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Inhibitor studies with isolated mitochondria demonstrated that complex I (CI) and III (CIII) of the electron transport chain (ETC) can act as relevant sources of mitochondrial reactive oxygen species (ROS). Here we studied ROS generation and oxidative stress induction during chronic (24 h) inhibition of CI and CIII using rotenone (ROT) and antimycin A (AA), respectively, in intact HEK293 cells. Both inhibitors stimulated oxidation of the ROS sensor hydroethidine (HEt) and increased mitochondrial NAD(P)H levels without major effects on cell viability. Integrated analysis of cells stably expressing cytosolic- or mitochondria-targeted variants of the reporter molecules HyPer (H₂O₂-sensitive and pH-sensitive) and SypHer (H₂O₂-insensitive and pH-sensitive), revealed that CI- and CIII inhibition increased cytosolic but not mitochondrial H₂O₂ levels. Total and mitochondria-specific lipid peroxidation was not increased in the inhibited cells as reported by the C11-BODIPY581/591 and MitoPerOx biosensors. Also expression of the superoxide-detoxifying enzymes CuZnSOD (cytosolic) and MnSOD (mitochondrial) was not affected. Oxyblot analysis revealed that protein carbonylation was not stimulated by CI and CIII inhibition. Our findings suggest that chronic inhibition of CI and CIII: (i) increases the levels of HEt-oxidizing ROS and (ii) specifically elevates cytosolic but not mitochondrial H₂O₂ levels, (iii) does not induce oxidative stress or substantial cell death. We conclude that the increased ROS levels are below the stress-inducing level and might play a role in redox signaling.

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1. Introduction

Dysfunction of the mitochondrial electron transport chain (ETC) due to mutations in OXPHOS subunits or induced by chemical inhibitors is generally associated with increased mitochondrial reactive oxygen species (ROS) production [2,18,27,31]. These ROS include the superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and the extremely reactive hydroxyl radical (OH*), with each species displaying specific reactivity, specific range of action and distinct molecular targets [11,12]. When exceeding the capacity of the cell’s antioxidant systems, increased ROS production triggers oxidative stress and cell death. Mitochondria contain both ETC-linked and non ETC-linked pathways for ROS production [46]. For example, isolated mitochondria can generate ROS via ETC...
complexes I, II and III (CI, CII, CIII), monoamino oxidase, α-ke-tonolurate dehydrogenase, glycerocephosphate dehydrogenase, di-hydro-orotate dehydrogenase, electron transfer flavoprotein dehydrogenase, branched-chain 2-oxoacid dehydrogenase, pyruvate dehydrogenase and p66shc/cytochrome c [7,49,56,58,66]. Importantly, the relevance of each of these enzymes during increased mitochondrial ROS production is tissue-specific and depends on the metabolic and experimental condition [68].

In case of the ETC, CI and CIII are generally considered the most important contributors to mitochondrial ROS production in intact cells [7,49]. The chemical CI inhibitor rotenone (ROT) was demonstrated to stimulate \( \text{O}_2^- \) production at the NADH oxidizing flavin group and/or at the CoQ-binding site [7]. However, when the PMF (proton motive force) is sufficiently large, CI can also generate large quantities of \( \text{O}_2^- \) via reverse electron transfer (RET) from CII to CI that is inhibited by ROT [44,49]. Moreover, it appears that CI can also directly catalyse \( \text{H}_2\text{O}_2 \) formation [29]. Evidence was provided that \( \text{O}_2^- \) produced by CI is specifically released towards the mitochondrial matrix, whereas inhibition of the Qi site of CIII with antimycin A (AA) in the presence of reduced coenzyme Q10 releases large amounts of \( \text{O}_2^- \) from the Qo site into both the mitochondrial matrix and the intermembrane space [7,70]. However, CI appears to contribute the main source of mitochondrial \( \text{O}_2^- \) under physiological conditions [28,49,57].

While ROS quantification in isolated mitochondria generally yields consistent results, proper interpretation of live-cell ROS measurements still remains challenging. This relates to the fact that mitochondrial ROS can be produced using different mitochondrial substrates, are removed by (local) antioxidant systems, and/or are difficult to specifically detect at the cellular level [23,32,78]. For instance, primary neuronal cell lines and immortalized mouse embryonic fibroblasts from mice with isolated CI deficiency (NDUFS4−/− mice; [10,72], as reported by oxidation of the ROS reporter molecule hydroethidine (HEt). In contrast, primary muscle or skin fibroblasts isolated from these mice exhibited increased HEt oxidation [73]. Similarly, oxidation of HEt and 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein (CM-\( \text{H}_2\text{DCF} \)) was increased in primary skin fibroblasts of patients with inherited CI deficiency [34,38,67,75]. Interestingly, increased HEt and CM-\( \text{H}_2\text{DCF} \) oxidation was not paralleled by alterations in thiol redox status and cellular lipid peroxidation in these cells [45,76]. This suggests that the ROS increase is still below the stress-inducing level and might play a signaling role [15,16,20,39,50,66,71,74,79].

We recently used HEK293 cells to analyze the bioenergetic consequences of chronic (24 h) CI and CIII inhibition by ROT or AA, respectively [24]. The latter study revealed that these treatments inhibited mitochondrial oxygen (\( \text{O}_2 \)) consumption and induced a glycolytic phenotype without off-target effects. Here this experimental model was used to determine whether CI and CIII inhibition increased ROS levels and triggered oxidative stress. Our results demonstrate that CI and CIII inhibition is associated with increased HEt-oxidation and elevated cytosolic but not mitochondrial \( \text{H}_2\text{O}_2 \) levels. However, no experimental evidence of oxidative stress, massive cell death or protein carbonylation was found. This suggests that the magnitude of the inhibition-induced increase in ROS level does not exceed the capacity of the cell's antioxidant systems.

2. Materials and methods

2.1. Generation of inducible HEK293 cell lines stably expressing cytosolic and mitochondria-targeted variants of HyPer and SypHer

Gateway® Entry vectors were generated by recombining Gateway-adapted PCR products containing the sequence encoding cyto-HyPer and mito-HyPer from pHyPer-cyo and pHyPer-dMito vectors (Evrogen, Moscow, Russia) with pDONR201 (Invitrogen, Breda, The Netherlands). A Gateway-adapted tag-less pcDNA/FRT/TO Destination vector was created by recombining the acGFP1-Destination vector [17] with a BacMamSVS-Destination vector [33]. Recombining the HyPer Entry vectors with this Destination vector using the LR Clonase II Enzyme Mix (Invitrogen) yielded the pcDNAS/FRT/TO/mito-HyPer and pcDNAS/FRT/TO/cyto-HyPer expression vectors. Targeting to the mitochondrial matrix was achieved by the presence of two N-terminally fused cox8 mitochondrial targeting sequences (2MTS). To create constructs encoding the pH sensor SypHer, the mito-HyPer and cyto-HyPer Entry vectors were mutated at a critical cysteine residue by site-directed mutagenesis as described previously [53]. These new Entry vectors were also recombined with the Gateway-adapted pcDNA/FRT/TO Destination vector to obtain mito-SypHer and cyto-SypHer expression vectors. Flp-In T-Rex293 cells (Invitrogen) were stably transfected with the expression vectors described above using the Superfect Transfection Reagent (Qiagen, Venlo, The Netherlands) and cultured for selection in the presence of 200 \( \mu \text{g}/\text{ml} \) hygromycin (Calbiochem, Bruenschwig, Amsterdam, The Netherlands) and 50 \( \mu \text{g}/\text{ml} \) blasticidin (Invitrogen). To induce expression of the biosensor, 1 \( \mu \text{g}/\text{ml} \) doxycyclin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the culture medium, followed by incubation for 24 h.

2.2. Cell culture and inhibitor treatment

Flp-In T-Rex293 cells were cultured in DMEM containing 25 mM glucose, 2 mM L-glutamine, 10% (v/v) fetal calf serum and 1% penicillin/streptomycin in a humidified atmosphere containing 5% \( \text{CO}_2 \) at 37°C. Prior to transfection, parental Flp-In T-Rex293 cells were cultured in the presence of 50 \( \mu \text{g}/\text{ml} \) blasticidin and 100 \( \mu \text{g}/\text{ml} \) Zeocin (Invitrogen). For fluorescence microscopy, 100,000 cells were seeded on 24-mm coverslips (Thermo Scientific, Etten-Leur, The Netherlands) placed in 35-mm CellStar tissue culture dishes (Sigma-Aldrich) two days prior to imaging to achieve a 70% confluence at the day of imaging. For the determination of superoxide dismutase protein levels, cells were grown to 80% confluence. One day after seeding, cells were treated with ROT or AA for 24 h and compared with vehicle (0.1% ethanol)-treated control (CT) cells.

2.3. Cell viability analysis

Cell viability was assessed using the crystal violet assay. Cells were seeded in 48 well plates and stained with crystal violet solution (0.5% crystal violet, 30% ethanol, 3% formaldehyde). Plates were rinsed with water and crystal violet internalized by the cells was re-solubilized in 1% SDS solution. Absorbance at 550 nm was measured using a microplate spectrophotometer (Benchmark Plus, Biorad, The Netherlands). Results were expressed as percentage of cell viability relative to untreated controls.

2.4. Fluorescence imaging of hydroethidine oxidation

Flp-In T-Rex293 cells were incubated with 10 \( \mu \text{M} \) HEt (Invitrogen) in harvested culture medium for 10 min at 37°C and 5% \( \text{CO}_2 \) in the dark. The HEt oxidation reaction was terminated by thoroughly washing the cells with PBS to remove excess HEt. Subsequently, the cells were covered by a colorless HEPES–Tris (HT) buffer (containing 132 mM NaCl, 10 mM HEPES, 42.2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\) and 25 mM d-glucose, adjusted to pH 7.4 with Tris salt). To quantify fluorescent HEt oxidation products, coverslips were mounted in an incubation chamber that was placed on the temperature-controlled (37°C) stage of an inverted microscope (Axiovert 200M, Carl Zeiss, Jena, Germany) equipped with a Zeiss ×40 1.3 NA Fluor objective.
The cells were excited for 100 ms at 490 nm using a monochromator (Polychrome IV, TILL Photonics, Gräfelfing, Germany). Fluorescence light from the cells was directed onto a CoolSNAP HQ monochrome CCD-camera (Roper Scientific, Vianen, The Netherlands) using a 525DRLP dichroic mirror (Omega Optical Inc., Brattleboro, VT, USA) and a 565ALP emission filter (Omega Optical Inc.). The hardware was controlled using Metafluor 6.0 software (Universal Imaging Corporation, Downingtown, PA, USA). Ten random fields of view were routinely analyzed per coverslip using MetaMorph 6.1 software (Universal Imaging Corporation). For time-lapse experiments involving the mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), images were recorded every 6 s.

2.5. Fluorescence imaging of HyPer and SypHer

For imaging the above described microscopy system was used. Five to 10 min prior to imaging, cells were covered by HT buffer. HyPer and SypHer were alternately excited at 420 nm and 470 nm for 200 ms using a 505DRLPXR and 505DRLPXR dichroic mirror (Omega Optical Inc.). Emission signals were directed through a 353AF45 (Omega Optical Inc.) emission filter onto the CCD camera. 10–15 Random fields of view were routinely analyzed per coverslip using MetaMorph 6.1 software (Universal Imaging Corporation).

2.6. Fluorescence imaging of lipid peroxidation

For imaging the above described microscopy system was used. Cellular and mitochondria-specific lipid peroxidation were assessed using the C11-BODIPY581/591 (Invitrogen) and a mitochondria-targeted variant of this molecule (MitoPerOx; [55]) biosensor, respectively. Cells were washed with PBS, covered with HT buffer (see above) and mounted onto an inverted microscope equipped with a ×40 1.3 NA Fluor oil-immersion objective. C11-BODIPY581/591 and MitoPerOx display similar spectral properties and were excited at 488 nm for 200 ms using a 505DRLPXR dichroic mirror (Omega Optical Inc.). Emission signals were detected using 510BW40 (oxidized form) and 565ALP (non-oxidized form) emission filters (Omega Optical Inc.). 10–15 Random fields of view were routinely analyzed per coverslip using MetaMorph 6.1 software (Universal Imaging Corporation).

2.7. Fluorescence imaging of NAD(P)H autofluorescence

Cellular NAD(P)H autofluorescence was excited for 1000 ms at 360 nm using a 430DCLP (Omega) dichroic mirror and 510BW40 (Omega) emission filter. A mitochondria-dense region of interest was used to quantify NAD(P)H autofluorescence intensity as described previously [76].

2.8. Superoxide dismutase protein levels

Cells (1–1.5 × 10^6) were harvested in ice-cold PBS. The cell suspension was centrifuged for 5 min at 1500 g and snap-frozen in liquid nitrogen. Cells were homogenized in three freeze-thaw cycles at −20°C. Following a boiling step for 5 min at 95°C in sample buffer, the samples were separated on a 10% SDS-PAGE gel. Next, the proteins were electrophoretically transferred to a PVDF membrane (Millipore, Amsterdam, The Netherlands). After blotting, the membranes were blocked for 45 min with one part Odyssey blocking buffer (Li-Cor, Lincoln, NE, USA) and one part 0.1% v/v Tween-20 containing PBS (PBS-T). The blots were rinsed twice with PBS-T and incubated with polyclonal anti-CuZnSOD (1:2000; ITK Diagnostics bv), polyclonal anti-MnSOD (1:2500; ITK diagnostics bv), polyclonal anti-Porin (1:1000; Calbiochem) or monoclonal anti-β-Actin (1:100,000; Sigma). For detection, IRDye 680-Conjugated Goat Anti-Mouse IgG, Highly Cross Adsorbed (Li-Cor) and IRDye 800CW-Conjugated Goat Anti-Rabbit IgG (Li-Cor) secondary antibodies were used. IRDye fluorescence was quantified using an Odyssey Imaging system (Li-Cor).

2.9. Protein carbonylation (Oxyblot)

At 40–60% confluence, cells were treated with 100 nM ROT or 100 nM AA. Cells were harvested after 24 h by centrifugation (5 min, 1000 g) and the cell pellet was washed once with 1 ml PBS containing 1 mM DTT and resuspended in 10 mM Tris–EDTA buffer containing 10 μg/ml DNase. After three freeze/thaw cycles in liquid nitrogen the suspension was stored at −20°C. Protein carbonyl levels were determined by immunoblotting using the Oxyblot assay kit (Merck Millipore, Temecula, CA, USA) with some slight modifications. 10 μl samples (~50 μg protein) were denatured with 10 μl 12% SDS and then derivatized with 20 μl 2,4-dinitrophenylhydrazone (DNPH) to the corresponding 2,4-dinitrophenylhydrazone (DNP). After 15 min at RT the reaction was stopped with 15 μl Neutralization buffer and the samples were diluted 2 times with 2 × SDS-sample buffer, further neutralized with TRIS, and 20–40 μl was run on a 10% SDS-PAGE gel. Next, protein was transferred electrophoretically to a PVDF membrane using the iBlot system (Life Technologies). After blotting membranes were blocked with Odyssey Blocking Buffer (Li-Cor) mixed 1:1 with PBS-Tween20 (0.1%, w/v) for 1 h at room temperature. Next, blots were incubated overnight at room temperature with the polyclonal antibody directed against DNP (Millipore) and the monoclonal antibody against β-actin (Sigma Aldrich) in the Odyssey-PBS-Tween buffer. Subsequently, blots were washed (3–5 times) with PBS-Tween and incubated with the secondary goat antibodies against rabbit and mouse IRDye 680 and IRDye 800 (Li-Cor) in the above mentioned buffer for 45 min at room temperature. After washing with PBS-Tween, PBS and water, the PVDF membranes were air dried in the dark. Finally, fluorescence signals were quantified using the Odyssey Imaging system (Li-Cor).

2.10. Data analysis

Fluorescence signals of HEt, HyPer, SypHer, MitoPerOx and C11-BODIPY581/591 were quantified by defining a region of interest (ROI) in the appropriate part of the cell (e.g. mitochondria, nucleus, cytosol) as described previously [24,36,55]. Cellular signals were background-corrected using an extracellular ROI close to the cell-of-interest. Curve fitting was performed using Origin Pro 6.1 (OriginLab Corp., Northampton, MA, USA). Unless stated otherwise, data is presented as mean ± SE (standard error) and statistical significance (*P < 0.05, **P < 0.01 and ***P < 0.001) was assessed using a Kruskal–Wallis 2-way ANOVA following Dunn’s multiple comparison test or a Mann–Whitney test using Graphpad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Inhibition of Cl⁻ or CII stimulates the rate of hydroethidine oxidation

Hydroethidine (HEt) is a cell permeable non-fluorescent reporter molecule that, upon oxidation, is converted into cationic fluorescent products [84]. Due to their positive charge, HEt oxidation products become cell impermeable and primarily accumulate in the mitochondrial matrix (Δψ) and the nucleus (‘Nuc’ by binding to negatively-charged nucleoli). HEK293 cells were incubated with 100 nM ROT or AA for
24 h [24] and, subsequently, the rate of HEt oxidation was measured [25]. To this end (Fig. 1A; inset), a mitochondria-dense region ("m") of interest (ROI) in the cytosol and a nuclear ("n") ROI was defined for each cell as described previously [75]. In the presence of extracellular HEt (10 μM), the fluorescence of the HEt oxidation products linearly increased over time in vehicle- (CT), ROT- and AA-treated cells during at least 10 min (Fig. 1A). This linearity demonstrates that HEt oxidation occurs via a 0th-order reaction mechanism [37] meaning that the rate of HEt-oxidizing ROS. The latter allows assessment of the fluorescence increase is a measure of the level of HEt-oxidizing ROS. The latter allows assessment of the fluorescence increase in the nuclear ("n") ROI was defined for each cell as described previously [75]. In the presence of extracellular HEt (10 μM), the fluorescence of the HEt oxidation products linearly increased over time in vehicle- (CT), ROT- and AA-treated cells during at least 10 min (Fig. 1A). This linearity demonstrates that HEt oxidation occurs via a 0th-order reaction mechanism [37] meaning that the rate of HEt-oxidizing ROS. The latter allows assessment of the fluorescence increase is a measure of the level of HEt-oxidizing ROS. The latter allows assessment of the fluorescence increase.
compartments. In contrast, AA-induced Δψ depolarization was associated with a slower increase in the mitochondrial than in the nuclear compartment (1.71- vs. 1.96-fold). This asymmetric increase is compatible with Δψ depolarization stimulating translocation of HET-oxidation products from the mitochondrial to nuclear compartment. Irrespective of the effect of Δψ hyperpolarization (ROT) and depolarization (AA), the larger increase in HET-oxidation in AA-treated relative to ROT-treated cells strongly suggests that the level of HET-oxidizing ROS is higher in the former cells. When cells were pre-incubated with HET (10 μM, 10 min), washed and subsequently imaged (endpoint measurement), ROT- and AA-treatment both increased the mitochondrial

![Image of microscopy images of HEK293 cells stably expressing HyPer in the cytosol (cyto-HyPer) and mitochondrial matrix (mito-HyPer).](image)

**Fig. 2.** Properties of targeted HyPer and SypHer variants in HEK 293 cells (A) Typical microscopy images of HEK293 cells stably expressing HyPer in the cytosol (cyto-HyPer) and mitochondrial matrix (mito-HyPer). (B) Effect of various extracellular H₂O₂ concentrations on the maximal cyto-HyPer ratio. The latter was calculated by dividing the maximal ratio value (R_max) by the pre-H₂O₂ ratio (R₀) for multiple cells (n=5) measured in two independent experiments. The experimental data was fitted using a sigmoidal (Logistic) fitting equation. (C) Effect of extracellular H₂O₂ application on the ratio signal (480 nm/420 nm) of cyto-HyPer and mito-HyPer (upper panel) and their individual 420 nm and 480 nm signals (lower panel). (D) Same as panel B but now for the effect of extracellular H₂O₂, the reducing agent dithiothreitol (DTT) and NH₄Cl on the signals of cyto-HyPer (left part) and cyto-SypHer (right part).
and nuclear fluorescence intensity (Fig. 1C). In summary, these results demonstrate that ROT- and AA-treatment increase the levels of HEt-oxidizing ROS and that this increase is larger for AA than for ROT.

3.2. Inhibition of CI or CIII does not induce substantial cell death and increases mitochondrial NAD(P)H level

We previously demonstrated that 24 h treatment with 100 nM ROT or AA greatly reduced mitochondrial O$_2$ consumption in HEK293 cells [24]. Visual inspection suggested that cell density was unaffected at 100 nM inhibitor concentration (Fig. 1D). Analysis of cell viability confirmed that ROT treatment did not affect this parameter, whereas AA induced a minor reduction (Fig. 1E). Under these conditions, ROT- and AA- treatment increased mitochondrial NAD(P)H auto-fluorescence (Fig. 1F), compatible with previous results obtained in cells with genetic and inhibitor-induced CI deficiency (e.g. [39,72,75]). Taken together, our results suggest that the increased levels of HEt-oxidizing ROS in ROT- or AA-treated HEK293 cells do not induce massive lethal oxidative stress, and are associated with increased mitochondrial NADH levels. Next we studied whether CI and CIII inhibition affected the levels of hydrogen peroxide ([H$_2$O$_2$]), lipid peroxidation and superoxide dismutases (SODs) in the mitochondrial and cytosolic compartment.

3.3. Characterization of the HyPer and SypHer biosensors in HEK293 cells

SODs convert superoxide (O$_2^-$) into H$_2$O$_2$, which can passively diffuse across membranes and thereby act as a cytosolic and extracellular signaling molecule [6,39,48,74]. To quantify H$_2$O$_2$ levels ([H$_2$O$_2$]) in the cytosol and mitochondrial matrix we used the protein-based reporter molecule “HyPer” [4]. HyPer consists of a circularly permuted (cpYFP) inserted into the H$_2$O$_2$-sensitive regulatory domain of the E. coli transcription regulator OxyR. This construct is also pH-sensitive and therefore we mutated a critical HyPer cysteine residue (C199S) to obtain the biosensor “SypHer”. The latter is H$_2$O$_2$-insensitive and can be used as a HyPer-control and pH sensor [24,53]. To allow subcellular analysis of H$_2$O$_2$ levels, we created four HEK293 cell lines that stably expressed HyPer (Fig. 2A) or SypHer (not shown) in the cytosol or mitochondrial matrix. Intracellular changes in [H$_2$O$_2$] are expected to occur within the nM to low mM range [9] and the minimal [H$_2$O$_2$]ext required to evoke a detectable change in the HyPer ratio signal was previously estimated to be ~5 μM [4]. In HEK293 cells the cytosolic HyPer ratio signal half-maximally increased at a concentration of 9.12 ± 3.68 μM of [H$_2$O$_2$]ext (Fig. 2B). Acute application of 200 μM extracellular H$_2$O$_2$ ([H$_2$O$_2$]ext) induced a 2-fold increase in cyto-Hyper and mito-Hyper ratio signal (Fig. 2C), demonstrating that exogenous H$_2$O$_2$ rapidly crosses cellular and mitochondrial membranes. Application of the reducing agent di-thiothreitol (DTT, 10 mM) returned the cyto-Hyper signal to levels slightly below basal in the presence of 50 μM [H$_2$O$_2$]ext (Fig. 2D; left panel). This demonstrates that DTT also crosses cellular membranes and lowers the levels of HyPer-oxidizing ROS. The SypHer ratio signal did not respond to a challenge with 50 μM [H$_2$O$_2$]ext but subsequently increased (at higher pH) and decreased (at lower pH) induced by transient extracellular NH$_4$Cl application (Fig. 2D; right panel). Importantly, HyPer and SypHer ratio changes were caused by changes in the emission from 420 and 470 nm
excitation in opposite directions, indicative of a cpYFP conformational change (Fig. 2C and D; lower panels). These results demonstrate that HyPer is both H$_2$O$_2$ and pH-sensitive whereas Sypher is H$_2$O$_2$-insensitive and pH-sensitive. In the next set of experiments we applied this information to specifically determine H$_2$O$_2$ levels and relative pH in the cytosol and mitochondrial matrix of inhibitor-treated cells.

3.4. Inhibition of CI or CIII increases cytosolic but not mitochondrial hydrogen peroxide levels

In CT cells, HyPer and Sypher ratios in the cytosol were lower than in the mitochondrial matrix (Fig. 3A; open bars; Sypher data taken from [24]). This is compatible with the mitochondrial matrix being more alkaline than the cytosol [47]. Given this pH-difference and the pH-sensitivity of HyPer the increased mitochondrial HyPer signal (Fig. 3A; filled bars) cannot be taken as direct evidence of a higher [H$_2$O$_2$]$_m$ in the mitochondrial matrix ([H$_2$O$_2$]$_{m}$). When correcting the HyPer signal for pH-induced changes (i.e. by dividing the mean HyPer ratio by the mean Sypher ratio) values of 0.69 (cyto-Hyper) and 0.82 were obtained (mito-Hyper). This suggests that the steady-state [H$_2$O$_2$]$_{m}$ is (slightly) higher than the steady state [H$_2$O$_2$] in the cytosol ([H$_2$O$_2$]$_{c}$). ROT treatment did not affect the cyto-HyPer signal whereas AA slightly increased this parameter (Fig. 3B; left panel). Both inhibitors reduced the mito-HyPer signal (Fig. 3B; right panel). ROT and AA treatment reduced the cyto-SypHer signal, whereas the mito-SypHer signal was only lowered in AA-treated cells (Fig. 3C; data taken from [24]). Correcting the HyPer signals for pH effects by dividing them by the corresponding Sypher signals revealed that ROT and AA significantly increased the cyto-HyPer signal (Fig. 3D; left panel). In agreement with the HEt results, this increase was larger in AA-treated than in ROT-treated cells. In contrast, no increase in mitochondrial HyPer signal was observed (Fig. 3D; right panel). These results suggest that ROT and AA inhibition do not elevate [H$_2$O$_2$]$_{c}$, albeit to a variable extent.

3.5. Inhibition of CI or CIII does not induce cellular or mitochondrial lipid peroxidation

Increased ROS levels are associated with peroxidation of cellular and mitochondrial lipids during oxidative stress induction [3]. Given the ROS increase detected by the HEt and HyPer sensors we next determined whether total and mitochondria-specific lipid peroxidation was affected by ROT- or AA-treatment in living HEK293 cells. To this end we applied the classical fluorescent lipid peroxidation probe C11-BODIPY$_{581/591}$ [19]. Moreover, we used a novel C11-BODIPY$_{581/591}$ (MitoPerOx; Fig. 3E) targeted to the mitochondrial inner membrane (MIM; [55]). Using primary human skin fibroblasts, we previously demonstrated the responsiveness of the C11-BODIPY$_{581/591}$ sensor towards exogenous H$_2$O$_2$ application (20 μM, 2 min) [23]. In HEK293 cells, treatment with H$_2$O$_2$ (50 μM, 50 min) slightly increased the MitoPerOx fluorescence ratio (Fig. 3F). Conversely, this ratio was slightly decreased upon treatment with the MIM-targeted antioxidant MitoQ$_{10}$ (10 nM, 24 h; Fig. 3F). ROT and AA treatment did not increase the C11-BODIPY$_{581/591}$ signal (Fig. 3G; left panel). The MitoPerOx ratio was slightly but significantly increased in AA but not in ROT-treated cells (Fig. 3G; right panel). These results suggest that peroxidation of cellular and MIM lipids is not greatly increased by inhibitor treatment, arguing against induction of oxidative stress.

3.6. Inhibition of CI or CIII does not increase superoxide dismutase protein levels

Under conditions of oxidative stress, high superoxide (O$_2^-$) levels are linked to increased expression of SODs [21,22,51,52]. These enzymes convert O$_2^-$ to hydrogen peroxide (H$_2$O$_2$) at very high rates [69]. The SOD family includes copper–zinc–SOD (CuZnSOD or SOD1) in the cytosol and mitochondrial intermembrane space (IMS), and manganese-SOD (MnSOD or SOD2) in the mitochondrial matrix. Western blot analysis revealed that neither ROT nor AA treatment induced a consistent and significant increase in CuZnSOD and MnSOD protein levels (Fig. 4A). These results suggest that ROT and AA treatment do not increase ROS to a level that is sufficiently high for induction of SOD expression, supporting our above conclusion that oxidative stress is virtually absent.

3.7. Inhibition of CI or CIII does not induce protein carbonylation

Both ROS and lipid peroxidation products can induce oxidation of specific amino acids, resulting in formation of stable carbonyl (-CO) groups (aldehydes and ketones) on protein side chains.
[5,13,65]. Protein carbonyls are generated by oxidative modification via direct reaction with ROS (Glu, Thr, Asp, Lys, Arg Pro; [42,43]), due to reaction (of Cys and Lys) with lipid peroxidation products like 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA; [82]), or by reaction with reducing sugars [83]. Upon carbonylation, protein function can be lost and/or proteins undergo proteasome-dependent degradation [82]. A frequently used method to detect protein carbonylation is the "Oxyblot" strategy [83], in which protein carbonyl groups are allowed to react with 2,4-dinitrophenylhydrazine (DNPH) yielding 2,4-dinitrophenylhydrazone (DNPH). The latter are subsequently detected using SDS-PAGE followed by Western blot analysis with a DNPH-specific antibody [81]. Neither ROT- nor AA-treatment detectably altered the Oxyblot signal (Fig. 4B), suggesting that ROT- and AA-treatment do not induce protein carbonylation in HEK293 cells.

4. Discussion

Mitochondrial dysfunction has been associated with increased ROS levels, either in the presence or absence of oxidative stress [21,23,40,49,51,52]. Here we demonstrate that chronic CI and CIII inhibition differentially stimulate HET-oxidation and cytosolic H$_2$O$_2$ levels in HEK293 cells and that this ROS increase is not associated with oxidative stress induction.

4.1. CI and CIII inhibition differentially stimulate HET oxidation

HET specifically reacts with O$_2^-$ to form 2-hydroxyethidium (2-OH-Et$^-$) or can act as a hydride acceptor to yield ethidium (Et$^+$). Both 2-OH-Et$^-$ and Et$^+$ display similar spectral properties ([55,60,61]). Therefore it is challenging to independently measure their fluorescence signal in single living cells. In this study we measured the combined fluorescence signal of 2-OH-Et$^-$ and Et$^+$ ("HET-oxidation products"). In the continuous presence of extracellular HET, its oxidation products progressively accumulated in the mitochondrial and nuclear compartment. In vehicle- and inhibitor-treated cells mitochondrial fluorescence signals increased faster than nuclear signals (Fig. 1A). As a consequence, mitochondrial signals were higher than nuclear signals suggesting that the HET-oxidation products residing in the nucleus are of mitochondrial origin [36]. In contrast to primary human skin fibroblasts [36], HEK293 cells displayed higher mitochondrial than nuclear signals, compatible with a mechanism in which HET is oxidized faster in HEK293 mitochondria or that these cells more effectively retain HET oxidation products in the mitochondrial matrix. The latter might suggest that HEK293 cells possess a more negative $\Delta\psi$ than human skin fibroblasts. The level of HET-oxidizing ROS were higher in AA- than in ROT-treated cells. This is compatible with previous results in isolated nerve terminals demonstrating that, when fully inhibited, the magnitude of CI-derived ROS is less than that observed for CIII-derived ROS [64]. AA-treatment fully blocked O$_2$ consumption in HEK293 cells, whereas ROT-treatment was associated with a low (< 10%) residual (CI-mediated) O$_2$ consumption [24]. In this sense, the higher NAD(P)H level in AA-treated relative to ROT-treated cells might reflect the more complete block of the ETC by AA, associated with enhanced stimulation of glycolysis and ensuing NADH production.

4.2. CI and CIII inhibition differentially increase cytosolic H$_2$O$_2$ levels

To quantify [H$_2$O$_2$] in the cytosol and mitochondrial matrix we used targeted variants of the cpYFP-based sensor HyPer. The pH-corrected HyPer ratio in the mitochondrial matrix was similar in vehicle- and inhibitor-treated cells, demonstrating that H$_2$O$_2$ levels in this compartment are not detectably increased by ROT- or AA-treatment. This result suggests that very little H$_2$O$_2$ is formed or that it is effectively removed from or within this compartment. The latter is in line with the idea that mitochondria can act as cellular sinks of H$_2$O$_2$ [8,66]. In contrast, CI and CII inhibition significantly increased cytosolic H$_2$O$_2$ levels. This might be explained by results obtained with isolated mitochondria, which demonstrate that CI releases O$_2^-$ into the IMS, whereas CII releases this ROS both into the matrix and mitochondrial intermembrane space at equal rates (see [7] and the references there in). Since O$_2^-$ cannot freely cross the IMM it will form H$_2$O$_2$ in the compartment in which it has been generated, after which it freely permeates through biological membranes [39]. Compatible with the HET measurements, cytosolic H$_2$O$_2$ levels increased to a greater extent in CIII- than in CI-inhibited cells. In case of CI, the (minimal) cytosolic H$_2$O$_2$ increase might originate from CI-derived O$_2^-$ that is formed in the matrix and converted into H$_2$O$_2$ reaching the cytosol. In case of CIII, the cytosolic H$_2$O$_2$ likely derives from O$_2^-$ that is released by CIII into the IMS and converted in H$_2$O$_2$. Under certain conditions RET from CI to CIII can lead to CI-mediated ROS production. However, this RET and ensuing H$_2$O$_2$ production is inhibited by the CI inhibitor ROT [56,63]. Since we here observed that ROT- and AA-treatment increased HET-oxidation and cytosolic H$_2$O$_2$ levels it is unlikely that RET mediates the ROS increases.

4.3. ROS generation and the role of $\Delta\psi$ in CI- and CIII-inhibited cells

Using conditions identical to this study, we previously demonstrated that ROT- and AA-treatment induce a mild $\Delta\psi$ hyperpolarization and a relatively large $\Delta\psi$ depolarization, respectively [24]. Experimental evidence suggests that CI-mediated ROS production during ROT inhibition is independent of the mitochondrial proton motive force (PMF) and stimulated by an increase in NADH/NAD$^+$ ratio [49,63,77]. This is compatible with the observed $\Delta\psi$ hyperpolarization, increased NAD(P)H autofluorescence and elevated HET-oxidation in ROT-treated HEK293 cells. Furthermore, this suggests that CI-mediated ROS production might contribute to the increased HET-oxidation in AA-treated cells. ROS production by CIII increases exponentially with $\Delta\psi$ in the absence of AA [62]. In contrast, CI-mediated ROS production is virtually $\Delta\psi$-independent in the presence of AA [30]. Combined experimental and in silico analysis suggested a bell-shaped dependency of the rate of CIII-mediated O$_2^-$ generation on the fraction of reduced quinone, with maximum O$_2^-$ generation reached at a fraction of ~ 0.6 [30]. Taken together, this explains why $\Delta\psi$ hyperpolarization (ROT) and depolarization (AA) are both associated with increased ROS levels in HEK293 cells.

4.4. CI and CIII inhibition does not stimulate SOD expression, lipid peroxidation and protein carbonylation

ROT- and AA-treated cells did not display increased levels of CuZnSOD and MnSOD, cellular/mitochondrial lipid peroxidation or protein carbonylation. Compatible with these results, cellular lipid peroxidation was not increased in fibroblasts of patients with isolated CI deficiency that displayed increased ROS levels [76]. However, as illustrated by our results with exogenous H$_2$O$_2$ and MitoQ, live-cell analysis of CI1-BODIPY581/591 and MitoPerOx might be not sensitive enough to detect small increases in lipid peroxidation [23,55].

Both ROS and lipid peroxidation products are capable to induce carbonylation of protein side chains [5,14,65]. In this sense, protein carbonylation was increased in cybrid cells bearing mtDNA mutations [54] and neuronal cells from NDUF5$^{-/-}$ mice with isolated CI deficiency (NesKO mice; [59]). Here we observed that ROT- and AA-treatment did not stimulate protein carbonylation. Similarly, short (10 min) AA treatment failed to stimulate protein carbonylation in...
143B osteosarcoma-derived cells [26]. Interestingly, high CuZnSOD levels are required to limit O$_2^-$ levels and cellular protein carbonylation is increased upon CuZnSOD knockout [11]. The same study revealed that ROT treatment (500 nM, 24 h) induced death in CuZnSOD knockout cells. Liver mitochondria from heterozygous MnSOD $^{-/-}$ mice displayed a reduction in MnSOD activity of 50% [80]. This reduction was paralleled by increased carbonylation of mitochondrial but not cytosolic proteins. In this study, inhibitor treatment did not stimulate CuZnSOD/MnSOD protein levels, did not markedly reduce cell attachment/viability, and did not increase lipid peroxidation or protein carbonylation. This suggests that CuZnSOD and MnSOD activities in HEK293 cells are high enough to prevent ROS-induced oxidative stress, and that the additional ROS generated in ROT- and AA-treated cells act not as stressors but might play a signaling role.

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References
