ERM or HyPer-PM alone. The plateaus, which were in total around 50 % lower than for HyPer-Peroxi, HyPer-ERM or HyPer-PM alone, were not reached up to H_2O_2 concentrations of around 250-350 µM (Fig. 2A-C). For even higher H_2O_2 concentrations (up to 50 mM) the oxidation of the HyPer sensor shows a dose-response relationship to H_2O_2 also in catalase co-expressing cells for all localisations. The HyPer ratios reach the same levels as in non-catalase expressing cells at concentrations between 30-50 mM H_2O_2 (Fig. S1).

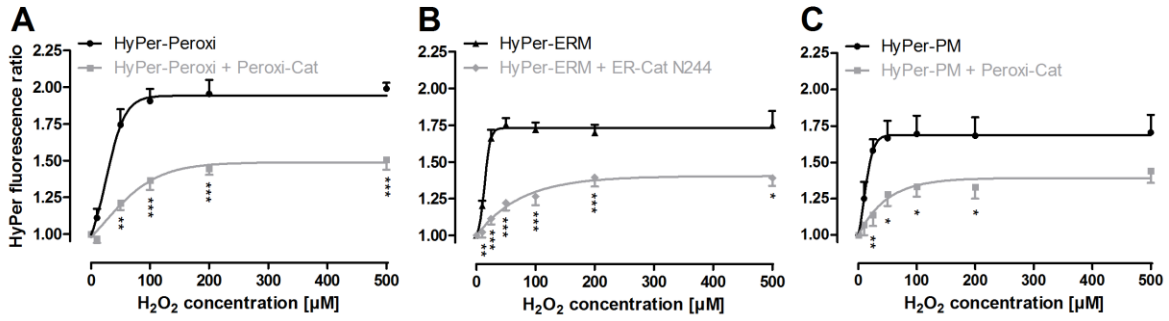

Fig. 2. Effects of co-expression of catalase on HyPer ratio changes in insulin-producing cells. Insulin-producing RINm5F cells stably expressing the H_2O_2 sensor protein HyPer (A) in the peroxisomes (HyPer-Peroxi) alone or with co-expressed peroxisomal catalase (Peroxi-Cat)), (B) at the cytosolic face of the ER membrane (HyPer-ERM) alone or with co-expressed ER catalase N244 (ER-Cat N244) or (C) at the cytosolic face of the plasma membrane (HyPer-PM) alone or with co-expressed peroxisomal catalase (Peroxi-Cat) were analysed by flow cytometry immediately after treatment with different concentrations of H_2O_2 (incubation time: 10 s). The HyPer fluorescence ratio, which increases with increasing H_2O_2 concentrations, was measured at

488/527 nm/405/527 nm. Data are means ± SEM from 4 to 23 independent experiments. *p<0.05, **p<0.01, ***p<0.001 (t-test, unpaired, two-tailed).

3.3 Analysis of reaction times of HyPer sensor proteins expressed at different subcellular sites in insulin-producing cells to exogenous H_2O_2

To determine whether HyPer sensor proteins localised at different subcellular sites differ in their reaction times to exogenously administered H_2O_2, HyPer expressing cells were perifused with medium containing 20 µM H_2O_2 and reaction times for the different localisations of the sensor were determined. The reaction times were approximately twice as fast ($p<0.01$) when the expressed HyPer sensors were located at the cytoplasmic face of the plasma membrane or at the ER membrane when compared to HyPer sensors expressed in the cytosol or in the inner space of the mitochondria or the peroxisomes (Table 4).

Table 4

Analysis of reaction times of HyPer sensor proteins expressed at different subcellular sites in insulin-producing cells to exogenous H_2O_2. RINm5F cells stably expressing the H_2O_2 sensor protein HyPer in the cytosol (HyPer-Cyto), the mitochondria (HyPer-Mito) or the peroxisomes (HyPer-Peroxi) as well as at the cytoplasmic face of the plasma membrane (HyPer-PM) or the ER membrane (HyPer-ERM) were seeded on collagen-coated glass coverslips 24 hours prior to analysis. Cells were perifused with medium containing 20 µM H_2O_2. Live cell fluorescence microscopic imaging of the HyPer sensor was performed by excitation at 500/24 nm and 436/10 nm and emission at 542/27 nm. Reaction time of the H_2O_2 sensor protein HyPer was defined as the minimum turning point of the HyPer fluorescence ratio. Data are means ± SEM of 8-17 independent experiments.

	HyPer reaction time [s]
HyPer-Cyto	8.7 ± 1.4 (8)
HyPer-Mito	9.4 ± 0.9 (11)
HyPer-Peroxi	7.3 ± 0.6 (10)
HyPer-PM	4.5 ± 0.6 (17)
HyPer-ERM	3.5 ± 0.9 (14)

Since the HyPer fluorescence may be dependent on pH, the effect of incubation with H_2O_2 on the pH was analysed using the fluorescence protein SypHer. Incubation with H_2O_2 had no influence on the pH as shown for cytosolic and peroxisomal localisation in Fig. S2 and Fig. S3.

3.4 Co-expression of peroxisomal catalase in insulin-producing cells lead to changes in the reaction of the HyPer H_2O_2 sensor localised in the peroxisomes in response to exogenous H_2O_2

To analyse the effect of H_2O_2-detoxifying peroxisomal catalase overexpression on the reaction of the peroxisomal HyPer sensor protein a perifusion with exogenous H_2O_2 was performed. Co-expression of Peroxi-Cat reduced the total HyPer-Peroxi fluorescence ratio increase by a factor of 7.6 (Fig. 3A). On the other hand, the reaction time of the peroxisomal HyPer sensor was prolonged by 49% ($p<0.01$) in the case of peroxisomal catalase co-expression (Fig. 3B&C).

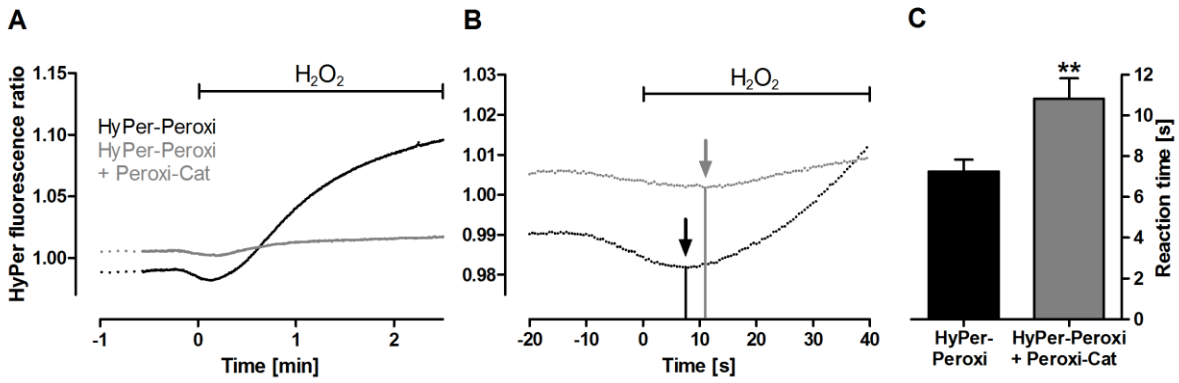

Fig. 3. Co-expression of peroxisomal catalase in insulin-producing cells lead to changes in the reaction of the HyPer H_2O_2 sensor localised in the peroxisomes in response to exogenous H_2O_2. RINm5F insulin-producing cells stably expressing the specific H_2O_2 sensor protein HyPer in the peroxisomes (HyPer-Peroxi) alone or with co-expressed peroxisomal catalase (Peroxi-Cat) were seeded on collagen-coated glass coverslips 24 hours prior to analysis. (A) Cells were perifused with medium containing 20 µM H_2O_2. Live cell fluorescence microscopic imaging of the HyPer sensor was performed by excitation at 500/24 nm and 436/10 nm and emission at 542/27 nm. (B, C) Reaction time of the H_2O_2 sensor protein HyPer was defined as the minimum turning point of the HyPer fluorescence ratio. Data are means ± SEM of 6-10 independent experiments. **$p<0.01$ (t-test, unpaired, two-tailed).

3.5 Co-expression of ER catalase in insulin-producing cells lead to changes in the reaction of the HyPer H_2O_2 sensor localised at the cytosolic face of the ER membrane in response to exogenous H_2O_2

To analyse the effect of H_2O_2-detoxifying ER-catalase expression on the reaction of the

HyPer sensor protein localised at the cytoplasmic face of the ER membrane a perifusion with exogenous H_2O_2 was performed. Co-expression of ER-Cat reduced the total HyPer-ERM fluorescence ratio increase by a factor of 2.3 (Fig. 4A). The reaction time of the HyPer sensor at the ER membrane showed a tendency towards an increase in the case of ER-Cat co-expression (Fig. 4B&C).

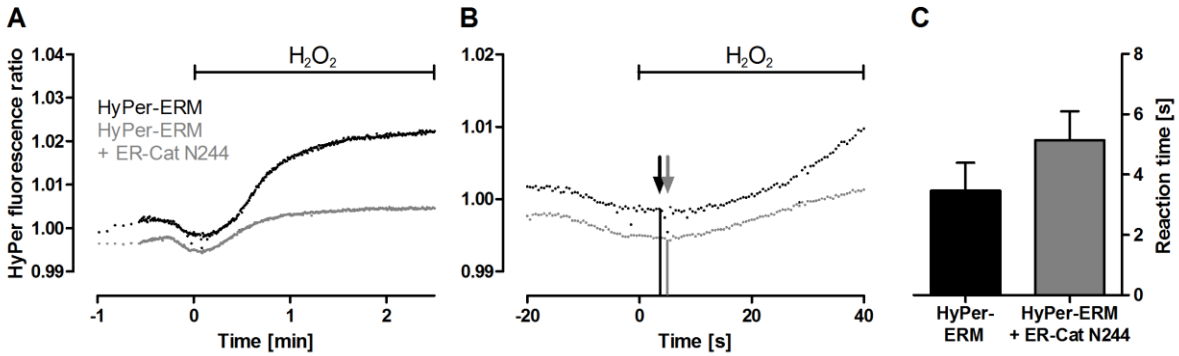

Fig. 4. Co-expression of ER catalase in insulin-producing cells lead to changes in the reaction of the HyPer H_2O_2 sensor localised at the cytosolic face of the ER membrane in response to exogenous H_2O_2. RINm5F insulin-producing cells stably expressing the specific H_2O_2 sensor protein HyPer at the cytosolic face of the ER membrane (HyPer-ERM) alone or with co-expressed ER catalase (ER-Cat N244) were seeded on collagen-coated glass coverslips 24 hours prior to analysis. (A) Cells were perifused with medium containing 20 µM H_2O_2. Live cell fluorescence microscopic imaging of the HyPer sensor was performed by excitation at 500/24 nm and 436/10 nm and emission at 542/27 nm. (B, C) Reaction time of the H_2O_2 sensor protein HyPer was defined as the minimum turning point of the HyPer fluorescence ratio. Data are means ± SEM of 7-14 independent experiments. No significance (t-test, unpaired, two-tailed).

3.6 Co-expression of peroxisomal catalase in insulin-producing cells lead to changes in the reaction of the HyPer H_2O_2 sensor localised at the cytosolic face of the plasma membrane in response to exogenous H_2O_2

To investigate whether the expression of catalase exclusively inside the peroxisomes can also influence the reaction of the HyPer sensor protein localised at the cytoplasmic face perifusion with exogenous H_2O_2 was performed. In this experimental setup the sensor protein and the antioxidative enzyme were separated by of the cytosolic compartment and the

peroxisomal membrane. Interestingly co-expression of Peroxi-Cat still reduced the total HyPer-PM fluorescence ratio increase by 20% (Fig. 5A). The reaction time of the HyPer sensor at the plasma membrane also showed a tendency towards an increase in the case of Peroxi-Cat co-expression (Fig. 5B&C) as shown before for the peroxisomal compartment and the ER.

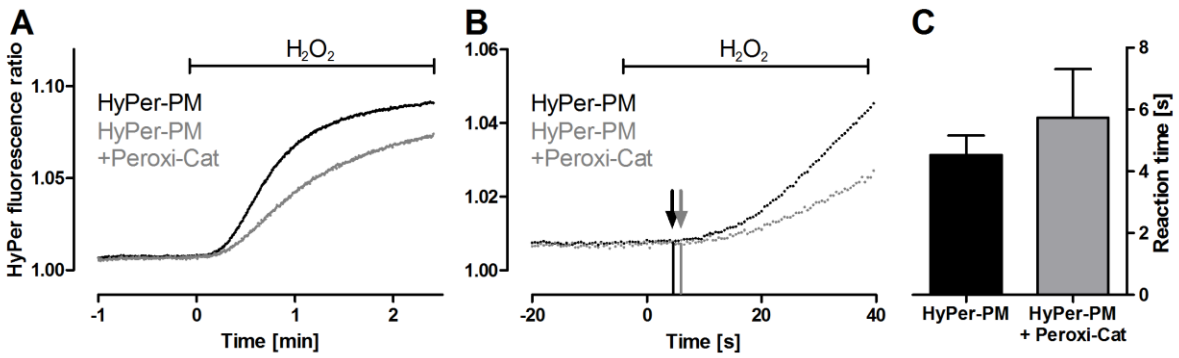

Fig. 5. Co-expression of peroxisomal catalase in insulin-producing cells lead to changes in the reaction of the HyPer H_2O_2 sensor localised at the cytosolic face of the plasma membrane in response to exogenous H_2O_2. RINm5F insulin-producing cells stably expressing the specific H_2O_2 sensor protein HyPer at the cytosolic face of the plasma membrane (HyPer-PM) alone or with co-expressed peroxisomal catalase (Peroxi-Cat) were seeded on collagen-coated glass coverslips 24 hours prior to analysis. (A) Cells were perifused with medium containing 20 µM H_2O_2. Live cell fluorescence microscopic imaging of the HyPer sensor was performed by excitation at 500/24 nm and 436/10 nm and emission at 542/27 nm. (B, C) Reaction time of the H_2O_2 sensor protein HyPer was defined as the minimum turning point of the HyPer fluorescence ratio. Data are means ± SEM of 11-17 independent experiments. No significance (t-test, unpaired, two-tailed).

3.7 Incubation of insulin-producing cells with palmitic acid reduced peroxisomal membrane permeability for H_2O_2

In addition to the overexpression of H_2O_2 detoxifying catalase the effect of the saturated long-chain free fatty acid palmitic acid (PA) and of the mono-unsaturated long-chain free fatty acid oleic acid (OA) on the H_2O_2 membrane permeability of the peroxisomal membrane in insulin-producing cells was examined. For this purpose the cells were incubated with PA (50 µM) or OA (50 µM) for 24 h and subsequently perifused with exogenous H_2O_2. Pre-

incubation with PA did not affect the total HyPer-Peroxi fluorescence ratio increase (Fig. 6A), whereas the total ratio increase of the sensor after OA incubation was reduced by around 50% compared to control cells (Fig. 6A). However, the reaction time of the peroxisomal HyPer sensor did not change after OA incubation but it was significantly reduced ($p<0.01$) after exposure of the cells to PA (Fig. 6B&C).

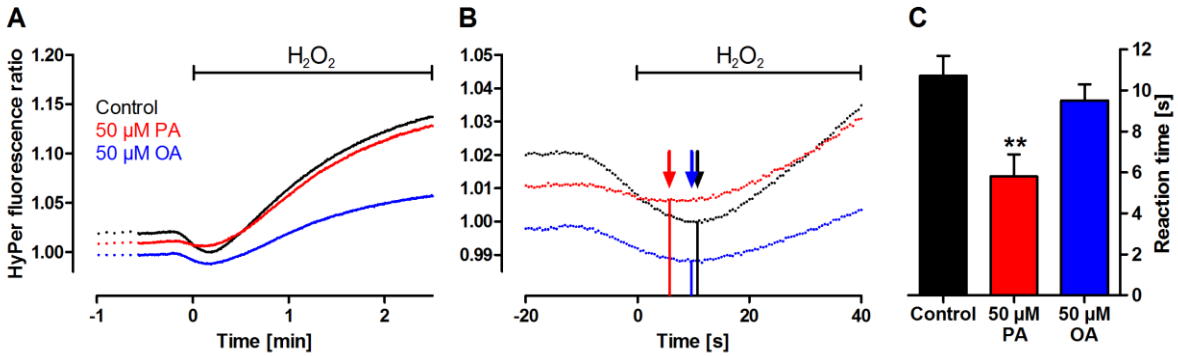

Fig. 6. Incubation of insulin-producing cells with PA increased peroxisomal membrane permeability for H_2O_2. RINm5F cells stably expressing the specific H_2O_2 sensor protein HyPer in the peroxisomes (HyPer-Peroxi) were seeded on collagen-coated glass coverslips 48 hours prior to analysis. The cells were incubated with palmitic acid (PA; red) or oleic acid (OA; blue) dissolved in 90% ethanol (50 mM) and used at a final concentration of 50 µM in RPMI 1640 with 0.1% BSA and 1% fetal calf serum for 24 hours. Control cells (Control; black) were treated with the same amount of ethanol and BSA but without PA or OA. (A) Cells were perifused with medium containing 20 µM H_2O_2. Live cell fluorescence microscopic imaging of the HyPer sensor was performed by excitation at 500/24 nm and 436/10 nm and emission at 542/27 nm. (B, C) Reaction time of the H_2O_2 sensor protein HyPer was defined as the minimum turning point of the HyPer fluorescence ratio. Data are means ± SEM of 5-7 independent experiments. **$p<0.01$ compared with control cells (t-test, unpaired, two-tailed).

This effect of PA was abolished in cells co-expressing catalase in the peroxisomes (Fig. S4). Reaction times of HyPer sensors localised in the cytosol, the mitochondria or at the cytoplasmic face of the plasma membrane or the ER membrane to exogenously administered H_2O_2 did not change significantly upon PA or OA pre-incubation (data not shown).

4. Discussion

The delicate equilibrium between redox signalling and oxidative stress is of great interest as various pathways and their regulation or dysregulation are not completely elucidated [3, 4, 30]. The biologically important ROS H_2O_2 plays a major role both as a signalling molecule and as a stressor [4, 8]. Oxidative stress mediated by H_2O_2 is especially problematic for pancreatic beta-cells because of their low expression of antioxidative enzymes [8, 14, 15, 31]. Being relatively inert to reactions with biomolecules H_2O_2 can travel significant distances through the cell thereby passing several biological membranes [2, 32]. These have a limited permeability for H_2O_2, which is amongst others dependent on the membrane composition and on the expression of aquaporins [18, 33]. But there is not so much known about the factors influencing the barrier function of biological membranes towards H_2O_2. Therefore the aim of the present study was to analyse the diffusion of H_2O_2 through membranes of several subcellular compartments in insulin-producing RINm5F cells with high time resolution. Reaction times of the genetically encoded H_2O_2 sensor protein HyPer localised at different subcellular sites were determined. Additionally the influence of an overexpression of the antioxidative enzyme catalase on the HyPer reaction was studied. With respect to the metabolic situation in type 2 diabetes the influence of the FFAs PA and OA on the membrane permeability to H_2O_2 was also analysed.

4.1 H_2O_2 detection in insulin-producing cells expressing the H_2O_2 sensor protein HyPer at different subcellular localisations

To detect H_2O_2 at different subcellular localisations the genetically encoded sensor protein HyPer was expressed not only inside subcellular compartments such as the cytosol, the peroxisomes and the mitochondria, but additionally on membrane surfaces, such as the cytoplasmic face of the plasma membrane and the ER membrane. The correct subcellular

localisation was confirmed by immunocytochemical staining (Fig. S5). An analysis of the functionality of the sensor protein at the different subcellular localisations revealed small differences between the sensors. Most importantly they all showed a significant increase of the HyPer fluorescence ratio upon exposure to H_2O_2 proving their functional expression. The independence of the sensor reaction from pH changes was shown by the example of cytosolic and peroxisomal localisation using the pH sensor protein SypHer (Fig. S2 & S3). Sensors localised in the cytosol or at the cytoplasmic face of the plasma membrane showed the highest increase of the HyPer fluorescence ratio after incubation with 100 µM H_2O_2. Compared to that, the ratio increase of the peroxisomal sensor was around 10% lower. This can be explained by a very low level of endogenous expression of catalase in the peroxisomes of RINm5F cells [15]. Although it is not comparable to catalase expression levels in other cell types, it might still detoxify at least a small proportion of the supplied H_2O_2 thereby shifting the reaction of the sensor to higher H_2O_2 concentrations and keeping the maximal ratio slightly lower. HyPer sensors localised inside the mitochondria or at the cytoplasmic face of the ER membrane exhibit around 25% lower HyPer ratio values after incubation with 100 µM H_2O_2 compared to the other localisations. Inside the ER of insulin-producing cells also a small proportion of antioxidative enzymes is present, namely glutathione peroxidases 7 and 8 [31]. It can be speculated that the intraorganellar detoxification of at least a small proportion of H_2O_2 creates a gradient into the organelle thereby minimizing the effective H_2O_2 concentration at the ER surface leading to a smaller total ratio increase of the HyPer sensor protein. In mitochondria a different pH compared to other cellular compartments might influence the HyPer reaction [34]. In which way the response of the sensor changes at higher pH levels has not yet been studied. However, it has been

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shown that the HyPer ratio increases with higher pH values, leaving a smaller range for the further ratio response to H_2O_2 [16].

The determination of reaction times of the various HyPer sensors revealed that membrane bound sensors directed towards the cytosol react around twice as fast to exogenous H_2O_2 than sensors localised inside organelles like the mitochondria or the peroxisomes. This can be interpreted as an indication for the easier accessibility of the membrane attached HyPer sensors in the cytosol for the exogenously administered H_2O_2 when compared to intraorganellar locations requiring the passage of an additional lipid membrane with a limited permeability for H_2O_2 [18]. Peroxisomal and mitochondrial membranes appear to exhibit a comparable permeability for H_2O_2 since the reaction times of the sensors inside the organelles show no significant differences.

A comparison between the four investigated HyPer localisations and the sensor expressed in the cytosol with respect to reaction times is difficult for several reasons. The measured reaction time of the cytosolic sensor is in the range of the intraorganellar sensors but twice as long as compared with the membrane bound cytosolic sensors. This can be explained by the fact that the cytosolic sensor is not exclusively expressed in the cytosol but also in the nucleus. The nucleus has been shown to be the compartment with the highest reducing potential with regard to the HyPer ratio, taking longer to be maximally oxidized [35]. Re-analysis of the obtained microscopic images from perifusion in the basal state differentiating between the nucleus and the periphery of the cell also showed a significantly reduced HyPer ratio for the nucleus (Fig. S6). As the nuclear and the cytosolic compartment can in general be seen as one entity in our experimental setup, this could explain the delay in cytosolic HyPer reaction compared to the membrane bound cytosolic HyPer sensors.

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4.2 Co-expression of antioxidative enzymes lead to changes in HyPer reaction to exogenous H_2O_2 in RINm5F cells

To analyse the influence of catalase expression on the HyPer sensor reaction to H_2O_2 in insulin-producing RINm5F cells, the enzyme was overexpressed inside the peroxisomes with co-expression of peroxisomal or plasma membrane attached HyPer or inside the ER lumen with co-expression of ER membrane attached HyPer. The overexpression of catalase reduced the maximal ratio increase of the respective HyPer sensors in FACS studies by around 50%. This and the fact, that higher H_2O_2 concentrations were necessary to reach a plateau of the fluorescence ratio can be explained with the H_2O_2 decomposing action of the enzyme. Since the activity of the overexpressed catalase inside the peroxisomes or the ER is high (Fig. S7A, S8A, S9A) and overexpressing RINm5F cells are well protected against toxicity of high concentrations of exogenously administered H_2O_2 (Fig. S7B&C, S8B&C, S9B&C) it can be concluded, that the effective H_2O_2 concentration at the site of catalase expression is much lower than in control RINm5F cells. This will in turn lead to a lower total ratio increase of the sensor, which additionally can only be reached at higher H_2O_2 concentrations. This hypothesis does not only appear to be true for co-expression of the sensor and the enzyme in the same compartment, as shown here for the peroxisomes, but also with a membrane separating sensor and enzyme, shown for the ER lumen and the outer face of the ER membrane, and also with a membrane and the cytosol separating sensor and enzyme, shown for the peroxisomal lumen and the cytosolic face of the ER membrane.

Furthermore perifusion experiments with exogenously administered H_2O_2 revealed, that for all three combinations of enzyme and sensor the co-expression of catalase reduced the total increase of the respective HyPer fluorescence ratio drastically, which is again due to the H_2O_2

detoxification capacity of catalase. At the used concentration of 20 µM H_2O_2 catalase quenched the HyPer signal. The differences in the quenching effects are due to differences in catalase activity (Fig. S7A, S8A, S9A). The detoxification of H_2O_2 did not only cause a much smaller increase in the fluorescence ratio but also lead to a delayed reaction of the sensor to exogenous H_2O_2. The shift of the reaction time to higher values was significant for co-expression of catalase and HyPer both localised inside the peroxisomes. The sensor expressed at the outer surface of the ER membrane with co-expressed ER catalase and the sensor expressed at the cytosolic face of the plasma membrane with co-expressed peroxisomal catalase indicated a similar tendency for a delayed reaction. The detoxification of H_2O_2 inside the ER causes the formation of a gradient of the H_2O_2 concentration across the membrane [36] thereby accelerating H_2O_2 diffusion into the ER lumen and reducing the effective H_2O_2 concentration at the membrane surface. This finally leads to a decrease of the HyPer ratio increase at similar concentrations compared to controls and to a retarded reaction time of the HyPer sensor at the ER membrane. The same seems to be true for the HyPer sensor at the plasma membrane with co-expressed peroxisomal catalase. The concentration gradient starting from the peroxisomes as site of catalase expression seems to stretch out through the cytosol to the plasma membrane, still reducing the effective H_2O_2 concentration leading to a delay in HyPer reaction time.

4.3 Pre-incubation with FFAs lead to changes in HyPer reaction to exogenous H_2O_2 at specific intracellular localisations

FFAs are an important stressor for pancreatic beta-cells especially during the development of type 2 diabetes mellitus [8]. Especially long-chain and very long-chain FFAs are known to be metabolized in the peroxisomal beta oxidation thereby generating H_2O_2 [9, 10, 37, 38]. Here the effects of FFAs on the membrane permeability for H_2O_2 were investigated with the

saturated PA (C16:0) and the monounsaturated OA (C18:1) as examples, which are the physiologically most prevalent FFAs [39]. Therefore RINm5F cells expressing the HyPer sensor in the cytosol, the peroxisomes, the mitochondria and at the cytoplasmic face of the plasma membrane or the ER membrane were incubated with the FFAs for 24 hours prior to perifusion with H_2O_2. The only significant effect of the FFAs on the reaction time of one of the sensors was observed for peroxisomal HyPer incubated with PA. OA incubation caused a reduction in the HyPer fluorescence ratio increase similar to but not as strong as peroxisomal catalase overexpression. This can possibly be explained by the ROS scavenging capacity of the monounsaturated FFA OA, as shown before for superoxide radical quenching [40]. Nevertheless, this had no effect on the reaction time of the HyPer sensor. Only PA pre-incubation caused a significant acceleration of the HyPer sensor reaction to H_2O_2. As shown before the membrane composition can influence the membrane permeability [33, 41]. It can be assumed that PA incorporation into the peroxisomal membrane makes it more permeable for H_2O_2.

We have observed in several of our perifusion experiments an initial decrease in the HyPer fluorescence ratio – a phenomenon we cannot explain. To exclude a pH dependency control experiments with the pH sensitive fluorescent protein SypHer were performed which revealed no fluorescence changes of the pH sensor (Fig. S2). Furthermore we can exclude effects by the temperature, changes in medium composition or the flow rate. However, based on the results shown in Fig. 3 and Fig. 6 for HyPer-Peroxi we can exclude that this phenomenon significantly influenced the measured reaction times. The initial decrease of the HyPer ratio is less pronounced in the PA condition in comparison to control cells (Fig. 6). A similar effect can be seen in cells expressing catalase in the peroxisomes (Fig. 3). Independently of this effect the PA incubation leads to a faster reaction time in comparison to the control condition whereas the

catalase co-expression results in a significantly slower reaction time of the HyPer sensor. Therefore we conclude that the observed effects on the sensor do not depend on the extent of the initial ratio decrease.

In conclusion, the present results show that using the genetically encoded H_2O_2 sensor protein HyPer expressed at different subcellular sites allows an examination of H_2O_2 movement within different compartments of mammalian cells with high time resolution. The plasma membrane as well as the peroxisomal and the mitochondrial membrane of insulin-producing RINm5F cells are permeable for H_2O_2, though the organelle membranes represent a certain barrier for H_2O_2 diffusion, shown by extended reaction times of the intraorganellar sensors. Also an increased permeability of the peroxisomal membrane for H_2O_2 caused by PA was discovered.

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Author Disclosure Statement

Declarations of interest: none.

Author Contributions

AL, SLo, CS, SL and ME were responsible for the study design and concept. AL conducted all experiments and analysed the data together with CS. AL, SLo, CS, SL and ME interpreted the results. AL, ME and SL wrote the manuscript. SLo and CS critically revised the content. All authors approved the final version of the paper.

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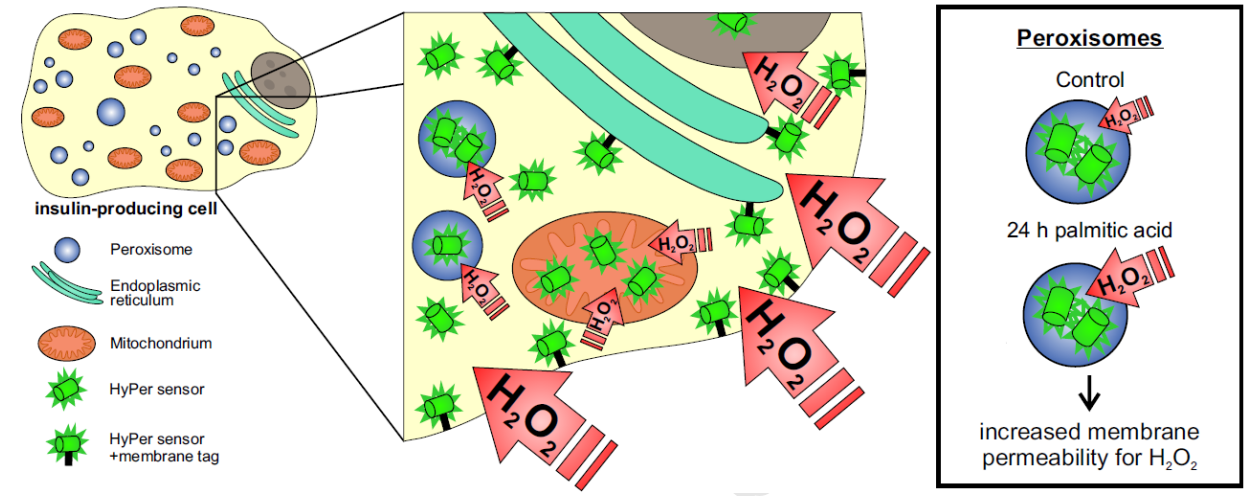
Graphical abstract



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Highlights

- H_2O_2 can travel within compartments of insulin-producing cells
- Membranes of the cell and organelles represent a diffusion barrier for H_2O_2
- Accelerated decomposition of H_2O_2 by catalase retards HyPer sensor reaction to H_2O_2
- Incorporation of PA into the peroxisomal membrane makes it more permeable for H_2O_2

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